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Ibuprofen effects on Alzheimer pathology and open field activity in APPsw transgenic mice

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Abstract

We previously showed the non-steroidal anti-inflammatory drug (NSAID) ibuprofen suppresses inflammation and amyloid in the APPsw (Tg2576) Tg2576 transgenic mouse. The mechanism for these effects and the impact on behavior are unknown. We now show ibuprofen's effects were not mediated by alterations in amyloid precursor protein (APP) expression or oxidative damage (carbonyls). Six months ibuprofen treatment in Tg+ females caused a decrease in open field behavior ($p < 0.05$), restoring values similar to Tg- mice. Reduced caspase activation per plaque provided further evidence for a neuroprotective action of ibuprofen.

The impact of a shorter 3 month duration ibuprofen trial, beginning at a later age (from 14 to 17 months), was also investigated. Repeated measures ANOVA of A β levels (soluble and insoluble) demonstrated a significant ibuprofen treatment effect ($p < 0.05$). Post-hoc analysis showed that ibuprofen-dependent reductions of both soluble A β and A β 42 were most marked in entorhinal cortex ($p < 0.05$). Although interleukin-1 β and insoluble A β were more effectively reduced with longer treatment, the magnitude of the effect on soluble A β was not dependent on treatment duration. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce risk for Alzheimer's disease (AD) in multiple epidemiological studies [31]. Chronic CNS inflammation in AD brain is implicated in the pathology [1,35], but how NSAIDs impact the pathogenic AD pathways or behavior are unclear [12]. Of the various NSAIDs associated with reduced AD risk, the over-the-counter drug, ibuprofen, has been among the most widely used, notably in the Baltimore Longitudinal and twin studies [3,36]. Transgenic mice bearing the "Swedish mutation" for human APP (HuAPPsw, line Tg2576 [17]) develop age-related amyloid plaques with neuritic pathology, gliosis

and inflammation [10,19]. Treating Tg2576 mice from 10 to 16 months with chronic dietary ibuprofen significantly delayed or reduced transgene- and age-dependent increases in interleukin-1 β , microgliosis, GFAP, total A β by ELISA, amyloid deposition and ubiquitin positive abnormal neurites [27]. A reduction in amyloid plaques with ibuprofen and a related NSAID, nitroflurbiprofen, has also recently been reported [20]. These results provide evidence that NSAIDs can effectively attenuate aspects of AD pathogenesis, including plaque formation, and are consistent with a causal role for NSAID consumption in delaying onset and reducing risk of AD.

Since oxidative damage and alterations in APP have been hypothesized to be attenuated by ibuprofen effects, the present paper now includes an assessment of associated effects on APP and oxidative damage to proteins in the 6 month (from 10–16 months of age) treatment group. Because an increase in open field activity has been reported in this model, we also evaluated chronic ibuprofen effects on open field behavior. Finally, epidemiological studies sug-

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gest that NSAIDs may be more efficacious with earlier and longer use [18,36]. In one study, little or no benefit has been found from conventional NSAID use beginning in the year immediately preceding the onset of dementia [18]. For this reason, we sought to determine the effect of later and more acute ibuprofen treatment for 3 months (from 14 to 17 months) in contrast with earlier and more chronic treatment beginning at the age of 10 months (from 10 to 16 months).

2. Materials and methods

2.1. Animals

In our initial published report (“chronic group”), ten-month old male and female Tg2576 Tg+ and Tg− mice from twelve litters were randomly split between treatment groups. Tg+ mice were fed either control chow (Tg+, n = 9) or chow containing 375 ppm ibuprofen (Tg+ ibuprofen, n = 8) for six months before being sacrificed. Studies have shown CNS PGE2 reduction with an ibuprofen ED50 at doses of 15–20 mg/kg body weight (bw). The human plasma half life is on the order of 2.5 hours but CSF half-life has been reported to be longer, approximately 8 hours [2]. Plasma half-life values for ibuprofen in rodents are similar, but CNS values are unavailable. Considering CNS half life and ED50, doses from 45–60 mg/kg bw/day should fall within the therapeutic range for continuous cyclooxygenase inhibition. Our dose of 375 ppm in chow was originally estimated to be 56 mg/kg bw/day based on textbook numbers, but using direct measurements of food consumption and actual average weights, we now find it to be equivalent to a total daily amount of approximately 46 mg/kg bw/day. Both doses are in the range expected for continuous cyclooxygenase inhibition. Mice are nocturnal and eat more at night, so some plasma fluctuations in ibuprofen are likely. Tg− control mice were fed chow containing no drug (n = 6). For the new “short-term” study, additional groups of animals were similarly randomized and placed on control or ibuprofen diet from 14 to 17 months of age (Tg+ control, n = 9, 5 females and 4 males, Tg+ ibuprofen, n = 8, 5 females and 3 males). At the time of sacrifice, neither the weights nor the ages of the mice were significantly different. Animals were sacrificed with a lethal dose of anesthetic using an Institutional Review Board approved protocol and perfused before brain dissection with 0.9% normal saline followed by HEPES buffer (pH 7.2) containing 5 mg/ml each of leupeptin and aprotinin and 2 mg/ml of pepstatin A. Brain regions were dissected from one hemisphere using mouse brain atlas coordinates [9] as previously described [27]. Thalamic, cortical, and hippocampal regions, as well as entorhinal cortex and piriform cortex/amygdala sections, were dissected out and snap frozen in liquid nitrogen. Biochemical measurements were performed in the hippocam-

