NEUROBIOLOGY

α_7 Nicotinic Receptor Agonist Enhances Cognition in Aged 3xTg-AD Mice with Robust Plaques and Tangles

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Accepted for publication
October 23, 2013.

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Alzheimer disease (AD) is a progressive neurodegenerative disorder with associated memory loss, spatial disorientation, and other psychiatric problems. Cholinergic system dysfunction is an early and salient feature of AD, and enhancing cholinergic signaling with acetylcholinesterase inhibitors is currently the primary strategy for improving cognition. The beneficial effects of acetylcholinesterase inhibitors, however, are typically short-lived and accompanied by adverse effects. Recent evidence suggests that activating α_7 nicotinic acetylcholine receptors (α_7 nAChR) may facilitate the specific modulation of brain cholinergic signaling, leading to cognitive enhancement and possibly to amelioration of AD pathologic findings. In the present study, we determined the effect of long-term treatment with the selective α_7 nAChR agonist A-582941 in aged 3xTg-AD mice with robust AD-like pathology, which is particularly significant not only because this is the only mouse model that co-develops amyloid plaques and neurofibrillary tangles but also because it enabled us to explore whether A-582941 is able to restore brain function after the severe damage associated with AD. Analysis of β -amyloid deposits, tau phosphorylation, and inflammatory cells revealed that, overall, pathologic findings were unchanged. Rather, α_7 nAChR activation induced expression of c-Fos and brain-derived neurotrophic factor and phosphorylation of cyclic adenosine monophosphate response element binding and neurotrophic tyrosine receptor kinase type 2. More important, A-582941 completely restored cognition in aged 3xTg-AD mice to the level of that in age-matched nontransgenic mice. These novel findings indicate that activating α_7 nAChR is a promising treatment for cognitive impairment in AD. (*Am J Pathol* 2014, 184: 520–529; <http://dx.doi.org/10.1016/j.ajpath.2013.10.010>)

Alzheimer disease (AD) is a devastating neurodegenerative disorder that impairs memory and causes cognitive and psychiatric deficits. The buildup of β -amyloid (A β) is considered a central feature in the pathogenesis of AD and facilitates induction of tau pathology. Together, A β and tau are considered the two pathologic hallmarks of AD.¹ However, other critical molecular and neurochemical alterations occur as well. Of these, cholinergic dysfunction is particularly relevant because it emerges early in the disease process.² Traditionally, two different therapeutic strategies have been used to modulate the cholinergic system within the degenerative brain. One approach involves use of acetylcholinesterase inhibitors to prevent degradation of acetylcholine and thereby boost overall brain levels.³ An alternative approach involves direct stimulation of cholinergic receptors.^{4–7} Targeting cholinergic dysfunction has been the principal approach for

improving cognitive decline in AD because, with the notable exception of memantine, most of the US Food and Drug Administration–approved therapies for AD affect the cholinergic system.

Among the cholinergic receptors, α_7 nicotinic acetylcholine receptors (α_7 nAChRs) have recently received considerable attention because of their implication in neuropsychiatric, neurodegenerative, and inflammatory diseases.^{8–10} α_7 nAChRs assemble as a homopentamer composed of five α_7

Supported by AbbVie Inc. (North Chicago, Illinois; formerly the proprietary pharmaceutical business of Abbott Laboratories).

Disclosures: T.A.E., R.S.B., and M.W.D. are employed by AbbVie Inc., which funded the study and provided the α_7 nAChR agonist A-582941, and own stock in Abbott Laboratories and AbbVie Inc.

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subunits.¹¹ This receptor subtype is expressed on neurons, macrophages, microglia, and astrocytes.^{10,12} Neuronal α_7 nAChRs are abundant in many brain regions, notably in the hippocampus at presynaptic and postsynaptic neurons.^{13,14} α_7 nAChR activation triggers high calcium permeability and modulates other neurotransmitters (eg, glutamate), fast synaptic transmission, and cognitive function.^{15–18} In the AD-affected brain, α_7 nAChR protein levels are reduced in the cerebral cortex.¹⁹ A β_{42} binds with high affinity to α_7 nAChR, and both co-localize with neuritic plaques in postmortem brain tissue from patients with AD.^{20,21} Moreover, it has been suggested that A β_{42} accumulates in the intracellular compartment of neurons through its interaction with α_7 nAChR, followed by endocytosis of the resulting complex and its accumulation within the lysosomal compartment.²² The binding of A β to α_7 nAChRs is noncompetitive, specific, and reversible and occurs with high affinity through the N-terminal extracellular portion of the receptor.²³ However, whether this interaction between A β and α_7 nAChRs is capable of eliciting cellular signal transduction has not been fully established.^{23–27}

