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Research Report

Ibuprofen reduces Aβ, hyperphosphorylated tau and memory deficits in Alzheimer mice

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ABSTRACT

We examined the effects of ibuprofen on cognitive deficits, Aβ and tau accumulation in young triple transgenic (3xTg-AD) mice. 3xTg-AD mice were fed ibuprofen-supplemented chow between 1 and 6 months. Untreated 3xTg-AD mice showed significant impairment in the ability to learn the Morris water maze (MWM) task compared to age-matched wild-type (WT) mice. The performance of 3xTg-AD mice was significantly improved with ibuprofen treatment compared to untreated 3xTg-AD mice. Ibuprofen-treated transgenic mice showed a significant decrease in intraneuronal oligomeric Aβ and hyperphosphorylated tau (AT8) immunoreactivity in the hippocampus. Confocal microscopy demonstrated co-localization of conformationally altered (MC1) and early phosphorylated tau (CP-13) with oligomeric Aβ, and less co-localization of oligomeric Aβ and later forms of phosphorylated tau (AT8 and PHF-1) in untreated 3xTg-AD mice. Our findings show that prophylactic treatment of young 3xTg-AD mice with ibuprofen reduces intraneuronal oligomeric Aβ, reduces cognitive deficits, and prevents hyperphosphorylated tau immunoreactivity. These findings provide further support for intraneuronal Aβ as a cause of cognitive impairment, and suggest that pathological alterations of tau are associated with intraneuronal oligomeric Aβ accumulation.

1. Introduction

Alzheimer's disease (AD), the most common age-dependent neurodegenerative disorder, is clinically characterized by progressive memory loss and cognitive decline. The neuropathological hallmarks of AD are neurofibrillary tangles, intra neuronal filaments composed of aggregated hyperphosphorylated tau protein, and senile plaques, extracellular accumulations of amyloid β protein (Aβ) (Blennow et al., 2006). Tau pathology in AD evolves in a sequential pattern, first involving the somatodendritic accumulation of conformationally altered, nonfibrillar tau, recognized by the antibody MC1 (Haroutunian

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et al., 2007; Jicha et al., 1997a; Jicha et al., 1997b; Weaver et al., 2000), followed by immunoreactivity for early forms of hyperphosphorylated tau, such as CP-13 (Duff et al., 2000; Klein et al., 2004; Lewis et al., 2000), and later by immunoreactivity for other forms of hyperphosphorylated tau, such as AT8, and PHF-1, that form fibrillar inclusions in neuronal perikarya and neuritic processes (Greenberg et al., 1992; Lewis et al., 2001; Otvos et al., 1994). Aβ peptide is derived by sequential proteolytic cleavage of APP by β-secretase and presenilin dependent γ-secretase to yield $A\beta_{42}$ and $A\beta_{40}$ (Bayer et al., 2001). Mutations in the genes of amyloid precursor protein (APP) (Goate et al., 1991), presenilin-1 and presenilin-2 (PS-1 and PS-2) (Rogaev et al., 1995; Sherrington et al., 1995) account for most familial early onset cases of AD by enhancing Aβ production. Some cases of late-onset Alzheimer's disease are associated with variants in at least 2 genes that result in increased Aβ, including the e4 allele of the *apolipoprotein E* gene (Price and Sisodia, 1998), and recently, variants in the *SORL1* gene that directs trafficking of APP into Aβ-generating compartments (Rogaeva et al., 2007). Although multiple other complex systems are likely involved in the pathogenesis of AD, there is substantial evidence that Aβ neurotoxicity, especially nonfibrillar oligomers derived from $A\beta_{42}$, is a fundamental event (Koo, 2002; Selkoe, 2004). $A\beta_{42}$ exists in several physical states, including monomers, oligomers and fibrils. In vitro studies show that synthetic $A\beta_{42}$ monomers aggregate over time to form oligomers, and eventually, fibrils (Chromy et al., 2003; Glabe, 2005; Klein, 2002a,b; Pike et al., 1995). Experimental evidence in vivo and in vitro supports soluble $A\beta_{42}$ oligomers as the predominant toxic species for neurons (Glabe, 2005; Lambert et al., 1998; Oddo et al., 2003a,b, 2006b). $A\beta_{42}$ oligomers have been found in brains of AD patients in concentrations up to 70-fold higher than control brains (Gong et al., 2003), and they have been observed within abnormal processes and synaptic compartments of human AD brains (Takahashi et al., 2004).

Post-mortem studies of human brain indicate that intraneuronal accumulation of $A\beta_{42}$ is an early event in AD pathogenesis, preceding extracellular plaque formation (Gouras et al., 2000; Gyure et al., 2001; Iwatsubo et al., 1994; Mori et al., 2002; Skovronsky et al., 1998). Intraneuronal Aβ accumulation is also one of the earliest age-related pathological alterations in transgenic mouse models of AD (Blanchard et al., 2003; Shie et al., 2003; Takahashi et al., 2002; Wirths et al., 2001). In the triple transgenic (3xTg-AD) mouse model developed by La Ferla and colleagues (Oddo et al., 2003b), mice equivalently overexpress three mutant human genes: APP_{swe}, $PS1_{M146V}$ and tau_{P301L} and accumulate Aβ with age, but unlike many other mouse models of AD, the 3xTg-AD mice also develop tau pathology and tangle formation in the hippocampus and amygdala (Oddo et al., 2003a,b). In 3xTg-AD mice, $A\beta_{42}$ first appears intraneuronally in the neocortex at 4 months, and in the CA1 subfield of the hippocampus by 6 months (Oddo et al., 2003b, 2006b). Extracellular Aβ oligomers appear before the detection of extracellular thioflavin-S-positive (fibrillar) plaques, supporting an intermediate role for extracellular Aβ oligomers in plaque pathogenesis (Oddo et al., 2006b). $A\beta_{42}$ oligomerization and protofibril formation are associated with microglial and astrocytic activation, which elicit an inflammatory response including the release of cytokines that may contribute to neuronal degeneration and cell death (Bossy-Wetzel et al., 2004; Tan et al., 1999).