pus, entorhinal cortex, piriform cortex/amygdala, and residual cortex (cortex region without frontal, entorhinal, or piriform areas). The remaining hemibrain was immersion fixed in 4% paraformaldehyde for fractin immunohistochemistry.

2.2. Tissue preparation

Tissue sample preparation methods have been previously described [27]. Briefly, samples were homogenized in 10 volumes of TBS containing a cocktail of protease inhibitors (20 μ g/ml each of pepstatin A, aprotinin, phosphoramidon, and leupeptin, 0.5 mM PMSF, 1 mM EGTA). Samples were sonicated briefly and centrifuged at $100,000 \times g$ for 20 min at 4°C. The soluble fraction (supernatant) was used for IL-1 β or A β ELISAs while the TBS-insoluble pellet was sonicated in 10 volumes of 2% SDS. The resulting homogenate was centrifuged at $100,000 \times g$ for 20 min at 20°C. To analyze insoluble A β , the SDS insoluble pellet was solubilized and sonicated in 70% formic acid. The resulting extract was neutralized with 0.25 M Tris containing 30% acetonitrile and 5 M NaOH and assayed as the insoluble amyloid fraction.

2.3. Sandwich ELISA for A β (total A β) and A β 42

The details of our total A β sandwich ELISA have been previously described [4,27]. Briefly, we use monoclonal 4G8 against A β 17–24 (Senetek, Napa, CA) as the capture antibody at 3 μ g/ml, biotinylated 10G4 anti-A β 1–15 for detection, and a reporter system with streptavidin-alkaline phosphatase using AttoPhos (JBL Scientific Inc, San Luis Obispo, CA) as the substrate (excitation 450 nm/emission 580 nm). The minimal detectable quantity of A β was 40 pg under most conditions. Sandwich ELISA for A β 42 was performed on some samples. Polyclonal capture antibody against A β 37–42 [30] was loaded onto Reacti-bind goat anti-rabbit coated plates (Pierce, Rockford, Illinois) at 5 μ g/ml in 0.1 M carbonate buffer, pH 9.6. Blocking was completed in 2% bovine serum albumin in Tris-buffered saline for 3 h at room temperature. After the addition of processed brain samples, biotinylated 10G4 (1:1500) was used as the detection antibody and streptavidin-alkaline phosphatase as the reporter system with Attophos as the substrate. A representative standard curve of A β 42 is shown in Fig. 5E with sensitivity of 110 pg using A β 37–42 capture.

2.4. Sandwich ELISA for IL-1 β

IL-1 β was measured by sandwich ELISA as previously described [27], utilizing polyclonal antibody against mouse IL-1 β (Endogen, Woburn, MA) for capture and monoclonal

anti-mouse IL1- β (Endogen) for detection. Assay sensitivity was approximately 0.5 pg.

2.5. Western blot analysis

Supernatants from the 2% SDS extract of brain samples (40 μ g) were electrophoresed on a 10% acrylamide gel under reducing conditions. Proteins were transferred to a PVDF membrane (400 mA \times 2h) before blocking in 10% nonfat dry milk, 0.1% gelatin in PBS for 1.5 h. Blots were co-incubated with a cocktail of monoclonal antibodies against synaptophysin, SNAP-25, and actin, and polyclonal antibodies against APP(679–695) C-terminus [5] overnight at 4°C. All antibodies were diluted to 1:1000. Blots were incubated in HRP-conjugated goat anti-mouse or anti-rabbit (1:10,000) for 45 min before development with SuperSignal (Pierce, Rockford, IL). Bands were quantified using densitometric software (Molecular Analyst II) and analyzed by unpaired Student t-tests.

2.6. Oxidative damage to proteins

Carbonyl groups on oxidized proteins were measured using an Oxyblot kit (Intergen, Purchase, NY). Briefly, 10 μ g of protein from the SDS extract was reacted with dinitrophenylhydrazine (DNPH) for 15–30 min, followed by neutralization with a solution containing glycerol and β -mercaptoethanol. These samples were electrophoresed on a 10% Tris-glycine gel and blotted. After blocking, the blot was incubated overnight with a rabbit anti-DNP antibody (1:150) at 4°C, followed by incubation in HRP conjugated goat anti-rabbit (1:300) for 1 h at RT and chemiluminescent detection as for Western blots.

