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β-Amyloid$_{1-42}$ Binds to α7 Nicotinic Acetylcholine Receptor with High Affinity

IMPLICATIONS FOR ALZHEIMER'S DISEASE PATHOLOGY*

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Hoau-Yan Wang‡, Daniel H. S. Lee‡§, Michael R. D'Andrea, Per A. Peterson, Richard P. Shank, and Allen B. Reitz

From the R. W. Johnson Pharmaceutical Research Institute, Spring House, Pennsylvania 19477-0776

Alzheimer's disease pathology is characterized by the presence of neuritic plaques and the loss of cholinergic neurons in the brain. The underlying mechanisms leading to these events are unclear, but the 42-amino acid β-amyloid peptide (Aβ_{1-42}) is involved. Immunohistochemical studies on human sporadic Alzheimer's disease brains demonstrate that Aβ_{1-42} and a neuronal pentameric cation channel, the α7 nicotinic acetylcholine receptor (α7nAChR), are both present in neuritic plaques and co-localize in individual cortical neurons. Using human brain tissues and cells that overexpress either α7nAChR or amyloid precursor protein as the starting material, Aβ_{1-42} and α7nAChR can be co-immunoprecipitated by the respective specific antibodies, suggesting that they are tightly associated. The formation of the α7nAChR·Aβ_{1-42} complex can be efficiently suppressed by Aβ_{12-28}, implying that this Aβ sequence region contains the binding epitope. Receptor binding experiments show that Aβ_{1-42} and α7nAChR bind with high affinity, and this interaction can be inhibited by α7nAChR ligands. Human neuroblastoma cells overexpressing α7nAChR are readily killed by Aβ_{1-42}, whereas α7nAChR agonists such as nicotine and epibatidine offered protection. Because Aβ_{1-42} inhibits α7nAChR-dependent calcium activation and acetylcholine release, two processes critically involved in memory and cognitive functions, and the distribution of α7nAChR correlates with neuritic plaques in Alzheimer's disease brains, we propose that interaction of the α7nAChR and Aβ_{1-42} is a pivotal mechanism involved in the pathophysiology of Alzheimer's disease.

Evidence obtained from familial Alzheimer's disease (AD)[1] patients (1–3) and transgenic animals (4–7) overexpressing amyloid precursor protein (APP) indicates that Aβ_{1-42} is a causal factor of neuritic plaque formation and neurodegeneration in AD. Aβ peptides are known to bind specifically to the scavenger receptors expressed in microglia and macrophages (8, 9) and the ubiquitous receptor for advanced glycation end products (RAGE) (10, 11). Aβ binding to scavenger receptors may induce cell death by generating free radicals (12, 13), whereas binding to RAGE may promote Aβ clearance (10, 11). However, the respective receptor function and distribution pattern for scavenger receptors and RAGE cannot fully explain the Aβ_{1-42}-induced pathophysiology observed in AD such as cognitive and memory impairment, in which cholinergic neurons are involved. This suggests that additional physiologically relevant neuronal receptors for Aβ_{1-42} may exist.

The α7 nicotinic acetylcholine receptor (α7nAChR) is highly expressed in the basal forebrain cholinergic neurons that project to the hippocampus and cortex of normal and AD brains (14, 15), correlating well with brain areas that exhibit neuritic plaques in AD. More importantly, the α7nAChR modulates calcium homeostasis and release of the neurotransmitter acetylcholine, two important parameters involved in cognition and memory. We thus investigated the molecular and biochemical relationships of α7nAChR with Aβ_{1-42}. Both proteins were found to co-localize in cortical and hippocampal neurons in AD brains and neuritic plaques. The two proteins were tightly associated together, which may be explained by their specific high affinity interaction. Because this α7nAChR-Aβ_{1-42} interaction also leads to the inhibition of acetylcholine release and calcium flux, and even cell death *in vitro*, we speculate that the α7nAChR-Aβ_{1-42} interaction may be one of the key events in the pathogenesis of AD.

MATERIALS AND METHODS

Tissue Samples—Postmortem human brain tissues (cortex and hippocampus) from 12 patients with a clinical diagnosis of AD and 12 age-matched 61–81-year-old control individuals with no history of neurological or psychiatric disorders were used in this study (the mean ages of the AD and control subjects ± S.E. were 73.9 ± 1.5 and 71.6 ± 1.4, respectively). The postmortem time intervals for collecting these brains were ≤ 24 h (the mean postmortem intervals for the collection of AD and control brain samples were 11.7 ± 1.4 h and 10.0 ± 2.0 h, respectively). Tissues were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA) and Analytical Biological Services, Inc. (Wilmington, DE). AD was diagnosed according to the criteria defined by the National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's disease (16). The presence of both amyloid plaques and neurofibrillary tangles in all AD brains was confirmed by Nissl and Bielschowsky staining and anti-neurofibrillary tangle staining. All control brain samples exhibited minimal histopathological changes (0–3 amyloid plaques/low power field and 0–6 neurofibrillary tangles/low power field).