Aβ neurotoxicity is theorized to be the critical event in AD pathogenesis and there have been numerous experimental attempts to reduce Aβ accumulation and/or speed its clearance in transgenic mouse models. One of the more promising treatments has been with the non-steroidal anti-inflammatory drug (NSAID) ibuprofen. Ibuprofen may directly reduce $A\beta_{42}$ production and processing by inhibition of γ-secretase (Weggen et al., 2001), reduce Aβ pathology independent of its effect on production (Morihara et al., 2005), and lessen inflammation due to cyclooxygenase inhibition (Vane, 2003). Ibuprofen also inhibits PPAR-γ, which reduces β-secretase activity (Sastre et al., 2006) and inhibits cytokines such as IL1β, α-1-antichymotrypsin (Morihara et al., 2005) and the Rho cascade (Zhou et al., 2003), all of which may reduce Aβ production and diminish the neurotoxic effects of Aβ. In several single and double transgenic mouse models of AD, ibuprofen reduces $A\beta_{42}$ deposition (Dedeoglu et al., 2002, 2003; Heneka et al., 2005; Jantzen et al., 2002; Lim et al., 2000, 2001; Yan et al., 2003). 4–6 month treatment with ibuprofen in APP_{Tg2576} mice decreased Aβ deposition by 40–60% and significantly improved performance on behavioral tasks (Lim et al., 2000, 2001; Yan et al., 2003). Similarly, double transgenic APP/PS-1 mice, whose level of Aβ production is 5–15-fold higher than single transgenic APP mice, showed 20–25% reduction in Aβ load after 5 months treatment with ibuprofen (Jantzen et al., 2002). In addition, Heneka and colleagues reported that acute, 7 day treatment of 10-month old APPV717I mice with ibuprofen resulted in a significant reduction of the total area and staining intensity of $A\beta_{42}$ amyloid deposits in the hippocampus and cortex in association with reduced glial inflammation (Heneka et al., 2005).

The therapeutic benefits of ibuprofen to individuals with AD have been more controversial. Epidemiological studies suggest that long-term use of NSAIDs is associated with reduced risk of developing AD (Akiyama et al., 2000; McGeer and McGeer, 1996), whereas placebo-controlled clinical trials of NSAIDs aimed at reducing inflammation in the brain of AD patients have produced negative results (Imbimbo, 2004). Post-mortem examination of individuals who used NSAIDs has also been reported to show significantly fewer activated microglia in the brain compared to non-users (Mackenzie and Munoz, 1998).

Although ibuprofen treatment has been associated with decreased Aβ, the effect of ibuprofen on tau accumulation has not been investigated. We studied the prophylactic effects of ibuprofen treatment on cognitive impairment, Aβ and tau accumulation in 3xTg-AD mice, a murine model that develops Aβ pathology before tau pathology and tangle formation, and whose pattern of conformational and phosphorylation changes of tau protein parallels the sequence in human brain (Oddo et al., 2003b).

2. Results

2.1. *Morris water maze (MWM)*

Untreated and ibuprofen-treated 3xTg-AD mice together with age-matched untreated WT mice from the same background strain were simultaneously tested in MWM. In the MWM task, mice were trained to escape on to a submerged platform for a

maximum of 60 s giving 4 trials/day to each mouse during a 7 consecutive day period. The escape latency for the ibuprofen-treated 3xTg-AD and WT mice decreased at a comparable rate and both groups reached the pre-set criteria (average mean latency of the group <25 s) for learning the MWM task on the same day, training trial day 4 (Fig. 1, A). After reaching criteria, WT mice kept learning and mastering the MWM task with progressively shorter escape latencies; their average escape latency on day 7 was <10 s. Ibuprofen-treated mice maintained their escape latency at the criteria mark on trial days 5 through 7, although their performance showed no additional improvement. Ibuprofen-treated mice performance became significantly different from WT mice on day 6 and significance was maintained on day 7. Six month old untreated 3xTg-AD mice, but not 2 month old untreated 3xTg-AD mice, were unable to reach the pre-set criteria for learning the MWM task after the 7 days of training (Fig. 1, A). Performance of untreated 6 month old 3xTg-AD mice was significantly different from WT mice on days 5 through 7 and significantly different from ibuprofen-treated 3xTg-AD mice on days 6 and 7 (Fig. 1, A).

Probe analysis in the absence of platform was performed for all 3 groups of mice on day 4, (1.5 h after reaching criteria) and on day 5 (24 h after reaching criteria). Ibuprofen-treated 3xTg-AD mice performed better than untreated 3xTg-AD in all probe measures even though the differences did not reach significance. Ibuprofen-treated transgenic mice performed as well or better than WT mice in all probe measures at the 1.5 h probe, however, at the 24 h probe, the performance of the ibuprofen-treated transgenic mice tended to decline, while that of the WT mice tended to improve (Figs. 1, B–D).

Swim speed was monitored during the training and probe trials and no speed differences were detected among animal groups.

2.2. *Aβ pathology*

To determine whether the differences in the cognitive behavior correlated with differences in AD-related pathology in the CA1/subiculum, mice were euthanized immediately after behavioral testing and the brains were removed for immunohistological