2.7. Immunohistochemistry

Samples were stained for fractin, which recognizes caspase cleaved actin, as previously described [38]. Briefly, 10 μ M coronal cryostat sections were mounted and pre-treated to kill endogenous peroxidase, blocked and incubated with affinity purified rabbit polyclonal anti-KYE “fractin” antibody (1 μ g/ml) overnight in the cold. Sections were washed, incubated with Vectastain ABC reagents (Vector Labs, Burlingame, Ca.) and developed using peroxidase/diaminobenzidine (Pierce, Rockford, Ill.) Light double labeling of plaques was achieved by formic acid treatment to enhance A β labeling and denature the fractin and secondary antibodies, followed by washing and incubation with 10G4 monoclonal to A β 1–15 described above and development with alkaline phosphatase kit III and Vector Blue (Vector Labs, Burlingame, Ca.). This is analyzed using NIH image analysis of fractin per plaque in rings of multiple plaque radii around plaques which was carried out as previously described [10].

2.8. Statistical analysis

2-way ANOVA (diet \times region or transgene \times region) were performed to determine any differences in levels of IL-1 β , synaptic markers, and protein carbonyls using Statview 4.5 (Abacus, Berkeley). Repeated measures ANOVA was applied to measurement of soluble and insoluble A β in multiple brain regions per mouse. Logarithmic or square root transformations were sometimes necessary to establish homogeneity of variance (see figure legends for details). Western blot APP and A β 42 analyses were performed using unpaired Student's t-tests.

2.9. Open field analysis

In mice from the study on ibuprofen diet from 10–16 months of age, the open field tests were performed from 14–16 months of age. The open field was constructed of a 62 cm by 62 cm laminated wood floor surrounded by clear acrylic walls of 30 cm height. A 4 \times 4 grid matrix was painted on the floor of the open field, dividing the field into 16 squares (12 outer, 4 inner) each measuring 15.5 cm by 15.5 cm. The experimenter was blinded to mouse transgene or diet status. At the beginning of the open field session, mice were placed on the right front corner square (relative to the position of the experimenter) and were retained inside a clear acrylic tube (diameter = 3.5”). After 30 seconds, the tube was lifted and the mouse was allowed to move freely about the open field for a period of 5 minutes, and then returned to its home cage. The open field was washed with Windex™ between each session.

The behavioral parameter measured was total number of grid crossings made during the 5-minute trial. A crossing was defined as all four paws moving out of one of the 16 squares and into another. This measure is expected to correlate highly with the distance traveled in the open field, and it reflects locomotor activity, exploratory behavior, and anxiety. Open field sessions were videotaped and performance was analyzed after the testing took place.

3. Results

3.1. Chronic ibuprofen treatment

We previously reported that chronic ibuprofen treatment reduced plaque burden by 50% [27]. However, it was unclear whether ibuprofen altered plaque deposition by reducing APP expression. Fig. 1 demonstrates that ibuprofen did not significantly affect APP levels in any region as detected by immunoblot. Analysis of synaptic markers revealed no age- or transgene-dependent differences in synaptophysin or SNAP-25 in any region examined (data not shown). A significant transgene-dependent elevation in oxidized pro-

