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The N-terminal region of myelin basic protein reduces fibrillar amyloid- β deposition in Tg-5xFAD mice

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by extensive deposition of fibrillar amyloid β (A β) in the brain. Previously, myelin basic protein (MBP) was identified to be a potent inhibitor to A β fibril formation and this inhibitory activity was localized to the N-terminal residues 1–64, a fragment designated MBP1. Here we show that modest neuronal expression of a fusion protein of the biologically active MBP1 fragment and the enhanced green fluorescent protein (MBP1-EGFP) significantly improved the performance of spatial learning memory in Tg-5xFAD mice, a model of pathologic A β accumulation in brain. The levels of insoluble A β and fibrillar amyloid were significantly reduced in bigenic Tg-5xFAD/Tg-MBP1-EGFP mice. Quantitative stereological analysis revealed that the reduction in amyloid was due to a reduction in the size of fibrillar plaques, rather than a decrease in plaque numbers. The current findings support previous studies showing that MBP1 inhibits A β fibril formation in vitro, and demonstrate the ability of MBP1 to reduce A β pathology and improve behavioral performance.

Keywords

Alzheimer's disease; myelin basic protein; transgenic mice; amyloid β -protein

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Conflict of interest statement

None of the authors have a conflict of interest.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative condition and a major cause of dementia and death in developed countries. One of the prominent features of AD is the aggregation and extracellular deposition of amyloid β (A β) in the brain. A β is a proteolytic product generated from sequential cleavages of the amyloid precursor protein (A β PP) by β -secretase and γ -secretase (De Strooper et al., 1998; Edbauer et al., 2003; Vassar et al., 1999). A β has a tendency to self-assemble into a hierarchy of soluble oligomeric forms and fibrils that have been linked to synaptic dysfunction, neuroinflammation, and memory decline suggesting that they play an important role in the progression of AD (Haass and Selkoe, 2007; McLellan et al., 2003; Selkoe, 2008; Urbanc et al., 2002).

The aggregation status of A β *in vivo* is influenced by naturally occurring brain proteins, known as A β chaperones that bind to A β and modulate its assembly. Some of the more commonly known A β chaperones include α_1 -anti-chymotrypsin (Yamamoto et al., 2005), transthyretin (Buxbaum et al., 2008; Schwarzman and Goldgaber, 1996), clusterin (DeMattos et al., 2002b; Matsubara et al., 1995), proteoglycans (van Horssen et al., 2003) and the well-known apolipoprotein E (apoE) family (LaDu et al., 1994; Strittmatter et al., 1993). More specifically, the apoE2 and apoE3 isoforms disrupt A β fibril assembly, whereas apoE4 is reported to promote fibril formation (Aleshkov et al., 1997; Castano et al., 1995; Wisniewski et al., 1994). Studies using transgenic mice have demonstrated that by modulating the levels of these A β chaperones, A β deposition can be delayed or cleared (Holtzman, 2001).

Previously, we showed that myelin basic protein (MBP) has a strong affinity to bind A β and can potentially inhibit A β fibril assembly *in vitro* (Hoos et al., 2009). Encoded by the Golli-MBP gene and expressed by oligodendrocytes and neurons in the brain, MBP is one of the major structural proteins of the myelin sheaths. In addition, recent studies have implicated Golli-MBP proteins as intracellular multifunctional scaffolds that can bind a number of intracellular proteins and small molecule ligands affecting diverse cellular processes (reviewed in (Boggs, 2006)). Expression of MBP isoforms is developmentally regulated, with the 18.5 kDa species being the most prevalent isoform in mature human brain (de Ferra et al., 1985; Harauz et al., 2004). While endogenous MBP proteins possess numerous post-translational modifications to give rise to various charged isomers, the use of recombinant MBP suggested the inhibition of A β fibril assembly was independent of these modifications (Liao et al., 2010). Further, using deletion mutants it was shown that the N-terminal 1–64 amino acids of MBP (MBP1), harbored the A β interacting site and is capable of inhibiting A β fibril assembly (Liao et al., 2010). In addition, synthetic MBP1 protected cultured primary rat neurons from the cytotoxic effects of A β (Liao et al., 2010).

In the present study, we aimed to investigate whether the biologically active fragment MBP1 could influence A β assembly *in vivo*. To address this we generated a novel transgenic mouse model that modestly expressed the active MBP1 fragment as a fusion protein with enhanced green fluorescent protein, MBP1-EGFP. To study the effect of MBP1-EGFP, the Tg-MBP1-EGFP mice were crossed to Tg-5xFAD mice, a model of early-onset AD-like parenchymal amyloid pathology and cognitive impairment (Oakley et al., 2006). In bigenic Tg-MBP1-

EGFP/Tg-5xFAD mice, we observed improved spatial memory performance accompanied by a reduction in fibrillar amyloid. These findings indicate that MBP1, even at relatively low levels, reduced fibrillar A β pathology and improved cognitive performance *in vivo* and suggest that MBP and derived active fragments could provide novel therapeutic agents for AD and related disorders.