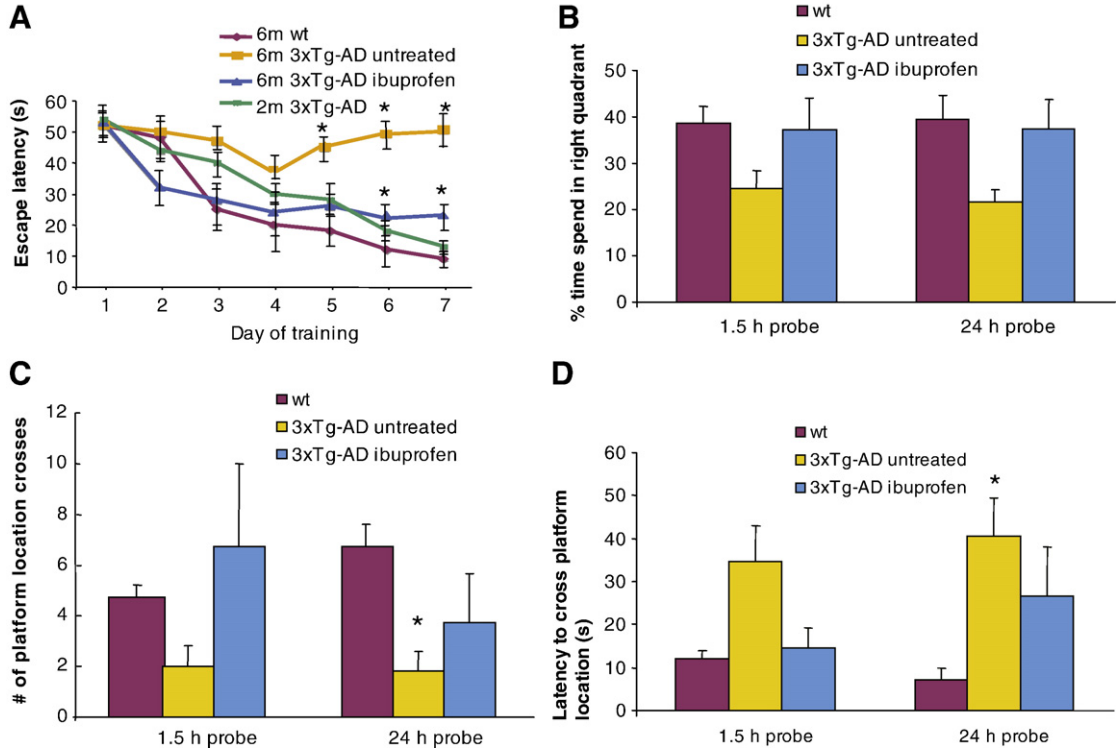

Fig. 1 – Comparison of MWM performance among 3xTg-AD mice, ibuprofen-treated 3xTg-AD mice and WT mice. Asterisks denote significant differences with respect to WT mice at a $p<0.05$ level. Error bars indicate SEM. A. Six month old ibuprofen-treated 3xTg-AD mice learned the MWM task at the same rate as age-matched WT mice reaching the pre-set criteria for learning (mean latency<25 s) on the same day (day 4). On days 6 and 7 WT mice reached the platform with decreasing escape latencies (escape latency of <10 s on day 7) while ibuprofen-treated 3xTg-AD mice maintain their escape latency around 25 s. Untreated 6 month old 3xTg-AD mice were unable to learn the MWM task, as their escape latency did not progressively decrease during the 7 consecutive days of training. On the other hand, untreated 2 month old 3xTg-AD mice performed similarly to 6 month WT mice. B, C and D. Probe analysis was performed on day 4 (1.5 h after training day 4) and day 5 (24 h after training day 4). Ibuprofen-treated 3xTg-AD mice spent as much time as WT mice swimming in the quadrant where the platform was supposed to be at both 1.5 and 24 h probe. While WT mice improved their performance at the 24 h probe compared to the 1.5 h probe (increased the number of platform location crosses and decreased the latency to cross the platform location), ibuprofen-treated 3xTg-AD mice did not. Untreated 3xTg-AD mice performed significantly worse than WT mice based on the number of platform location crosses and the latency to cross the platform location at the 24 h probe test.

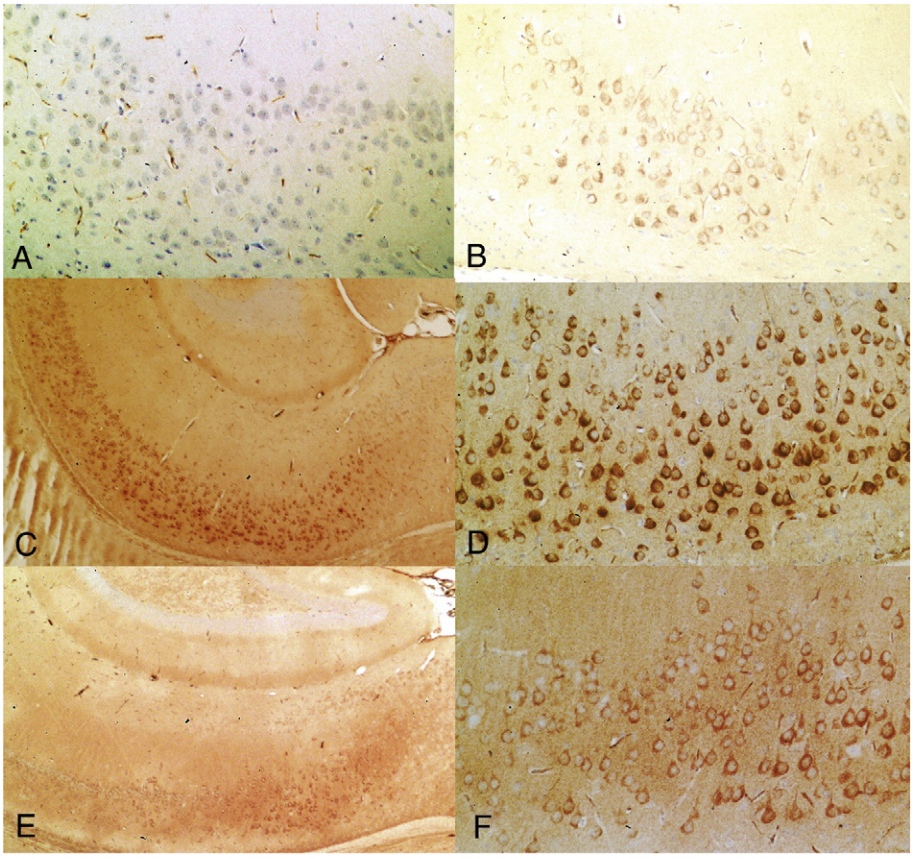

Fig. 2 – Sections of hippocampus CA1 and subiculum immunostained for Aβ. A. Six month wild-type mouse shows no immunoreactivity for 6E10 in CA1. B. Untreated 2 month 3xTg-AD mouse shows scant granular immunoreactivity for NU-1 in the perikarya of CA1 neurons. C, D. Untreated 6 month 3xTg-AD mouse shows intense immunoreactivity for NU-1 in the perikarya of CA1 and subicular neurons. E, F. Ibuprofen-treated 6 month 3xTg-AD mouse shows markedly reduced immunoreactivity for NU-1 in the same region. A, B, D, F: Original magnification × 300. C, E: Original magnification × 38.

analysis. In 2 month and 6 month old WT mice, there was no immunoreactivity for Aβ, using 6E10 or NU-1 antibodies (Fig. 2A). In 2 month old untreated 3xTg-AD mice, there was clear granular intraneuronal immunoreactivity for 6E10 in the hippocampal subfields and subiculum, whereas NU-1 immunostaining was only weakly present (Fig. 2B). In 6 month old untreated 3xTg-AD mice, both antibodies, 6E10 and NU-1, showed robust intraneuronal immunoreactivity in the hippocampal CA fields, subiculum, amygdala and cortex (Figs. 2, C,D). Intraneuronal immunoreactivity for NU-1 was generally more intense than for 6E10, but both appeared as dense granular immunostained deposits within the perikarya and proximal apical dendrite of neurons in the hippocampal CA1 and CA2 fields, subiculum, amygdala, frontal, somatosensory association and retrosplenial cortex of the untreated 6 month 3xTg-AD mice. Thioflavine S staining was negative indicating the absence of fibrillar Aβ. Occasional extracellular Aβ plaques were also found in the somatosensory association cortex with 6E10. The regional and cellular pattern of immunostaining for 6E10 and NU-1 was similar in the ibuprofen-treated mice, although the intensity was less (Figs. 2, E,F). Image analysis determined that 6E10 immunoreactivity was reduced 40.8% and NU-1 immunoreactivity was reduced 44.1% in the 6 month ibuprofen-treated 3xTg-AD mice compared to untreated littermate controls ($p<0.05$) (Table 1).

