Published in final edited form as:

Brain Res. 2013 December 6; 1541: . doi:10.1016/j.brainres.2013.10.025.

R-flurbiprofen improves tau, but not A β pathology in a triple transgenic model of Alzheimer's disease

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Abstract

We have previously reported that chronic ibuprofen treatment improves cognition and decreases intracellular A β and phosphorylated-tau levels in 3xTg-AD mice. Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that independently of its anti-inflammatory effects has anti-amyloidogenic activity as a gamma-secretase modulator (GSM) and both activities have the potential to decrease A β pathology. To further understand the effects of NSAIDs in 3xTg-AD mice, we treated 3xTg-AD mice with R-flurbiprofen, an enantiomer of the NSAID flurbiprofen that maintains the GSM activity but has greatly reduced anti-inflammatory activity, and analyzed its effect on cognition, A β , tau, and the neurochemical profile of the hippocampus. Treatment with R-flurbiprofen from 5 to 7 months of age resulted in improved cognition on the radial arm water maze (RAWM) test and decreased the level of hyperphosphorylated tau immunostained with AT8 and PHF-1 antibodies. No significant changes in the level of A β (using 6E10 and NU-1 antibodies) were detected. Using magnetic resonance spectroscopy (MRS) we found that R-flurbiprofen treatment decreased the elevated level of glutamine in 3xTg-AD mice down to the level detected in non-transgenic mice. Glutamine levels correlated with PHF-1 immunostained hyperphosphorylated tau. We also found an inverse correlation between the concentration of glutamate and learning across all the mice in the study. Glutamine and glutamate, neurochemicals that shuttles between neurons and astrocytes to maintain glutamate homeostasis in the synapses, deserve further attention as MR markers of cognitive function.

Keywords

R-flurbiprofen; hyperphosphorylated tau; A β ; 3xTg-AD mice; magnetic resonance spectroscopy

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1. Introduction

Alzheimer's disease (AD) is an age dependent neurodegenerative disorder clinically characterized by progressive memory loss and cognitive decline. AD is the most common cause of dementia. It affects 5% of the population over the age of 65 and more than 30% of individuals over 80 years of age (Brookmeyer et al., 2007). There is no effective treatment for AD and as the society ages, AD is prone to become a major health problem. AD pathology develops over the course of decades (Braak and Braak, 1997; Jack et al.; Price and Morris, 1999) but early diagnostic is not available and the definitive diagnosis only comes from the post-mortem examination of the brain.

The neuropathological hallmarks of AD are extracellular plaques, intraneuronal neurofibrillary tangles (NFT) and the degeneration and loss of neurons and synapses (Blennow et al., 2006; Selkoe, 2001). Plaques are mainly composed of fibrils of beta-amyloid (A β) peptides surrounded by dystrophic neurites, reactive astrocytes and activated microglia. A β peptides are produced by the sequential proteolytic cleavage of the transmembrane amyloid precursor protein (APP) by the action β -secretase at the amino-termini and by γ -secretase at the carboxy-termini (Bayer et al., 2001). γ -secretase is a multi-unit enzyme that cleaves APP (and other type I transmembrane proteins such as Notch) within its transmembrane domain (Lichtenthaler et al., 2002). γ -secretase does not provide strict sequence specificity and generates A β peptides of different lengths, mainly peptides of 40 (A β ₄₀) and 42 (A β ₄₂) residues. The longer A β ₄₂ peptide is more prone to aggregation and despite of being much less abundant than A β ₄₀ is the initial and the most predominant A species in plaques. Soluble A β ₄₂ peptides and oligomers are neurotoxic and thought to be central to AD pathogenesis (Lacor et al., 2007; Shankar et al., 2008). The amyloid hypothesis, the most popular working hypothesis for AD pathogenesis, supports that the modest increase of A β ₄₂ peptide in the brain is sufficient to cause AD with complete penetrance (Hardy and Selkoe, 2002). NFT are intracellular fibrillar aggregates primarily composed of abnormally hyperphosphorylated forms of the microtubule-associated protein tau. Phosphorylated tau sequesters normal tau and other microtubule-associated proteins causing disassembly of microtubules and impaired axonal transport, compromising neuronal/synaptic function (Iqbal et al., 2005). Tau pathology is initially evidenced by the somatodendritic accumulation of conformationally altered, non-fibrillar tau, followed by the accumulation of progressively hyperphosphorylated tau detected by CP-13 antibody first and later by AT8 and PHF-1 antibodies. PHF-1 labels the fibrillar NFT in neuronal perikarya and neuritic processes (Greenberg et al., 1992; Lewis et al., 2001). It is widely considered that in AD tau phosphorylation is a consequence of A β accumulation despite the fact that hyperphosphorylation and accumulation of pathological forms of tau is found in other neurodegenerative diseases in the absence of A β and has been extensively linked to neurodegeneration (Lee et al., 2001).

Another important feature of AD pathology is the local inflammatory response, particularly involving microglia. A β plaques are consistently surrounded by microglia that display an activated phenotype characterized by enhanced expression of immune cell surface markers and the production of proinflammatory cytokines and chemokines (Akiyama et al., 2000). The finding in multiple epidemiological studies that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) was associated with a reduced risk of developing AD was initially taken as proof that inflammation is a pathogenic factor in AD (Aisen, 2002; McGeer and Rogers, 1992; McGeer et al., 1996; McGeer and McGeer, 2007; Townsend and Pratico, 2005). However, the results of pharmacological studies with anti-inflammatory agents have been inconsistent in both mice and humans (Imbimbo, 2009a).

Interest in the effects of NSAIDs on AD grew faster after a subset of NSAIDs was found to decrease the production of the more amyloidogenic A β ₄₂ peptide at the expense of shorter and less toxic A β forms (Eriksen et al., 2003; Weggen et al., 2001). The A β ₄₂-lowering action of some NSAIDs is independent of their anti-inflammatory action on cyclooxygenase (COX) and appears to involve the allosteric modulation of γ -secretase (Lleo et al., 2004). The effect of A β ₄₂-lowering NSAIDs, unlike the action of γ -secretase inhibitors that uncovered the essential role of γ -secretase activity in the proteolytic processing of the Notch receptor (Behr et al., 2004), is specific to the production of A β without significantly perturbing the action of γ -secretase on other substrates (Eriksen et al., 2003; Weggen et al., 2001; Weggen et al., 2003). The increasing number of small molecules able to decrease production of A β ₄₂ is collectively termed γ -secretase modulators (GSMs) (Bulic et al., 2011). Among NSAIDs, ibuprofen and flurbiprofen are among the most prominent GSM. Treatment with ibuprofen, that has dual anti-inflammatory and GSM activity, has shown striking preventive effects in several mouse models of AD reducing A β ₄₂ deposition and/or A β load (Choi et al., 2010; Dedeoglu et al., 2004; Heneka et al., 2005; Jantzen et al., 2002; Lim et al., 2000; Lim et al., 2001; Yan et al., 2003), and significantly improving performance on behavioural tasks (Lim et al., 2000; Lim et al., 2001; Yan et al., 2003). In the triple transgenic mouse model of AD (3xTg-AD) that develops A β and tau pathology (Oddo et al., 2003), ibuprofen reduced both the level of A β accumulation and tau phosphorylation together with improved spatial learning and memory (McKee et al., 2008). Treatment with R-flurbiprofen, an enantiomer of flurbiprofen that maintains the GSM activity in vitro and in vivo (Eriksen et al., 2003; Morihara et al., 2002), but lacks anti-inflammatory activity (Geisslinger et al., 1994; Wechter et al., 1994), attenuated learning impairments in the APP Tg2576 mouse model of AD (Kukar et al., 2007) and lowered A β ₄₂ in vivo (Eriksen et al., 2003; Morihara et al., 2002). A phase 2 trial of R-flurbiprofen suggested that patients with mild AD, but not moderate AD, had a dose-related slower rate of decline than those treated with placebo, and that the drug was well tolerated with few adverse effects (Wilcock et al., 2008). On the basis of these promising results, a large phase 3, randomized, placebo controlled trial of tarenflurbil was conducted in patients with mild AD. Disappointing results of the phase 3 trial showed no effect of R-flurbiprofen slowing cognitive decline or loss of activities of daily living in patients with mild AD nor did any secondary outcome measure or post hoc analysis favor the drug. In the present study, we treated young pre-plaque 3xTg-AD mice with R-flurbiprofen (from 5 to 7 months of age) and analyzed the effect of treatment on cognition and A β and tau pathology. Because there is a great need for early in vivo biomarkers to diagnose AD and for methods to monitor the disease progression and therapy with a high degree of specificity and sensitivity, we also applied magnetic resonance spectroscopy (MRS) to detect the effect of R-flurbiprofen on the neurochemical profile of the brain. Since each observable metabolite is sensitive to a different aspect of the pathology at the molecular or cellular level, the spectroscopic profile provides a wealth of information of the in vivo process. With technical advances in the field, the potential clinical application of MRS in ageing and dementia is rapidly growing.