Immunohistochemistry—For immunohistochemical studies, brain tissues were immersion-fixed in cold 10% phosphate-buffered (0.15 M) formalin, pH 7.4, for 2 weeks before use. 5-μm sections prepared from paraffin-fixed human hippocampus of AD (n = 12) as well as age- and postmortem interval-matched, nondemented control subjects (n = 12) were cut serially onto SuperFrost Plus+ (Fisher) for silver staining.

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‡ The first two authors contributed equally to this work.

§ To whom correspondence should be addressed: R. W. Johnson Pharmaceutical Research Institute, Rm. 345, Research Bldg., McKean and Welsh Roads, Spring House, PA 19477-0776. Tel.: 215-540-4910; Fax: 215-628-3297; E-mail: dlee@prius.jnj.com.

[1] The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; RAGE, receptor for advanced glycation end products; Aβ_{1-42}, 42-amino acid β-amyloid peptide; α7nAChR, α7 nicotinic acetylcholine receptor; α7SK-N-MC cells, human neuroblastoma SK-N-MC cells overexpressing α7nAChR; α-BTX, α-bungarotoxin.

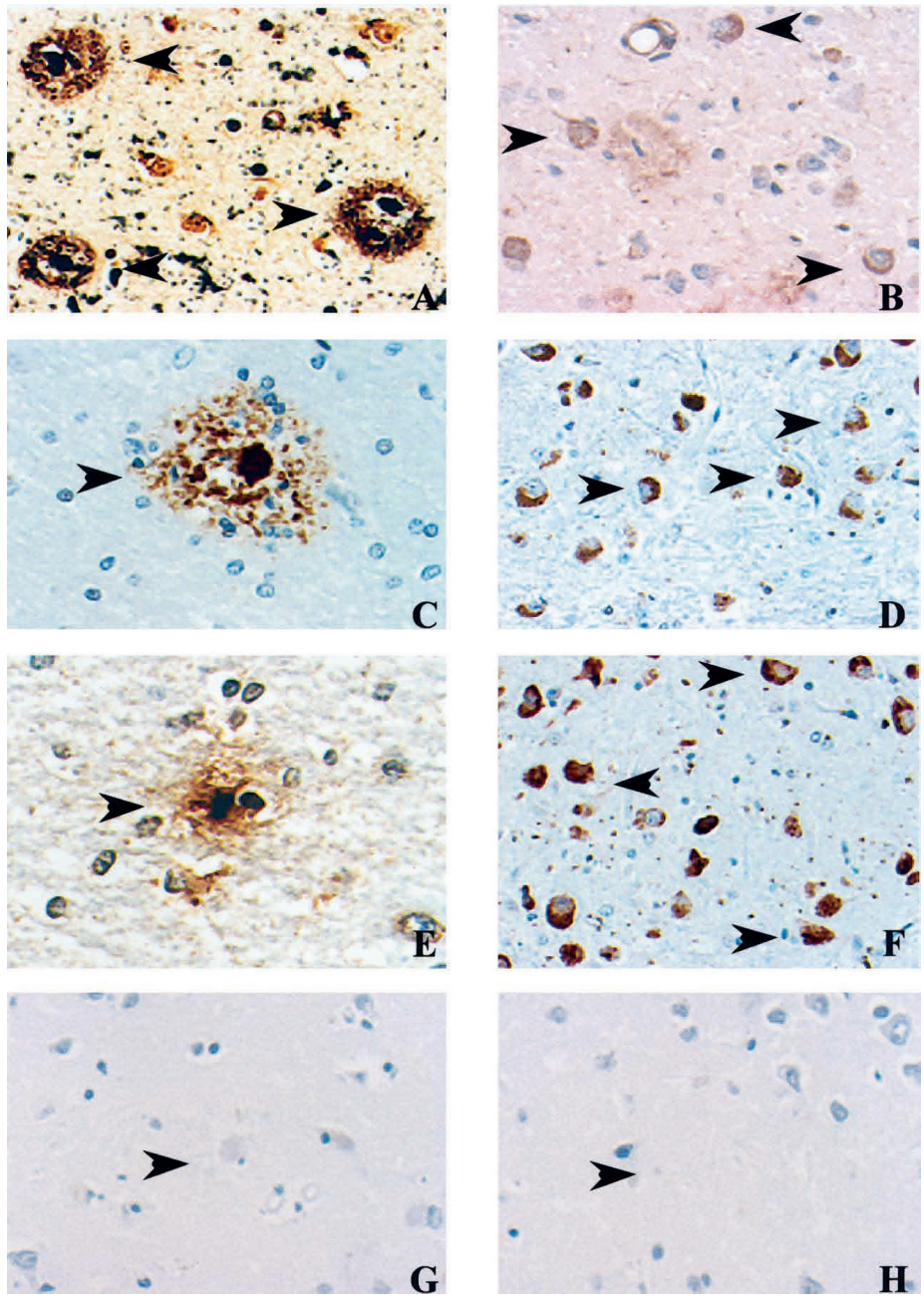

FIG. 1. **Immunohistochemical detection of α7nAChR in neuritic plaques of AD hippocampus.** *Panel A*, tissues were stained with a modified Bielschowsky silver stain technique. *Arrowheads* indicate areas of neuritic plaques. Tissues were then stained with the appropriate antibodies. *Panel B*, *arrowheads* indicate neurofibrillary tangles. *Panel C*, presence of $A\beta_{1-42}$ (*arrowheads*) in a dense core plaque and (*panel D*) in neurons. *Panel E*, presence of α7nAChR (*arrowheads*) in a neuritic plaque and (*panel F*) in neurons. *Panel G*, lack of α4nAChR immunoreactivity in a plaque (*arrowhead*). *Panel H*, lack of *N*-methyl-D-aspartate R1 glutamate receptor immunoreactivity in a plaque (*arrowhead*). Magnification: *panel A*, × 20; *panels B–H*, × 40. All 12 AD brain samples showed identical results, and representative data from two cases of sporadic AD are shown.

Single labeling protocols for immunocytochemistry were performed as described previously (17) except that the dilutions of the primary antibodies were: rabbit anti-$A\beta_{1-42}$, 1:25 (Quality Control Biochemicals, Hopkinton, MA); rat anti-α4nAChR, 1:25 (Research Biochemicals, Inc., Natick, MA); mouse anti-α7nAChR, 1:600 (Research Biochemicals); rat anti-*N*-methyl-D-aspartate R1 glutamate receptor, 1:10 (Research Biochemicals). Specifically, the anti-$A\beta_{1-42}$ was tested in a preabsorption experiment using a 100-fold excess of $A\beta_{1-42}$, $A\beta_{1-40}$, or $A\beta_{40-1}$ antigens verifying the specificity of anti-$A\beta_{1-42}$. Anti-$A\beta_{1-42}$ obtained from two other vendors, Pharmingen (San Diego, CA) and Calbiochem also generated identical results. Negative controls included the replacement of the primary antibody with similar species isotype antibody. Positive control antibody included glial fibrillary-associated protein (Chemicon, Temecula, CA).