2. Methods

2.1 Recombinant MBP1-EGFP expression and purification

MBP1–64 (MBP1) cDNA was PCR amplified with primers containing the XhoI 5'-linker and KpnI 3'-linker from pcDNA3.1 vector containing full length MBP. The PCR product was digested with XhoI and KpnI and subcloned into pEGFP-N1 (Clontech) in frame with EGFP. Using MBP1/pEGFP as template, the region MBP1-EGFP was PCR amplified. The PCR product was cleaned and subcloned into pProEx HT (Invitrogen), between the restriction sites EcoRI and SpeI. MBP1-EGFP/pProEx HT was transformed into competent BL21 DE3 with heat shock. The cells were grown in LB with ampicillin at 37°C until absorbance of 600 nm reached 0.5 then induced with 0.5 mM IPTG for 3 hours at 37°C. Cells were sonicated and cracked by French press in Ni-EQ buffer (5 mM imidazol, 20 mM Tris, 500 mM NaCl, 6 M urea, pH 7.9). After centrifugation, supernatant was applied to HisBind resin (Invitrogen), washed with Ni-EQ containing 20 mM imidazole and eluted with Ni-EQ containing 1 M imidazole. Eluent from Ni column was dialyzed against CM-EQ buffer (80 mM glycine, 6 M urea, pH9.0) then loaded on Whatman CM52 column equilibrated with CM-EQ, washed and eluted with CM-EQ containing 0.2 M NaCl. Eluent was dialyzed against with water, lyophilized and stored in –80°C until use. The expression and purification of recombinant MBP1 peptide was performed as described (Kotarba et al., 2013).

2.2 Thioflavin T Fluorescence Assay

A β 40 with Dutch and Iowa mutation (A β 40DI) was synthesized on an ABI 430A solid phase peptide synthesizer (Applied Biosystems, Foster City, CA) and purified as previously described (Burdick et al., 1992). A β peptide was prepared in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), flash frozen, lyophilized and stored in –80°C. Stock solution of A β was dissolved in DMSO to 2.5mM. Lyophilized MBP1 or MBP1-EGFP was dissolved in PBS and concentration was determined by BCA assay (Thermo). A β stock was diluted to 12.5 μ M with PBS and rocked at 37°C with or without the incubation of 1.56 μ M MBP or MBP1-EGFP. At each time point, 100 μ l of each sample was removed and loaded in triplicate onto 96-well microplate. Thioflavin T (Sigma-Aldrich) was dissolved in water at a concentration of 10 mM and diluted 1:100 again with water before use. From the diluted 0.1 mM thioflavin T solution, 10 μ l was taken to add to each well. Plate was mixed on plate shaker followed by 10 min incubation in the dark before reading. Fluorescence was measured at 490 nm using an excitation wavelength of 446 nm in a SpectraMax spectrofluorometer (Molecular Devices) by SoftMax Pro control software.

2.3 Animals

All work with mice followed National Institutes of Health guidelines and was approved by the Stony Brook University Institutional Animal Care and Use Committee. Tg-5xFAD mice were obtained from Jackson Laboratories. Tg-5xFAD mice co-express human A β PP and human presenilin 1 with five familial AD mutations (A β PP K670N/M671L + I716V + V717I and PS1 M146L + L286V) and develop early-onset A β accumulation and fibrillar A β plaques in the brain starting at about two months of age (Oakley et al., 2006). Tg-MBP1-EGFP mice, generated as described below, were maintained as homozygous. The two transgenic lines were crossed for over two generations before the het/het mice used in this study were generated. Twenty mice of each group with equal numbers from both sexes were tested in behavioral measures prior to brain tissue collection.

2.4 Generation of Tg-MBP1-EGFP Mice

MBP1–64 (MBP1) cDNA was PCR amplified with primers containing the XhoI 5'-linker and KpnI 3'-linker from pcDNA3.1 vector containing full length MBP. The PCR product was digested with XhoI and KpnI and subcloned into pEGFP-N1 in frame with EGFP (Clontech). Sequenced clone of MBP1/pEGFP-N1 was used as template for PCR amplification at the MBP1-EGFP region using primers containing XhoI 5'-linker and NheI 3'-linker. The amplified MBP1-EGFP cDNA, without a signal peptide leader sequence, was digested and subcloned into exon 2 of the mouse Thy1.2 expression cassette between *XhoI* and *NheI*. The 6.5kb Thy1.2 cassette lacks exon 3 and its flanking introns required for expression in non-neural cells and limited the construct expression in neural cells (Caroni, 1997; Vidal et al., 1990). The transgene was released by *EcoRI*/*PvuII* digestion, purified and sent to the transgenic mouse facility at University of California in Irvine where it was microinjected into CB6 (Balbc x BL6) embryos. The founder transgenic mice were identified by Southern blot analysis of tail DNA. Transgenic offspring were determined by PCR analysis of tail DNA using the forward primer in the MBP region 5'–CACGGATCCAAGTACCTGGC–3' and reverse primer in the EGFP region 5'–CGCTCCTGGACGTAGCCTTCG–3', generating a about 500-bp product in positive offspring.