2. Results

2.1 Administration of R-flurbiprofen in 3xTg-AD mice

Eight 3xTg-AD mice were orally treated for 2 months, from 5 to 7 months of age, with R-flurbiprofen (tarenflurbil, the generic name, was donated by Myriad Pharmaceuticals) at a dose of 10 mg/kg/day. R-flurbiprofen was formulated into the regular chow food. 3xTg-AD mice tolerated well the R-flurbiprofen treatment with no apparent side effects. During the 2 months treatment we detected comparable body weight and food consumption among the R-flurbiprofen-treated mice and the untreated littermate controls.

The treatment dose of 10 mg/kg/day was chosen based on the results from the study by Kukar et al., in APP Tg2576 transgenic mice (Kukar et al., 2007). In that study the authors reported that R-flurbiprofen treatment of APP Tg2576 at doses 25 and 50 mg/kg/day resulted in 85% and 100% mortality, respectively, within 2 weeks while the long-term treatment with a dose of 10 mg/kg/day showed beneficial effects without detrimental side effects.

2.2 Effect of R-flurbiprofen treatment on learning and memory retention in 3xTg-AD mice

At seven months of age, and while still being treated, R-flurbiprofen-treated and untreated 3xTg-AD mice were tested on a radial arm water maze (RAWM) protocol to evaluate the cognitive function of 3xTg-AD mice and the effect of R-flurbiprofen treatment on the hippocampal-dependent spatial memory. The difference between the RAWM and the most classic Morris water maze (MWM) is that performance in the RAWM entails finding a platform submerged in one of the several arms in the pool, compared to MWM in which the swim field is open. Besides in the RAWM the platform location changes everyday. These differences makes the task a bit more difficult in the RAWM forcing the animal to use spatial cues and working memory to keep track of the arms already visited in order to escape onto the platform.

Mice were tested during consecutive days until the average number of errors committed by one of the groups during the last acquisition trial of the day (T4) was less than two. R-flurbiprofen-treated mice reached the milestone of committing less than 2 errors in T4 on Day 9 of testing (Figure 1A). On Day 9, the number of errors of R-flurbiprofen-treated mice in T4 was 1.5 ± 0.8 , which was significantly smaller from the number of errors committed by the untreated mice group, 3.3 ± 0.3 ($p < 0.05$). Results from the test also showed that on Day 6 of testing R-flurbiprofen-treated mice started to commit significantly less number of errors in T4 than on Day 1. In contrast, it was not until Day 7 that the number of errors committed by untreated mice in T4 was significantly different than those committed in T1 (Figure 1A). As expected, control WT mice performed better than transgenic mice and shown by a faster learning curve with significantly less number of errors. Figure 1B presents the average number of errors committed by R-flurbiprofen-treated and untreated transgenic and WT mice in T1, T4 and T5 (performed 30 minutes after T4) during the last 3 days of testing (Days 7 - 9). The graph shows the learning curve as the difference in the number of errors between T1 and T4, which is a function of both reference memory to retain the general rules of the task across days and working memory to retain information for a short time. The graph also illustrates the working memory as the difference in the number of errors between T4 and T5. Based on the criteria of the RAWM test used in the study, 3xTg-AD mice were able to learn the task (the number of errors in T4 is significantly smaller than the number of errors in T1). Despite the ability to learn, 3xTg-AD mice showed dysfunctional spatial reference memory because the number of errors made in T4 was significantly higher than the number of errors made by control age matched WT mice in T4. We did not detect however impaired working memory in transgenic mice, as the number of errors in T5 (after a lapse of 30 minutes) was still significantly smaller than the number of errors in T1 although there was a trend for transgenic mice to make more errors in T5 than in T4. R-flurbiprofen reversed the spatial memory deficit of transgenic mice. The learning curve of R-flurbiprofen-treated mice is significantly steeper than for untreated transgenic mice and is not different than the learning curve of WT mice. The difference in the number of errors between T4 and T5 was however, not significantly different in either group of transgenic mice indicating that 3xTg-AD mice have no significantly impaired working memory at this age.

2.3 Plasma and Brain levels of R-flurbiprofen in 3xTg-AD mice

Immediately after behavioral testing, mice were euthanized and blood and brains were obtained for analysis. The concentration of R-flurbiprofen in plasma and in the brain was measured by Myriad Pharmaceuticals using a combination of liquid chromatography and mass spectrometry (Kukar et al., 2007). The concentration of R-flurbiprofen in plasma was 3542.7 ± 416 ng/g and in the brain was 30.4 ± 6 ng/g. The concentration in the brain was only 0.86% of that found in plasma, which corresponds to a brain-to-plasma (B/P) ratio of approximately 0.01. The poor penetration of R-flurbiprofen into the central nervous system has been blamed for the negative results found in clinical trials with R-flurbiprofen (Imbimbo, 2009b).

2.4 Effect R-flurbiprofen treatment on A β and tau pathology in 3xTg-AD mice

To determine whether the effect of R-flurbiprofen treatment on the cognitive behavior correlated with differences in AD-related pathology in the CA1/subiculum, brains were analyzed by immunohistochemistry. Assessment of A accumulation was performed using two antibodies with different specificity: monoclonal 6E10, which recognizes amino acid residues 1–16 of A β , and immunoreacts with all forms of A β including monomeric A β as well as the non-proteolytically processed APP; and monoclonal NU-1 antibody, which recognizes oligomeric and fibrillar but not monomeric A β_{42} . The immunohistological analysis of 7 months old 3xTg-AD mice showed a robust intraneuronal immunoreactivity for both A β antibodies in the hippocampal CA1 and CA2 fields, subiculum, amygdala, frontal, somatosensory and retrosplenial cortex. Rare extracellular immunoreactivity with either A β antibody was found. Staining with Thioflavine S was negative indicating the absence of fibrillar A β . R-flurbiprofen-treated 3xTg-AD mice had similar regional and cellular pattern of immunostaining than untreated mice for both antibodies. Image analysis of the CA1/subiculum area of the hippocampus showed no significant signal intensity with either antibody between R-flurbiprofen-treated and untreated mice (Figure 2, B,C,E,F,G). In 7 month old WT mice there was no immunoreactivity for A β using 6E10 or NU-1 antibodies (Figure 2, A,D). Assessment of tau phosphorylation was performed with 3 different antibodies that recognize specific pathological forms of tau, CP-13, AT8 and PHF-1. Immunostaining for CP-13 that recognizes an early form of pathological tau (pSer202), was prominent in neurons of the CA1/subiculum area of the hippocampus, in the amygdala and to a less extent in cortical neurons. CP13 immunoreactivity in the CA1/subiculum was shown in neuronal perikarya as a diffuse, finely granular staining, extending into apical dendrites and dendritic arbors (Figure 3). Although immunoreactivity for CP-13 appeared reduced in R-flurbiprofen-treated 3xTg-AD mice compared to untreated mice, densitometric image analysis of the subiculum area revealed no staining in WT mice and no significant differences between the R-Flurbiprofen treated and untreated 3xTg-AD mice (Figure 3 D). Staining with AT8 and PHF-1 antibodies that recognize late hyperphosphorylated forms of tau (pSer202/205 and pSer396/404, respectively) identified scattered neurons and neuronal processes in the CA1/subiculum area and in the amygdala. AT8 and PHF-1 immunoreactive neurons often showed dense staining at both the basal and apical poles of the perikaryon, with finely granular immunostaining of apical and other dendritic neuronal processes (Figure 4). We counted the number of AT8 and PHF-1 immunostained neurons in the CA1/subiculum area in 3 sections per mouse, as detailed in the Experimental Procedure section below, and detected a reduced number of neurons in the brains of R-flurbiprofen-treated mice compared to untreated controls and no immunostaining in WT mice. For PHF-1 antibody the number of immunoreactive neurons was significantly reduced in R-flurbiprofen treated 3xTg-AD mice ($p < 0.01$). In the CA1/subiculum area of untreated 3xTg-AD mice, the number of immunostained PHF1 neurons ranged from 0 to 3 neurons/section with most of the sections showing 1 or 2 stained neurons and only in 1 mouse we observed no immunoreactivity. The number of PHF1 immunostained neurons in the CA1/subiculum of

R-flurbiprofen treated mice on the other hand, ranged from 0 to 1 cells/section. For AT8 there was a strong trend towards reduced number of AT8 positive neurons in the R-flurbiprofen treated mice compared to untreated mice but the difference did not reach significance.