2.3. *Tau pathology*

Diffuse, finely granular somatodendritic MC1 and CP-13 immunoreactivity was prominent in neurons of the CA1 and

Table 1 – Effects of ibuprofen on Aβ, AT8, CP-13 and MC1 immunoreactivity in 3xTg-AD mice

	Untreated	Ibuprofen	% Change
Aβ(6E10)	48.1% ± 0.061	28.5% ± 0.061	−40.8
Aβ(NU-1)	41.1% ± 0.037	23.0% ± 0.065	−44.1
AT8	12.6 ± 5.3	1.4 ± 1.4*	−88.9
CP-13	5.2 ± 7.1	1.7 ± 0.7	Ns
MC1	1.10 ± 0.4	0.87 ± 0.2	Ns

All values are mean ± SEM. 6E10 and NU-1 immunoreactivity were detected in the CA1/subiculum region of posterior hippocampus, quantified by pixel counting and compared by Student's *t*-test. AT8 immunopositive cells and CP-13 and MC1 immunoreactivity were compared by Wilcoxon–Mann–Whitney Rank Sum Test. *$p<0.05$, Ns = not significant.

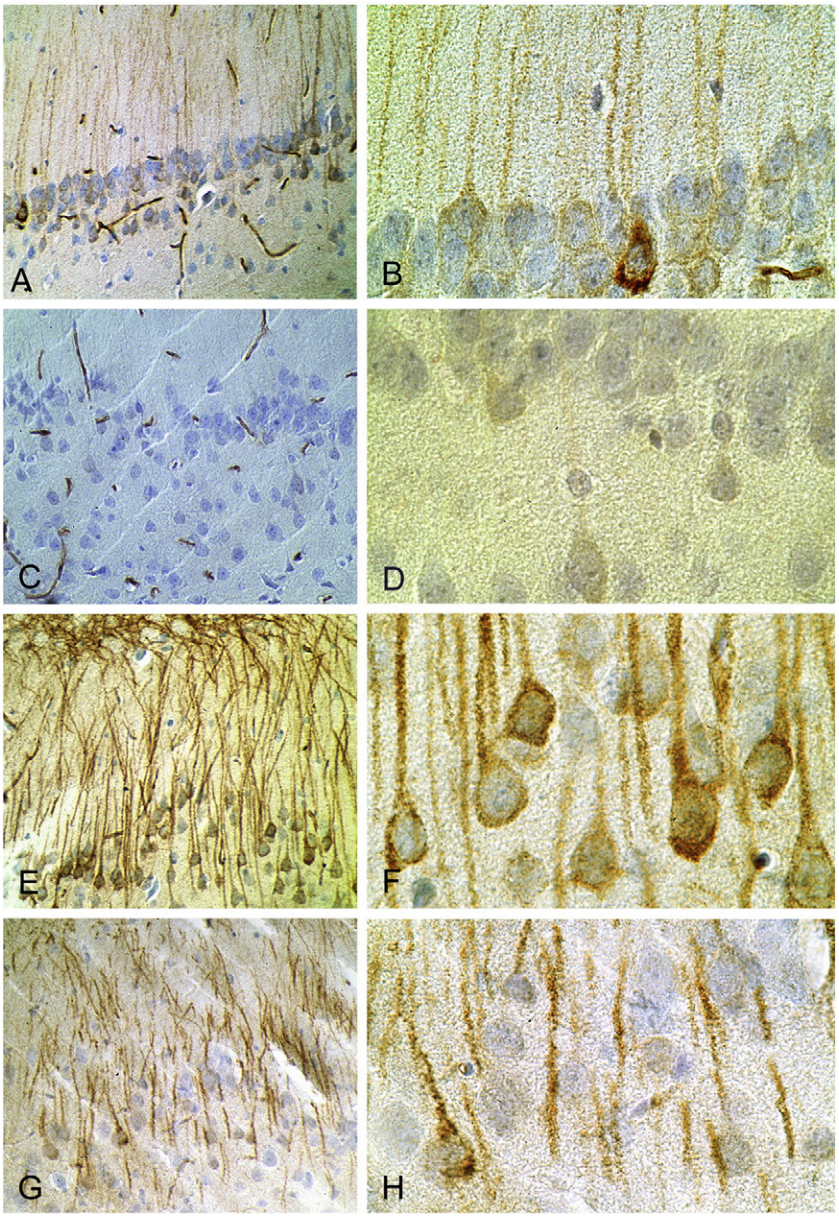

Fig. 3 – Sections of hippocampal CA1 neurons immunostained for MC1 and CP-13. A, B. Untreated 6 month 3xTg-AD mouse shows immunoreactivity for MC1 in the neuronal perikarya extending into the apical dendrite. A. Original magnification × 150. B. Occasional neurons show intense granular staining for MC1 in the perikaryon, as well as diffuse dendritic staining, original magnification × 945. C, D. Ibuprofen-treated 6 month 3xTg-AD mouse shows nearly absent immunoreactivity for MC1. C. Original magnification × 150. D. Original magnification × 945. E, F. Untreated 6 month 3xTg-AD mouse shows intense immunoreactivity for finely granular CP-13 in the perikarya extending into the apical dendrites and dendritic arbors. E. Original magnification × 150. F. Original magnification × 945. G, H. Ibuprofen-treated 6 month 3xTg-AD mouse shows marked reduction in immunoreactivity for CP-13, immunoreactivity is primarily dendritic. G. Original magnification × 150. H. Original magnification × 945.

subiculum of the hippocampus in the untreated 3xTg-AD mice (Fig. 3, A–D). CP-13 immunoreactivity was also evident in the somatodendritic domains of neurons in the amygdala and, less frequently, in the cortex. In the untreated 3xTg-AD mice, double immunostaining for NU-1 and MC1, and NU-1 and CP-13 showed NU-1 immunoreactivity in the cell body, with MC1 and CP13 immunoreactivity in long dendritic processes and dendritic arbors (Fig. 4, A,B,E). Neither CP-13 nor MC1 dendritic immunoreactivity was found in any region without intraneuronal NU-1. Both MC1 and CP13 immunoreactivity appeared reduced in ibuprofen-treated 3xTg-AD mice, although the difference was not significant (Figs. 3, E–H, Table 1).