Both the blockade and activation of α_7 nAChRs have been proposed as potential therapeutic strategies for AD. Genetic deletion of α_7 nAChR subtype in PDAPP_{Swe/Ind} mice protected the brain from synaptic loss, reduced gliosis, reversed long-term potentiation dysfunction, and improved cognitive function without altering total A β levels.²⁸ Conversely, α_7 nAChR activation initiates signaling through the phosphatidylinositol 3-kinase/Akt cascade, which reduces A β -induced neurotoxicity *in vitro*.²⁹ In addition, activation of α_7 nAChRs rescued long-term potentiation impairment in A β -infused rats and in nontransgenic (nTg) mice but not in APP_{Swe}/PS1 Δ E9 mice.^{30,31} Of note, selective α_7 nAChRs agonists modulate glycogen synthase kinase 3 β (GSK3 β) activity and reduce tau phosphorylation *in vitro* and *in vivo*, which suggests that it is a potential target to reduce tau pathology.^{32,33}

In the present study, we sought to determine the role of α_7 nAChRs in AD by evaluating the cognitive, biochemical, and histopathologic effects of the α_7 nAChR agonist A-582941 in aged 3xTg-AD mice with severe plaques, tangles, and neuroinflammation.^{34–37} A-582941 binds to α_7 nAChRs with high affinity (K_i = 10.8 nmol/L in rat and 17 nmol/L in humans) but has at least 250-fold lower affinity for the other predominant neuronal nAChRs, $\alpha_4\beta_2^*$ and $\alpha_3\beta_4^*$, and for neuromuscular-type nAChRs.³⁸ Moreover, A-582941 has partial agonist activity *in vitro*, evoking peak currents of 60% of the maximum produced by acetylcholine in oocytes expressing rat α_7 nAChRs. Like many other α_7 nAChR agonists (eg, MEM-3454 and EVP-6124^{39,40}), A-582941 has some affinity for 5-HT₃ receptors, with affinity approximately 10-fold lower than for α_7 nAChR.

Materials and Methods

Animal Treatments

3xTg-AD mice harbor a presenilin1 mutation (PS1_{M146V}), the Swedish double mutation in APP (APP_{KM670/671ML}), and a

frontotemporal dementia mutation in tau (tau_{P301L}).³⁵ Strain-matched nTg mice, 129/C57BL/6, were used as controls.

Male 3xTg-AD mice were treated with selective α_7 nAChR agonist A-582941 [octahydro-2-methyl-5-(6-phenyl-3-pyridazinyl)-pyrrolo(3,4-c)pyrrole] (AbbVie, North Chicago, IL) dissolved in 2% sucrose and administered via drinking water at a dose of either 1.2 mg/kg/day or 12 mg/kg/day.⁴¹ A-582941 is orally bioavailable in mice (100%), with good brain penetration (brain-plasma ratio, 20:1) and a half-life of 1.4 hours.³⁸ In pilot pharmacokinetic studies conducted in 6-month-old male 3xTg-AD mice, the 12 mg/kg/day A-582941 dose administered via *ad lib* drinking water for 7 and 14 days produced plasma levels ranging from 3 to 45 ng/mL, consistent with plasma concentrations (3 to 30 ng/mL) associated with efficacy in cognition and *in vivo* signaling models (data not shown).^{33,41} In the present study, 3xTg-AD mice were treated for 3 months, from 15 to 18 months of age. At this age, 3xTg-AD mice show robust detergent-soluble and detergent-insoluble A β levels, intense phosphorylation of tau at multiple phosphorylated epitopes, increased microglia and astrocytes cell numbers, and presence of thioflavin-S–positive plaques and Gallyas silver–positive tangles (reviewed by Medeiros et al³⁴). Animals continued to receive drug or vehicle for the duration of the behavioral studies. No changes in total water consumption and body weight were found between vehicle- and A-582941–treated mice. All procedures used in the present study followed the Principles of Laboratory Animal Care from NIH publication 85-23 and were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Behavior Paradigms

3xTg-AD mice were tested using novel object recognition, Morris water maze, and contextual fear conditioning. The behavior testing procedures used have been described previously⁴² and are briefly described as follows.

Novel Object Recognition

Each mouse was habituated to an empty arena for 3 consecutive days. On the first day of testing, mice were exposed to two identical objects placed at opposite ends of the arena for 5 minutes. After 24 hours, mice were returned to the test box, which now contained one familiar object and one novel object. Time spent exploring the objects was recorded for 5 minutes. The recognition index represents the percentage of time that mice spend exploring the novel object. Objects used in this task were carefully selected to prevent preference or phobic behavior.