Human AD cortical sections were incubated with anti-α7nAChR (Research Biochemicals), which was detected using a fluorescein isothiocyanate-conjugated secondary antibody (Vector Laboratories, Burlingham, CA). These were incubated sequentially with anti-$A\beta_{1-42}$ and detected using a Texas red (Vector)-conjugated secondary antibody with thorough washes in between. The slides were mounted with an antifade water-based mounting medium with 4,6-diamidino-2-phenylindole (Vector) and analyzed under an BX50 Olympus fluorescent microscope.

Immunoprecipitation—Membranes were prepared from human AD (n = 12) as well as age- and postmortem interval-matched, nondemented control subjects (n = 12), and the protein contents were determined using Bio-Rad reagent. Hippocampal membrane proteins (200 μg) were solubilized for 60 min at 4 °C in a medium containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% digitonin, 0.2% sodium deoxycholate, 0.5% Nonidet P-40, and protease and protein phosphatase inhibitors. Solubilized proteins (1 ml) were immunoprecipitated with anti-$A\beta_{1-42}$ or nonimmune rabbit serum, 1:250, for 16 h at 4 °C. The immunoprecipitates were enriched by protein A/G-agarose beads (20 μl, Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to 8–16% SDS-polyacrylamide gel electrophoresis. Western analyses using the appropriate monoclonal antibodies (anti-α1,3,5nAChR, anti-α4nAChR, anti-α7nAChR, anti-α8nAChR, and anti-β2nAChR; Research Biochemicals) were performed and detected by chemiluminescence. A reciprocal study employing anti-α7nAChR in the immunoprecipitation and anti-$A\beta_{1-42}$ in the Western analyses was performed similarly. In a separate study, membranes (200 μg of protein) prepared from hippocampus of a 51-year-old male control subject were preincubated with 100 nM $A\beta_{1-42}$ for 15 min, solubilized, and immunoprecipitated with anti-$A\beta_{1-42}$. The immunoprecipitates were subjected to 8–16% SDS-polyacrylamide gel electrophoresis and West-

ern analyses using the monoclonal antibodies stated above. α7nAChR but not α1nAChR, α3nAChR, α4nAChR, α5nAChR, α8nAChR, or β2nAChR co-immunoprecipitated with $A\beta_{1-42}$. All protein bands were subsequently characterized by NH_2-terminal amino acid sequencing. The intensities of the protein bands were quantitated in a Zeineh Soft laser scanning densitometer (Fullerton, CA).

Similarly, α7SK-N-MC (see below) cell membranes were incubated with 100 nM $A\beta_{1-42}$ (Palomar Research Inc., Carlsbad, CA) or rat $A\beta_{1-42}$ (Calbiochem) before co-immunoprecipitation and Western analyses using anti-$A\beta_{1-42}$ and anti-α7nAChR, respectively, in reciprocal studies. Using the same experimental setup, the effects of galanin, neuropeptide Y (Palomar Research Inc.), $A\beta_{1-11}$, $A\beta_{10-20}$, $A\beta_{12-28}$, $A\beta_{22-35}$, or $A\beta_{40-1}$ (Sigma) on complex formation were evaluated separately by including a 100 nM concentration of each peptide in the incubation mixture.