In the untreated 3xTg-AD mice, AT8 and PHF immunoreactivity were found in scattered neurons and neuronal processes of CA1 and subiculum of the hippocampus, and less frequently in abnormal neuritic processes in the amygdala (Figs. 4 C,D, 5). AT8 and PHF-1 immunoreactivity were only found in regions that were also sites of pronounced

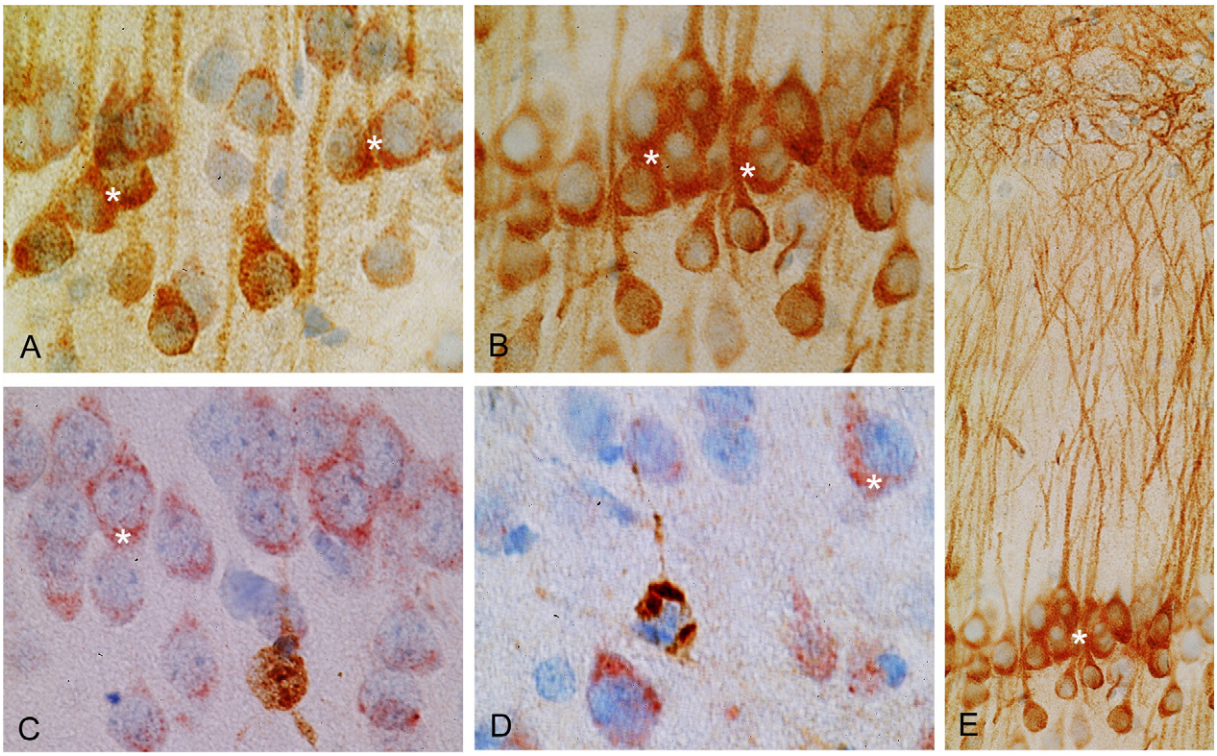

Fig. 4 – Double immunostained sections of hippocampal CA1 neurons in untreated 6 month 3xTg-AD mice. A. Double immunostained sections for NU-1 and MC1 show diffuse MC1 immunoreactivity (brown) extending into the apical dendrite of neurons with granular perikaryal NU-1 (red, asterisks), original magnification × 945. B. Double immunostained sections for NU-1 and CP-13 shows diffuse CP-13 immunoreactivity (brown) extending into the apical dendrite of neurons with granular perikaryal NU-1 (red, asterisks), original magnification × 945. C. Double immunostained sections for NU-1 and AT8 shows granular AT8 immunoreactivity (brown) in occasional neurons, while neighboring neurons show granular perikaryal NU-1 (red, asterisk), original magnification × 945. D. Double immunostained sections for NU-1 and PHF-1 shows dense aggregates of PHF-1 immunoreactivity (brown) in the perikaryon and dendrite of occasional neurons, while neighboring neurons show granular perikaryal NU-1 (red, asterisk), original magnification × 945. E. Double immunostained sections for NU-1 and CP-13 show diffuse CP-13 immunoreactivity (brown) extending into the apical dendrites and lush dendritic arbors of neurons with granular perikaryal NU-1 (red, asterisks), original magnification × 945.

intraneuronal NU-1 accumulation. AT8 and PHF-1 immunoreactive neurons often showed dense immunoreactivity at both the basal and apical poles of the perikaryon, with finely granular immunostaining of the apical dendrite and other proximal neuronal processes.

Confocal microscopy revealed co-localization of punctuate granular immunoreactivity for NU-1 and MC1, CP13, AT8 and PHF-1 within hippocampal CA1 neuronal perikarya (Figs. 6 A–S) of 6 month untreated 3xTg-AD mice. Co-localization of the later forms of phosphorylated tau, AT8 and PHF-1, with NU-1 was less than that found with early forms of phosphorylated and conformationally altered tau, CP-13 and MC1 (Fig. 6). There was also a significant reduction in the number of AT8 immunostained neurons in ibuprofen-treated 3xTg-AD mice (12.6 ±

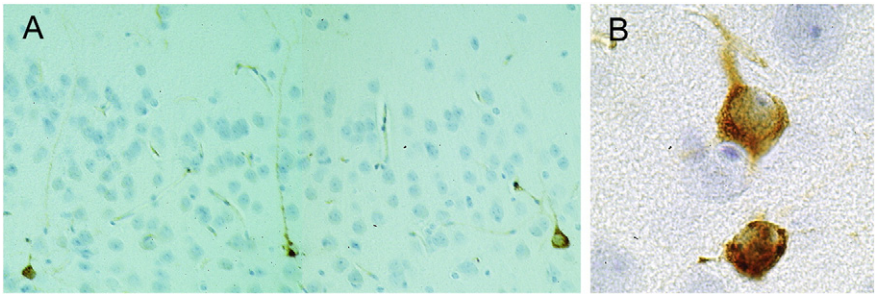

Fig. 5 – Sections of CA1 hippocampus from untreated 6 month 3xTg-AD mouse shows neurons immunoreactive for AT8. A. Original magnification × 150. B. Original magnification × 945.