Morris Water Maze

Mice were trained to swim to a circular clear acrylic glass (Plexiglas) platform submerged 1.5 cm beneath the surface. Four 60-second trials were performed per day, with 5 minutes between trials. Mice were trained for as many days as needed for the group to reach the training criterion of 25 seconds. The

probe test was assessed at 24 hours after the last trial, with the platform removed. Performance was monitored using the EthoVision XT video tracking system (Noldus Information Technology, Inc., Leesburg, VA).

Contextual Fear Conditioning

During training, mice were placed in the fear conditioning chamber and allowed to explore for 2 minutes before receiving three electric foot shocks (duration, 1 second; intensity, 0.2 mA; intershock interval, 2 minutes). Animals were returned to the home cage 30 seconds after the last foot shock. After 24 hours, behavior in the conditioning chamber was video recorded for 5 minutes and subsequently analyzed for freezing behavior.

Tissue Preparation

Mice were deeply anesthetized with sodium pentobarbital and sacrificed via transcardial perfusion with 0.1 mmol/L PBS solution (pH 7.4). The right brain hemispheres were fixed for 48 hours in 4% paraformaldehyde and cryoprotected in 30% sucrose for immunohistochemical analysis. Frozen brains were sectioned coronally into 40- μ m sections using a Leica SM2010R freezing microtome (Leica Biosystems GmbH, Wetzlar, Germany), serially collected in cold 0.02% sodium azide, and stored at 4°C. The left hemispheres were snap-frozen on dry ice after removal of cerebellum, brainstem, and olfactory bulb and were subjected to protein extraction sequentially using T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Inc., Rockford, IL) and 70% formic acid. The supernatant was aliquoted and stored at -80°C. Protein concentration in the supernatant was determined using the Bradford assay.

Immunoblotting

Equal protein amounts were separated on a 4% to 12% gradient SDS-PAGE, transferred to a nitrocellulose membrane, and incubated overnight at 4°C with primary antibody. The following primary antibodies were used in this study: phospho-GSK3 β (Ser⁹), human APP-CT20, ADAM10, ADAM17, BACE1, GSK3 β , and CDK5 (Calbiochem, San Diego, CA); human tau (HT7), phospho-tau AT8 (phospho-Ser²⁰²/Thr²⁰⁵), phospho-tau AT180 (phospho-Thr²³¹), and phospho-tau AT270 (phospho-Thr¹⁸¹) (Thermo Fisher Scientific); A β ₁₋₁₆ (6E10) (Covance Research Products, Inc., Denver, PA); PP2A (Sigma-Aldrich Corp., St. Louis, MO); p35, neprilysin, IDE, phospho-TrkB, BDNF, and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); TrkB and CREB (Cell Signaling Technology, Inc., Danvers, MA); and PHF-1 (phospho-S³⁹⁶/S⁴⁰⁴) (Peter Davies, PhD, Albert Einstein College of Medicine, Manhasset, NY). After washing, the membranes were incubated with adjusted secondary antibodies coupled to horseradish peroxidase. The immunocomplexes were visualized using the SuperSignal West Pico Kit (Thermo Fisher Scientific). Band density

measurements were made using ImageJ 1.36b imaging software (NIH, Bethesda, MD).

ELISA

For quantification of the A β levels, T-Per soluble fractions were loaded directly onto ELISA plates, and the formic acid supernatants (insoluble fractions) were diluted 1:20 in a neutralization buffer (1 mmol/L Tris base and 0.5 mmol/L NaH₂PO₄) before loading. MaxiSorp immunoplates (Nalge Nunc International, Rochester, NY) were coated with mAb20.1 antibody (William E. Van Nostrand, PhD, Stony Brook University, Stony Brook, NY) at a concentration of 25 μ g/mL in coating buffer [0.1 mmol/L Na₂CO₃ (pH 9.6)] and blocked with 3% bovine serum albumin (BSA). Standard solutions for both A β ₄₀ and A β ₄₂ were made in the antigen capture buffer (20 mmol/L NaH₂PO₄, 2 mmol/L EDTA, 0.4 mol/L NaCl, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, and 1% BSA (pH 7.0) and loaded onto ELISA plates in duplicate. Samples were loaded, also in duplicate, and incubated overnight at 4°C. Plates were then washed and probed using either horseradish peroxidase-conjugated anti-A β ₄₀ (C49) or anti-A β ₄₂ (D32) (Alzheimer's Disease Research Center, University of California, Irvine, CA) overnight at 4°C. The chromogen was 3,3',5,5'-tetramethylbenzidine, and the reaction was stopped with 30% phosphoric acid. The plates were read at 450 nm using a plate reader (Molecular Dynamics, Inc., Sunnyvale, CA). The readings were normalized to protein concentrations of the samples.