Cell Lines—PC12 and SK-N-MC were obtained from American Type Tissue Center (Rockville, MD). PC12 overexpressing APP was provided by B. Zhao (18). Full-length human α7nAChR cDNA inserted into the stable expression vector pcDNA3 was obtained from Dr. P. Groot Kormelink (19). This was transfected into the human neuroblastoma SK-N-MC cell line using Superfect transfection reagent according to the manufacturer's protocol (Qiagen, Valencia, CA). Transfectants were selected in medium supplemented with 2 mg/ml Geneticin (Life Technologies, Inc.) and cloned (α7SK-N-MC). The expression level of α7nAChR increased by ~5-fold as assessed by Western analysis and ^{125}I-α-BTX (500 pM, 2,000 Ci/mmol, Amersham Pharmacia Biotech) binding.

Receptor Binding—Membranes from α7SK-N-MC cells were prepared according to standard procedures except that the membranes were washed five times to remove soluble factors such as endoplasmic reticulum amyloid-binding protein (20) that might have affected Aβ activity. For each assay, membranes (20 μg) were suspended in 50 mM HEPES (pH 7.5) containing 0.01% bovine serum albumin, 2 mM Mg^{2+}, 2 mM Ca^{2+}, and protease inhibitors (Roche Molecular Biochemicals, Germany). $A\beta_{1-42}$, α-BTX, methyllycaconitine, or epibatidine (the last three from Research Biochemicals) were added followed by ^{125}I-α-BTX or ^{125}I-$A\beta_{1-40}$ (40 pM, 2,000 Ci/mmol, Amersham Pharmacia Biotech). The final volume was 150 μl, and each assay was tested in triplicate. At the end of the incubation (30 min), unbound ligand was removed by filtration. Bound radioactivity was measured in a TopCount (Packard, Meriden, CT). IC_{50} values were calculated using Prism (GraphPad Software, San Diego, CA). ^{125}I-$A\beta_{1-40}$ or ^{125}I-α-BTX did not bind to control human Bowes melanoma (21) membranes that do not express α7nAChR as verified by Western analyses (data not shown).

RESULTS

To investigate the possible involvement of α7nAChR in AD pathology, we attempted first to detect its presence in neuritic plaques (enriched in $A\beta_{1-42}$) of sporadic AD brains. The pathology of the human AD hippocampal sections was defined by the presence of neuritic plaques (Fig. 1*A*) and neurofibrillary tangles (Fig. 1*B*). Immunohistochemical analyses on 12 sporadic AD hippocampal samples all showed that $A\beta_{1-42}$ was abundantly present in neuritic plaques and neurons (Fig. 1, *C* and *D*). The α7nAChR was also detected in all of the identified neuritic plaques and in neurons (Fig. 1, *E* and *F*), whereas the α4nAChR (another neuronal nicotinic acetylcholine receptor) or *N*-methyl-D-aspartate R1 glutamate receptor (Fig. 1, *G* and *H*) were not detected. Similar results were observed in the AD cerebral cortical sections obtained from the same subjects (data not shown).

Because both $A\beta_{1-42}$ and α7nAChR are present in neuritic plaques and in hippocampal neurons, we examined the nature of the relationship of these proteins in individual AD neurons. Double immunofluorescence studies using serial AD cortical sections prepared from 12 sporadic AD brain samples all showed that in human AD cortical neurons where α7nAChR was expressed (*green*) (Fig. 2*A*), $A\beta_{1-42}$ was also present (*red*) (Fig. 2*B*). The relative spatial distribution of $A\beta_{1-42}$ and α7nAChR in these neurons was further demonstrated when the two filters were overlaid (*yellow*) (Fig. 2*C*), suggesting a close association between $A\beta_{1-42}$ and α7nAChR.

To study the potential molecular association between $A\beta_{1-42}$ and α7nAChR, hippocampal membranes from 12 sporadic AD brains were prepared and subjected to immunoprecipitation and Western analyses. A protein of ~57 kDa which was reactive to anti-α7nAChR co-immunoprecipitated with endogenous $A\beta_{1-42}$, which is elevated in AD (Fig. 3*A*). Replacing anti-α7nAChR with anti-α1, -α3, -α4, -α5, -α8, or -β2 failed to yield any detectable co-immunoprecipitate with anti-$A\beta_{1-42}$ (data not shown). When hippocampal membranes prepared from age-matched nondemented control subjects (n = 12) were studied similarly, the amount of α7nAChR immunoprecipitate obtained was ~20-fold lower (Fig. 3*A*). In a reciprocal experiment, membrane proteins prepared from AD or control brain samples were first immunoprecipitated by anti-α7nAChR and then subjected to Western analyses using anti-$A\beta_{1-42}$. Two protein bands were detected, a heavier band of ~57 kDa and a lighter

