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Reductions in A β -derived neuroinflammation, with minocycline, restore cognition but do not significantly affect tau hyperphosphorylation

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Abstract

Cognitive decline in Alzheimer's disease (AD) occurs as a result of the buildup of pathological proteins, and downstream events including an elevated and altered inflammatory response. Inflammation has previously been linked to increased abnormal phosphorylation of tau protein. To determine if endogenous A β -induced neuroinflammation drives tau phosphorylation *in vivo* we treated 8-month-old 3xTg-AD with minocycline, an anti-inflammatory agent, to assess how it influenced cognitive decline and development of pathology. 4-months of treatment restored cognition to non-transgenic performance. Inflammatory profiling revealed a marked decrease in GFAP, TNF α as well as IL6 and an increase in the CXCL1 chemokines KC and MIP1a. Minocycline also reduced levels of insoluble A β and soluble fibrils. Despite reducing levels of the tau kinase cdk5 coactivator p25, minocycline did not have wide effects on tau pathology with only one phospho-epitope showing reduction with treatment (S212/S214). The sum of these findings shows that reduction of the inflammatory events in an AD mouse model prevents cognitive deficits associated with pathology, but that endogenous A β -derived neuroinflammation does not contribute significantly to the development of tau pathology.

Keywords

Alzheimer's disease; inflammation; tau phosphorylation; therapeutic; cognition; p25

Introduction

Alzheimer's disease (AD) is the most common dementia of the elderly. It is a progressive neurodegenerative disease, which is characterized by the aggregation of the amyloid- β peptide (A β) into extracellular plaque deposits, and the hyperphosphorylation of the

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microtubule stabilizing protein tau, causing it to aggregate into neurofibrillary tangles (NFTs) in the neuronal cytosol. The plaque and tangle pathologies are associated with an inflammatory response characterized by increased activated microglia [1], astrocytes [2], cytokines [3, 4], chemokines [5] and complement factors [6].

These features of AD have been recapitulated in the 3xTg-AD mouse model, which develops age dependent A β and tau pathologies [7], along with increased activated microglia and astrocytes in response to plaque deposition [8], and progressive cognitive decline [9]. This mouse model has been useful in elucidating several pathways by which A β pathology controls the development of tau pathology – for example immunotherapy, which removes A β was also shown to remove somatodendritic accumulation of tau [10], suggesting that A β levels lie upstream of tau accumulation, at least in this model. Increased inflammation, via lipopolysaccharide (LPS) injection, has been shown to enhance tau phosphorylation through increased p25 generation, a coactivator for the tau kinase cyclin dependent kinase 5 (cdk5) [8] while immunosuppression of tau overexpressing P301S mice, with FK506, resulted in decreased microglia and tau pathology [11]. Hence, brain inflammation may play a central role in mediating the development of tau pathology in response to A β . Furthermore, it has been shown that aspects of a dysregulated inflammatory response, in both transgenic mouse models of AD [12] and AD patients [13], can lead to cognitive decline, while anti-inflammatory Tumour Necrosis Factor α (TNF α) antagonists have been reported to show remarkable benefits to cognition [14], although in a very small patient sample. Therefore, we set out to determine if dampening inflammation in a mouse model of AD during the early formation of pathological proteins would be beneficial for cognition and if it could affect the progression of the pathology.

Multiple studies have shown that minocycline, a second-generation derivative of tetracycline, is neuroprotective in animal models of CNS injury and neurodegenerative diseases [15–18]. These neuroprotective effects appear to be distinct from the antimicrobial activity of minocycline, but appear to be associated with the ability of minocycline to suppress p38 mitogen-activated protein kinase activity in microglia [19]. Indeed, this minocycline-mediated suppression of p38, in both microglia and other cell types, appears to confer protection in a number of insult models (i.e. [20–22]). In a cerebral ischemia model, minocycline reduced glial activation, inflammation and decreased the size of the infarct [23]. In a traumatic brain injury model minocycline was shown to reduce the size of the injury, inhibit caspase-1, and block expression of inducible nitric oxide synthase (iNOS) [24]. Moreover, minocycline delayed death and inhibited caspases-1 and caspases-3 expression in a Huntington disease transgenic mouse model [16, 25], slowed disease progression in a mouse model of amyotrophic lateral sclerosis (ALS) [15], and attenuated LPS-induced white matter injury in the neonatal rat brain [26]. However, a recent clinical trial with minocycline for ALS demonstrated a worsening of patients on the drug [27], cautioning the translatability of the effects of minocycline in mouse models to the human disease. *In vitro* studies have begun to provide insights into the mechanisms involved in the broad spectrum of minocycline-mediated neuroprotection [28–32].