2.5 Effect of R-flurbiprofen treatment on hippocampal chemical profile and the correlative analysis between the chemical profile and the effects of R-flurbiprofen on AD related cognition and pathology in 3xTg-AD mice

The MRS of the hippocampus region of R-flurbiprofen treated and untreated transgenic mice was measured by high-resolution magic angle spinning (HRMAS). HRMAS was used because it allows for small punches (1mm diameter) that would be difficult to obtain in vivo or using tissue extracts. This reduces partial volume averaging and allows for better sensitivity to neurochemical deficits. The only significant difference in all of the twenty chemicals quantified was a decrease of glutamine in the R-flurbiprofen treated mice back to levels noted in wild-type mice (0.38 ± 0.03 vs. 0.34 ± 0.01 in untreated vs. R-flurbiprofen; $p < 0.05$) (Table I). Correlative analysis between the chemical profile and the performance in the cognitive test shows a significant correlation between learning on the RAWM and glutamate concentrations ($R = 0.72$; $p < 0.05$) across all the animals (Figure 5). The score in PHF-1 correlated with both glutamine and with learning on the radial arm water maze (Figure 6).

3. Discussion

We have previously showed that ibuprofen decreases intracellular A β and phosphorylated-tau levels in young 3xTg-AD mice resulting in improved memory and learning capacity. Ibuprofen is an NSAID with anti-inflammatory and anti-amyloidogenic GSM that selectively lowers the level of A β_{42} . Here we treated young 3xTg-AD mice with R-flurbiprofen, a compound with similar GSM activity but with practically null anti-inflammatory action. Results show that R-flurbiprofen treatment improves cognition and reduces the pathological levels of tau hyperphosphorylation and glutamine without significantly affecting the level of A β . Although the reasons for the elevated cortical levels of A β in patients with typical late-onset AD is not known, leading theories in the field centers the overproduction of A β , specifically of A β_{42} , on the pathogenesis of AD. It is proposed that increased level of A β precedes tau phosphorylation and that both processes contribute to the cognitive decline and neurodegeneration that characterize the disease. Elevated levels of soluble A β_{42} monomers enable formation of soluble oligomers that can diffuse into synaptic clefts. At picomolar concentrations, these soluble oligomers decrease dendritic spine density in organotypic hippocampal slice cultures, disrupt hippocampal LTP, and in vivo impair the memory of a complex learned behavior (Selkoe, 2008). Elevated levels of intraneuronal A β appear to activate the kinases responsible for the hyperphosphorylation of tau. The abnormal phosphorylation of tau promotes its misfolding, decreases its degradation, sequesters normal tau, and induces their assembly into tangles of paired helical filaments, which alter the microtubule stability and normal neuronal functions (Iqbal et al., 2009). In 3xTg-AD mice, we have previously showed the intraneuronal co-localization of oligomeric A β and phosphorylated tau supporting the idea that oligomeric forms of A β are associated with tau phosphorylation (McKee et al., 2008). In this 3xTg-AD model, reduction of soluble A and soluble abnormally hyperphosphorylated tau, but not soluble A alone, was found to ameliorate the cognitive decline (Oddo et al., 2006). The disjunction between A β levels and hippocampal synaptic plasticity in APP transgenic mice has also been described (Townsend et al., 2010). Failure of GSM to reduce the level of A β but still improve cognition has been previously reported (Balducci et al. 2011; Imbimbo et al., 2007; Kukar et al., 2007). This apparent paradox could be explained by the relative increase of specific synaptotoxic forms of soluble A β more than by the absolute level of A β .

Based on recent studies (Giuffrida et al., 2009; Kim et al., 2007; Kuperstein et al. 2010), one could imagine that subtle changes in the A β ₄₂/A β ₄₀ ratio within neurons, rather than a reduction of the total brain A β content, might be more closely associated with the therapeutic action of R-flurbiprofen in young 3xTg-AD mice. To determine the effect of R-flurbiprofen on A β we analyzed the hippocampal region of the brain by immunohistochemistry using two different antibodies that recognize different forms of A β , 6E10 and NU-1. 6E10 is a monoclonal antibody that recognizes all forms of A β including monomers (Covance). NU-1 on the other hand is a monoclonal antibody raised against A β ₄₂ oligomers that recognizes A β oligomers and fibrils but not A β monomers (courtesy of Dr. William Klein). No significant differences in the level of immunoreactivity to 6E10 or NU-1 were detected between R-flurbiprofen treated and untreated mice. Unfortunately, we could not measure the effect of R-flurbiprofen treatment on the concentration of A β ₄₂ and A β ₄₀ because at 7 months of age 3xTg-AD mice do not hold enough A β to be detected by ELISA. Kukar et al., reported (Kukar et al., 2007) that treatment of Tg2576 mice with R-flurbiprofen lessened the cognitive decline of mice with a non-statistically significant reduction of A β ₄₂ and A β ₄₀ levels or formic acid-soluble levels of A β . The intracellular accumulation of A β was proposed to be the initiator of AD pathogenesis in 3xTg-AD mice but this idea has been challenged by a report using 3xTg-AD/BACE(–/–) mice that do not produce A β peptides (Winton et al., 2011). In these mice the intraneuronal immunoreactivity was identified as A β epitopes on the full-length precursor APP and not as free A β peptides. In the absence of A β , 3xTg-AD/BACE(–/–) mice developed tau pathology with the same temporal and abundance as 3xTg-AD mice implying that tau pathology develops independently from the generation of A β and suggest a role for tau in AD neurodegeneration. Our data using the oligomer specific NU-1 antibody together with 6E10 reveal the intracellular accumulation of A β aggregates in 3xTg-AD mice. Staining brain tissue from 3xTg-AD/BACE(–/–) mice with an oligomer specific antibody like NU-1 could clarify the nature of the intraneuronal material in these mice. Our results on the effect of R-flurbiprofen on tau but not A β that correlates with behavioral and other neurochemical markers of AD support Winton's contention for the independent development of tau pathology and for tau-mediated effects in neurodegeneration.

Learning and memory of untreated, R-flurbiprofen treated 3xTg-AD and age matched WT mice were analysed in the RAWM test. Treatment with R-flurbiprofen improved the long-term memory of 3xTg-AD mice allowing them to learn the task faster than untreated littermates. Unlike the standard Morris water maze, which typically tests spatial reference memory (memory for spatial information that does not change over time), the RAWM protocol used in the present study analyses simultaneously spatial reference and short-term memory retention (working memory) of a submerged platform, the arm location of which changes each day. This is the first report we are aware of testing 3xTg-AD mice in the RAWM. Our results indicate that young 7 months old 3xTg-AD mice have impaired spatial reference memory but not working memory based on the criteria established by the RAWM test used in the study. Although transgenic mice committed more errors than WT mice in the working memory test, the number of errors committed by transgenic mice in T5 (after the short lapse of 30 minutes) was not different than the number of errors committed by the same group of mice in T4. Other studies also reported that young 3xTg-AD mice have reduced long-term memory (from day to day) but not short-term memory dysfunction (Billings et al., 2005) although synaptic dysfunction confirmed by deficits in LTP were detected at 6 months of age (Oddo et al., 2003). Impaired spatial reference memory but not working memory has also been described for young (but not for old) double transgenic APPxPS1 mice (Arendash et al., 2004). It appears thus that loss of long-term memory precedes loss of short-term memory in this and other mouse models of AD.