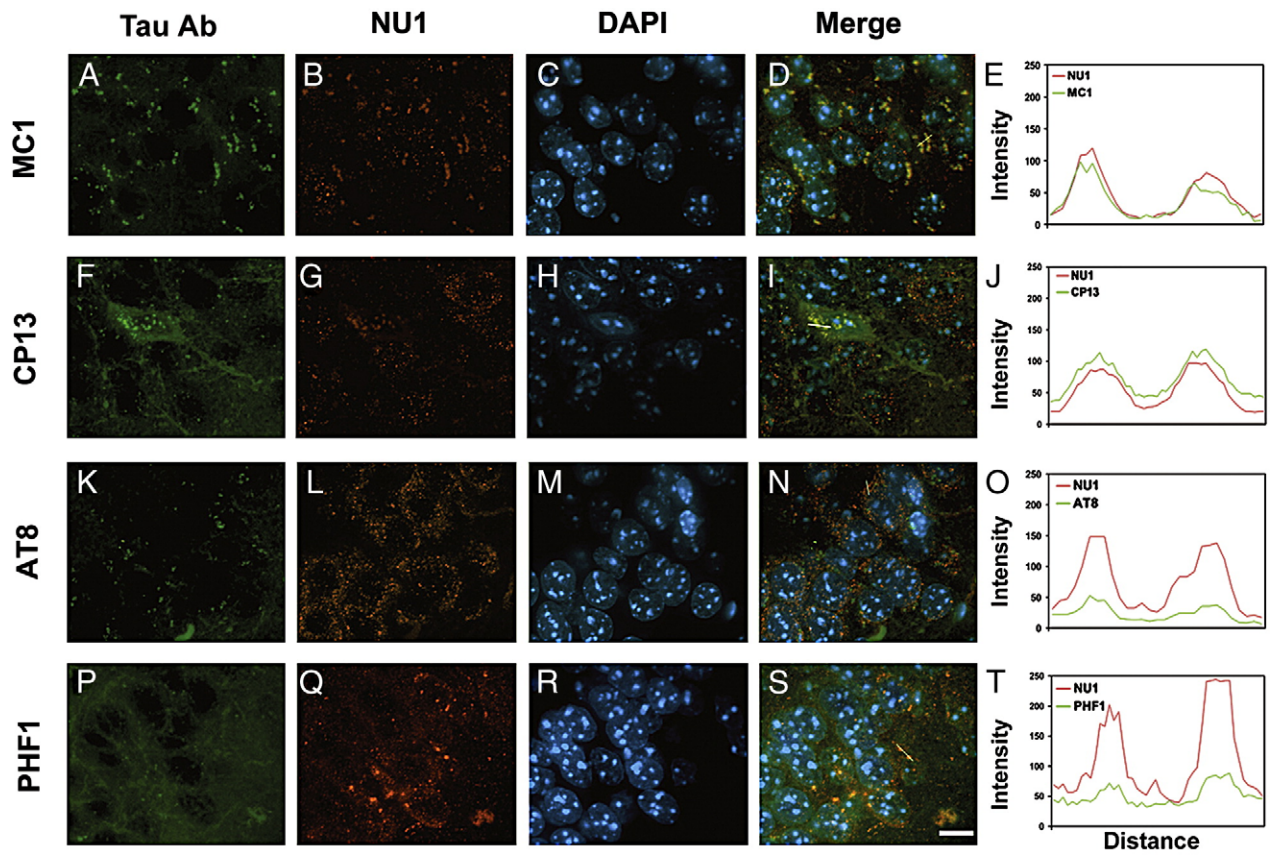

Fig. 6 – The co-localization of tau and NU-1 using confocal microscopy in 6 month untreated 3xTg-AD mice. The co-localization intensity of tau [stained with MC1 (A) (green), CP13 (F) (green), AT8 (K) (green), and PHF1 (P) (green)] and NU-1 (B, G, L, and Q) (red) were determined by Disk Confocal Microscopy (Olympus, Tokyo, Japan) and the line measurement (AQI-X-COMBO-CWF, MediaCybernetics Inc. Bethesda, MD). The image analysis data shown in panel E, J, O, and T demonstrate strong co-localization of NU1 with MC1 and CP13 and less co-localization with AT8 and PHF1. Scale bar: 10 mm.

5.3) compared to untreated 3xTg-AD mice (1.4±1.4) ($p<0.05$) (Table 1). No AT8 immunopositive neurons were found in the 6 month old WT mice.

3. Discussion

We demonstrate that prophylactic treatment of young 3xTg-AD mice with ibuprofen is associated with a significant reduction of intraneuronal Aβ accumulation and improved cognitive performance. 3xTg-AD mice treated with ibuprofen were able to learn the MWM task at the same rate as their WT littermates, but unlike WT mice, were unable to excel and master the task. The MWM task is highly dependent on hippocampal integrity, and is considered a measure for deficits in spatial reference and memory (Davis, 1992; Nakazawa et al., 2004; Sutherland et al., 1989). The inability of ibuprofen-treated 3xTg-AD mice to master the task may reflect a diminished ability for long-term memory retention. The low number of mice in each group may have contributed to the lack of significance found for some cognitive parameters.

Although it is possible that ibuprofen interfered with the recognition of Aβ by antibodies because of competition for PET ligand ^{18}FFDDNP (Agdeppa et al., 2003), the Ki for inhibition of the ^{18}FFDDNP to $A\beta_{40}$ fibrils is approximately 40 μM for ibuprofen, suggesting a relatively low affinity. Also, the plasma half-life of ibuprofen at doses used for this study is approximately 2.8 h (Shah and Jung, 1987). We collected and processed the brain tissue more than three half-lives after the last ibuprofen dose, making competitive interference for Aβ highly unlikely. Moreover, the 2 different Aβ antibodies, which recognize two different molecular targets, were equally decreased (40%) by ibuprofen; it is unlikely that competitive interference would affect 2 different molecular targets equally.