Immunohistochemistry

Antigen retrieval was optimized using a 90% formic acid solution for 7 minutes for A β staining. Free-floating sections were pretreated with 3% hydrogen peroxide and 10% methanol in Tris-buffered saline solution (TBS) for 30 minutes to block endogenous peroxidase activity. After a TBS wash, sections were incubated once in 0.1% Triton X-100 in TBS for 15 minutes and once with 2% BSA in Triton X-100 in TBS for 30 minutes. Sections were then incubated overnight at 4°C with the following primary antibodies: A β ₄₂ (D32) (Alzheimer's Disease Research Center), CD45 (AbD Serotec, Inc., Raleigh, NC), A β ₁₋₁₆ (6E10) (Covance Research Products), phospho-tau AT8 (phospho-Ser²⁰²/Thr²⁰⁵) (Thermo Fisher Scientific), p-CREB (Cell Signaling Technology), and c-Fos (Santa Cruz Biotechnology) with 5% normal serum in TBS. After the appropriate biotinylated secondary antibody (1:200 in TBS + 2% BSA + 5% normal serum), sections were processed using the Vectastain Elite ABC reagent and 3,3'-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene, and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, England). All sections were processed under the same conditions. Negative control experiments included

omission of primary antibody as well as substitution of the primary antibody with equivalent dilutions of nonimmune serum, using the same staining protocol, and were devoid of specific immunoreaction product.

Immunostaining was assessed at six brain coronal levels. Specifically, six alternate 40- μ m sections of brain with an individual distance of approximately 160 μ m were obtained between 1.34 and 2.54 mm posterior to the bregma. Images of stained hippocampal and entorhinal cortex area were acquired using an Axiocam digital camera and AxioVision 4.6 software connected to an Axioskop 50 microscope (Carl Zeiss MicroImaging, Thornwood, NY). Settings for image acquisition were identical for vehicle- and A-582941-treated tissues.

Staining analyses were calculated as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels) using NIH ImageJ 1.36b imaging software, complying with strict standards.^{4,43,44}

Immunofluorescence

Sections were first blocked with 3% normal serum, 2% BSA, and 0.1% Triton X-100 in TBS for 1 hour at room temperature. Using the same buffer solution, sections were incubated overnight at 4°C with the following primary antibodies: GFAP (Millipore Corp., Billerica, MA), human tau (HT7) (Thermo Fisher Scientific), phospho-tau PHF-1 (phospho-S³⁹⁶/S⁴⁰⁴) (Peter Davies, PhD), Iba1 (Wako Chemicals USA, Inc.), and A β ₁₋₁₆ (6E10) (Covance Research Products). Sections were then rinsed and incubated for 1 hour with secondary Alexa Fluor-conjugated antibodies (Invitrogen Corp., Carlsbad, CA) at room temperature. Finally, sections were mounted onto gelatin-coated slides in Fluoromount-G (Southern Biotech Associates, Birmingham, AL) and examined under a Leica DM2500 confocal laser microscope using the Leica Application Suite Advanced Fluorescence software version 2.6.0 (Leica Microsystems, Inc., Bannockburn, IL). Immunofluorescence was assessed at the same brain coronal levels as previously described. Confocal images were acquired via sequential scanning using a z-separation of 0.5 μ m using the same Leica software. Volumetric image measurements were made in the hippocampus and entorhinal cortex using Imaris software version 7.5.2 (Bitplane, Inc., South Windsor, CT).

Protein Kinase Activity Assay

Protein kinase activity was measured using the nonradioactive protein kinase assay kit (Calbiochem) according to the manufacturer's instructions, as described earlier.⁴

Statistical Analysis

All data are expressed as means $\pm$ SEM. Statistical evaluation of the results was performed using 1- or 2-way analysis of variance. After significant analysis of variance, multiple *post hoc* comparisons were performed using the

Bonferroni test. Some data were analyzed using the unpaired *t*-test. The accepted level of significance for the tests was $P < 0.05$. All tests were performed using the Statistica software package version 5.1 (StatSoft, Inc., Tulsa, OK).