Failure of R-flurbiprofen to slow cognitive decline in patients with mild AD in the phase 3 trial was attributed in part to the weak pharmacological activity of R-flurbiprofen as a GSM and to its poor brain penetration (Imbimbo, 2009b). The penetration of R-flurbiprofen into the brain appears to be severely limited by plasma protein binding, which reduces the plasma-free fraction in the blood circulation by >90% and significantly reduces the amount of drug that can cross the blood–brain barrier (Szpunar et al., 1989). In our study R-flurbiprofen was well absorbed by 3xTg-AD mice (plasma concentration of R-flurbiprofen was 3542.7±416 ng/g) but had a very low penetration rate into the brain (brain tissue concentration of R-flurbiprofen was 30.4±6 ng/g) with a brain to plasma ratio of approximately 0.01. The low level of R-flurbiprofen achieved in the brain seems far from the concentration needed to impact the activity of γ -secretase ($IC_{50} \approx 250 \mu M$) (Eriksen et al., 2003). Furthermore, 3xTg-AD mice overexpress the human PS1 gene carrying the M146V mutation, which further decrease the sensitivity of γ -secretase to GSM (Hahn et al., 2011). Specie specific traits of R-flurbiprofen and targets other than γ -secretase may help to explain the beneficial effects of R-flurbiprofen in 3xTg-AD mice. R-flurbiprofen is considered the ‘inactive’ isomer of flurbiprofen because it does not inhibit the activity of cyclooxygenase (COX). However in mice, different than in human and rats, 22–30% of the (R)-enantiomer is converted to the S-flurbiprofen that maintains a potent anti-COX activity (Wechter et al., 1994). Using the whole blood system that take into account the protein-binding factor, reports indicate that in human there is a selectivity for COX-1 vs. COX-2, with the IC_{50} for COX-1 being 0.06 μM and for COX-2 1.8 μM (van Haeringen et al., 2000) but in guinea pig the IC_{50} value of S-flurbiprofen is about 0.5 μM for both COX-1 and COX-2 (Carabaza et al., 1996). S-flurbiprofen but not R-Flurbiprofen has been shown to inhibit microglia neurotoxicity to neuroblastoma SH-SY5Y cells at low concentrations, that appears to be consistent with a COX-dependent mechanism (Klegeris et al., 2004). In addition, R-flurbiprofen has anti-inflammatory activity on its own through the inhibition of NF- κ B and AP-1 which are key regulatory transcription factors in inflammatory processes (Tegeder et al., 2001). The anti-inflammatory action of the drug could easily explain the decrease in tau phosphorylation detected in R-flurbiprofen treated mice because all evidence points towards the deleterious relationship between neuroinflammation and tau pathology. For example, IL-1 was shown to increase tau phosphorylation *in vitro* using neuronal–microglial co-culture experiments (Li et al., 2003), and *in vivo* (Ghosh et al. 2013; Sheng et al., 2000). Moreover, parenchymal LPS injections worsened tau pathology in a transgenic murine model of forebrain-specific P301L tau overexpression (Lee et al. 2010) and led to exacerbated tau pathology in 3xTgAD mice (Kitazawa et al., 2005). Ablation of CX3CR1 in mice and resulting increases in microglial activation were associated with exacerbated tau pathology in hTau mice (Bhaskar et al. 2010). There is also evidence that tau kinases (i.e., GSK3 β and p38MAPK) are activated by pro-inflammatory cytokines and thus it is may be that R-flurbiprofen inhibits them. In contrast to tau pathology, there have been many confounds over the nature of the relationship between amyloid and neuroinflammation and some studies indicate the divergent effect of neuroinflammation on tau and A β pathology (Kitazawa et al., 2005) (Ghosh et al. 2013). Activation of microglia in 3xTg-AD mice did not affect A β level or processing (Kitazawa et al., 2005). R-flurbiprofen at clinically relevant concentrations has also been shown to upregulate NGF and BDNF *in vitro*, which could potentially offer neuroprotection (Zhao et al., 2008). Taking into account the poor penetration of R-flurbiprofen into the brain and the low brain to plasma ratio in treated 3xTg-AD mice, it is likely that brain γ -secretase inhibition does not explain the effects we observed. It is possible that other CNS or peripheral targets of R-flurbiprofen are responsible for this effect. R-flurbiprofen clearly reached the concentration necessary to activate COX1/2 in the periphery but not in the brain. It is possible therefore that R-flurbiprofen acts peripherally rather than centrally. Growing evidence suggests that the brain and immune system are intricately connected and engaged in significant crosstalk and that altering

peripheral inflammation during neurodegenerative disease can significantly alter disease course (Lucin and Wyss-Coray, 2009). Changes detected in the brain of R-flurbiprofen treated 3xTg-AD mice may be the result of a systemic effect in the pattern of soluble communication factors in the periphery due to the large concentration of R-flurbiprofen in plasma. Several peripheral COX-independent targets have been described for R-flurbiprofen in association with its anti-cancer activity in diverse tissues such as colon and prostate (Grosch et al., 2003) (Wynne and Djakiew, 2010). Genetic differences between mice and humans that translates in differential expression and affinity for target proteins most probably account for the disconnect effect of R-flurbiprofen in the 3xTg mouse and in human. Unfortunately there are many examples showing that the effects on animals are not always predictive of the effects in humans.

MRS studies indicate that there is a decrease in NAA and an increase in myo-inositol with the progression AD (Klunk et al., 1996; Pettegrew et al., 1997; Shonk et al., 1995). By combining the increase in myo-inositol with the decrease in NAA, Ross and colleagues were able to distinguish AD from other dementias (Ross et al., 1997). We also found increased glutamine and decreased glutamate in AD (Choi et al.; Jenkins et al., 2000). This may reflect a change in the balance of neuronal/glial volume as neurons degenerate, or shrink (Jenkins et al., 2005) or a change in energy metabolism as shown by PET studies which demonstrate good correlations between glutamate levels and glucose utilization (Pfund et al., 2000). Our pre-clinical studies with ibuprofen have shown that the long-term treatment with ibuprofen in a double transgenic APP/PS1 mice provided significant protection against NAA and glutamate loss suggesting the protective effect of ibuprofen to neuronal pathology in AD (Choi et al., 2010). At 6 months of age, when there is small but significant plaque accumulation in the PS1xAPP mice there were no significant neurochemical changes in any of the 16 metabolites analyzed (Choi et al., 2010). 3xTg-AD mice display accumulation of intraneuronal A β at 3 months, tau accumulation at 6 months and extracellular plaques start to develop at 12 months of age (Billings et al., 2005; Oddo et al., 2003; Oddo et al., 2006). The earliest cognitive impairment manifests at 4 months as a deficit in long-term retention that correlates with the accumulation of intraneuronal A β in the hippocampus (Billings et al., 2005). While astrogliosis is exclusively associated with the A β plaques in old 3xTg-AD mice, a generalized atrophy of GFAP-positive astrocytes was found in the plaque-free hippocampus of 6 months old 3xTg-AD mice (Olabarria et al.). Astroglia plays a crucial role in brain homeostasis and particularly important for homeostasis and turnover of the main excitatory neurotransmitter glutamate. Astroglial failure may disrupt neural networks connectivity and stimulate neurotransmitters imbalance and hence be instrumental in early cognitive deficits. In agreement with this idea, our studies show that R-flurbiprofen significantly decreases glutamine back to levels noted in wild-type mice. We found that glutamine levels directly correlated with increased PHF-1 immunoreactivity and hence with increased number of errors in the RAWM test. Whereas glutamate levels inversely correlated with learning on the maze. Glutamine and glutamate may represent the earliest MR markers of cognitive function.

3. Experimental Procedure

3.1 Mice and treatment

Homozygous 3xTg-AD mice expressing mutant human genes APP_{swe}, PS1_{M146V} and tau_{p301L}, previously characterized by Oddo et al., (Oddo et al., 2003) and wild-type (WT) mice from the same hybrid background strain, 129/C57BL6, were used in the study. At 5 months of age, eight 3xTg-AD mice were orally treated with R-flurbiprofen (donated by Myriad Pharmaceuticals) for 2 months at a dose of 10 mg/kg/day. R-flurbiprofen was formulated in the food chow by Research diets. Ten untreated 3xTg-AD and 10 untreated WT mice of the same age were used as control. Mice weight and food consumption were

monitored weekly for the length of the experiment. At 7 months of age, and while still being treated, treated mice together with untreated controls were tested on the radial arm water maze testing and subsequently euthanized to collect plasma and brains for analysis.