In addition to improved cognition, prophylactic treatment of 3xTg-AD mice was associated with a significant reduction in intraneuronal oligomeric Aβ and significantly fewer hyperphosphorylated tau immunoreactive hippocampal neurons. Previous studies have shown that 3xTg-AD mice develop age-related accumulation of plaques and tangles in disease relevant regions. We found accumulation of intraneuronal oligomeric Aβ in hippocampal pyramidal neurons at 6 months in association with deficits in water maze spatial memory, findings that are consistent with previous reports (Billings et al., 2005). Unlike prior studies (Oddo et al., 2004, 2006b), we also found many pathological forms of tau present at 6 months in

untreated 3xTg-AD mice, including conformationally altered, pre-fibrillar tau (MC1), early hyperphosphorylated tau (CP-13), and other, later forms of hyperphosphorylated tau (AT8) and PHF-tau (PHF-1). Moreover, co-localization of oligomeric Aβ with later forms of hyperphosphorylated tau (AT8) and PHF-tau (PHF-1), was less than that observed with early forms of altered and phosphorylated tau (MC1 and CP-13). Why we found immunoreactivity for multiple forms of tau at a younger age of 3xTg-AD mice than previous reports is unclear, but is likely best explained by decades of immunocytochemical experience using similar antibodies in post-mortem human brain (McKee et al., 1989).

The monoclonal antibody, MC1, identifies early conformational changes in tau, and formation of its epitopes is considered one of the earliest pathological alterations of tau in AD (Vincent et al., 1998; Weaver et al., 2000). MC1 recognition is dependent on epitopes on both the extreme N-terminus and third microtubule-binding domain of tau. This soluble, conformational variant of tau is also phosphorylated and ubiquitinated (Cripps et al., 2006). In the unified model of tau aggregation proposed by Barghorn and Mandelkow, conformational alterations in tau are a major factor leading to tau fibrillization (Barghorn and Mandelkow, 2002). Vulnerable hippocampal neurons show MC1 immunoreactivity prior to the formation of paired helical filaments (PHF) in individuals with preclinical AD, and levels of conformationally altered tau increase regionally in mildly demented subjects prior to the detection of PHFs (Haroutunian et al., 2007; Weaver et al., 2000). The monoclonal antibody CP-13 recognizes phosphorylated tau at serine 202. Within 1 month of localized gene transfer of human tau carrying the P301L mutation to adult rats, diffuse cytoplasmic immunoreactivity for CP-13 was found in neuronal perikarya, indicating that CP-13 is an early marker of hyperphosphorylated tau (Klein et al., 2004). In the untreated 3xTg-AD mice, we found intense immunoreactivity for MC1 and CP13 in the hippocampal CA1/subiculum subfields. This finely granular cytoplasmic immunoreactivity co-localized with oligomeric Aβ, and appeared less intense in mice treated with ibuprofen. These changes suggest that the accumulation of conformational and early phosphorylation alterations in tau in perikarya and proximal dendrites is associated with intraneuronal oligomeric Aβ.

Recent studies using soluble extracts from AD and control human brain tissue support simultaneous evolution of Aβ and tau accumulation. De Felice and colleagues recently showed that Aβ oligomers stimulate tau phosphorylation in hippocampal neuronal cultures (Lambert et al., 2007). Guo and colleagues reported that intracellular soluble Aβ binds to soluble tau and that these soluble Aβ–tau complexes in human brain promote tau phosphorylation by GSK-3β, and the production of insoluble tau aggregates (Guo et al., 2006), consistent with our finding of co-localized oligomeric Aβ and conformationally altered and nonfibrillar, hyperphosphorylated tau. As intraneuronal phosphorylated tau accumulated, Guo and colleagues found dissociation of the Aβ–tau complexes (Guo et al., 2006), which may explain our observation of less co-localized fibrillar forms of tau, AT8 and PHF-1, with NU-1.

Under physiologically normal conditions, tau is a highly soluble protein that binds microtubules and is critical to axonal integrity and neuronal outgrowth (Ramsden et al., 2005; Weingarten et al., 1975). Recent studies show that exposure of pre-fibrillar $A\beta_{42}$ to neurons that express endogenous tau initially results in neuritic abnormalities, including neuritic varicosities, and acute microtubular disassembly prior to cell death (King et al., 2006). Loss of microtubule integrity most likely results in reduced axonal transport necessary for basic cellular functions such as maintenance of synapses and repair of membrane holes, both known to be produced by oligomeric Aβ (Glabe and Kayed, 2006; Kayed et al., 2003; Shen et al., 2005).

Oligomeric Aβ and synaptic loss correlate with cognitive dysfunction in AD (Selkoe, 2002), but tau immunoreactive NFT and neuropil threads also predict overall cognitive function in AD (McKee et al., 1991). Oligomeric intraneuronal Aβ may produce early cognitive abnormalities in AD and serve as the trigger of the initial tau pathology. Once tau pathology becomes established, either by Aβ-induced tau aggregation, or perhaps, self-promoted tau aggregation, aggregated tau itself may further exacerbate cognitive decline (Oddo et al., 2006b).

A novel aspect of this study is that hyperphosphorylated tau is significantly reduced by prophylactic ibuprofen administration to young triple transgenic mice; to our knowledge there are no other similar reports of NSAID treatment reducing tau pathology. Although it remains to be determined whether ibuprofen can reduce tau pathology directly in the absence of reduced Aβ accumulation, previous studies in 3xTg-AD mice support a causal relationship between Aβ and tau. Aβ immunotherapy in 3xTg-AD mice clears not only intraneuronal and extracellular Aβ pathology, but also clears somatodendritic tau (Oddo et al., 2006a). Furthermore single immunization with anti-oligomeric specific antibody reduces tau as well as Aβ pathology (Oddo et al., 2006b). These results suggest that the reduction in hyperphosphorylated tau we found after ibuprofen treatment was secondary to reduced Aβ accumulation. On the other hand, anti-inflammatory agents such as ibuprofen are associated with reduced activated microglia in human brain tissue (Mackenzie and Munoz, 1998), and microglia releases cytokines that activate tau kinases, e.g. cyclin-dependent kinase (cdk5), glycogen synthase kinase 3β (GSK-3β), and p38-mitogen activated protein kinase (p38-MAPK), that are candidates for tau phosphorylation (Griffin et al., 2006; Kitazawa et al., 2005; Li et al., 2003; Sheng et al., 2000). To clearly resolve whether ibuprofen directly reduces hyperphosphorylated tau or is secondary to reduced Aβ accumulation, further studies will be required in transgenic mouse models expressing only mutant tau genes.

4. Experimental procedures

4.1. *Mice*

In this study, 10 homozygous 3xTg-AD mice expressing mutant human genes APP_{swe}, $PS1_{M146V}$ and tau_{P301L}, previously characterized by Oddo et al. (2003b), and 5 wild-type mice from the same hybrid background strain, 129/C57BL6, were used. Starting at weaning (1 month of age), a group of 5 3xTg-AD mice and 5 WT mice were fed regular chow diet while another group of 5 3xTg-AD mice were given chow supplemented with ibuprofen (375 ppm). Mice were housed on a 12 h light:12 h dark schedule. All mice were given access to food and water ad libitum. At 6 months of age, the cognitive ability of the three groups of

mice (untreated 3xTg-AD, ibuprofen-treated 3xTg-AD and untreated WT mice) was tested in the Morris water maze (MWM) spatial memory test. Upon completion of the MWM test, mice were euthanized and brains removed for histological analysis.