Results

Activation of α_7 nAChR Restores Learning and Memory in Aged 3xTg-AD Mice

Impairment of cognitive capacities is the most feared consequence of AD. The underlying mechanism of cognitive decline in AD involves, at least in part, degeneration of cholinergic transmission. Therefore, we analyzed the effect of α_7 nAChR stimulation on AD-like cognitive impairment in aged 3xTg-AD mice. As previously observed, 18-month-old 3xTg-AD mice performed significantly worse relative to age-matched nTg mice in the reference spatial memory version of the Morris water maze (Figure 1, A and B), novel object recognition (Figure 1C), and contextual fear conditioning (Figure 1D). In the hippocampal-dependent Morris water maze, 3xTg-AD mice exhibited longer latencies to find

Figure 1 A-582941 improves cognition in aged 3xTg-AD mice. **A:** Eighteen-month-old 3xTg-AD and nTg control mice were treated orally with vehicle, 1.2 mg/kg/day, or A-582941, 12 mg/kg/day. 3xTg-AD mice show a dose-dependent decrease in escape latency times to the platform in the Morris water maze. Area under the curve for each individual group: nTg: vehicle = 221, A582941 1.2 mg/kg = 224, A-582941 12 mg/kg = 211; 3xTg-AD: vehicle = 276, A-582941 1.2 mg/kg = 256, A-582941 12 mg/kg = 217. **B:** Similarly, compared with vehicle-treated 3xTg-AD mice, A-582941-treated 3xTg-AD mice spend an increased amount of time in the target quadrant during the water maze probe trial. **C:** In novel object recognition, a cortical-based task, A-582941-treated 3xTg-AD mice spend more time exploring the novel object, showing memory retention similar to nTg levels, whereas vehicle-treated 3xTg-AD mice show no preference for the novel object. **D:** 3xTg-AD mice treated with A-582941 exhibit dosage-dependent increased contextual fear memory, with the 12 mg/kg/day group performing at nTg levels. **A–D:** Vehicle-treated 3xTgAD mice perform significantly worse than nTg mice in all tasks. Error bars represent means $\pm$ SEM, 1- or 2-way analysis of variance followed by the *post hoc* Bonferroni test. * $P < 0.05$ and ** $P < 0.01$ between nTg and 3xTgAD mice, †† $P < 0.01$ between vehicle- and A-582941-treated 3xTgAD mice.

the platform in the training session (Figure 1A) and reduced target quadrant preference during the probe trial (Figure 1B), compared with age-matched nTg mice. Of note, treatment with 12 mg/kg/day A-582941 improved the cognitive function of 3xTg-AD mice during the training and probe sessions of the Morris water maze. Novel object recognition has been described as a task primarily dependent on the cortex.⁴⁵ In this task, A-582941-treated 3xTg-AD mice also performed substantially better than vehicle-treated 3xTg-AD mice, as demonstrated by the significant increase in the exploration of the unfamiliar object (Figure 1C). Finally, mice were tested in a contextual fear conditioning task, an amygdala- and hippocampal-dependent form of learning characterized by the association of a normally innocuous environment or context with an aversive stimulus, in this case a mild foot shock. Animals that learned to associate the conditioning chamber with the shock exhibited increased freezing during subsequent exposures to that chamber (Figure 1D). Compared with nTg mice, 3xTg-AD mice manifested a significant deficit in freezing in contextual memory when tested 1 day after training. A-582941-treated mice demonstrated a reduced degree of mobility in the conditioning chamber during the probe session, compared with vehicle-treated 3xTg-AD mice. No significant changes in learning and memory functions were found between vehicle- and A-582941-treated nTg mice in the different cognitive tasks (Figure 1). Moreover, the improvement in cognitive performance in the A-582941-treated 3xTgAD mice was not directly related to changes in motor function inasmuch as no significant alterations in swimming speed and total distance traveled in the water maze or the total squares crossed and rearing behavior in the open-field arena were observed when compared with vehicle-treated 3xTgAD mice (results not shown).

Activation of α_7 nAChR Raises Levels of Memory-Related Proteins in Brains of 3xTg-AD Mice

At the molecular level, the increase in c-Fos levels and phosphorylation of CREB at Ser¹³³ are highly involved in

many forms of experience-dependent plasticity, which is necessary for learning and memory. Thus, we analyzed levels of these transcriptional factors after stimulation of α_7 nAChR in the 3xTg-AD mice (Figure 2, A and C). Immunohistochemistry revealed that treatment with 12 mg/kg/day A-582941 resulted in a significant increase in the levels of c-Fos and p-CREB in the brain of 3xTg-AD mice compared with vehicle-treated mice. Likewise, stimulation of α_7 nAChR raised activation of protein kinase A, a major upstream kinase involved in CREB phosphorylation (Figure 2D). To further explore the effect of α_7 nAChR activation on cognition, we assessed the levels of p-TrkB and BDNF. Treatment with selective α_7 nAChR agonist A-582941 significantly increased steady-state levels of these proteins in the brain of 3xTg-AD mice (Figure 2, B and C).