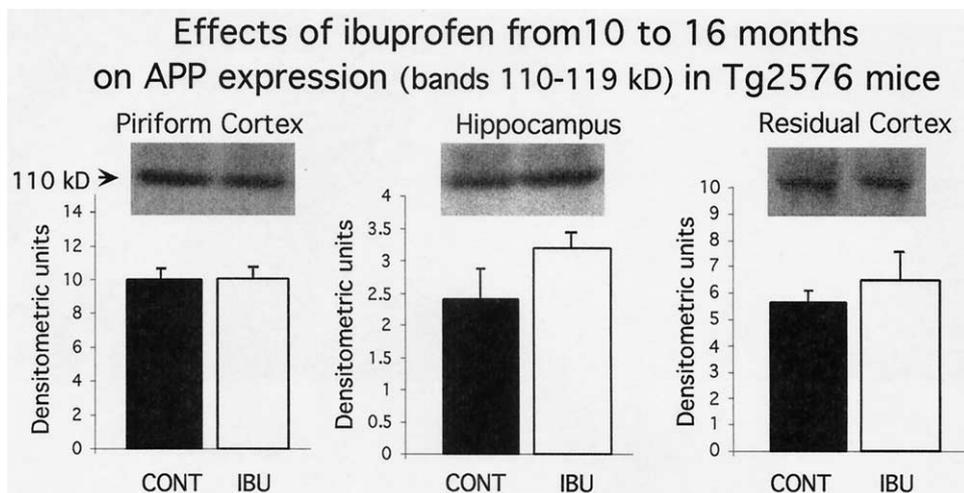


Fig. 1. Effect of chronic ibuprofen treatment on APP expression in APPsw mice as measured by Western blot (OD of bands 110–120 kD). Protein levels were measured in SDS-soluble fractions of piriform cortex, hippocampus, and residual cortex of Tg+ control (n = 8) and Tg+ ibuprofen (n = 8) mice. Representative examples of APP bands (110 kD band) are shown above graphs. Error bars represent standard error. Student's t-tests revealed no significant reductions in APP protein levels in any brain region of ibuprofen-treated mice.

teins, measured as protein carbonyls on Oxyblots, was observed in cortex and hippocampus (Fig. 2B). Chronic ibuprofen treatment did not reduce the level of oxidized proteins (Fig. 2C).

3.2. Gender dependent effects of ibuprofen on open field activity

Open field activity was used to evaluate locomotor activity, exploratory behavior, and anxiety in Tg– control, Tg+ control, and Tg+ ibuprofen mice (Fig. 3). There was a non-significant trend for a transgene-dependent increase in open field activity in the 16 month-old mice. However, the sample size required in our study to detect significant transgene-dependent differences of 34 grid crosses would have to be 22 mice per group assuming an estimated population standard deviation of approximately 40 ($p < 0.05$, power 80%). There was, however, sufficient power to estimate

diet-dependent differences in Tg+ females who showed large responses (73 points) to ibuprofen ($p < 0.05$). Diet effects of Tg+ males could not be determined because they showed smaller changes to ibuprofen and had large standard deviations.

3.3. Short term ibuprofen effects on fractin-positive and ubiquitin-positive dystrophic neurites

A second group (n = 5) of Tg2576 mice was placed on control or 375 ppm ibuprofen from 14 to 17 months of age. Fig. 4A, low magnification, and Fig. 4C, high magnification, show a cluster of particulate fractin (caspase-cleaved actin [38]) positive staining. A β and thioflavin double labeling showed that all such clusters were plaque-associated [7]. Image analysis of large and small fractin-immunoreactive particles per plaque revealed significant reductions in the number of large and small particles per plaque (Fig. 4D)

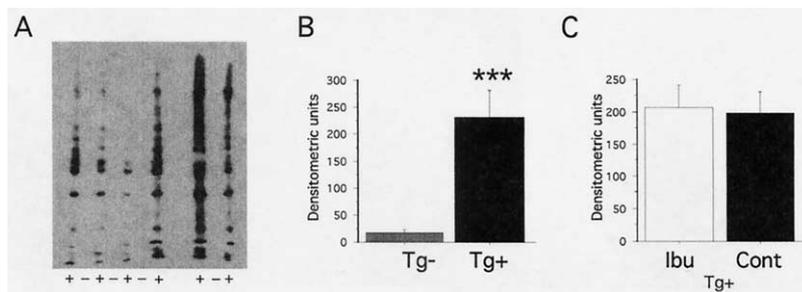


Fig. 2. Effect of chronic ibuprofen treatment on oxidative damage in APPsw brains—Oxidative damage was assessed using Oxyblot kits (Intergen) on SDS-soluble fractions (10 μ g). **A.** Representative example of typical Oxyblot. Lanes from Tg+ and Tg– are marked. **B.** Effect of transgene on oxidative damage in hippocampal and cortical regions of Tg– and Tg+ mice. Two-way ANOVA revealed a significant increase in the level of oxidized proteins in Tg+ animals compared to Tg– animals. *** indicates a P-value less than 0.001. **C.** Effect of chronic ibuprofen treatment on oxidized protein levels in hippocampus and cortex of Tg+ control and Tg+ ibuprofen animals. Two-way ANOVA showed no significant reduction in oxidative damage in ibuprofen-treated groups.