Previously minocycline has been shown to protect against A β -induced neuronal loss in the rat hippocampus [33], prevent hippocampal long-term potentiation (LTP) deficits induced

by A β [34], reduce aggregation of A β into fibrils [32], and improve cognitive deficits in mouse models of AD [35, 36], and in A β infused rats [37]. Here we set out to investigate the role that AD-pathology induced inflammation has on tau pathology progression, and also cognitive decline. We found that minocycline treatment in the 3xTg-AD mouse model of AD prevents cognitive decline associated with pathology, reverses the inflammatory response to wild type levels, reduces insoluble and fibrillar A β and exhibits differential effects on phosphorylation of tau despite reductions in p25.

Materials and Methods

Animal Treatments

All rodent experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of California, Irvine (UCI). The triple-transgenic mice (3xTg-AD) have been described previously (Oddo et al., 2003). Briefly, these mice harbor a knock-in mutation of presenilin 1 (PS1_{M146V}), the Swedish double mutation of amyloid precursor protein (APP_{KM670/671NL}), and a frontotemporal dementia mutation in tau (tau_{p301L}) on a 129/C57BL/6 background. In order to investigate how reducing the inflammatory system affected the accumulation of pathologies and resultant cognitive decline, we treated 8-month old 3xTg-AD and wild-type non-transgenic (nonTg) mice with minocycline in AIN17 chow (55 mg/kg/day based on average mouse body weight and average daily consumption; manufactured as a custom diet at Bio-Serv (Frenchtown, NJ)), or AIN17 diet alone, for 4 months. Diet was replaced twice weekly, and stored at 4°C protected from light. After the treatment, the animals were sacrificed and the brains removed. The brains were immediately dissected in half along the coronal line and one-half frozen for biochemical analysis and the other half fixed in 4% paraformaldehyde. 48 h later brains were sliced into 40 μ M sections using a vibrotome.

Behavioral Assays

Novel Object Recognition (NOR)—This task is based on the spontaneous tendency of rodents to explore a novel object more often than a familiar object (Ennaceur and Delacour, 1988). For this task, mice were first familiarized with an empty open field for 10 minutes. On the following day, mice were exposed in the same open field with two identical objects (Object A; two identical balls). 1.5 and 24 hours later, animals were subjected to a 3-minute retention phase test where they were exposed to one object A and one Object B (at 1.5 hours) and one Object C (at 24 hours). The time spent exploring the familiar and the novel object at each 1.5 and 24 hours was calculated where exploration equals touching the object with nose or paws, or sniffing within 1.5 cm of the object. Time spent with the novel object as compared to time spent with the familiar object was used as memory index.

Morris Water Maze (Adapted from [38])—The Morris Water Maze (MWM) is a test for spatial memory (i.e. hippocampus dependent) and cued learning (i.e. non-hippocampal) in rodents. Many studies in the last two decades have used this test as a reliable measure of hippocampal-dependent learning [39], including several transgenic models [40, 41].

The water maze is a circular pool filled with opaque water. Mice were pre-trained by swimming to a plexiglass platform submerged 1.5cm beneath the surface of the water. The location of the platform was selected randomly for each individual mouse throughout training. The maze is located in a room containing several visual, extra-maze cues. For spatial training, mice were subjected to four trials per day for three consecutive days. Before the first trial, the mouse was placed on the platform for 30s. On each trial, the mouse was then placed into the tank at one of four designated starting points in a random order. Mice were then allowed to find and escape onto the submerged platform. If an animal failed to find the platform within 60s, it was manually guided to the platform and remained there for 15s.

Retention of spatial training was assessed 1.5 and 24 hours after the last training trial. Both of these probe trials consisted of a 60s free swim in the pool with the platform removed. Mice were monitored by a camera mounted in the ceiling directly above the pool for subsequent analysis. The parameters measured during the probe trial include (1) time spent in the quadrant containing the platform during training and (2) initial latency to cross platform location. The escape data was examined with a multifactor analysis of variance (ANOVA) including genotype (transgenic vs. non-transgenic), and probe trial (1.5 and 24 hours).

Passive Inhibitory Avoidance (IA)—The inhibitory avoidance task is used in mice to assess primarily amygdala-dependent learning [42–44]. IA testing consists of a training session followed by testing 1.5 and 24 hours post training. During the training session, a mouse was placed in a lightened chamber and when the mouse crossed to the dark compartment, it received a mild footshock (0.15 mA/1 s). During testing, the mouse was placed again in the light compartment and the latency to cross over to the dark compartment measured. This latency measure is used as an index of passive fear avoidance.