Activation of α_7 nAChR Does Not Change A β Pathology in Brains of 3xTg-AD Mice

We also sought to evaluate whether stimulation of α_7 nAChR modulates A β formation in 3xTg-AD mice. We found that treatment with selective α_7 nAChR agonist A-582941 did not change levels of A β ₄₀ and A β ₄₂ peptides in both soluble- and insoluble-detergent fractions of 3xTgAD mice brains (Figure 3, A–D). Similar results were found via immunohistochemistry using anti-A β ₄₂ and anti-6E10 antibodies. Brains of A-582941- and vehicle-treated 3xTg-AD mice exhibited comparable levels of immunostained A β (Figure 3E). Consistently, no changes were detected in the APP processing cascade (Figure 3, F and G). A-582941 did not alter APP steady-state levels and its proteolytic fragments CTF β [99-amino acid long C-terminal APP fragment produced by β -secretase cleavage (C99)] and CTF α [83-amino acid long C-terminal APP fragment produced by α -secretase cleavage (C83)]. Moreover, levels of α -APP cleaving enzymes ADAM10 and ADAM17, the putative β -secretase enzyme BACE1, and the major A β -degrading enzymes insulin-degrading enzyme and neprilysin were unaffected by stimulation of α_7 nAChR.

Figure 2 A-582941 increases levels of memory proteins in aged 3xTgAD mice. In brain samples from 3xTg-AD mice, those treated with 12 mg/kg/day A-582941 show higher levels of c-Fos, p-CREB Ser¹³³ (A) and p-TrkB and mature BDNF (B). C: Quantification of the immunostaining intensity in A shows a significant increase in c-Fos and p-CREB intensity in A-582941 compared with vehicle. Quantification of Western blots in B was performed via densitometric analysis and is presented as a percentage of control, normalized to GAPDH. D: A-582941 treatment results in increased protein kinase A activity. Error bars represent means $\pm$ SEM, unpaired Student's *t*-test, **P* < 0.05, ***P* < 0.01.

Figure 3 A-582941 does not reduce levels of Aβ in 3xTg-AD mice. **A–D:** ELISA analysis reveals that levels of soluble and insoluble Aβ₄₀ and Aβ₄₂ do not change in 3xTg-AD mice treated with α_7 nAChR agonist. **E:** In brain samples from 3xTg-AD mice, those treated with 12 mg/kg/day A-582941 exhibited equivalent levels of Aβ₄₂ and 6E10 immunostaining compared with vehicle-treated 3xTg-AD mice. **F:** APP processing is not affected by A-582941. Levels of APP, C99/C83, ADAM10, ADAM17, BACE1, IDE, and neprilysin are similar between A-582941- and vehicle-treated 3xTgAD mice. **G:** Quantification of western blots in **F** was performed via densitometric analysis and is presented as a percentage of control, normalized to GAPDH. Error bars represent means $\pm$ SEM.

Activation of α_7 nAChR Does Not Change Tau Pathology in Brains of 3xTg-AD Mice

Tau pathology is another important pathologic hallmark of AD. Despite activation of α_7 nAChR, we found that phosphorylation of tau was unchanged, as demonstrated by the equivalent levels of phospho-tau AT8 (phospho-Ser²⁰²/Thr²⁰⁵), phospho-tau AT180 (phospho-Thr²³¹), phospho-tau AT270 (phospho-Thr¹⁸¹), and phospho-tau PHF-1 (phospho-S³⁹⁶/S⁴⁰⁴) between A-582941- and vehicle-treated 3xTg-AD mice (Figure 4, A and B). Corroborating these findings, no changes in AT8 staining (Figure 2C) or in co-localization among total tau and PHF-1 (Figure 4D) were observed in the brains of 3xTg-AD mice treated with α_7 nAChR agonist. Moreover, A-582941 did not change the level of insoluble tau (Figure 4E). Of note, although stimulation of α_7 nAChR did not modify activity of tau kinase CDK5, evaluated by analyzing levels of p35 and p25, and level of the major tau phosphatase PP2A, it significantly decreased activation of tau kinase GSK3β, as demonstrated by the increase in GSK3β phosphorylation at Ser⁹ (Figure 4, F and G).