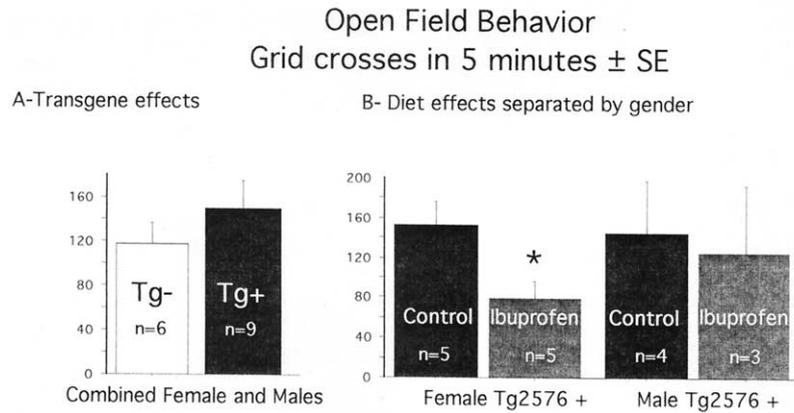


Fig. 3. **A.** Open field activity as measured by the number of grid crossings in a 5-minute period was elevated 34 points in the Tg+ group compared to Tg- group. The standard deviation of population requires a sample size of 22 animals to show a significant difference. There were not enough males in the Tg- group to perform gender analysis, but similar trends were observed for both sexes (data not shown). **B.** The ibuprofen diet significantly reduced open field activity as measured by number of grid crossings only in Tg+ females, and not in Tg+ males, suppressing the elevated levels in the Tg+ mice.

and a reduction in the size of large particles (Fig. 4E) in the ibuprofen treated group. Ubiquitin particles correlated with the number of plaques per section in both groups ($r^2 = 0.942$, $p < 0.05$ in the control group and $r^2 = 0.843$, $p < 0.002$ in the ibuprofen group).

3.4. Short term ibuprofen effects on A β levels

The results of TBS-soluble A β and formic acid-extracted SDS insoluble A β by ELISA of dissected brain regions are illustrated in Fig. 5A–D. Repeated measures ANOVA (sol-

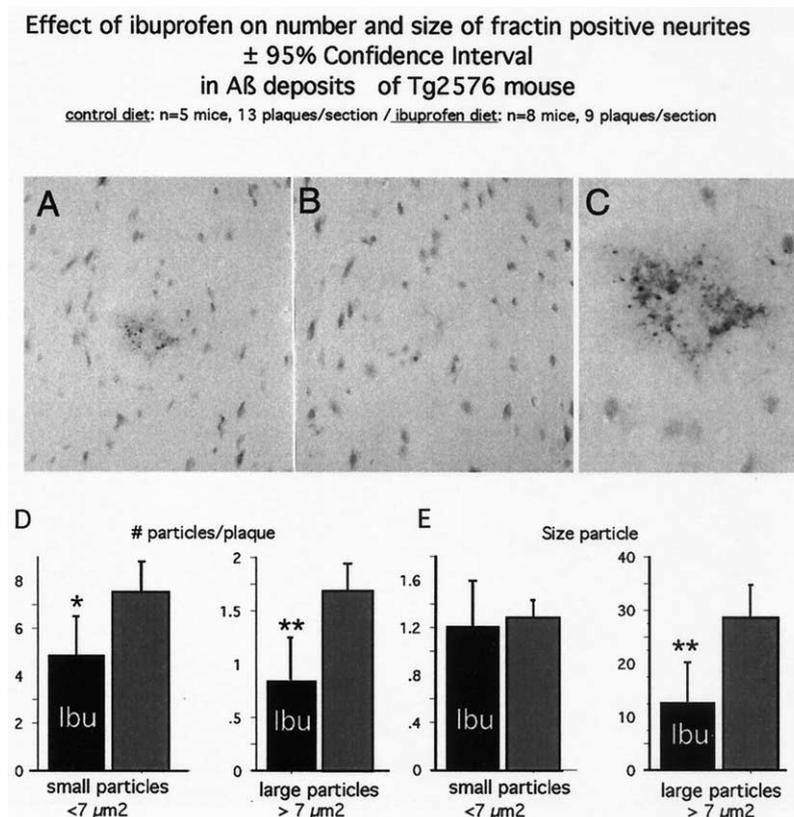


Fig. 4. Caspase activity per plaque is suppressed by ibuprofen. **A.** Profiles immunolabeled for a caspase cleavage product, fragment of actin (fractin), were observed in amyloid plaques. **B.** Preabsorption of antiserum with the fractin peptide prevented staining in an adjacent section, demonstrating antibody specificity. **C.** A higher magnification of deposits reveals punctate nature of fractin positive profiles. **D.** Ibuprofen treatment resulted in significant reductions in per plaque numbers of both small profiles (<7 μ m²) and large profiles per plaque. **E.** Ibuprofen treatment reduced the average size of large profiles, but not the smaller profiles. * $p < 0.05$, ** $p < 0.01$.