2.5 Behavioral Analysis

Barnes maze testing was performed as described (Xu et al., 2014). Briefly, the Barnes maze apparatus consisted of a sealed wooden disk 91 cm in diameter, elevated 75 cm off the floor, with eight escape holes, spaced 24.5 cm apart. An escape box measuring 10 cm×8.5 cm×4 cm was used. Visible distal cues were placed around the room and remained constant throughout each day of testing. An observer sat 1.5 m away from the maze during trials. At the start of each trial, each mouse was picked up from their home cages by the base of their tail and placed onto the center of the maze. A timer was then started and the mouse was allowed a maximum of 5 min to find a randomized designated hole and escape box. When the mouse entered the box, the timer was stopped and they were given 1 min inside the box before being transferred back to the home cage. If the mouse did not find the escape hole within the 5 min limit, they were picked up by the base of their tail and placed into the hole

and left there for 1 minute. Each mouse was given 2 trials per day with a 15 min ITI. Testing spanned 5 consecutive days.

2.6 Brain Tissue Preparation

After behavioral testing was completed the mice were euthanized with 2.5% avertin. Brains were perfused with PBS and bisected along the mid-sagittal plane. One hemisphere was fixed in 70% ethanol and embedded in paraffin or alternatively fixed in 4% paraformaldehyde and embedded in OCT for immunohistological analysis. The other hemisphere was stored at -80°C for use in ELISA and immunoblotting studies.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA) of $\text{A}\beta_{40}$ and $\text{A}\beta_{42}$

Extractions of different pools of $\text{A}\beta$ and procedures of sandwich $\text{A}\beta$ ELISA were described in (Ou-Yang and Van Nostrand, 2013). Briefly, pulverized brain aliquots were extracted sequentially with Tris-buffer, Tris-buffer with 1% Triton X-100 and 5 M guanidine-HCl for soluble, membrane and insoluble fractions. Each fraction was subjected to sandwich ELISAs where $\text{A}\beta_{40}$ and $\text{A}\beta_{42}$ were captured using their respective carboxyl-terminal specific antibodies m2G3 and m21F12 and biotinylated m3D6, specific for human $\text{A}\beta$, was used for detection (DeMattos et al., 2002a). To determine the relative levels of oligomeric $\text{A}\beta$, soluble TBS extracts prepared from above were used in sandwich ELISA that uses m3D6 to capture and biotinylated m3D6 to detect $\text{A}\beta$ oligomers larger than dimers (DeMattos et al., 2002a; Xu et al., 2014; Yang et al., 2013). Each brain lysate fraction was measured in triplicate and compared to linear standard curves generated with known concentrations of human $\text{A}\beta$ using a Spectramax M2 plate reader (Molecular Devices).

2.8 Quantitative Immunoblotting

Pulverized brain aliquots extracted with TBS containing 1% Triton X-100 was used for immunoblot. Equal amount of total protein was separated on SDS-PAGE or 16% Tricine (Invitrogen) for $\text{A}\beta\text{PP}$ C-terminal fragments (CTF). Gel was transferred onto nitrocellulose membrane (Amersham Hybond-ECL). Membrane was blocked with 5% non-fat milk and incubated overnight at 4°C with anti-human $\text{A}\beta\text{PP}$ antibody (mouse mAb, P2-1, 1:1000), anti- $\text{A}\beta\text{PP}$ CTF (rabbit pAb, 1:1000), anti-MBP (rabbit pAb, 61B 1:1000), anti-neurospecific β -tubulin (Abcam ab18207, 1:2000). Horse-radish peroxidase conjugated secondary antibodies (Amersham) were used for detection at 1:5000 dilution. Membrane was developed using ECL (Pierce) and chemiluminescence signals were quantitated with VersaDoc (BioRad Model 3000). Chemiluminescence signals were normalized to that of the internal control and presented as percentage to the level of Tg-5xFAD.

2.9 Dot Blot Analysis of Soluble $\text{A}\beta$ Oligomers

Ten μg of total protein from TBS soluble fraction of each brain lysates was loaded on wet nitrocellulose membrane using a bio-dot apparatus (Bio-Rad). The membrane was then blocked in 5% milk/PBST and incubated with OC antibody (a gift from Dr. Charles Glabe, UC Irvine, rabbit pAb, 1:5000) overnight at 4°C . Horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham) was used for detection at 1:5000 dilution. The

membrane was developed using ECL (Pierce) and chemiluminescence signals were quantitated with VersaDoc (BioRad Model 3000).

2.10 Immunohistochemical Analysis

10 μ m paraffin sections or 20 μ m frozen sections were blocked in SuperBlock blocking buffer (Thermo #37515) and incubated with primary antibodies at 4°C overnight. Antibodies, chemicals and working dilutions used for this study are listed below: anti-A β 1–28 (rabbit pAb, 1:500), rat anti-MBP (AbD serotec MCA408S, 1:200), NeuN (Millipore MAB377, 1:1000), OC (rabbit pAb, 1:1000), Nissl stain (Life Technologies, N-21482). For intraneuronal A β staining, sections were treated by 88% formic acid for 15 min at room temperature prior to incubation with OC antibody. For immunofluorescence, Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 350 (Life Technologies) were used for secondary antibodies. Biotinylated secondary antibodies (Vector laboratories) followed by Vectastain ABC kit (Vector laboratories) were used for DAB staining.