Immunoblotting: Protein extracts were prepared from whole brain samples by homogenizing in T-per (Pierce Biotechnology, Rockford, IL, USA) extraction buffer and Complete Mini Protease Inhibitor Tablets (Roche, Indianapolis, IN, USA) followed by high-speed centrifugation at 100,000 g for 1 h. The supernatant was taken as the protein extract. Protein concentrations were determined by the Bradford method. Equal amounts of protein (20 µg – 50 µg depending on protein of interest) were separated by SDS/PAGE on a 10% Bis/Tris gel (Invitrogen, Carlsbad, CA, USA), transferred to 0.45 µM nitrocellulose membranes, blocked for 1 hour in 5% (vol/vol) nonfat milk in Tris-buffered saline (pH 7.5) supplemented with 0.2% Tween20, and processed as described. Antibodies and dilutions used in this study include 6E10 (1:1000 Signet, Dedham, MA, USA), CT20 (1:5,000; Calbiochem, San Diego, CA, USA) for C99 and C83, HT7 (1:3000, Innogenetics, Gent, Belgium), AT8 (1:1000, Pierce Biotechnology, Rockford, IL, USA), AT180 (1:1000, Pierce Biotechnology, Rockford, IL, USA), AT270 (1:1000, Pierce Biotechnology, Rockford, IL, USA), anti-C'-term p35 (1:2000, Santa Cruz Biotechnology) for p25 and p35, IBA1 WB (1:1000; Wako Chemicals USA) and βActin (1:10,000; Sigma-Aldrich, USA). Quantitative densitometric analyses were performed on digitised images of immunoblots with Scion Image 4.0 (Scion Corporation, Frederick, MD, USA).

A β ELISA: A β ₁₋₄₀ and A β ₁₋₄₂ were measured using a sensitive sandwich ELISA system. Soluble and insoluble A β was isolated from whole brain homogenates using T-per Extraction Buffer (Pierce Biotechnology, Rockford, IL, USA) and 70% formic acid (FA) respectively. Soluble fractions were loaded directly onto ELISA plates and FA fractions were diluted 1:20 in neutralization buffer (1 M Tris base; 0.5 M NaH₄PO₄) prior to loading. MaxiSorp immunoplates (Nunc, Rochester, NY, USA) were coated with mAB20.1 (William Van Nostrand, Stony Brook University, NY) antibody at a concentration of 25 μ g/ml in Coating Buffer (0.1 M NaCO₃ buffer, pH 9.6), and blocked with 3% BSA. Standards of both A β ₄₀ and ₄₂ were made in Antigen Capture Buffer (ACB; 20 mM NaH₂PO₄; 2 mM EDTA, 0.4 M NaCl; 0.5 g CHAPS; 1% BSA, pH 7.0), and loaded onto ELISA plates in duplicate. Samples were then loaded in duplicate and incubated overnight at 4°C. Plates were washed and then probed with either biotinylated-anti-A β 35–40 (C49 for A β ₁₋₄₀) or anti-A β 35–42 (D32 for A β ₁₋₄₂) overnight at 4°C. Following this plates were washed with PBS and then incubated with streptavidin-HRP (Pierce Biotechnology, Rockford, IL, USA) for 4 hours at 37°C. 3,3',5,5'-tetramethylbenzidine was used as the chromagen, and the reaction stopped by 30% O-phosphoric acid, and read at 450 nm on a Molecular Dynamics plate reader.

Immunostaining: Light-level immunohistochemistry was performed using an avidin-biotin immunoperoxidase technique (ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) and was visualized with diaminobenzidine as previously described [7]. The following antibodies were used: anti-A β , 6E10 (Signet Laboratories, Dedham, MA, USA), anti-Tau HT7 (Innogenetics, Gent, Belgium). Primary antibodies were applied at dilutions of 1:1000 for 6E10; 1:1000 for HT7.

Confocal microscopy: Fluorescent immunolabeling followed a standard two-way technique (primary antibody followed by fluorescent secondary antibody). Free-floating sections were rinsed in TBS (pH 7.4) and then blocked (0.25% Triton X-100, 5% normal goat serum in TBS) for 1 h. Sections were incubated in primary antibody overnight (4°C), rinsed in PBS, and incubated (1 h) in either fluorescently labeled anti-rabbit (Alexa 555, 1:200; Molecular Probes Inc., Eugene, OR, USA) or anti-mouse secondary antibodies (Alexa 488, 1:200; Molecular Probes Inc., Eugene, OR, USA). Antibodies were diluted as follows: GFAP, 1:1,000 (Dako, Glostrup, Denmark); IBA1, 1:1,000 (Wako, Richmond, MA). Omission of primary antibody or use of pre-immune IgG eliminated all labeling (data not shown). Confocal images were captured on a Biorad Radance 2100 (Bio-Rad, Hercules, California, USA) confocal system. To prevent signal bleed-through, all fluorophores were excited and scanned separately using lambda strobing.