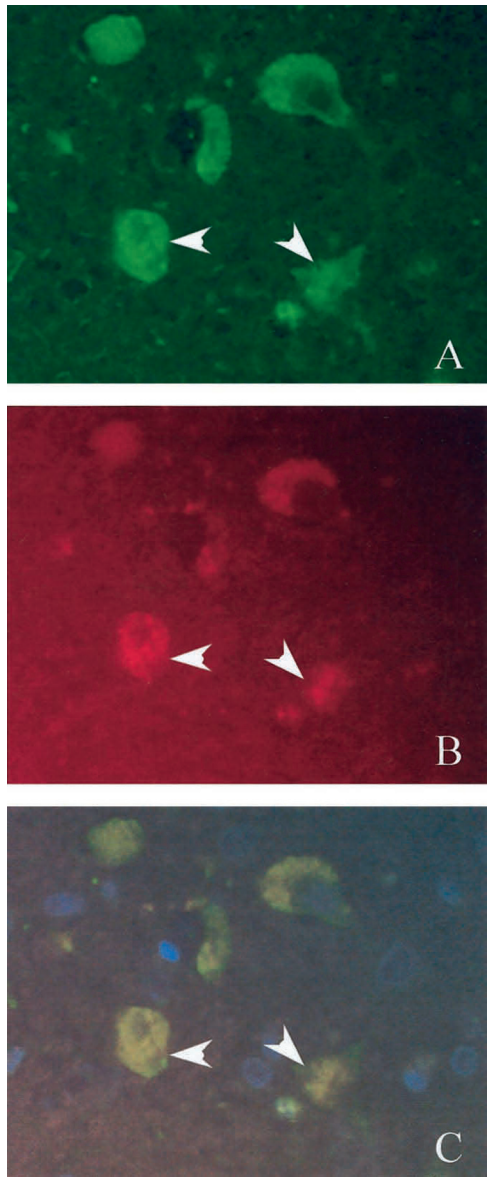

FIG. 2. **Double immunofluorescence performed on AD cortical neurons.** *Panel A*, *arrowheads* indicate the presence of α7nAChR detected using an fluorescein isothiocyanate-conjugated secondary antibody. *Panel B*, *arrowheads* indicate the presence of $A\beta_{1-42}$ detected using a Texas red-conjugated secondary antibody. *Panel C*, a triple fluorescent cube is used to detect both fluorochromes simultaneously. *Arrowheads* indicate the co-localization or close proximity of $A\beta_{1-42}$ and α7nAChR, yielding a *yellow* color, which is the summation of the *red* and *green* fluorochrome. *Blue* 4,6-diamidino-2-phenylindole-dyed nuclei are also present (magnification: × 100). All 12 AD brain samples showed identical results, and representative data from two cases of sporadic AD are shown.