2.11 Stereological Analysis

From the library containing a hundred continuous sections for each brain, every tenth section was selected for A β immunolabeling or thioflavin S staining. The images of cortex, subiculum, hippocampus and thalamus area were obtained under 4x magnification and analyzed using ImageJ to measure the area of positive staining. For plaque size and plaque density, comparable sections were labeled for A β or thioflavin S and 15–20 frames in the cortex were randomly selected. The size of the plaques in the frames was measured using Stereologer (systems planning and analysis, INC.). For measuring area fraction of intraneuronal A β , comparable sections were stained for intraneuronal A β using OC antibody as described above and counter-stained with Nissl (Life Technologies N-21482). Images were collected across cortex under high magnification. Neurons containing intracellular A β and were devoid of extracellular A β plaques were selected. Intracellular A β were measured as area fraction to the neuron. The final area fraction of intraneuronal A β was an average from 35–45 neurons.

2.12 Statistical Analysis

Overall differences on ten trials in Barnes maze were analyzed by repeated measures ANOVA. Single day performances between Tg-5xFAD and Tg-5xFAD/Tg-MBP1-EGFP mice were compared using Student's t test. A significance level was taken at p value less than 0.05. Error bars represent standard error of mean (SEM).

3. Results

3.1 Purified recombinant MBP1-EGFP inhibits A β fibrillar assembly

Previously, we showed that purified recombinant MBP1 inhibited A β fibrillar assembly in a comparable manner as purified intact MBP (Liao et al., 2010). Here we expressed, purified and tested recombinant MBP1 and MBP1 with EGFP attached to its C-terminus (MBP1-EGFP). As shown in Fig. 1A, purified MBP1 migrated at $\approx$ 9 kDa on SDS-PAGE whereas MBP1-EGFP migrated at $\approx$ 32 kDa due to the presence of the C-terminal EGFP moiety. We next tested the ability of purified MBP1-EGFP to inhibit A β fibrillar assembly using a

thioflavin T fluorescence assay (Kotarba et al., 2013). Fig. 1B shows that A β rapidly assembles into fibrils evident by the sharp increase in thioflavin T fluorescence. On the other hand, the presence of a substoichiometric concentration of purified MBP1 blocked A β fibrillar assembly as reported earlier (Liao et al., 2010). Similarly, the same substoichiometric concentration of purified MBP1-EGFP comparably inhibited A β fibrillar assembly. These findings indicate that the presence of the EGFP moiety at the C-terminus of MBP1 does not interfere with its ability to inhibit A β fibrillar assembly.

3.2 Transgenic expression of MBP1-EGFP in a majority of cortical and hippocampal neurons

The goal of the present study was to investigate the effect of MBP1 on brain fibrillar amyloid deposition and downstream consequences in transgenic mice. Therefore, we generated transgenic mice that express MBP1-EGFP in brain under control of the mouse Thy1.2 promoter, which has been widely used for neuronal specific expression (Caroni, 1997). Overall protein expression of MBP1-EGFP was confirmed by robust EGFP fluorescence (Fig. 2E) and detected by strong immunostaining using a rat anti-human MBP antibody (Fig. 2F) compared to age matched wild-type controls (Fig. 2A, B). Under higher magnification, MBP1-EGFP expression was detected in cell body and neurites in the cortex (Fig. 2G) and CA1 of hippocampus (Fig. 2H) but not in wild-type mice (Fig 2C, D). To confirm that MBP1-EGFP expression was neuronal specific, we evaluated EGFP fluorescence (Fig. 2I, M), immunolabeling for MBP1 (Fig. 2J, N) and immunolabeling for NeuN (Fig. 2K, O), a marker for neurons. In the merged images MBP1-EGFP expression is clearly seen in cell bodies and neurites of most of NeuN labeled neurons in cortex (Fig. 2L) and in the CA1 region of the hippocampus (Fig. 2P). It was estimated that $\approx$ 80–90% of the cortical and hippocampal neurons expressed MBP1-EGFP. Interestingly, little, if any, MBP1-EGFP protein was detected in cerebrospinal fluid suggesting that it was largely retained within the neurons (data not shown).

To investigate the cellular distribution of MBP1-EGFP, pulverized brains were sequentially extracted with Tris-buffer to obtain a soluble brain fraction followed by Tris Buffer containing 1% triton X-100 to obtain a membrane fraction. In contrast to endogenous MBP, which was predominantly present in the membrane fraction, MBP1-EGFP was largely present in the soluble fraction (Fig. 3A). This was consistent to the primarily cytoplasmic staining for MBP1-EGFP in Figure 2. Based on the immunoblot shown in Fig. 3A, neuronal expression of MBP1-EGFP did not seem to interfere with the levels of endogenous mouse MBP. Quantitative immunoblot analyses revealed that the level of MBP1-EGFP was modest and estimated to be $\approx$ 20% of the endogenous mouse MBP (14kDa + 18.5kDa) (Fig. 3B). The level of protein expression of MBP1-EGFP and its distribution between soluble/detergent-soluble fractions did not change with age (data not shown). Together, these findings indicate that MBP1-EGFP is widely, but modestly, expressed and largely retained in cortical and hippocampal neurons, primarily in a soluble form, in Tg-MBP1-EGFP mice.