Inflammatory profiling: Profile of brain homogenate levels of inflammatory markers were performed in all 4 groups of animals – nonTg, nonTg treated with minocycline, 3xTg-AD and 3xTg-AD treated with minocycline. Bio-plex multi-plex analysis (Bio-Rad, Hercules, California, USA) was performed using a Bio-Rad Bio-Plex 200 reader according to manufacturer's instructions in a custom kit designed to detect multiple inflammatory markers (IL12p70, TNF α , RANTES, IL5, IL4, IL6, IL12p40, IL17, IL13, GMCSF, EOTAXIN, IL2 and KC). This multi-plex technology uses polystyrene beads internally dyed with differing ratios of two spectrally distinct fluorophores. Dyed beads are labeled with

antibodies for each marker and the antibody-conjugated beads are allowed to react with sample and a secondary antibody in a 96-well plate to form a capture sandwich immunoassay. The assay solution is read by a Bio-Plex array reader, which distinguishes the beads' spectral address. Using the amount of green fluorescence emitted by the phycoerythrin-tagged detection antibody, the array reader extrapolates the concentration to the appropriate standard curve.

Statistical Analysis—Behavioral data was analyzed using repeated measures ANOVA. Statistical analysis of biochemical data employed unpaired student T test to compare between minocycline treated and control 3xTg-AD mice with $P < 0.05$.

Results

We previously developed a transgenic mouse model of AD which develops both A β and tau pathologies in a hierarchical fashion, referred to as the 3xTg-AD mice [7]. In order to investigate how reducing the inflammatory system affected the accumulation of pathologies and resultant cognitive decline, we treated 8-month old 3xTg-AD and wild-type non-transgenic (nonTg) mice with minocycline in AIN17 chow (55 mg/kg/day; based on average weight of mice and average chow consumption), or AIN17 diet alone. This treatment with minocycline has been previously shown to significantly reduce microglial activation [35], and pro-inflammatory cytokine levels [45] in mice. Following 4-months of treatment mice were assessed on a battery of cognitive tasks, which test hippocampal, amygdala and cortical functioning – areas that are impacted by pathology in the 3xTg-AD mice. 3xTg-AD mice show spatial memory impairments from as young as 4-months of age [46], and demonstrate progressive cognitive deterioration as they age and pathology progresses. Hence, at the start of treatment at 8 months of age, these animals show significant impairments in learning and memory compared to nonTg animals [47].

Minocycline treatment restores hippocampus-, cortex- and amygdala- dependent learning and memory deficits in the 3xTg-AD mice

Morris Water Maze (MWM)—3xTg-AD and NonTg mice (which included minocycline treated and untreated groups; $n=8/\text{group}$) were tested on the Morris water maze (MWM), a spatial task that is highly dependent upon the hippocampus [48]. Treatment of mice with minocycline continued throughout the behavioral tasks. Mice were trained to meet the criterion (escape latency <25 s) in the spatial reference version of the MWM to find the location of a hidden platform [46]. Mice were subjected to 4 daily trials to locate the hidden platform in the water maze, and the mean escape latencies plotted on a day-to-day basis (Figure 1A). NonTg mice steadily improved and were able to acquire a mean escape latency of less than 25 seconds by the 5th day of training. 3xTg-AD mice, which have established intraneuronal accumulation of both A β and tau/hyperphosphorylated tau by this age, showed reduced acquisition and failed to reach escape criterion by day 5. NonTg mice treated with minocycline showed identical acquisition curves to untreated nonTg mice, and hence had significantly faster escape latencies than 3xTg-AD on days 3–5. Notably 3xTg-AD mice treated with minocycline performed at nonTg levels, reaching escape latencies of less than 25 seconds by day 5, showing that treatment with minocycline restored spatial acquisition to

nonTg levels. To ensure that all groups of animals started out equally we analyzed escape latencies from the first 2 trials on day one of training. No differences were seen between the groups (Figure 1B), indicating that all mice started out equally, but that their subsequent rates of spatial acquisition differed.

Spatial reference memory probe trials were conducted at 1.5-h and 24-h after the last training trial to examine short and long-term memory, respectively. NonTg mice and minocycline treated nonTg mice performed comparably on all trials including the latency to cross the platform location (Figure 1C), the number of platform crosses (Figure 1D), the time spent in the target quadrant (Figure 1E), and also the time spent in the opposite quadrant (Figure 1F). As expected treated and untreated nonTg mice had significantly reduced latencies to reach the platform location compared to untreated 3xTg-AD mice, and then crossed the platform location significantly more times than the untreated 3xTg-AD mice at both the 1.5 h and 24 h probes. 3xTg-AD mice treated with minocycline had similar latencies to reach the platform location as nonTg mice, but significantly faster latencies than untreated 3xTg-AD mice at both probes. Likewise, minocycline treated 3xTg-AD mice crossed the platform location significantly more times than untreated 3xTg-AD mice, and on par with the nonTg mice, at both probes. Untreated 3xTg-AD mice also spent less time in the target quadrant than nonTg and minocycline treated 3xTg-AD mice (Figure 1E), and more time in the opposite quadrant (Figure 1F). These results show that minocycline improved 3xTg-AD acquisition to nonTg levels and also restored both short- and long- term retention memory.