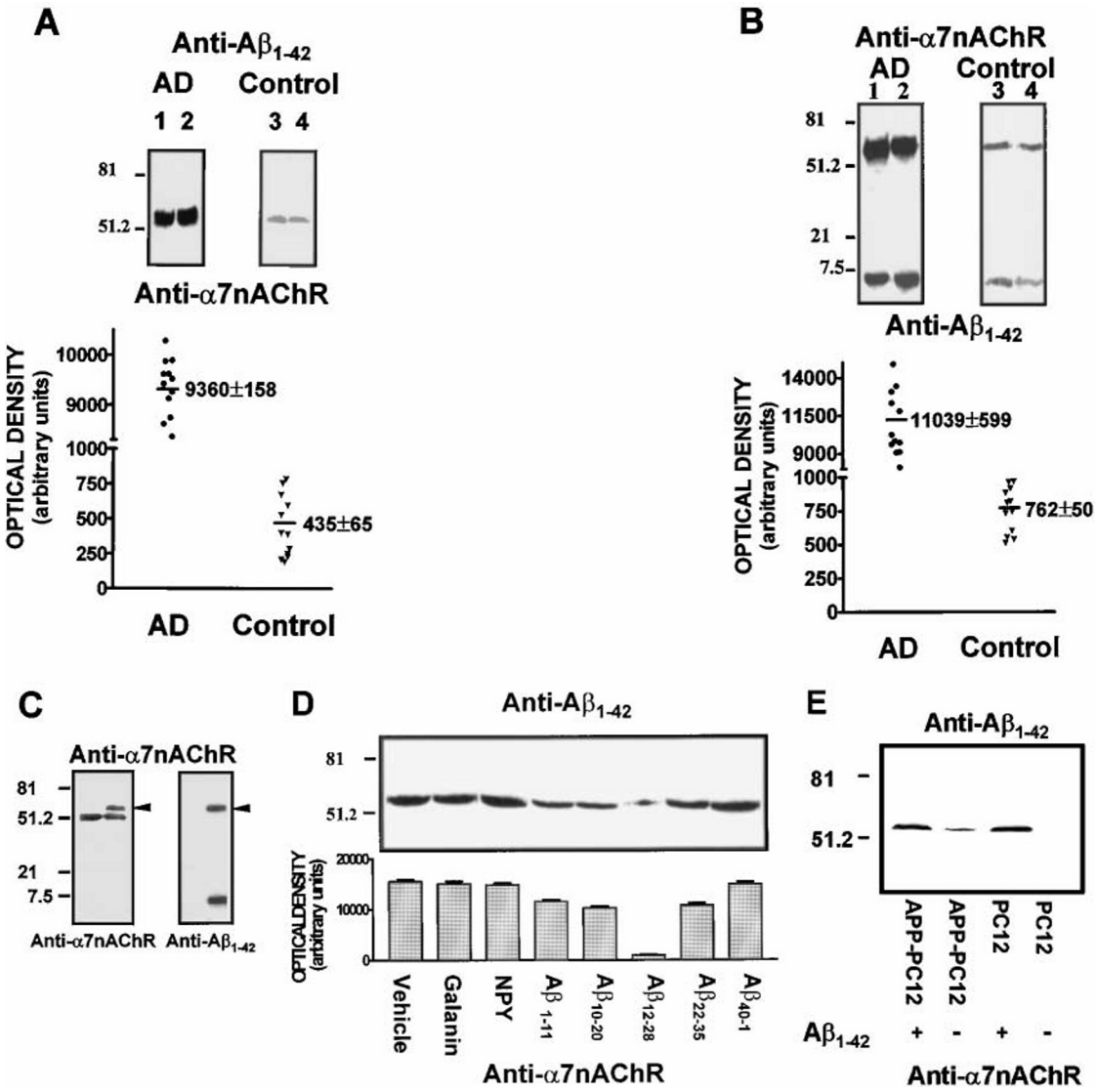

FIG. 3. **Co-immunoprecipitation of α7nAChR with $A\beta_{1-42}$.** *Panel A. Upper*, equal amounts of hippocampal membrane proteins prepared from two female sporadic AD patients (*lane 1*, 73 years old; *lane 2*, 78 years old) and two age-, sex-, postmortem interval-matched, nondemented controls (*lanes 3* and *4*) were immunoprecipitated with anti-$A\beta_{1-42}$ and subjected to Western analysis with anti-α7nAChR. Molecular masses are in kDa. *Lower*, the intensity of the α7nAChR band for each individual AD ($n = 12$) and control ($n = 12$) hippocampal sample was measured by a densitometer and is presented as mean optical density ± S.E. in arbitrary units. *Panel B. Upper*, equal amounts of hippocampal membrane proteins prepared from two female sporadic AD patients (*lane 1*, 73 years old; *lane 2*, 78 years old) and two age-, sex-, postmortem interval-matched, nondemented controls (*lanes 3* and *4*) were immunoprecipitated with anti-α7nAChR and subjected to Western analysis with anti-$A\beta_{1-42}$. Molecular masses are in kDa. *Lower*, the intensity of the $A\beta_{1-42}$ associated with the α7nAChR for each individual AD ($n = 12$) and control ($n = 12$) hippocampal sample was measured by a densitometer and is presented as mean optical density ± S.E. in arbitrary units. *Panel C*, the immunopurified α7nAChR prepared from α7SK-N-MC cells was incubated with 100 nM $A\beta_{1-42}$, solubilized at 25 °C for 1 h, and then subjected to SDS-polyacrylamide gel electrophoresis. Western analyses using anti-α7nAChR and anti-$A\beta_{1-42}$ are shown. *Arrowheads* indicate the protein bands that contain both $A\beta_{1-42}$ and α7nAChR. Control membrane proteins prepared from human Bowes melanoma cells that do not express α7nAChR failed to show any detectable protein. *Panel D*, immunoprecipitation and Western analyses were performed in a manner similar to those described above except that various peptides (100 nM) were included in the incubation. Molecular masses are in kDa. *Error bars* in *lower panel* indicate S.E. Relative reduction in band intensities are: $A\beta_{1-11}$, 20% ($p < 0.0001$); $A\beta_{10-20}$, 22% ($p < 0.05$); $A\beta_{12-28}$, 92% ($p < 0.001$); $A\beta_{22-35}$, 21% ($p < 0.001$). *Panel E*, immunoprecipitation and Western analyses were performed in a similar manner using an equal amount of PC12 cells or PC12 overexpressing APP cell membrane proteins as the starting material in the presence or absence of exogenous $A\beta_{1-42}$ (100 nM). Molecular masses are in kDa.

band of ~5 kDa (Fig. 3*B*). Based on the observed molecular mass, the lighter band may represent $A\beta_{1-42}$, whereas the heavier band seems to have a molecular mass that is slightly higher than that of α7nAChR (~52 kDa), probably representing an α7nAChR complex. Thus, this reciprocal experiment provides additional evidence that α7nAChR and $A\beta_{1-42}$ forms a stable complex.