3.3 Bigenic Tg-5xFAD/Tg-MBP1-EGFP mice show improved spatial memory performance

Previously, Tg-5xFAD mice were shown to exhibit impaired spatial learning in the Barnes maze task at six months old (Xu et al., 2014). To determine whether MBP1-EGFP

expression affected spatial learning, age matched six-month old wild-type, Tg-MBP1-EGFP, Tg-5xFAD and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice were tested in performance on the Barnes maze. Prior to spatial learning testing examination of all of the mice at this age showed that they had comparable motor activity and physical strength (data not shown). The latencies of mice finding the escape hole were plotted against the trials (Fig. 4). All of the mice started between 2 to 3 minutes on the first two trials. Wild-type mice showed decreased latencies over the testing period and completed the task in ≈ 20 seconds at the last trial. Tg-MBP1-EGFP mice showed a similar learning curve to wild-type mice, suggesting that transgene expression of MBP1-EGFP did not affect the spatial memory. On the other hand, Tg-5xFAD mice showed little improvement after five days evident by the nearly flat learning curve. However, the learning curve of bigenic Tg-5xFAD/Tg-MBP1-EGFP mice suggested an improvement in spatial memory in Tg-5xFAD mice when MBP1-EGFP was expressed. In particular, the latencies of bigenic Tg-5xFAD/Tg-MBP1-EGFP mice were significantly shorter than that of Tg-5xFAD on the 8th ($p = 0.031$) and 10th trial ($p = 0.024$).

3.4 Insoluble A β was significantly reduced in bigenic Tg-5xFAD/Tg-MBP1-EGFP mice

After the behavioral testing was completed, the mice were euthanized and brains were collected for biochemical and pathological analyses. To measure brain A β , aliquots of pulverized forebrain were sequentially extracted with TBS, TBS with 1% triton X-100 and guanidine to obtain soluble, membrane and insoluble fractions, respectively, and analyzed by ELISA. While there was no change in the levels of soluble and membrane A β , there was a significant 25% reduction in insoluble A β_{40} and A β_{42} in bigenic Tg-5xFAD/Tg-MBP1-EGFP mice compared to Tg-5xFAD mice (Fig. 5A–C).

Since soluble A β oligomers have been implicated as an important toxic species (Lesne et al., 2006; Selkoe, 2008; Shankar et al., 2008; Tomic et al., 2009), we further investigated whether there was a change in the levels of A β oligomers in the soluble fraction of the brain lysates using two complimentary approaches. First, we used a sandwich ELISA format detect all forms of oligomers larger than monomer in soluble brain fraction (DeMattos et al., 2002b; Landry et al., 1996; Xu et al., 2014; Yang et al., 2013). Second, we performed quantitative dot blot analysis using the conformational OC antibody that recognizes a common fibrillar epitope in fibrils and fibrillar oligomers (Kayed et al., 2007). In both cases, we observed no differences in the signals between Tg-5xFAD mice and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice (Fig. 5D–F). Together, these findings suggest that although the presence of MBP1-EGFP reduced insoluble A β levels in Tg-5xFAD mice it had no effect on total soluble A β or soluble oligomeric A β levels.

3.5 Presence of MBP1-EGFP does not alter A β PP processing

To determine whether the reduction of insoluble A β in the presence of MBP1-EGFP was the result of a change in expression or processing of human A β PP in Tg-5xFAD mice we measured the levels of human A β PP and its processing fragments in Tg-5xFAD mice and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice. As shown in Fig. 6, although the levels of human A β PP trended to be lower in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice this was not statistically significant ($p = 0.11$). However, the levels of C99 (the C-terminal fragment of β secretase processing) and C83 (the C-terminal fragment of α secretase processing) were

essentially the same in the Tg-5xFAD mice in the presence or absence of MBP1-EGFP suggesting that A β PP processing was unaffected.

3.6 Analysis of intracellular and extracellular A β accumulation in Tg-5xFAD mice and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice

Since we found a decrease in the amount of insoluble A β in Tg-5xFAD mice in the presence of MBP1-EGFP (Fig. 5C) we next determined if there was a compartmental change in A β accumulation and deposition in the bigenic mice. The majority of MBP1-EGFP appeared to be retained within neurons in soluble form (Figs. 2 and 3) suggesting that it may interact with intracellular A β . Therefore, we labeled intraneuronal oligomeric A β using the OC antibody (Kayed et al., 2007) along with Nissl staining to visualize the neurons. In addition to extracellular deposited A β , punctate intraneuronal A β labeling can be observed in both Tg-5xFAD mice and Tg-5xFAD/Tg-MBP1EGFP mice. Neurons that were devoid of extracellular plaques were individually selected and the percent area fractions of intracellular A β were measured revealing no difference between Tg-5xFAD mice and Tg-5xFAD/Tg-MBP1-EGFP mice (data not shown).

We next evaluated the gross neuroanatomical distribution of extracellular A β and fibrillar amyloid deposition in the mice. The overall pattern of total immunolabeled A β (Fig. 7A, C) and fibrillar A β stained by thioflavin S (Fig. 7B, D) were very similar between Tg-5xFAD mice and Tg-5xFAD/Tg-MBP1-EGFP mice with numerous deposits seen throughout the cortex and in the subiculum, hippocampal and thalamic regions. However, under higher magnification, it was obvious that for comparably sized A β plaques, the thioflavin S-positive cores were smaller in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice (Fig. 8D–F) than in Tg-5xFAD mice (Fig. 8A–C). Based on this observation we next sought to measure this apparent change in thioflavin S staining using quantitative image analysis. No significant difference in the area of A β immunostaining was observed in different brain regions between the two groups of mice (Fig. 8G). On the other hand, the thioflavin S-staining area was reduced 50% in the cortex and hippocampus and 30% in the subiculum and thalamus of Tg-5xFAD/Tg-MBP1-EGFP mice compared to the Tg-5xFAD mice (Fig. 8H). To further understand this reduction in the observed thioflavin S-staining we then measured the numbers and the size of thioflavin S-positive plaques in the cortex using quantitative stereological methods. While there was no significant change in the numbers of thioflavin S-positive plaques between the mice (Fig. 8I), the size of the thioflavin S-positive plaques in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice was $\approx$ 50% smaller than those seen in the Tg-5xFAD mice (Fig. 8J). Together, these results suggest that the expression of MBP1-EGFP in the bigenic mice does not reduce the numbers of A β plaques in the brain but rather reduces the amount of fibrillar amyloid that is present in the plaques.