Novel Object Recognition—We next examined the effects of minocycline treatment in 3xTg-AD and nonTg mice on the primarily cortex dependent novel object recognition task (Aggleton et al., 1997; Wan et al., 1999). NonTg mice (minocycline treated and untreated) spent significantly more time with the novel object as compared to the familiar object at both 1.5 and 24 hours (Figure 1G). Minocycline treatment in nonTg mice also significantly improved their recognition index at 24 hours suggesting that minocycline treatment in nonTg mice might have some cortex dependent long-term memory benefits. Compared to nonTg mice, 3xTg-AD mice spent significantly less time with the novel object at both 1.5 and 24 hours. In contrast, minocycline treatment in 3xTg-AD mice restored these memory deficits to nonTg levels.

Contextual fear conditioning—Lastly, we evaluated the effects of minocycline treatment on contextual fear conditioning, an amygdala- and hippocampal-dependent task, using passive inhibitory avoidance test. NonTg mice tended to avoid the shock-associated dark compartment at both the 1.5- and 24h probes, which test for short- and long-term retention memory respectively, having latencies of ~120 sec before crossing over (Figure 1H). Treatment of nonTg mice with minocycline had no effect on their latencies to enter the shock associated dark compartment. Untreated 3xTg-AD mice, which show prominent pathology in both the hippocampus and the amygdala at this age, had a much weaker association with the fear conditioning induced by an electric shock in the dark compartment, and had significantly lower latencies to enter the shock associated dark compartment (~30 sec; Figure 1G) than nonTg mice. On the contrary, 3xTg-AD mice treated with minocycline

demonstrated the same strong association with fear conditioning as the nonTg mice, taking ~120 sec before crossing into the shock associated dark compartment (Figure 1H) on both short- and long- term memory probes. These results show that minocycline restores contextual learning on an amygdala- and hippocampal-dependent task in the 3xTg-AD mice.

These collective results show that minocycline treatment restores the cognitive deficits, induced by accumulation of both A β and tau pathologies in the 3xTg-AD mice, back to nonTg performance levels.

Inflammatory profiling of minocycline treated 3xTg-AD mice

Minocycline is known to have anti-inflammatory properties, by reducing microglial activation and a number of cytokines and chemokines. Analysis of several markers of inflammation showed a reduction in the astrocyte marker Glial fibrillary acidic protein (GFAP) and the microglial marker IBA1 (ionized calcium binding adaptor molecule 1) in the hippocampus of minocycline treated 3xTg-AD mice as compared to untreated transgenic mice (Figure 2A; representative images shown). However, western blot analyses of IBA1 revealed no significant changes in steady state levels in 3xTg-AD mice, compared to nonTg, or to 3xTg-AD mice treated with minocycline. although trends were seen. Further analysis revealed a significant reduction in steady state levels of GFAP in 3xTg-AD mice treated with minocycline (Figure 2B, C).

Using multiplex technology (BioPlex) we screened brain homogenates from treated and untreated 3xTg-AD and nonTg mice for inflammatory markers (Figure 3A–C). Compared to nonTg mice, 3xTg-AD mice exhibited increased levels of the proinflammatory TNF α as well as Interleukin-12 p70 (IL12p70), RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted or Chemokine (C-C motif) ligand 5 (CCL5)), Interleukin 5 (IL5) and Interleukin 4 (IL4) and showed decreased Eotaxin, KC (chemokine (CXC motif) ligand 1 (CXCL1)), Macrophage Inflammatory Protein 1 a (MIP1a) and Interleukin 2 (IL2) (Figure 3A). Minocycline treatment in 3xTg-AD mice resulted in altered inflammatory profile affecting cytokine and chemokine levels (Figure 3B). Notably minocycline treatment decreased levels of the pro-inflammatory cytokine TNF α , which is increased in AD [3] and mouse models of AD [49] and contributes to cognitive decline [50]. Minocycline treatment also decreased levels of RANTES/CCL5, a chemokine involved in recruiting T cells into sites of inflammation [51]. In contrast, minocycline treatment increased MIP1, Eotaxin and the CXCL1 chemokine KC, which has been previously shown to induce neuroprotection from A β *in vitro* [52]. Comparing the inflammatory profile in minocycline treated 3xTg-AD mice and untreated nonTg mice, reveals that minocycline treatment in 3xTg-AD mice restores their profile to levels close to nonTg (Figure 3C). Additionally, minocycline treatment of nonTg mice was also able to reduce inflammatory markers (Figure 3D), compared to untreated nonTg mice.