To characterize the composition of the heavier protein band observed above, a complementary set of experiments was performed. α7nAChR was first purified from the α7SK-N-MC cells that overexpress α7nAChR, incubated with $A\beta_{1-42}$, and then subjected to Western analysis using anti-α7nAChR. Two protein bands were detected similarly: a 52-kDa band (the expected molecular mass of α7nAChR) and a 57-kDa band (Fig. 3*C*). When the filter was stripped and re-probed with anti-$A\beta_{1-42}$, only the 57-kDa band could be detected. This suggests that the heavier protein band contained both $A\beta_{1-42}$ (~5 kDa) and the α7nAChR (~52 kDa). Similar results were obtained using guinea pig hippocampal membranes (enriched in α7nAChR), whereas striatal membranes devoid of α7nAChR failed to show detectable co-immunoprecipitate (data not shown). When control peptides, neuropeptide Y or $A\beta_{40-1}$, were used in place of

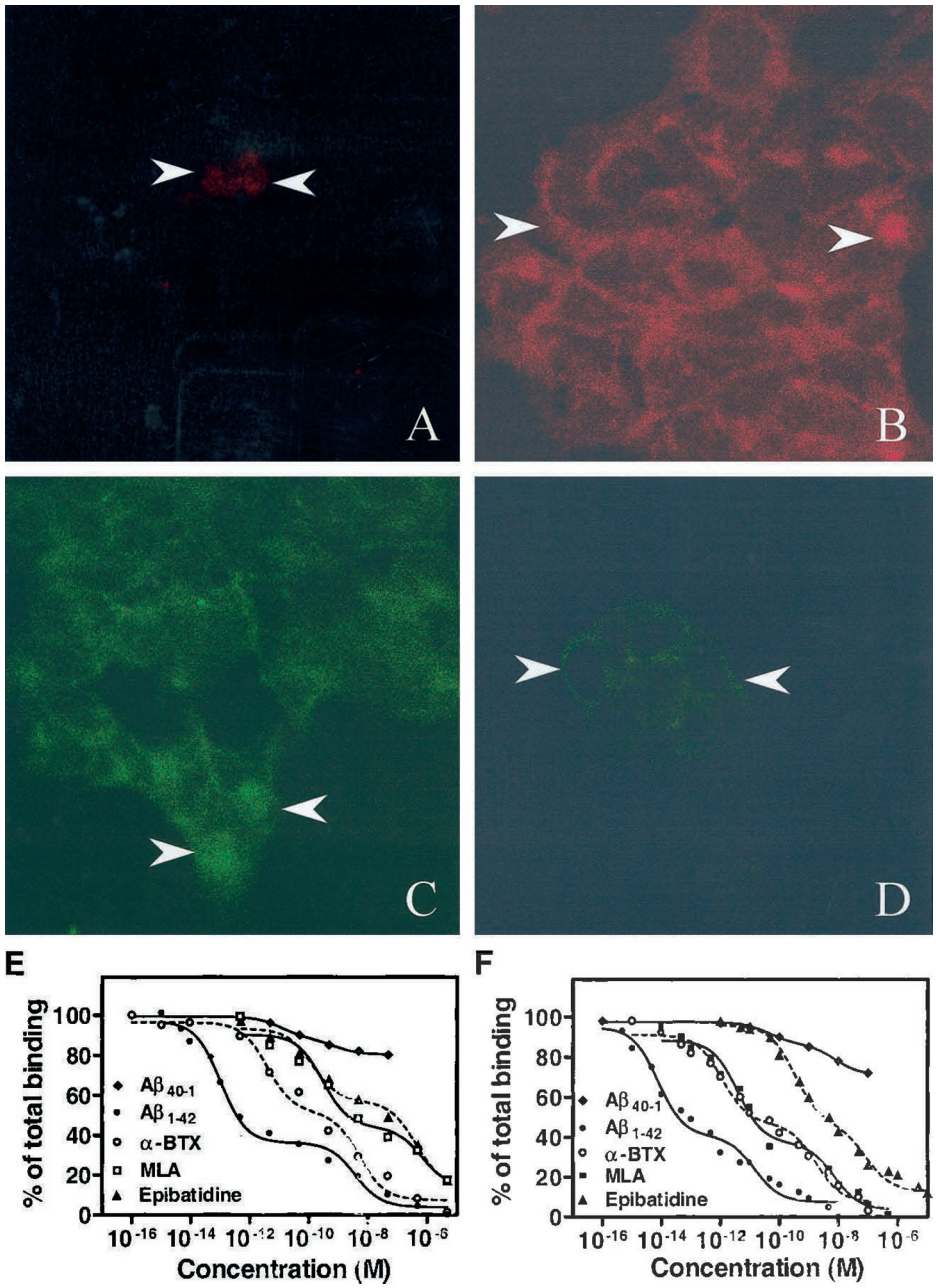

FIG. 4. **Fluo-Aβ_{1-42} binds to α7nAChR.** α7SK-N-MC cells were grown on 22-mm^2 plastic coverslips (Nalge Nunc, Naperville, IL). Fluo-Aβ_{1-42} (100 nM; Advanced Bioconcept, Montreal, Canada) was added to the cells and incubated for 30 min at 37 °C in the presence or absence of 1 μM α-BTX. Fluo-Aβ_{1-42} added to wild type cells was used as a control (data not shown). The slides were mounted with an anti-fade 4,6-diamidino-2-phenylindole fluorescent medium and examined under a Zeiss confocal laser scanning fluorescent microscope. Texas red staining for α7nAChR (*arrowheads*) is shown in wild type SK-N-MC cells (*panel A*) and α7SK-N-MC cells (*panel B*). *Panel C*, Fluo-Aβ_{1-42} visualized with fluorescence microscopy on α7SK-N-MC (*arrowheads*). *Panel D*, reduced Fluo-Aβ_{1-42} fluorescence in the presence of 1 μM of α-BTX (magnification: × 100). Similar results were obtained in six separate experiments. In other studies, the interaction of Aβ peptides with α7nAChR was assessed by ligand receptor binding assay. *Panel E*, ^{125}I-α-BTX binding to α7SK-N-MC membranes. *Panel F*, ^{125}I-Aβ_{1-40} binding to α7SK-N-MC membranes. Mean data from at least three experiments are presented. Nonlinear regression data curve fit was performed by Prism.

Aβ_{1-42} in the experiment, no detectable immunoprecipitate with anti-α7nAChR could be obtained (data not shown). Taken together, these observations suggest that the two proteins may be tightly associated, and this phenomenon can be observed in human AD and control brains, animal brains and even human cell lines in culture.

To define further the specificity of the α7nAChR-Aβ_{1-42} interaction, a different immunoprecipitation experiment was performed. Exogenous Aβ_{1-42} was mixed with an equimolar amount (100 nM) of various Aβ peptide fragments and neuropeptides and then incubated with membrane proteins prepared from the α7SK-N-MC cells before being subjected to immunoprecipitation and Western studies. Fig. 3*D* shows that galanin, neuropeptide Y, and Aβ_{40-1} all failed to