4.2. *Morris water maze spatial reference task*

A white pool of 4 ft in diameter filled with water tinted with non-toxic white paint and maintained at 24 °C±2 was used. In the water maze test mice were trained to find in the pool an invisible submerged platform of 14 cm of diameter using a variety of visual extra maze cues located on the white curtain surrounding the pool. Prior to water maze testing, a pre-training procedure was implemented to habituate mice to the water and to train them to escape from the water by climbing on to a visible platform, as previously described (Frick et al., 2002). The pre-training protocol consisted of four trials in which each mouse was first placed on the visible platform for 10 s and then placed at three progressively further distances from the platform where it was allowed 30 s to escape onto it. No data were collected during this procedure. For the MWM test, mice were trained in four trials per day during 7 consecutive days. The 4 ft pool was imaginarily divided into 4 quadrants and north, west, south and east positions located at the intersections of the quadrants. The submerged platform was standing on the west position of the tank 20 cm from the wall. The starting position was randomized among the north, west and east positions such that over the 4 trials only one starting position was randomly repeated. In each trial mice were given 60 s to locate the platform and if a mouse failed to find the platform it was placed on the platform for 15 s. Probe trials (trials without platform) were run for all groups, 1.5 h and 24 h after the training trial, once a group reached in the training trial a preset criteria for learning (average latency to reach the platform <25 s). During probe trials all animals started from the east position and were allowed to swim for 60 s. Data were recorded using an HVS 2020 automated tracking system (HVS Image, Hampton, UK). Multiple measures of water maze performance were recorded during the training and probe trials: latency to reach platform (s), swim distance (cm), swim speed (cm/s) were recorded during the training trial. Moreover during the probe trial, the number of platform crossings, the latency to first cross, and the percent time spent in each quadrant was recorded. The recorded data were used to analyze mouse performance.

4.3. *Tissue preparation and immunohistochemistry*

Immediately after mice were euthanized by CO_2 asphyxiation, the brains were removed. The right hemibrains were cut into 3 coronal sections, 2 mm in thickness, using a coronal matrix. The 3 coronal sections, which spanned from 3 mm anterior to the bregma to 3 mm posterior to the bregma, were fixed in 4% paraformaldehyde at room temperature for 2 h and embedded in a single paraffin block. The paraffin-blocks were cut serially in 10 μm sections, each section consisted of 3 anterior–posterior coronal levels and each block encompassed the entire mid section of the hemibrain. Sections were immunostained using the following antibodies: 6E10 (Signet Laboratories, 1:1000 dilution, pretreated with formic acid), $A\beta_{42}$ (Biosource, 1:2000 dilution), NU-1, which recognizes oligomeric and fibrillar, not monomeric $A\beta_{42}$ (Lambert et al., 2001, 2007) (courtesy of William Klein, 1:1000, pretreated with formic acid), MC1, directed against conformationally altered tau (Klein et al., 2004; Lewis et al., 2000, 2001; Weaver et al., 2000), CP13, directed against phosphoserine 202 of tau (Klein et al., 2004; Lewis et al., 2000) (courtesy of Peter Davies, 1:200,), AT8, directed against phosphoserine 202 and phosphothreonine 205 (Innogenetics. 1:2000), and PHF-1, directed against phosphoserine 396 and phosphoserine 404 (Klein et al., 2004; Lewis et al., 2000, 2001; Weaver et al., 2000) (courtesy of P. Davies, 1:1000). Sections for immunostaining were processed using the Vectastain Elite ABC Kit (Vector Labs, Burlingame, CA). Following the appropriate biotinylated secondary antibody, slides were developed with diaminobenzidine (DAB) for the exact same amount of time and counterstained with hematoxylin. For double immunostained sections, the tissue was blocked with avidin and biotin before each primary antibody (Multiple Antigen Labeling, Vector Labs). The first primary antibody was visualized with DAB, the second primary antibody was visualized with aminoethylcarbazole. For thioflavine S (Sigma) staining, slides were hydrated in 1% thioflavine S diluted in distilled water for 3 min, dehydrated and mounted.

4.4. *Confocal microscopy*

Indirect double immunofluorescence staining methods were used to determine the localization of $A\beta_{42}$ and AT8 in the untreated 3xTg-AD mice. Tissue sections were blocked for 1 h with blocking solution containing 0.3% Triton X-100, 5% BSA, and 3% goat serum and then incubated with a 1:500 dilution of $A\beta_{42}$, and a 1:500 dilution of AT8 overnight at 4 °C. After three washes with PBS, the specimens were incubated for 1 h with fluorescence (FITC)-conjugated goat anti-mouse IgG antibody (Vector Labs), and Cy3-conjugated anti-rabbit IgG antibody (Jackson Laboratories). The tissue sections were washed three times with PBS and mounted with fluorochrome mounting solution (Vector). Images were analyzed using a Spinning Disk Confocal microscope (IX2-DSU, Olympus). Control experiments were performed in the absence of primary antibody.

4.5. *Quantitation and statistical analysis*

To quantify intraneuronal Aβ immunoreactivity for 6E10 and NU-1, and tau immunoreactivity for MC1 and CP-13, photomicrographs (five mice per group, two sections per mouse, three photographs per section) were taken with a Nikon Eclipse 80i microscope using an Optronics digital camera. The images were processed in Photoshop by extracting the region of interest, which was defined as the posterior hippocampal CA1/subicular region. Each image was normalized for color and brightness using an unaffected region of the section. Quantification was performed using Image J (NIH, Bethesda, MD). The images were thresholded at fixed, pre-determined settings and the resultant pixels were counted. The total number of pixels in the unthresholded region of interest was also counted. The percentage of thresholded pixels to total pixels in the region of interest were calculated for each image and presented as the percentage of affected tissue. 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 quantitation of AT8 immunoreactive neurons, every 6th serially collected slide from each mouse hemisphere was analyzed microscopically, and the number of AT8 immunoreactive neurons in the hippocampal CA1/subicular region was tabulated. The number of AT8 positive neurons in the untreated 3xTg-AD mice was compared to the number of AT8 positive neurons in the ibuprofen-treated 3xTg-AD mice and analyzed between the groups using the Wilcoxon–Mann–Whitney Rank Sum Test. Statistical significance was defined as $p<0.05$.

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