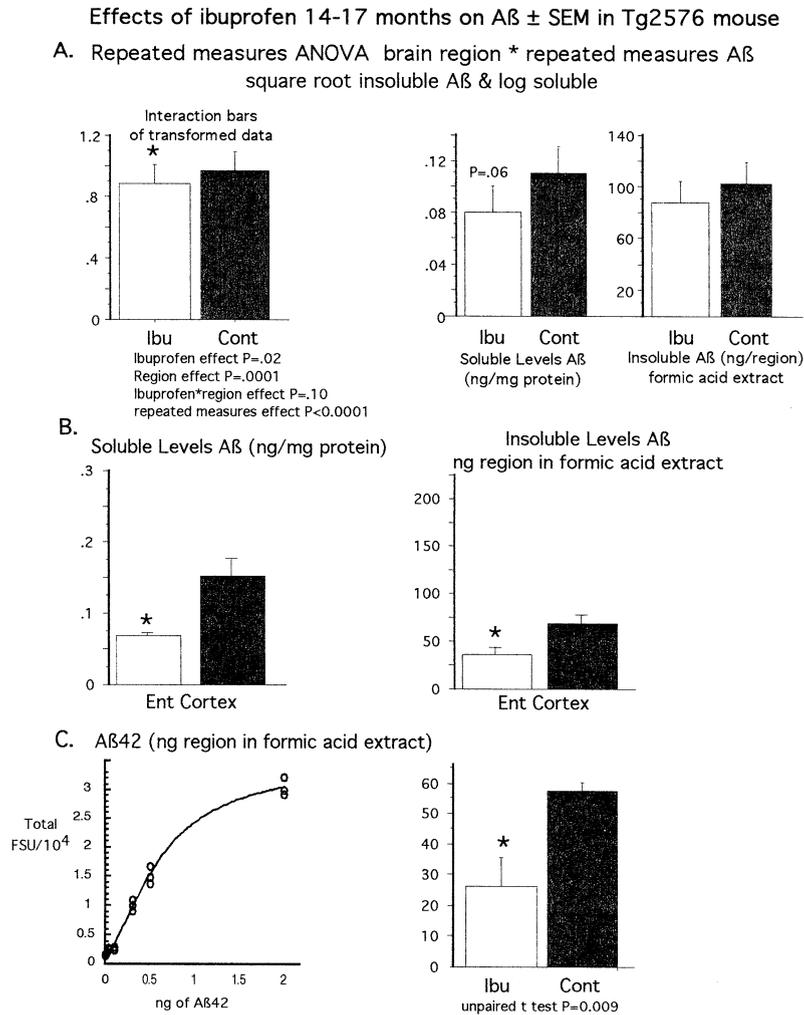


Fig. 5. Effect of short-term (3 month duration) ibuprofen treatment from 14–17 months of age on soluble A β and insoluble amyloid. Panels A and B show results of repeated measures ANOVA. Formic acid-extracted SDS insoluble A β (total ng/region) and soluble A β levels (ng/mg protein) were compacted to adjust for within animal variation. Analysis required log transformation of insoluble A β and square root transformation of soluble A β to establish homogeneity of variance, which are reflected in panel A left graph interaction bars. Treatment (diet) effect was significant ($p < 0.05$); treatment \times region interaction was not significant ($p = .10$); region effect, compacted repeated measures variable and interaction of compacted repeated measures variable with brain region were all significant ($p < .0001$). The second and third graphs in panel A show interaction bars of soluble and insoluble A β levels, respectively. **B.** Soluble and insoluble A β changes in entorhinal cortex in response to ibuprofen, which was the region most affected ($p < 0.05$). **C.** A β 42 (total ng/region). The standard curve for A β 42 is also shown (panel C).

soluble and insoluble A β levels in 5 regions within an animal) showed a significant ibuprofen treatment diet effect ($p < 0.05$). Although the treatment \times region interaction did not reach significance ($p = 0.10$), the ibuprofen-associated reduction in total A β levels (SDS insoluble and TBS soluble) was most pronounced in the entorhinal cortex ($p < 0.05$). A β 42 levels by ELISA in the SDS insoluble, formic acid fraction were similarly reduced by ibuprofen in the entorhinal cortex ($p < 0.05$, Fig. 5E–F).

3.5. Short term ibuprofen effects on Interleukin-1 β levels

ELISA of IL-1 β revealed a significant ibuprofen treatment effect ($p < 0.005$). There was a treatment by region interaction, reflecting the large reduction of IL-1 β in the piriform cortex/amygdala ($p < 0.05$, Fig. 6).

4. Discussion

4.1. Lack of long term ibuprofen effects on oxidative damage and APP

It has been suggested that IL-1 β can drive the endogenous APP promoter [14], and that ibuprofen-induced reductions in IL-1 β could reduce APP and A β production. Our data argues against this hypothesis in Tg2576 since APP is not reduced; in fact, mice subjected to ibuprofen treatment showed a slight (33%) increase in APP in the hippocampus that did not reach statistical significance with our sample size. This observation raises the possibility that ibuprofen may increase APP slightly despite decreasing A β . Thus, effects on APP levels cannot explain the ibuprofen-mediated reduction in amyloid and soluble A β in Tg2576. While