4. Discussion

The loss of myelin and degradation of MBP are correlated with AD risk factors (e.g. aging, apoE4, traumatic brain injury) and increased levels of A β peptides in humans and mouse models (Bartzokis et al., 2006; Bartzokis et al., 2007; Bartzokis et al., 2004; Liu et al., 2006; Marnier et al., 2003; Roher et al., 2002). Previous work from our laboratory demonstrated that MBP has a strong affinity for A β and is a potent inhibitor of A β fibril formation *in vitro*

(Hoos et al., 2007; Hoos et al., 2009). Further, the region responsible for this activity was localized to the N-terminal residues 1–64 of MBP, a fragment termed MBP1 (Liao et al., 2010). However, direct evidence for a role of this region in MBP in regulating brain amyloid pathology *in vivo* has been lacking. To address this issue, we generated a novel mouse model designated Tg-MBP1-EGFP, which expresses the biologically active MBP1 fragment fused to EGFP at its C-terminus, in order to breed to human A β PP transgenic mice that develop amyloid pathology. A neuronal promoter was chosen in order to express MBP1-EGFP in the same cellular origin where A β is generated. Further, although MBP is largely expressed in oligodendrocytes certain Golli-MBP proteins are expressed in other cell types including neurons (Landry et al., 1996; Landry et al., 1998). Based on immunoblotting analysis, we showed that the transgenic expression of MBP1-EGFP was modest and did not alter the levels of endogenous mouse MBP. Additionally, expression of neuronal MBP1-EGFP did not appear to affect myelination in brain (data not shown). Finally, behavioral testing of Tg-MBP1-EGFP mice revealed overall activity, motor coordination and physical strength performance that were comparable to age-matched, non-transgenic wild-type mice. Thus, modest neuronal expression of MBP1-EGFP appeared to have no overt consequences on the health or function of the mice.

In the present study, we found that the expression of MBP1-EGFP significantly improved the behavioral performance of Tg-5xFAD mice in a task of spatial learning memory (Fig. 4). To determine if the improvement in behavioral performance was correlated with changes in the levels of A β peptides we performed ELISA analysis. The soluble and membrane associated pools of A β ₄₀ and A β ₄₂ were unaffected by the expression of MBP1-EGFP. Previous studies have reported that the levels of soluble A β oligomers correlate with memory deficits (Lesne et al., 2006; Selkoe, 2008; Shankar et al., 2008; Tomic et al., 2009). However, our analysis of soluble A β oligomers revealed no significant change in the presence of MBP1-EGFP expression (Fig. 5C–F). Based on our earlier structural data, we found that the MBP1 fragment still allows the formation of low MW oligomers with a height of ~2 nm but appears to prevent their stacking and further elongation into proto-fibrils and fibrils (Liao et al., 2010). We also demonstrated that MBP1 blocked the cytotoxic effects of A β ₄₂ in cultured primary neurons (Liao et al., 2010). Based on these findings, it is possible that MBP1 binds LMW A β oligomers causing a loss of their pathogenic nature or reduced toxicity that resulted in the improvement in the cognitive performance of the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice. Such a mechanism is supported by studies showing resveratrol, a natural A β assembly inhibitor, attenuated A β cytotoxicity in human neuroblastoma cells without preventing A β oligomer formation (Feng et al., 2009).

Consistent with the fact that MBP1 is a potent inhibitor of A β fibril assembly, we observed a significant reduction in fibrillar A β in brain evident by ELISA and thioflavin S staining in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice (Fig. 4 and Fig. 8). Previous studies have shown that fibrillar assemblies of A β are toxic to neuronal and cerebral vascular cells, can activate complement, and can stimulate potent neuroinflammatory responses (Rogers et al., 1992; Van Nostrand et al., 1998; Urbanc et al., 2002; Van Nostrand and Melchor, 2001; Floden et al., 2005; Xu et al., 2014). Along these lines, thioflavin S-positive plaques have been shown to be a source of reactive oxygen species that can promote oxidative damage in

AD (McLellan et al., 2003). More recent *in vivo* imaging studies in a mouse model of AD showed fibrillar amyloid plaques, but not soluble oligomers, significantly increased oxidative stress in surrounding neurites, which was positively correlated to the size of amyloid plaques (Xie et al., 2013). This link between oxidative stress and fibrillar plaques demonstrated in these studies suggests a possible cause for the improved behavioral performance in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice, which exhibited a 50% reduction in thioflavin-S positive plaque load.