3.2 Radial arm water maze

At 7 months of age, 3xTg-AD treated mice and control mice were trained to find an invisible submerged platform of 14 cm in diameter, in a 6-arm-maze created inside a 4 feet diameter pool, using a variety of visual extra maze cues. Each testing day consisted in four acquisition trials (T1-4) and one memory retention trial (T5) 30 minutes after the last acquisition trial. For any given day of testing, the submerged escape platform was positioned near the end of a randomly designated goal arm for that day and the starting position for T1-4 was chosen in a semi-random sequence from the remaining five arms. The memory retention trial T5 started from the same arm as the last acquisition trial (T4) of the day. Mice were allowed to swim for up to 1 minute to find the submerged platform and then they were allowed to remain on the platform for 30 seconds. Alternatively, if a mouse did not find the platform in the 60 seconds trial, it was guided to the submerged platform where it stayed for 30 seconds. Errors were charged upon entering all four paws in an incorrect arm, upon entering the goal arm but failing to find the submerged platform, or upon failing to select an arm after 20 seconds. Every time an error occurred, the mouse was gently pulled back to the start arm for that trial. The number of errors during all trials was recorded and used to calculate the index of daily learning (the difference of errors between the first, T1, and the last acquisition trial, T4) and the index of memory (the difference in errors between the last acquisition trial, T4, and the memory retention trial, T5). Testing lasted until a group reached the pre-set criteria of committing less than two errors in the last acquisition trial of the day (T4).

3.3 Blood and brain tissue samples

Immediately after the behavioural test, mice were euthanized by CO₂ asphyxiation and brains and blood were obtained. Blood was centrifuged to get the plasma samples that were stored at -80°C until analyzed for the concentration of R-flurbiprofen. Brains were cut into three 2 mm thick coronal sections using a coronal matrix. The three 2 mm sections spanned from 3 mm anterior to the bregma to 3 mm posterior to the bregma. Each coronal section was cut sagittally in two hemisections. The left hemisections were immediately frozen and a tissue punch of 1 mm diameter in the hippocampus was obtained for MRS analysis. The rest of the hippocampus was dissected out, homogenized in 2 volumes of HPLC-grade water (1 µl per mg) followed by centrifugation at 2,000g, 4°C for 10 min and supernatants stored at -80°C until analyzed for the concentration of R-flurbiprofen. The three right hemisections were fixed in 4% paraformaldehyde at room temperature for 2 h and embedded in a single paraffin block to be analyzed by immunohistochemistry.

3.4 Analysis of R-flurbiprofen levels in blood and brains

Concentration of R-flurbiprofen in plasma and brain extracts was determined by Myriad Pharmaceuticals using tandem liquid chromatography-mass spectrometry mass, as previously described (Kukar et al., 2007). Methanol (200 µL) was used to precipitate proteins from 100 µL of sample followed by fortification with an internal standard (deuterated racemic flurbiprofen). Samples were mixed for two minutes in a Captiva filter plate on a plate shaker before being transferred to a vacuum apparatus. Vacuum (1 mm Hg) was applied for three minutes and filtered extracts were collected in a new 96-well plate ready for LC-MS/MS analysis.

A calibration curve was made in the range of 1 to 1000 ng/ml. Each 10xspiking solution (10 µl) was added to 90 µl of blank sample prepared as above to generate the curve. Three QC samples were made at each different concentration of 80, 320 and 800 ng/ml to determine

the validity of the calibration curve. Standard curve and QC points were prepared in the same manner as the samples for analysis.

Following sample preparation, 10 μ l of sample extract was injected onto a Daicel ChiralPAK AD-RH 4 x150 mm column and eluted at 0.55 ml/min using the following isocratic mobile phase: 90% methanol, 5% acetonitrile, 5% water and 0.1% acetic acid. Compounds were detected using an ABI 4000 Q-Trap linear ion trap mass spectrometer in Multiple Reaction Monitoring mode with the following mass transitions monitored: m/z 243.3&199.1 for 7869 and S-flurbiprofen and m/z 246.3&202.1 for the deuterated 7869 and S-flurbiprofen internal standard.

3.5 Immunohistochemistry

Paraffin-embedded hemibrains were serially cut in 10 μ m thick sections. Sections were immunostained for A β and tau using the following antibodies. For A β we used 6E10 (Covance, 1:1000, pre-treated with formic acid) and NU-1, which recognizes oligomeric and fibrillar but not monomeric A β 42 (courtesy of William Klein, 1:1000). For tau we used CP13 directed against phosphoserine (courtesy of Peter Davies, 1:200), PHF-1 directed against phosphoserine 396 and phosphoserine 404 (courtesy of Peter Davies, 1:200), and AT8 directed against phosphoserine 202 and phosphothreonine 205 (Innogenetics 1:2000). Sections were processed using the Vectastain Elite ABC Kit (Vector Labs, Burlingame, CA). Following the appropriate biotinylated secondary antibody, slides were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. For the quantitation of immunoreactivity, three sections per mouse (130 μ m apart) encompassing the posterior hippocampus were used. To quantify intraneuronal A β immunoreactivity for 6E10 and NU-1 and tau immunoreactivity for CP-13, densitometric analysis was performed using a house-written Matlab program. Pictures of the posterior hippocampus were taken with Nikon Eclipse 80i microscope using an Optronics digital camera. Each image was processed in Photoshop to extract the region of interest (ROI), the posterior hippocampal CA1/subicular region, and an unaffected area used to define the unspecific background stain. Both images were processed together in the Matlab program to provide the percent of specific immunoreactivity. The data were subsequently analyzed using a one-way ANOVA performed with a Tukey-Post hoc comparison. Statistical significance was defined as $p < 0.05$. For the quantitation of AT8 and PHF-1, we tabulated the number of AT8 and PHF-1 immunoreactive neurons in the hippocampal CA1/subicular region. The number of positive neurons in the untreated 3xTg-AD mice was compared to the number of positive neurons in the R-flurbiprofen-treated 3xTg-AD mice and analyzed between the groups using the Wilcoxon-Mann-Whitney Rank Sum Test. Statistical significance was defined as $p < 0.05$.

3.6 Magnetic Resonance Spectroscopy

The neurochemical profile was collected from hippocampal tissue punches using high-resolution magic angle spinning (HRMAS) on a Bruker 14T magnet. HRMAS was used because it allows for small punches (1mm diameter) that would be difficult to obtain in vivo or using tissue extracts. We used tissue punches of 1 mm diameter from hippocampus. The punches were performed on freshly frozen tissue as described in the section above. The dissected tissue sample was placed into a glass cylinder positioned in a 3 mm zirconium oxide MAS rotor (volume 50 μ L). HRMAS measurements were performed using a sample spinning rate, of 2.5 kHz selected to push the spinning side bands outside the frequency region of the metabolites. The experiments were performed at 4°C to minimize tissue degradation. Data was acquired using a rotor synchronized, T2-filtered Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [90 -(t- 180)_n acquisition] with two different effective TEs (100ms/10ms). The longer TE serves to remove the lipid/macromolecular resonances and the short TE retains them. We also collected a spectrum without water pre-

saturation to use water to assess linewidths. The interpulse delay, t , was synchronized to the rotor frequency, and was 272 μ s. The n value for the relatively short T2 filter was 36 and for the long TE was 360. The short t value removes all the T2-like effects on the lineshapes. The long T2 filter yields approximately 95% of the total spectral intensity of all metabolites of interest compared to the short TE. Other acquisition parameters are a 90 pulse of 5–10 μ s, a spectral width of 8 kHz, 16K complex points, 256 averages and a TR of 5s. Samples were placed in the rotor with a small amount of D₂O to which DSS was added to provide for locking and chemical shift reference.

Analysis of the in vitro HRMAS spectra was performed using the Chenomx NMR Suite 4.6 (Edmonton, Alberta). This package has a library of over 350 chemical metabolite spectra to which new spectra can be added. The spectra were then corrected for lineshapes using a standard (selected from the experimental spectrum) and the data was fit to the full model metabolite spectrum. We took ratios of the chemicals to both creatine and the internal DSS standard.