Minocycline reduces fibrillar A β levels

Brain homogenates from untreated and treated 3xTg-AD mice were assessed for A β levels by sandwich ELISA. No changes were seen with treatment in the detergent soluble fraction, in either A β 40 or the more amyloidogenic A β 42 (Figure 4A), but analysis of the

detergentinsoluble fraction revealed a statistically significant reduction in A β 40 levels in minocycline treated 3xTg-AD mice (Figure 4B). Insoluble A β 42 levels were unchanged with treatment. Light microscopy revealed A β -like immunoreactivity present in the cell bodies of the hippocampus and layer V of the cortex, in both minocycline treated and untreated 3xTg-AD mice, using antibody 6E10, which recognizes amino acids 1–16 of A β . Intraneuronal A β has been previously characterized in these mice [46, 53]. No extracellular deposits of A β were seen at this age. We have previously shown that cognitive, and synaptic, deficits coincide with the appearance of intraneuronal A β in these mice [7, 46], which occurs up to 12 months before extensive extracellular deposition. Similar findings have been shown in other mouse models of AD, in which cognitive decline is observed many months prior to extracellular plaque deposition [13].

A β is produced via sequential cleavage – firstly from its parent protein APP by BACE into a 99 amino acid membrane attached stub named C99, which is then further cut by the γ -secretase complex via regulated intramembraneous proteolysis to release A β . We looked for evidence of altered APP processing, but western blot analysis of APP, or C99 showed no differences with minocycline treatment (Figure 4E, F). Further analysis of the aggregation state of A β , using conformation-specific antibodies, revealed no differences in soluble oligomers (~50–150kDa species [54] with antibody A11, but a significant reduction in soluble fibrils (Figure 4G, H), in line with reduced A β in the detergent insoluble fraction (Figure 4B), using conformation specific antibody OC [54].

Minocycline has differential effects on phosphorylation of tau, despite reductions in p25

Tau pathology occurs as a downstream event of A β pathologies in the 3xTg-AD mice [7]. Initially, tau becomes mislocalized to the somatodendritic compartment of neurons soon after the appearance of intraneuronal A β [7]. Tau then becomes hyperphosphorylated by the actions of a number of kinases such as cdk5 and GSK3 β . Eventually hyperphosphorylated tau aggregates in the cytosol into NFT's. Recent evidence has highlighted the soluble species of tau being important for cognitive decline, rather than aggregated tau species [55]. We previously showed that increasing microglial activation via LPS could increase tau phosphorylation, via increases in the cdk5 co-activator p25 [8]. Therefore we were interested in whether the opposite was also true – could reducing endogenous inflammation lead to the reduction of p25 and hyperphosphorylated tau? Analysis of steady state levels of human tau in treated and untreated 3xTg-AD mice revealed no differences (Figure 5A, C). Light microscopy revealed somatodendritic accumulation of human tau in both untreated and treated 3xTg-AD mice in the hippocampus (Figure 5B). Using phospho-specific antibodies, we found no differences in tau hyperphosphorylated at S199/S202 (AT8), T231 (AT180), or T181 (AT270) (Figure 5A, C). We found a significant reduction in tau phosphorylated at T212/S214 (AT100), but also an increase in tau phosphorylated at S396/S400/T403/S404 (PHF-1). Notably, levels of p25 – the coactivator for the tau kinase cdk5 were decreased with minocycline treatment, and corresponded to an increase in its parent protein p35. These results show that minocycline, and reduced inflammation, were unable to prevent a broad range of tau phosphorylations, and even led to increases in the PHF-1 site, despite reductions in p25.

Discussion

These results show that minocycline treatment is able to restore cognitive deficits in 3xTg-AD mice, and alters inflammation and reduces the formation of A β fibrils. Notably, it does not have wide-ranging positive effects on tau pathology, despite reductions in p25. Cognitive deficits occur in these mice due to the accumulation of multiple pathological proteins implicated in human AD. We have previously shown that deficits are first evident with the appearance of intraneuronal A β , and that removal of this A β pool recovers cognition [46]. Cognition worsens over time, as A β accumulation increases and leads to the cytosolic accumulation of tau protein, which becomes progressively hyperphosphorylated [7]. Recent evidence from inducible tau overexpressing mice have demonstrated that it is the soluble species of tau/hyperphosphorylated tau which induce cognitive decline, rather than the insoluble NFT's [55], and in accordance with this both soluble A β and soluble tau need to be reduced in order to improve cognition 3xTg-AD mice [56].