Activation of α_7 nAChR Does Not Change Neuroinflammation in Brains of 3xTg-AD Mice

Expression of α_7 nAChR is up-regulated in astrocytes and microglia in human brains affected by AD, which suggests a role for this receptor on neuroinflammation. To assess this hypothesis, we evaluated the levels of inflammatory cells

surrounding amyloid deposits in the brains of 3xTg-AD mice. We found that α_7 nAChR agonist A-582941 did not change the number of CD45- and Iba-1-positive microglia cells or the number of reactive GFAP-positive astrocytes in areas with Aβ deposits, when compared with vehicle-treated 3xTgAD mice (Figure 5).

Discussion

Activation of α_7 nAChRs restores cognition in aged 3xTg-AD mice with advanced AD-like disease. Synthesis and deposition of Aβ were not affected by activation of the α_7 nAChR pathway. Despite the clear demonstration that A-582941 reduces activation of GSK3β, as suggested by increased phosphorylation of Ser⁹, we did not find any significant changes in tau pathology. However, it is noteworthy that the increased GSK3β Ser⁹ phosphorylation produced by A-582941 provides clear molecular evidence of α_7 nAChR target engagement in 3xTg-AD-treated mice, inasmuch as short- and long-term administration of A-582941 increases Ser⁹ phosphorylation in nTg and AD transgenic mice but not α_7 knockout mice.^{33,40} Although this finding provides confirmation that cognitive enhancement was accompanied by α_7 activation in the present study, we cannot entirely discount some contribution of 5-HT₃ receptor antagonism to the effects because the binding affinity of A-542941 at 5-HT₃ receptors is only 10-fold lower than at α_7 . Finally, improvement in learning and memory functions was observed in animals of advanced age and with

Figure 4 A-582941 does not change tau phosphorylation in aged 3xTg-AD mice. **A:** α_7 nAChR agonist A-582941, 12 mg/kg/day, does not alter total tau levels and phosphorylation at several phospho-epitopes of tau in 3xTg-AD mice. **B:** Quantification of Western blots in **A** was performed via densitometric analysis and is presented as a percentage of control, normalized to GAPDH. **C:** Phosphorylation of tau residues Ser²⁰² and Thr²⁰⁵ does not change by A-582941, as shown at immunohistochemical analysis. **D:** Similar results were found in the analysis of phosphorylation of tau residues Ser³⁹⁶ and Ser⁴⁰⁴, as shown by PHF-1 staining by immunofluorescence. **E:** Levels of insoluble tau also remain unchanged between A-582941- and vehicle-treated 3xTg-AD mice. **F:** Stimulation of α_7 nAChR does not change CDK5, p35/p25, GSK3 β , and PP2A levels in the brains of 3xTgAD mice; however, it increases phosphorylation of GSK3 β at Ser⁹. **G:** Quantification of Western blots in **F** was performed via densitometric analysis and is presented as a percentage of control, normalized to GAPDH. Error bars represent means $\pm$ SEM. ** $P < 0.01$.

large amounts of amyloid and tau pathology. The underlying molecular mechanisms associated with restoration in cognition in brains with AD-like disease were activation of transcriptional factors c-Fos and CREB, up-regulation in BDNF levels, and phosphorylation of the TrkB receptor.

Changes in α_7 nAChR signaling have been implicated in the progression of AD. Previous studies have suggested that part of A β toxicity is mediated through its binding to α_7

nAChRs.²⁸ In addition, α_7 nAChRs seem to modulate GSK3 β activity, which in turn regulates tau phosphorylation.^{32,33} In the present study, we further explored the relationship between α_7 nAChR and AD by testing the effect of selective agonist A-582941 on 3xTg-AD mice. Our findings indicate that activation of α_7 nAChR does not affect APP processing or A β levels. Likewise, we did not find changes in tau pathology after treatment with A-582941,

Figure 5 A-582941 does not change neuroinflammation in 3xTg-AD mice. **A–C:** The number of CD45- (**A**), Iba-1- (**B**), and GFAP-positive cells (**C**) are not altered in mice treated with 12 mg/kg/day A-582941 compared with vehicle-treated 3xTg-AD mice. **D:** Quantification of immunoreactivity in **A** to **C** was performed via densitometric analysis and is presented as a percentage of control. Error bars represent means $\pm$ SEM.