Acknowledgments

This research is supported by grants from NIA (R01AG031896) to A. Dedeoglu, the Department of Veteran Affairs (Merit Award) to A. Dedeoglu and (CDA-2) to I. Carreras. There is no conflict of interest. The authors thank to Dr. Peter Davies for the generous gift of the antibodies used in these studies, Dr. Frank LaFerla for providing the 3xTg-AD mice, Carol A. Kubilus for her technical help in immunohistochemistry, Lokman Hossain for animal husbandry, and Dr. Kenton H. Zavitz for providing the R-flurbiprofen (tarenflurbil) and measuring R-flurbiprofen in plasma and brain.

References

- Aisen PS. The potential of anti-inflammatory drugs for the treatment of Alzheimer's disease. *Lancet Neurol.* 2002; 1:279–84. [PubMed: 12849425]
- Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. *Neurobiol Aging.* 2000; 21:383–421. [PubMed: 10858586]
- Arendash GW, Garcia MF, Costa DA, Cracchiolo JR, Wefes IM, Potter H. Environmental enrichment improves cognition in aged Alzheimer's transgenic mice despite stable beta-amyloid deposition. *Neuroreport.* 2004; 15:1751–4. [PubMed: 15257141]
- Balducci C, Mehdawy B, Mare L, Giuliani A, Lorenzini L, Sivilia S, Giardino L, Calza L, Lanzillotta A, Sarnico I, Pizzi M, Usiello A, Viscomi AR, Ottonello S, Villetti G, Imbimbo BP, Nistico G, Forloni G, Nistico R. The gamma-secretase modulator CHF5074 restores memory and hippocampal synaptic plasticity in plaque-free Tg2576 mice. *J Alzheimers Dis.* 24:799–816. [PubMed: 21321397]
- Bayer TA, Wirths O, Majtenyi K, Hartmann T, Multhaup G, Beyreuther K, Czech C. Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport. *Brain Pathol.* 2001; 11:1–11. [PubMed: 11145195]
- Behr D, Clarke EE, Wrigley JD, Martin AC, Nadin A, Churcher I, Shearman MS. Selected non-steroidal anti-inflammatory drugs and their derivatives target gamma-secretase at a novel site. Evidence for an allosteric mechanism. *J Biol Chem.* 2004; 279:43419–26. [PubMed: 15304503]
- Bhaskar K, Konerth M, Kokiko-Cochran ON, Cardona A, Ransohoff RM, Lamb BT. Regulation of tau pathology by the microglial fractalkine receptor. *Neuron.* 68:19–31. [PubMed: 20920788]
- Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM. Intraneuronal A β causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron.* 2005; 45:675–88. [PubMed: 15748844]

- Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. *Lancet*. 2006; 368:387–403. [PubMed: 16876668]
- Braak H, Braak E. Frequency of stages of Alzheimer-related lesions in different age categories. *Neurobiol Aging*. 1997; 18:351–7. [PubMed: 9330961]
- Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM. Forecasting the global burden of Alzheimer's disease. *Alzheimers Dement*. 2007; 3:186–91. [PubMed: 19595937]
- Bulic B, Ness J, Hahn S, Rennhack A, Jumpertz T, Weggen S. Chemical Biology, Molecular Mechanism and Clinical Perspective of gamma-Secretase Modulators in Alzheimer's Disease. *Curr Neuropharmacol*. 9:598–622. [PubMed: 22798753]
- Carabaza A, Cabre F, Rotllan E, Gomez M, Gutierrez M, Garcia ML, Mauleon D. Stereoselective inhibition of inducible cyclooxygenase by chiral nonsteroidal antiinflammatory drugs. *J Clin Pharmacol*. 1996; 36:505–12. [PubMed: 8809635]
- Choi J-K, Dedeoglu A, Jenkins BG. Application of MR spectroscopy to mouse models of neurodegeneration. *NMR Biomed*.
- Choi JK, Jenkins BG, Carreras I, Kaymakcalan S, Cormier K, Kowall NW, Dedeoglu A. Anti-inflammatory treatment in AD mice protects against neuronal pathology. *Exp Neurol*. 2010; 223:377–84. [PubMed: 19679126]
- Dedeoglu A, Choi JK, Cormier K, Kowall NW, Jenkins BG. Magnetic resonance spectroscopic analysis of Alzheimer's disease mouse brain that express mutant human APP shows altered neurochemical profile. *Brain Res*. 2004; 1012:60–5. [PubMed: 15158161]
- Eriksen JL, Sagi SA, Smith TE, Weggen S, Das P, McLendon DC, Ozols VV, Jessing KW, Zavitz KH, Koo EH, Golde TE. NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo. *J Clin Invest*. 2003; 112:440–9. [PubMed: 12897211]
- Geisslinger G, Lotsch J, Menzel S, Kobal G, Brune K. Stereoselective disposition of flurbiprofen in healthy subjects following administration of the single enantiomers. *Br J Clin Pharmacol*. 1994; 37:392–4. [PubMed: 8018462]
- Ghosh S, Wu MD, Shaftel SS, Kyrkanides S, LaFerla FM, Olschowka JA, O'Banion MK. Sustained interleukin-1 β overexpression exacerbates tau pathology despite reduced amyloid burden in an Alzheimer's mouse model. *J Neurosci*. 33:5053–64. [PubMed: 23486975]
- Giuffrida ML, Caraci F, Pignataro B, Cataldo S, De Bona P, Bruno V, Molinaro G, Pappalardo G, Messina A, Palmigiano A, Garozzo D, Nicoletti F, Rizzarelli E, Copani A. Beta-amyloid monomers are neuroprotective. *J Neurosci*. 2009; 29:10582–7. [PubMed: 19710311]
- Greenberg SG, Davies P, Schein JD, Binder LI. Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. *J Biol Chem*. 1992; 267:564–9. [PubMed: 1370450]
- Grosch S, Tegeder I, Schilling K, Maier TJ, Niederberger E, Geisslinger G. Activation of c-Jun-N-terminal-kinase is crucial for the induction of a cell cycle arrest in human colon carcinoma cells caused by flurbiprofen enantiomers. *FASEB J*. 2003; 17:1316–8. [PubMed: 12759338]
- Hahn S, Bruning T, Ness J, Czirr E, Baches S, Gijzen H, Korth C, Pietrzik CU, Bulic B, Weggen S. Presenilin-1 but not amyloid precursor protein mutations present in mouse models of Alzheimer's disease attenuate the response of cultured cells to gamma-secretase modulators regardless of their potency and structure. *J Neurochem*. 116:385–95. [PubMed: 21091478]
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–6. [PubMed: 12130773]
- Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, Van Leuven F. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. *J Neuroinflammation*. 2005; 2:22. [PubMed: 16212664]
- Imbimbo BP, Del Giudice E, Cenacchi V, Volta R, Villetti G, Facchinetti F, Riccardi B, Puccini P, Moretto N, Grassi F, Ottonello S, Leon A. In vitro and in vivo profiling of CHF5022 and CHF5074 Two beta-amyloid1-42 lowering agents. *Pharmacol Res*. 2007; 55:318–28. [PubMed: 17292621]
- Imbimbo BP. An update on the efficacy of non-steroidal anti-inflammatory drugs in Alzheimer's disease. *Expert Opin Investig Drugs*. 2009a; 18:1147–68.