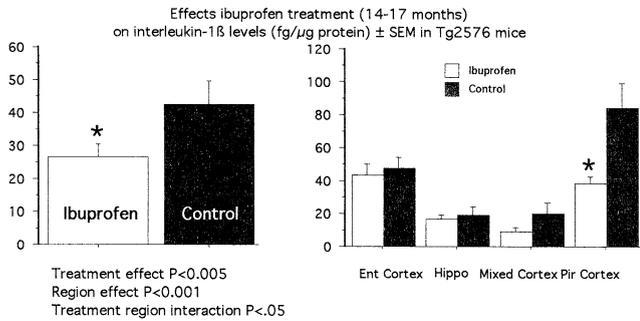


Fig. 6. Effect of short-term ibuprofen treatment on IL-1 β levels (fg/ μ g protein) from TBS-soluble fraction. Combined regional analysis of IL-1 β ELISA data showed a 33% reduction ($p < 0.005$) which was most pronounced in the piriform cortex ($n = 6$; $p < 0.05$).

expression of the APP transgene driven by the prion promoter in Tg2576 was not reduced, it remains possible that NSAID treatment in humans could attenuate expression of APP driven by the native APP promoter and provide an additional mechanism for suppression of A β .

While oxidative damage to proteins has been hypothesized to contribute to amyloid deposition [11,34], we did not detect a significant ibuprofen impact on protein carbonyls. This observation does not support the hypothesis that ibuprofen-mediated suppression of chronic inflammation [27] leads to reductions in oxidative damage which in turn cause a reduction of amyloid. Other mechanisms must be involved in the ibuprofen treatment-induced reductions in soluble A β and amyloid. Remaining possibilities include effects on pro-amyloidogenic factors (ApoE, ACT), processing of APP to A β , or A β clearance.

4.2. Ibuprofen suppresses microgliosis and IL-1 β without suppressing oxidative damage

Oxidative damage can be caused by increased reactive oxygen species and nitric oxide (NO) produced by inflammatory cells, notably microglia. Using protein carbonyls as an index, we detected significant microgliosis, IL-1 β and oxidative damage not in the Tg2576 mouse that is consistent with other cited reports. However, suppressing microglia and the cytokine IL-1 β with ibuprofen did not suppress oxidative damage, suggesting that reactive oxygen species from activated glia are not playing a major role in Tg2576. Protein nitration is another oxidative modification that could be secondary to inflammation and suppressed by NSAIDs. The possibility of a specific ibuprofen effect on protein nitration is currently being examined. However, our current data suggest that a combined antioxidant and traditional NSAID treatment may be more effective than treatment with NSAID alone.

4.3. Gender specific effects of ibuprofen on open field activity

Increased open field activity has been used as an index of locomotor and exploratory activity and anxiety in rodents

[8]. In AD patients, heightened agitation and anxiety are very frequent non-cognitive behavioral problems [32] that have not been adequately evaluated in transgenic models. A sex- and transgene-dependent increase in open field activity has been reported in young Tg2576 mice [22]. In our study, there was a trend for all Tg+ mice to show increased open field activity, but we were not able to evaluate whether this 34 point difference was statistically different because of the high standard deviations. Significant reductions in open field activity were observed in ibuprofen-treated Tg+ females ($p < 0.05$), restoring values to those of Tg- mice. Tg+ males showed a similar, but less dramatic and non-significant trend toward decreased spontaneous activity (21 points for males compared to 73 point differences for Tg+ females). It was not possible to evaluate small behavioral differences in a males only group with the small sample sizes in the study. Tg+ males showed higher standard deviations that may relate to their tendency to be more aggressive. Overall, these data raise the possibility that ibuprofen may influence behavioral alterations in this model and the effects may be greater in females (Fig. 3.).

Open field activity may be sensitive to hippocampal CA3/mossy fiber pathways [23] and the observed increase in Tg2576 may reflect electrophysiological alterations in CA3 stemming from increased NMDA receptor activity and synaptic loss reported in both young and old mutant APP transgenics [16]. Ultrastructural studies suggest that post-synaptic fractin labeling for caspase activation is increased in CA3 (A. Triller, unpublished data) in aged Tg2576 mice, but neither fractin labeling nor synapse loss in CA3 have been evaluated yet in aging or NSAID-treated Tg2576 mice.