A shortcoming of the Tg-MBP1-EGFP mice is that, with no signal peptide, the expressed MBP1-EGFP protein is largely retained within the neurons as demonstrated by immunolabeling (Fig. 2) and our inability to detect MBP1-EGFP in cerebrospinal fluid of the mice and nominal amounts of the protein in the media of cultured primary Tg-MBP1-EGFP neurons (data not shown). Since A β is largely released by neurons into the extracellular space where it assembles and deposits as fibrils the paucity of MBP1-EGFP in this environment diminishes its opportunity to interfere with the fibril assembly process. Although several studies have documented dynamic relationships between the intracellular and extracellular A β pools and that extracellular amyloid deposition can be changed through targeting intracellular A β (Oddo et al., 2004; Billings et al., 2005; Hu et al., 2009), we did not find differences in the levels of intracellular A β in the presence of MBP1 (data not shown). While we failed to detect any differences in intracellular oligomeric A β levels, the ability of MBP1 to cap A β oligomers, perhaps even intracellularly, could account for the observed reduction in extracellular fibrillar A β . Yet despite the largely intracellular retention and modest expression of MBP1-EGFP, the fact that there was a significant reduction in fibrillar amyloid deposition in the bigenic Tg-5xFAD/Tg-MBP1-EGFP mice (Fig. 8) suggests we might see a more dramatic effect on fibrillar amyloid assembly and deposition if MBP1-EGFP was present in the extracellular space and at higher levels.

Interestingly, a recent study showed a striking 70% reduction in fibrillar amyloid plaques in brain when a fusion protein of cholera toxin B subunit and full-length MBP (CTB-MBP) was orally delivered in a different A β PP transgenic mouse model (Kohli et al., 2013). In addition to its fibril assembly inhibiting activity, full length MBP also possesses proteinase activity that can degrade both soluble and fibrillar A β (Liao et al., 2009). In delivery of CTB-MBP to the brain, the authors speculated that the proteinase activity of MBP was likely responsible for the large reduction in amyloid deposition (Kohli et al., 2013). Based on our earlier *in vitro* work and the present study the strong effect of CTB-MBP on fibrillar amyloid deposition could result from a combination of the proteinase and fibril assembly inhibiting activities of intact MBP. Since MBP1 is devoid of this A β -degrading activity (Liao et al., 2010), it is possible that retaining this function could produce a more robust effect on reducing amyloid deposition. In future studies it will be interesting to determine if oral delivery of CTB-MBP1, resulting in higher extracellular levels of the protein, is more effective than the modest transgenic expression of MBP1 in the current model. In addition, comparisons of CTB oral delivery of native MBP, mutated MBP devoid of proteinase activity and mutated MBP devoid of fibril inhibiting activity could be performed to determine the contributions of each activity to the reduction in fibrillar amyloid deposition.

In conclusion, we have shown that modest neuronal expression of MBP1-EGFP significantly decreased fibrillar A β deposition and improved the spatial learning memory performance in Tg-5xFAD mice. Our study suggests that the N-terminal domain of MBP, and likely intact MBP, can indeed regulate amyloid pathology *in vivo* when it is soluble. While under normal conditions the majority of MBP is membrane imbedded in myelin sheaths, previous studies have shown increased MBP fragments in cerebrospinal upon brain injury and during progression of diseases suggesting opportunities for MBP and A β to interact and the potential importance of MBP in the pathology of AD (Landry et al., 1996; Landry et al., 1998; Beems et al., 2003). Future studies will be aimed at further evaluating the efficacy of MBP and extracellular MBP fragments as potential therapeutic interventions for amyloid assembly and deposition in AD and possibly other amyloid-depositing disorders.

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Highlights

- Transgenic mice express N-terminal fragment of myelin basic protein (MBP1) in brain
- Modest expression of MBP1 improved the behavioral performance of Tg-5xFAD mice
- Modest expression of MBP1 reduced fibrillar amyloid deposition in Tg-5xFAD mice
- Fragments of MBP could provide novel therapeutic agents against amyloid assembly

Fig. 1. Purified recombinant MBP1-EGFP inhibits A β assembly

(A) Recombinant MBP1 and MBP1-EGFP were expressed and purified and then aliquots were analyzed immunoblotting (B) A β was treated with HFIP, resuspended to a concentration of 2.5 mM in DMSO, and then diluted to a concentration of 12.5 μ M in PBS in the absence (white squares) or presence of 1.56 μ M MBP1 (black circles) or 1.56 μ M MBP1-EGFP (black squares). Samples were analyzed by thioflavin T binding and fluorescence to determine fibrillar assembly. Data are the mean $\pm$ S.D. of triplicate samples.

Fig. 2. MBP1-EGFP expression in neuron soma and neurites

Brains of Tg-MBP1-EGFP mice showed globally strong endogenous green fluorescence (**E**) and MBP1 immunostaining (**F**) compared to wild-type mice (**A, B**). MBP1-EGFP expression was detected in the cell bodies and neurites (as designated by the arrows) in the cortex (**G**) and CA1 of hippocampus (**H**) but not in the wild-type mice (**C, D**). MBP1-EGFP co-localized with neuronal NeuN immunolabeling in cortex (**I–L**) and CA1 region (**M–P**). Endogenous green fluorescence (**I, M**), MBP1 immunolabeling (**J, N**) and NeuN immunolabeling (**K, O**) were merged in (**L**) and (**P**). Scale bars = 1 mm in **A, B, E, F** and 50 μ m in the remaining panels.