which is in contrast with our previous finding that nicotine increases tau pathology in 3xTg-AD mice.³⁷ Thus, it seems that use of a more selective α_7 nAChR can prevent this potential liability of nicotine. The lack of effect of A-582941 on tau pathology is of particular interest because we demonstrated that activation of α_7 nAChR significantly inhibits the tau kinase GSK3 β , as demonstrated by the increase in its phosphorylation at Ser⁹ epitope. A hypothesis to explain such discrepancy may be that 3xTg-AD mice used in our study had extensive A β and neuroinflammation, which in turn triggered activation of multiple overlapping intracellular pathways that cause exacerbation of tau phosphorylation.^{34,35} In this sense, inhibiting GSK3 β may be insufficient to prevent progression of tau pathology in aged 3xTg-AD mice. Consistent with this hypothesis, A-582941 reduces tau hyperphosphorylation in 12- to 13-month-old 2xTg-APP/tau mice that exhibit robust tau, but not A β , pathology.^{33,46} However, future studies are required to better comprehend this phenomenon.

The leading hypothesis regarding progression of AD predicts that amyloid pathology is upstream from tau pathology and neuronal loss, and hence there has been an enormous effort to investigate and develop disease-modifying strategies targeting synthesis, aggregation, and clearance of A β .^{2,47} Regardless of the success of A β -based therapies in preclinical studies, the overwhelming rate of drugs failing clinical trials during the past decade has indicated that single-target therapies in patients with AD, even in those with mild stages of the disease, may be insufficient because of the numerous pathways and resultant damage triggered by accumulation of A β . Because patients with dementia also have extensive neuronal loss, in addition to the presence of plaques and tangles, it is unlikely that removing the proteinaceous pathologies will be beneficial without a simultaneous strategy to also help the brain compensate for the extensive loss of neurons. Thus, treatments that enhance cognition, especially in the presence of well-established amyloid and tau pathology, are urgently needed. In the present study, we found critical evidence that α_7 nAChRs are promising targets for development of cognitive enhancement therapy for AD. Activation of α_7 nAChRs completely restored learning and memory functions in 3xTg-AD mice to a level equivalent to that in nTg mice despite lack of changes in neuropathologic markers of the disease, namely, plaques, tangles, and neuroinflammation. Although α_7 nAChRs agonists have been reported to produce neuroprotective activity and to enhance cognition in a variety of experimental models, the present study is the first to show that activation of this pathway significantly recovers the cognitive decline in a model that co-develops amyloid and tau pathology.

Dysregulation in neuronal c-Fos, CREB, and BDNF signaling has been implicated as an underlying mechanism of learning and memory deficits in AD.^{48–52} c-Fos and CREB are early-immediate transcriptional factors that regulate the expression of multiple proteins linked to

cognitive function, including BDNF receptor TrkB. Likewise, BDNF signaling through TrkB stimulates the transcriptional activity of c-Fos and CREB. Activation of these cascades ultimately modulates protein synthesis and growth processes that yield synapse-specific structural changes to enable consolidation of experiences into a more durable long-term memory.^{53–56} Consistently, we recently showed that neural stem cell transplantation rescues the cognitive phenotype in 3xTg-AD mice that exhibit advanced AD-related disease. Like A-582941, the beneficial effects of neural stem cells on cognition are not mediated by alteration of either A β or tau pathology. Rather, neural stem cell-derived cells elevate hippocampal BDNF, leading to increased synaptic density and restoring hippocampal-dependent cognition.⁵⁷ Of note, it has been suggested that the selective α_7 nAChR agonist A-582941 raises CREB phosphorylation and c-Fos expression in rodent brains.^{41,58} In addition, acetylcholinesterase inhibitors rapidly activate Trk neurotrophin receptors in mouse hippocampus.⁵⁹ Stimulation of α_7 nAChRs also produces higher expression of BDNF and increases phosphorylation of TrkB in brains with AD-like disease. Together, these studies indicate that selective activation of α_7 nAChRs is a promising strategy to reestablish learning- and memory-related pathways in patients with AD. Although A-582941 has served as a valuable preclinical tool to evaluate effects of α_7 agonism on memory processing, it is not being developed clinically because of compound-specific cardiovascular liabilities unrelated to its α_7 activity.^{38,41} However, preliminary reports of phase 2 clinical trial results in AD using other α_7 agonists, including MEM-3454, EVP-6124, and ABT-126, suggest signals of clinically relevant improvement in cognitive function with this mechanism.^{60–62}

As the number of patients with AD continues to rise, the need for efficacious therapeutics is becoming increasingly urgent. The present study provides clear functional and molecular preclinical evidence that selective α_7 nAChRs agonists are potential therapeutic tools for management of cognitive decline in patients with AD, including those with severe neuropathologic findings.

Acknowledgments

The authors thank Kathy Kohlhaas (AbbVie Inc.) for advice and technical assistance.

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