Our results here show a robust restoration of cognition, so that cognitive abilities in the 3xTg-AD mice were equal with nonTg mice, with minocycline treatment, with only modest changes in pathological proteins. This suggests that minocycline is improving cognition through multiple mechanisms – through reductions in soluble and insoluble A β fibrils and changes in inflammatory mediators. Recently, a lot of focus has been put on aspects of an altered inflammatory response in AD/AD mice mediating cognitive decline [13, 57], while altered peripheral inflammatory markers can be used as a diagnosis for AD [58]. In particular, TNF α has been highlighted as being detrimental to cognition in AD [3], as it is pro-inflammatory but also regulates synaptic activity [59, 60]. Of note, A β oligomers no longer induce LTP deficits in hippocampal slices from TNF α null mice [61], and minocycline prevents A β oligomer induced LTP deficits in wild type mice [34]. These observations, coupled with the results presented here, suggest that perhaps minocycline treatment is able to prevent downstream events from A β oligomers that impair cognition – in other words that A β could act on microglia, which then release an LTP/cognition modulating factor, such as TNF α [34], which is counteracted in the presence of minocycline. TNF α has been shown to be upregulated in the 3xTg-AD brain from as early as 6 months of age [49] – a time point which also shows impaired LTP and synaptic deficits as well as impaired cognition [7, 46]. In our hands, minocycline treatment reduced TNF α levels as well as reducing GFAP, and several other cytokines and chemokines. This dampening of the inflammatory response is likely to have contributed to the cognitive recovery seen in minocycline treated mice and supports a role for the inflammatory response to AD pathology in mediating some of the cognitive decline, and for the possibility of anti-inflammatory therapeutics to alleviate some of the symptoms of the disease. Interestingly, we also observed an increase in KC, a ligand to the CXCR2 receptor, which has been suggested to provide neuroprotection against A β -induced cell death. Therefore this increase in KC could potentially also contribute to the cognitive amelioration seen in minocycline treated 3xTg-AD mice.

We found that minocycline also lowered levels of insoluble A β 40, as well as soluble fibrils. These findings are consistent with *in vitro* experiments showing that minocycline (and to a lesser extent tetracycline) could inhibit fibril formation directly [32]. Minocycline has also

been shown to decrease fibrillar A β associated with the vasculature in APP/Tg Sw-DI mice [36], consistent with a reduction in insoluble A β 40 shown here in the 3xTg-AD mice. Notably, we see a selective reduction in insoluble A β 40, but not A β 42. It is not clear how minocycline could affect A β 40, but not A β 42, but perhaps it is specifically inhibiting fibril formation from A β 40 resulting in both the reduction in insoluble A β 40 seen, as well reductions in soluble fibrils. Soluble fibrils are aggregates detected in the detergent soluble fraction with the conformation specific antibody OC [54], which does not differentiate between A β 40 and 42 but recognizes only fibrillar conformations. Recent evidence has shown that lower molecular weight oligomeric A β species mediate cognitive decline and LTP deficits, rather than the fibrillar species – indeed mouse models with enhanced fibrillogenesis have reduced oligomer levels and show cognitive improvements [62]. However, fibril formation is a keen inducer of microglial activation [63], which our previous results showed could lead to an increase in tau phosphorylation [8] due to an upregulation of p25. Given this, we were expecting a decrease in both p25 and tau phosphorylation with minocycline treatment as it is an anti-inflammatory agent. In keeping with our previous data we did observe a reduction in the tau kinase cdk5 co-activator p25, but this did not appear to be sufficient to reduce tau phosphorylation at a broad range of cdk5 phosphorylation sites, such as S199/S202 (AT8), although we did find a reduction in a single tau epitope phosphorylated at T212/S214 (AT100), which is a cdk5 site. These results suggest that the endogenous inflammatory response evoked by the presence of AD neuropathology in the 3xTg-AD mice does not significantly facilitate tau hyperphosphorylation, at least not in this age animal. However, it should be noted that the human tau produced in the 3xTg-AD mice contains a mutation at P301L, which is not found in human AD, and therefore may not be fully indicative of the relationship between inflammation and tau in human disease. Despite finding that reducing inflammation with minocycline only had modest effects on tau pathology, cognitive deficits were reversed by the treatment. It suggests that minocycline works mainly by preventing the downstream events from A β and tau pathology that mediate cognitive decline, which are likely to be inflammatory modulators such as TNF α . Although some reductions in A β were seen with treatment they were not in A β species usually associated with cognitive decline (i.e. A β oligomers, A β 42), which therefore makes it unlikely that these reductions were completely restoring cognition in these mice. Furthermore, as cognition was restored without impacting tau, it suggests that tau pathology does not contribute significantly to cognitive decline, at least at this age/stage of disease pathology.

Given our data showing that minocycline can reverse cognitive deficits in the 3xTg-AD, should minocycline be considered as a therapeutic agent for testing in humans? It has previously shown great promise in transgenic mouse models for a variety of neurodegenerative diseases such as ALS [64], Huntington's disease [25] and Parkinson's disease [65]. Despite these promising data *in vivo*, minocycline has been shown to worsen ALS patients in a clinical trial [27], but this could be due to the high dose of minocycline used in this trial (400mg). Other trials in Huntington's disease and Parkinson's disease have used 1–200mg/day, a dose that is tolerated. Initial trials in Huntington's disease, however, have shown promise with improvements in motor function and a slower rate of cognitive decline compared to placebo [66]. These trials ran for 6 months, and so it is unknown if

long-term treatment will result in side effects as longer treatments with minocycline have been shown to cause hyperpigmentation (1–5 years; [67]). It may be that minocycline could show clinical efficacy in AD, but perhaps at a lower dose and in combination with other disease modifying compounds. Given the data from animal models and acute treatment of slices with minocycline prior to LTP recordings, it suggests that minocycline should be effective within a short period of time on cognitive outcomes.