Fig 3. MBP1-EGFP is predominantly present in the soluble brain fraction

Brain was sequentially extracted with Tris buffer and Tris buffer containing 1% Triton X-100 for soluble fraction (denoted as S) and membrane fraction (denoted as M), respectively. Equal amounts of total protein were separated on SDS-PAGE. **(A)** The majority of MBP1-EGFP was extracted with Tris buffer, unlike membrane associated endogenous MBP which was largely detergent soluble. **(B)** The expression level of MBP1-EGFP was estimated by quantitative immunoblotting to be approximately 20% of endogenous MBP. Data presented are the mean $\pm$ S.E.M. of $n = 3$ per group.

Fig. 4. Bigenic Tg-5xFAD/Tg-MBP1-EGFP mice showed improved spatial memory performance in the Barnes maze

Latencies were plotted over ten trials and exponential trend lines were fitted to the graph. The latency of Tg-5xFAD/Tg-MBP1-EGFP mice was significantly shorter than that of Tg-5xFAD mice in trial 8 and trial 10 with $p = 0.031$ and 0.024 , respectively. p value of repeated ANOVA < 0.03 .

Fig. 5. Significant reduction of detergent insoluble brain Aβ in bigenic Tg-5xFAD/Tg-MBP1-EGFP mice

Brains were extracted sequentially for soluble (A), membrane (B) and insoluble (C) fractions. The levels of Aβ₄₀ and Aβ₄₂ in these brain extracts were determined by ELISA analysis. Bigenic Tg-5xFAD/Tg-MBP1-EGFP mice exhibited a 25% reduction in insoluble Aβ. The data presented are the mean ± S.E.M. of n = 18–22 mice per group. *p < 0.03 for 40i. *p < 0.02 for 42i. The levels of Aβ oligomers present in soluble forebrain extracts were measured by ELISA (D). Representative immunoblots of soluble Aβ oligomers in Tg-5xFAD mice and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice probed with OC antibody (E). Quantitation of OC+ oligomers showed no significant differences in the mice (F). Data presented are the mean ± S.E.M. of n = 15 mice per group in (D) and n = 7–8 mice per group in (F).

Fig. 6. Presence of MBP1-EGFP does not alter human A β PP expression and processing in Tg-5xFAD mice

Equal amounts of total protein prepared from brain lysates of Tg-5xFAD mice and Tg-5xFAD/Tg-MBP1-EGFP mice were separated on SDS-PAGE and subjected to quantitative immunoblotting for human A β PP, A β PP C-terminal fragments (CTF) and β -tubulin. (A) Representative immunoblots. (B) The intensities of A β PP, C99 and C83 signals were normalized to β -tubulin, C99 and C83 and then normalized to A β PP. *p* values between Tg-5xFAD mice and Tg-5xFAD/Tg-MBP1-EGFP mice for A β PP, C99 and C83 are 0.11, 0.93 and 0.89, respectively. The data presented are as percentage to Tg-5xFAD in mean $\pm$ S.E.M. of *n* = 6 mice per group.

Fig. 7. Similar neuroanatomical distribution of A β and fibrillar amyloid deposition in Tg-5xFAD and bigenic Tg-5xFAD/Tg-MBP1-EGFP mice

Brains of age-matched Tg-5xFAD (**A, B**) and Tg-5xFAD/Tg-MBP1-EGFP mice (**C, D**) were immunolabeled with a polyclonal antibody against N-terminal A β ₁₋₂₈ (**A, C**) and stained for fibrillar amyloid using thioflavin S (**B, D**). Scale bars = 400 μ m.

Fig. 8. Reduced size of thioflavin-S positive fibrillar plaques in Tg-5xFAD/Tg-MBP1-EGFP mice
 Brains of age-matched Tg-5xFAD (A–C) and Tg-5xFAD/Tg-MBP1-EGFP mice (D–F) were immunolabeled with a polyclonal antibody against N-terminal A β_{1-28} (A, D) and stained for fibrillar amyloid using thioflavin S (B, E). With comparably sized A β plaques (A, D), the size of thioflavin S-positive core of fibrillar amyloid was noticeably reduced in the Tg-5xFAD/Tg-MBP1-EGFP mice (B) compared to Tg-5xFAD mice (E). Scale bars = 50 μ m. Quantitative image analysis was performed in different brain regions revealing no significant differences in the % area A β immunolabeling (G) but a significant reduction in the thioflavin S-positive area in Tg-5xFAD/Tg-MBP1-EGFP mice (H) in all brain regions examined (cx: cortex, sb: subiculum, hp: hippocampus, th: thalamus). Data presented are the mean $\pm$ S.E.M. of $n = 9$ –10 mice per group. *** $p < 0.001$ in cortex; ** $p < 0.01$ in subiculum; ** $p < 0.002$ in hippocampus; * $p < 0.02$ in thalamus. Quantitative stereological analysis revealed no significant difference in cortical plaque numbers (I) but a significant

reduction in fibrillar amyloid plaque volume in Tg-5xFAD/Tg-MBP1-EGFP mice (**J**). Data presented are mean $\pm$ S.E.M. of $n = 5-7$ mice per group. $**p < 0.002$.