4.4. Chronic ibuprofen, ubiquitin and fractin

We previously reported that ibuprofen suppressed ubiquitin-positive dystrophic neurites [27]. This reduction appeared to be simply proportional to the reduction in plaques because ubiquitin particles and plaques were similarly reduced (both by roughly 50%). With regression analysis of that data, ubiquitin particles and plaques are highly correlated in both groups with a similar slope. This observation suggested the ibuprofen effect on ubiquitin reflected fewer plaques rather than the response per plaque. We therefore sought to detect whether ibuprofen could reduce other neuritic changes on a per plaque basis. In this study, we found a significant ibuprofen-mediated suppression of immunostaining for fractin, a neo-epitope on caspase-cleaved actin, in the vicinity of plaques. While peri-plaque fractin labeling may represent both neuritic and glial alterations [38] based on immuno-ultrastructural observations, the most frequent fractin labeling in aged Tg2576 mice is postsynaptic and dendritic (A Triller, unpublished observations). The observed peri-plaque staining was typically distinct from phosphotau and ubiquitin labeling, but co-localized with synaptic markers using confocal double-labeling in Tg2576 mice [7]. The resolution obtained in our confocal micro-

scope at 40X cannot unambiguously distinguish presynaptic and postsynaptic localization. Similar punctate peri-plaque labeling for caspase-cleaved APP has been reported in AD brain [13] and is largely neuritic [6]. Both DNA fragmentation detected by TUNEL and caspase activation are increased in AD brain [25,26,28,37]. We hypothesize that caspase activation in synaptic compartments is part of a mechanism for synapse loss, but we could not detect age- and transgene-dependent synaptophysin or SNAP-25 loss by Western analysis at 16 months of age. However, we can detect significant synaptic marker loss in 20–30 month Tg+ mice (manuscript in preparation). Additional drug studies in older mice are required to determine whether ibuprofen treatment can prevent this loss.

4.5. *IL-1 β reductions by 16–17 months age after acute (3 months) and chronic (6 months) ibuprofen treatment*

We previously reported that chronic ibuprofen treatment reduced transgene-dependent elevations in IL-1 β (down 64.7%), plaque counts (down 52.6%) and A β by ELISA (down 40%) [27]. Regression analysis of that published data shows that with control diet, formic acid extracted SDS insoluble A β (but not soluble A β) correlates with cytosolic IL-1 β ($R^2 = .82$). This correlation was blocked by the ibuprofen diet ($R^2 = 0.003$), consistent with some suppression of amyloid as well as the inflammatory response to amyloid. We hypothesized that if chronic ibuprofen could reduce the inflammatory response to amyloid, acute ibuprofen effects might be obtained with a 3 month treatment. Our data demonstrated that acute ibuprofen treatment was also associated with reductions of interleukin 1 β levels, but were only 50% as effective.

4.6. *A β reductions by 16–17 months age after acute (3 months) and chronic (6 months) ibuprofen treatment*

Results revealed mild but significant reductions in insoluble and soluble A β that were most noticeable in the entorhinal cortex. A reduction in entorhinal cortex A β 42 paralleled the reduction in A β in the same region, suggesting that A β 42 was one species of A β being reduced. Although the magnitude of the reductions in soluble A β was not different between the 3 month vs 6 month duration of ibuprofen, the magnitude of the reduction in insoluble A β was much greater for the 6 month treatment. These data suggest that maximum reductions in insoluble A β and IL-1 β require early NSAID intervention, while reductions in soluble A β may be possible with acute NSAID treatment. Emerging data suggest that soluble A β oligomers or protofibrils may be toxic and linked to neurodegeneration [24,29,33], possibly involving electrophysiological changes consistent with NMDA activity and excitotoxicity [15,16]. If ibuprofen or other NSAIDs can reduce soluble oligomers in older animals or patients, it may have an A β based impact on

progression even in the absence of overall effects on amyloid burden.

One plausible explanation for a minimal effect of ibuprofen on the SDS insoluble, formic acid extracted fraction is that A β rises exponentially from 6 to 12 months, but appears to plateau over the 14 to 17 months of age in whole brain analysis of APPSw mice [21]. If the impact of ibuprofen is primarily to prevent the age-dependent buildup and not to stimulate amyloid removal, a plateau in the age-dependent increase could markedly lessen the impact. Alternatively, ibuprofen's impact on insoluble amyloid accumulation may act primarily at an early initiation stage of amyloid production or deposition and a consistent reduction in insoluble amyloid or plaque burden may only be achievable with early intervention.

4.7. *Summary*

In summary chronic ibuprofen can ameliorate transgene effects on open field behavior. The ibuprofen mediated reduction in amyloid is not dependent on reductions in oxidative damage or APP, but may instead be dependent on another pathway regulating A β production, deposition or clearance. Finally, compared to chronic ibuprofen treatment, acute treatment leads to only mild reductions in IL-1 β and insoluble A β including A β 42, while similar reductions in soluble A β are observed regardless of ibuprofen duration. Our data support the idea that ibuprofen or similar NSAIDs may be effective for prevention of AD. Acute effects on reducing soluble A β further suggest NSAIDs might also be important as an adjunct therapy for AD. Finally, since ibuprofen alone does not effectively reduce the oxidative damage in AD brain, prevention therapies involving NSAIDs are likely to be improved by addition of antioxidants.

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