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Figure 1.

Behavioral analysis of primarily hippocampus, cortex and amygdala dependent memory in minocycline treated and untreated 3xTg-AD mice and age-matched non-transgenic mice (n=8/group). Morris water maze, a primarily hippocampus dependent task, is depicted in (A–F). 3xTg-AD mice perform significantly worse than non-transgenic mice starting day 3 on the acquisition curve. In contrast, minocycline treatment in 3xTg-AD mice improved their performance to non-transgenic level (A). (B) All groups of mice started off from the same baseline (average of trials 1 and 2 on day 1 of testing). Minocycline treatment

significantly improved the performance of 3xTg-AD mice on latency to platform location (C), number of platform crosses (D), time spent in target quadrant (E) and time spent in opposite quadrant (F). (G) Performance on novel object recognition, primarily dependent on the cortex, showed that minocycline treatment in 3xTg-AD mice improved both short and long term memory while minocycline treatment in non-transgenic mice had beneficial effects only at the 24-hour time point. (H) Examining the effects of minocycline treatment on the primarily amygdala dependent task passive inhibitory avoidance demonstrated a significant behavioral improvement in treated 3xTg-AD mice compared to untreated 3xTg-AD mice at both 1.5 and 24 hour retention tests. * indicated $P < 0.05$

Figure 2.

Astrocyte and microglia analysis in minocycline treated and untreated 3xTg-AD mice. (A) Immunostaining for astrocyte and microglial cells using GFAP and IBA1 respectively in the hippocampus. Both the intensity and the number of GFAP and IBA1+ cells are decreased with minocycline treatment. (B, C) Steady state levels of GFAP and IBA1 as compared to loading control in whole brain extracts (3x = 3xTg-AD, 3x M = 3xTg-AD treated with minocycline, N = non-transgenic). (C) Quantification of (B) normalized to loading control * indicated $P < 0.05$ for 3xTg-AD vs. 3xTg-AD treated with minocycline.

Figure 3.

Analysis of inflammatory markers using multiplex technology in minocycline treated 3xTg-AD and non-transgenic mice in whole brain extracts. (A) Significantly altered levels of inflammatory markers in 3xTg-AD mice as compared to non-transgenic controls. (B) Significantly altered levels of inflammatory markers in minocycline treated 3xTg-AD mice as compared to untreated transgenic mice. (C) Significantly altered levels of inflammatory markers in minocycline 3xTg-AD mice as compared to non-transgenic controls. (D)

Significantly altered levels of inflammatory markers in minocycline treated non-transgenic mice as compared to non-transgenic controls.

Figure 4.

Amyloid load and production analysis in minocycline treated and untreated 3xTg-AD mice. Soluble and insoluble Aβ₄₀ and Aβ₄₂ levels were measured in whole brain homogenates. (A) Soluble Aβ₄₀ and Aβ₄₂ levels remain unaltered following minocycline treatment. (B) Minocycline treatment decreased insoluble Aβ₄₀ while exerting no effect on insoluble Aβ₄₂. (C, D) Aβ-like immunoreactivity (6E10) in the cortex and hippocampus of control and minocycline treated 3xTg-AD mice (scale bar = 250 μm). No overt difference in Aβ-like immunoreactivity was observed following minocycline treatment. High magnification of

intraneuronal A β -like immunoreactivity shown in panel (scale bar = 62.5 μ m). Analysis of APP processing is depicted in (E, F). (E) Steady state levels of APP, C99, C83 and β -actin control in representative samples of minocycline treated and untreated mice. (F) Analysis of APP, C99 and C83 steady state levels in whole brain extracts revealed no change following minocycline treatment. Evaluation of soluble oligomers and fibrils revealed that while minocycline treatment the in 3xTg-AD mice resulted in no change in soluble oligomers (G), it reduced soluble fibril levels (H). * indicated P<0.05

Figure 5.

The effect of minocycline treatment in 3xTg-AD mice on tau. **(A)** Steady state levels of total tau, a number of phospho epitopes for tau and β -actin control in whole brain extracts are depicted in representative samples. **(B)** Analysis of the Western blot revealed that minocycline treatment resulted in decreased tau phosphorylated at S212/S214 (AT100) while increasing the levels of the PHF1 phospho epitope. **(C)** Immunohistochemical analysis of total human tau using HT7 in the cortex and hippocampus of control and minocycline treated 3xTg-AD mice. No overt difference in tau levels was observed following

minocycline treatment. **(D)** Steady state levels of p35 and p25 in whole brain extracts. Minocycline treatment in the 3xTg-AD mice resulted in a decrease in the cdk-5 kinase coactivator p25 while increasing p35 levels **(E)**. * indicated $P < 0.05$