- Imbimbo BP. Why did tarenflurbil fail in Alzheimer's disease? *J Alzheimers Dis.* 2009b; 17:757–60. [PubMed: 19542625]
- Iqbal K, Alonso Adel C, Chen S, Chohan MO, El-Akkad E, Gong CX, Khatoon S, Li B, Liu F, Rahman A, Tanimukai H, Grundke-Iqbal I. Tau pathology in Alzheimer disease and other tauopathies. *Biochim Biophys Acta.* 2005; 1739:198–210. [PubMed: 15615638]
- Iqbal K, Liu F, Gong CX, Alonso Adel C, Grundke-Iqbal I. Mechanisms of tau-induced neurodegeneration. *Acta Neuropathol.* 2009; 118:53–69. [PubMed: 19184068]
- Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol.* 9:119–28. [PubMed: 20083042]
- Jantzen PT, Connor KE, DiCarlo G, Wenk GL, Wallace JL, Rojiani AM, Coppola D, Morgan D, Gordon MN. Microglial activation and beta -amyloid deposit reduction caused by a nitric oxide-releasing nonsteroidal anti-inflammatory drug in amyloid precursor protein plus presenilin-1 transgenic mice. *J Neurosci.* 2002; 22:2246–54. [PubMed: 11896164]
- Jenkins BG, Klivenyi P, Kustermann E, Andreassen OA, Ferrante RJ, Rosen BR, Beal MF. Nonlinear decrease over time in N-acetyl aspartate levels in the absence of neuronal loss and increases in glutamine and glucose in transgenic Huntington's disease mice. *J Neurochem.* 2000; 74:2108–19. [PubMed: 10800956]
- Jenkins BG, Andreassen OA, Dedeoglu A, Leavitt B, Hayden M, Borchelt D, Ross CA, Ferrante RJ, Beal MF. Effects of CAG repeat length, HTT protein length and protein context on cerebral metabolism measured using magnetic resonance spectroscopy in transgenic mouse models of Huntington's disease. *J Neurochem.* 2005; 95:553–62. [PubMed: 16135087]
- Kim J, Onstead L, Randle S, Price R, Smithson L, Zwizinski C, Dickson DW, Golde T, McGowan E. Abeta40 inhibits amyloid deposition in vivo. *J Neurosci.* 2007; 27:627–33. [PubMed: 17234594]
- Kitazawa M, Oddo S, Yamasaki TR, Green KN, LaFerla FM. Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. *J Neurosci.* 2005; 25:8843–53. [PubMed: 16192374]
- Klegeris A, Maguire J, McGeer PL. S- but not R-enantiomers of flurbiprofen and ibuprofen reduce human microglial and THP-1 cell neurotoxicity. *J Neuroimmunol.* 2004; 152:73–7. [PubMed: 15223239]
- Klunk WE, Xu C, Panchalingam K, McClure RJ, Pettigrew JW. Quantitative ¹H and ³¹P MRS of PCA extracts of postmortem Alzheimer's disease brain. *Neurobiol Aging.* 1996; 17:349–57. [PubMed: 8725895]
- Kukar T, Prescott S, Eriksen JL, Holloway V, Murphy MP, Koo EH, Golde TE, Nicolle MM. Chronic administration of R-flurbiprofen attenuates learning impairments in transgenic amyloid precursor protein mice. *BMC Neurosci.* 2007; 8:54. [PubMed: 17650315]
- Kuperstein I, Broersen K, Benilova I, Rozenski J, Jonckheere W, Debulpaep M, Vandersteen A, Segers-Nolten I, Van Der Werf K, Subramaniam V, Braeken D, Callewaert G, Bartic C, D'Hooge R, Martins IC, Rousseau F, Schymkowitz J, De Strooper B. Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio. *EMBO J.* 29:3408–20. [PubMed: 20818335]
- Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL. Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci.* 2007; 27:796–807. [PubMed: 17251419]
- Lee DC, Rizer J, Selenica ML, Reid P, Kraft C, Johnson A, Blair L, Gordon MN, Dickey CA, Morgan D. LPS- induced inflammation exacerbates phospho-tau pathology in rTg4510 mice. *J Neuroinflammation.* 7:56. [PubMed: 20846376]
- Lee VM, Goedert M, Trojanowski JQ. Neurodegenerative tauopathies. *Annu Rev Neurosci.* 2001; 24:1121–59. [PubMed: 11520930]
- Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science.* 2001; 293:1487–91. [PubMed: 11520987]

- Li Y, Liu L, Barger SW, Griffin WS. Interleukin-1 mediates pathological effects of microglia on tau phosphorylation and on synaptophysin synthesis in cortical neurons through a p38-MAPK pathway. *J Neurosci.* 2003; 23:1605–11. [PubMed: 12629164]
- Lichtenthaler SF, Beher D, Grimm HS, Wang R, Shearman MS, Masters CL, Beyreuther K. The intramembrane cleavage site of the amyloid precursor protein depends on the length of its transmembrane domain. *Proc Natl Acad Sci U S A.* 2002; 99:1365–70. [PubMed: 11805291]
- Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM. Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci.* 2000; 20:5709–14. [PubMed: 10908610]
- Lim GP, Yang F, Chu T, Gahtan E, Ubeda O, Beech W, Overmier JB, Hsiao-Ashe K, Frautschy SA, Cole GM. Ibuprofen effects on Alzheimer pathology and open field activity in APPsw transgenic mice. *Neurobiol Aging.* 2001; 22:983–91. [PubMed: 11755007]
- Lucin KM, Wyss-Coray T. Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron.* 2009; 64:110–22. [PubMed: 19840553]
- Lleo A, Berezhovska O, Herl L, Raju S, Deng A, Bacskaï BJ, Frosch MP, Irizarry M, Hyman BT. Nonsteroidal anti-inflammatory drugs lower Abeta42 and change presenilin 1 conformation. *Nat Med.* 2004; 10:1065–6. [PubMed: 15448688]
- McGeer PL, Rogers J. Anti-inflammatory agents as a therapeutic approach to Alzheimer's disease. *Neurology.* 1992; 42:447–9. [PubMed: 1736183]
- McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurology.* 1996; 47:425–32. [PubMed: 8757015]
- McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. *Neurobiol Aging.* 2007; 28:639–47. [PubMed: 16697488]
- McKee AC, Carreras I, Hossain L, Ryu H, Klein WL, Oddo S, LaFerla FM, Jenkins BG, Kowall NW, Dedeoglu A. Ibuprofen reduces Abeta, hyperphosphorylated tau and memory deficits in Alzheimer mice. *Brain Res.* 2008; 1207:225–36. [PubMed: 18374906]
- Morihara T, Chu T, Ubeda O, Beech W, Cole GM. Selective inhibition of Abeta42 production by NSAID R-enantiomers. *J Neurochem.* 2002; 83:1009–12. [PubMed: 12421374]
- Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron.* 2003; 39:409–21. [PubMed: 12895417]
- Oddo S, Vasilevko V, Caccamo A, Kitazawa M, Cribbs DH, LaFerla FM. Reduction of soluble Abeta and tau, but not soluble Abeta alone, ameliorates cognitive decline in transgenic mice with plaques and tangles. *J Biol Chem.* 2006; 281:39413–23. [PubMed: 17056594]
- Olabarria M, Noristani HN, Verkhratsky A, Rodriguez JJ. Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia.* 58:831–8. [PubMed: 20140958]
- Pettegrew JW, Klunk WE, Panchalingam K, McClure RJ, Stanley JA. Magnetic resonance spectroscopic changes in Alzheimer's disease. *Ann N Y Acad Sci.* 1997; 826:282–306. [PubMed: 9329700]
- Pfund Z, Chugani DC, Juhasz C, Muzik O, Chugani HT, Wilds IB, Seraji-Bozorgzad N, Moore GJ. Evidence for coupling between glucose metabolism and glutamate cycling using FDG PET and 1H magnetic resonance spectroscopy in patients with epilepsy. *J Cereb Blood Flow Metab.* 2000; 20:871–8. [PubMed: 10826538]
- Price JL, Morris JC. Tangles and plaques in nondemented aging and “preclinical” Alzheimer's disease. *Ann Neurol.* 1999; 45:358–68. [PubMed: 10072051]
- Ross BD, Bluml S, Cowan R, Danielsen E, Farrow N, Gruetter R. In vivo magnetic resonance spectroscopy of human brain: the biophysical basis of dementia. *Biophys Chem.* 1997; 68:161–72. [PubMed: 9468618]
- Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev.* 2001; 81:741–66. [PubMed: 11274343]
- Selkoe DJ. Soluble oligomers of the amyloid beta-protein impair synaptic plasticity and behavior. *Behav Brain Res.* 2008; 192:106–13. [PubMed: 18359102]

- Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med.* 2008; 14:837–42. [PubMed: 18568035]
- Sheng JG, Zhu SG, Jones RA, Griffin WS, Mrak RE. Interleukin-1 promotes expression and phosphorylation of neurofilament and tau proteins in vivo. *Exp Neurol.* 2000; 163:388–91. [PubMed: 10833312]
- Shonk TK, Moats RA, Gifford P, Michaelis T, Mandigo JC, Izumi J, Ross BD. Probable Alzheimer disease: diagnosis with proton MR spectroscopy [see comments]. *Radiology.* 1995; 195:65–72. [PubMed: 7892497]
- Szpunar GJ, Albert KS, Wagner JG. Pharmacokinetics of flurbiprofen in man. II. Plasma protein binding. *Res Commun Chem Pathol Pharmacol.* 1989; 64:17–30. [PubMed: 2749000]
- Tegeder I, Niederberger E, Israr E, Guhring H, Brune K, Euchenhofer C, Grosch S, Geisslinger G. Inhibition of NF-kappaB and AP-1 activation by R- and S-flurbiprofen. *FASEB J.* 2001; 15:2–4. [PubMed: 11099482]
- Townsend KP, Pratico D. Novel therapeutic opportunities for Alzheimer's disease: focus on nonsteroidal anti-inflammatory drugs. *FASEB J.* 2005; 19:1592–601. [PubMed: 16195368]
- Townsend M, Qu Y, Gray A, Wu Z, Seto T, Hutton M, Shearman MS, Middleton RE. Oral treatment with a gamma-secretase inhibitor improves long-term potentiation in a mouse model of Alzheimer's disease. *J Pharmacol Exp Ther.* 333:110–9. [PubMed: 20056779]
- van Haeringen NJ, van Sorge AA, Van Delft JL, Carballosa Core-Bodelier VM. Flurbiprofen and enantiomers in ophthalmic solution tested as inhibitors of prostanoid synthesis in human blood. *J Ocul Pharmacol Ther.* 2000; 16:345–52. [PubMed: 10977130]
- Wechter WJ, Bigornia AE, Murray ED Jr, Jee WS. Chiral pharmacokinetics of Rac-flurbiprofen and pharmacodynamics of anabolic bone response in the normal rat. *Chirality.* 1994; 6:457–9. [PubMed: 7946972]
- Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. *Nature.* 2001; 414:212–6. [PubMed: 11700559]
- Weggen S, Eriksen JL, Sagi SA, Pietrzik CU, Ozols V, Fauq A, Golde TE, Koo EH. Evidence that nonsteroidal anti-inflammatory drugs decrease amyloid beta 42 production by direct modulation of gamma-secretase activity. *J Biol Chem.* 2003; 278:31831–7. [PubMed: 12805356]
- Wilcock GK, Black SE, Hendrix SB, Zavitz KH, Swabb EA, Laughlin MA. Efficacy and safety of tarenflurbil in mild to moderate Alzheimer's disease: a randomised phase II trial. *Lancet Neurol.* 2008; 7:483–93. [PubMed: 18450517]
- Winton MJ, Lee EB, Sun E, Wong MM, Leight S, Zhang B, Trojanowski JQ, Lee VM. Intraneuronal APP, not free Abeta peptides in 3xTg-AD mice: implications for tau versus Abeta-mediated Alzheimer neurodegeneration. *J Neurosci.* 2011; 31:7691–9. [PubMed: 21613482]
- Wynne S, Djakiew D. NSAID inhibition of prostate cancer cell migration is mediated by Nag-1 Induction via the p38 MAPK-p75(NTR) pathway. *Mol Cancer Res.* 2010; 8:1656–64. [PubMed: 21097678]
- Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, Landreth G. Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. *J Neurosci.* 2003; 23:7504–9. [PubMed: 12930788]
- Zhao X, Rebeck GW, Hoe HS, Andrews PM. Tarenflurbil protection from cytotoxicity is associated with an upregulation of neurotrophins. *J Alzheimers Dis.* 2008; 15:397–407. [PubMed: 18997293]

- We examined the effect of R-flurbiprofen treatment in 3xTg-AD mice
- R-flurbiprofen treated mice learn faster on the radial arm water maze test.
- Learning inversely correlated with glutamate concentrations in the hippocampus.
- Treatment reduced the number of PHF1 stained neurons despite no changes in A β .
- Glutamine levels in the hippocampus decreased in R-flurbiprofen treated mice.

Figure 1. Comparison of radial arm water maze learning and memory retention among 3xTg-AD mice, R- flurbiprofen-treated 3xTg-AD mice and WT mice

A. Number of errors during the fourth trial of the day (T4). R-flurbiprofen treated mice committed less than 3 errors on day 6 and less than 2 errors on day 9. Untreated mice committed less than 3 errors on day 7 but did not reach the average of 2 errors/trial during the nine days of testing. WT mice performed significantly better than transgenic mice making less than 3 errors on day 4 and less than two by day 5. * significance compared to day 1, $p < 0.05$ **B.** Average number of errors for the last 3 days of testing in T1, T4, and T5. The average number of errors in T4 is significantly different than in T1 for all groups of mice indicating that three cohorts of mice have learned the task of the test. Untreated transgenic mice learned significantly less than WT mice because the number of errors committed in T4 is significantly different than the number of errors committed by WT mice. R-flurbiprofen treated mice learned the task similar than WT mice. During the memory retention test (T5) neither group of transgenic mice exhibited working memory impairment since the number of errors in T5 is not different than in T4 although both groups of transgenic mice committed significantly more errors than WT mice. * significance compared to day 1, # significance compared to WT, $p < 0.05$

Figure 2. Sections of hippocampus CA1 and subiculum immunostained for Aβ
A, D. Seven month wild-type mouse shows no immunoreactivity for 6E10 (A) and NU-1 (D) in CA1/subiculum region. **B, E.** Untreated 7 month 3xTg-AD mouse shows immunoreactivity for 6E10 (B) and NU-1 (E) in the perikarya of CA1 and subicular neurons. **C, F.** R-flurbiprofen treated 3xTg-AD mouse shows immunoreactivity for 6E10 (C) and NU-1 (F) in the perikarya of CA1 and subicular neurons. **G.** Densitometric analysis of the subiculum area of immunostained sections with 6E10 and NU-1 show no significant differences between untreated and R-flurbiprofen treated transgenic mice.

Figure 3. Sections of hippocampus CA1 and subiculum immunostained for CP13

A. Seven month wild-type mouse shows no immunoreactivity for CP13 in CA1/subiculum region. **B.** Untreated 7 month old 3xTg-AD mouse shows intense immunoreactivity for CP-13 in neurons of the CA1/subiculum area. Immunoreactivity is detected in the perikarya extending into the apical dendrites and dendritic arbors. **C.** Comparable immunoreactivity for CP13 is found in the CA1/subiculum area of an R-flurbiprofen treated 3xTg-AD mouse. **D.** No significant differences in the level of immunoreactivity for CP13 in the CA1/subiculum area is found between untreated and R-flurbiprofen treated mice.

Figure 4. Sections of hippocampus CA1 and subiculum immunostained for hyperphosphorylated tau with AT8 and PHF-1 antibodies

A, D. No immunoreactivity was detected in 7 months old WT mice with AT8 (A) or PHF-1 (D). **E-F** Dense aggregates of AT8 (B,C) and PHF-1 (E,F) immunoreactivity in the perikaryon and dendrites are detected in occasional neurons. All immunoreactive neurons for AT8 and PHF-1 were found in the CA-subiculum area. **G.** The number of PHF-1 immunostained neurons is significantly reduced in R-flurbiprofen treated mice compared to untreated.

Figure 5. Plot of glutamate levels in the hippocampus and their relation to learning

The learning was measured as the slope representing the change in the number of errors as a function of time. The correlation was highly significant ($R = 0.7$; $p < 0.01$).

Figure 6. Correlation between PHF-1, MRS, and learning

The score of PHF-1 immunoreactivity in the hippocampus correlated with both glutamine concentration and with learning on the radial arm water maze.

Table I

High Resolution Magic Angle Spinning Spectra from Hippocampus of R-flurbiprofen-treated and Untreated 7 Months 3×Tg-AD Mice

Chemical	Untreated	R-flurbiprofen
GABA	0.241 ± 0.045	0.238 ± 0.029
Acetate	0.043 ± 0.019	0.033 ± 0.009
Alanine	0.047 ± 0.009	0.058 ± 0.013
Aspartate	0.319 ± 0.087	0.287 ± 0.041
t-Cho	0.149 ± 0.035	0.141 ± 0.021
Glutamate	0.736 ± 0.051	0.707 ± 0.090
Glutamine	0.382 ± 0.034	0.346 ± 0.013 *
Glycine	0.122 ± 0.006	0.120 ± 0.011
Lactate	0.737 ± 0.138	0.768 ± 0.090
myo-Inositol	0.633 ± 0.090	0.600 ± 0.080
NAA	0.463 ± 0.056	0.435 ± 0.088
Scyllo-inositol	0.00022 ± 0.00006	0.00024 ± 0.00003
Taurine	1.102 ± 0.113	1.133 ± 0.143

* p<0.05; The only significant change was in glutamine at this time point. Values reported are molar ratios to creatine.