inhibit α7nAChR·Aβ_{1-42} complex formation. Treatment with Aβ peptide fragments, Aβ_{1-11}, Aβ_{10-20}, and Aβ_{22-35} all showed ~20% reduction in the amount of immunoprecipitate obtained. However, treatment with Aβ_{12-28} almost completely suppressed the complex formation. These data not only suggest the specificity of the α7nAChR-Aβ_{1-42} interaction, they also imply that a binding epitope for α7nAChR resides in the amino acid 12–28 sequence region of Aβ_{1-42}. In addition, rat Aβ_{1-42} that has three different amino acids in the NH_2 terminus compared with Aβ_{1-42} (Arg^5 → Gly^5, Tyr^{10} → Phe^{10}, and His^{13} → Arg^{13}) and can be recognized by anti-Aβ_{1-42}, also forms a rat Aβ_{1-42}·α7nAChR complex (data not shown). Human APP was overexpressed in a rodent PC12 cell line (18) which expresses the α7nAChR (22) and subjected to a similar immunoprecipitation experiment. Addition of exogenous Aβ_{1-42} facilitated the complex formation with α7nAChR in both the parent PC12 and the APP- transfected PC12 cells (Fig. 3*E*). However, in the absence of exogenous Aβ_{1-42}, only a small amount of α7nAChR/Aβ_{1-42} immunoprecipitate could be observed in the APP-overexpressing PC12 cells, whereas the parent PC12 cells produced no detectable immunoprecipitate. These results suggest that human Aβ_{1-42} deriving from APP in a cellular system maintains the tight association characteristic with α7nAChR and further substantiates the α7nAChR-Aβ_{1-42} tight association.

One possible explanation for the tight association of Aβ_{1-42} and α7nAChR is that Aβ_{1-42} may bind directly with high

affinity to α7nAChR. This hypothesis was evaluated by a series of binding studies. First, α7SK-N-MC cells maintained on chamber coverslips were incubated with Fluo-$A\beta_{1-42}$ in the presence or absence of α-BTX, a specific α7nAChR antagonist. Confocal laser scanning fluorescence microscopy showed that Fluo-$A\beta_{1-42}$ bound to α7SK-N-MC cells and that the addition of α-BTX significantly reduced Fluo-$A\beta_{1-42}$ binding (Fig. 4, *A–D*). Second, the effects of $A\beta_{1-42}$ on ^{125}I-α-BTX binding to α7SK-N-MC membranes were evaluated. $A\beta_{1-42}$ fully inhibited ^{125}I-α-BTX binding, as did methyllycaconitine (another specific α7nAChR antagonist) and the nonspecific nAChR agonist, epibatidine (Fig. 4*E*). For each compound, the saturation curve fit significantly better when the nonlinear regression curve-fit algorithm assumed the presence of two saturable sites rather than one site ($r^2 > 0.95$). A similar pattern of results was observed when rodent hippocampal membranes were used in the binding studies (data not shown). The calculated IC_{50} values for inhibiting ^{125}I-α-BTX binding in α7SK-N-MC membranes were: 0.2 pM and 5 nM for $A\beta_{1-42}$, 4 pM and 5 nM for α-BTX, 0.3 nM and 0.7 μM for methyllycaconitine, and 0.1 nM and 0.4 μM for epibatidine. A corresponding set of experiments using ^{125}I-$A\beta_{1-40}$ as the ligand generated similar saturation curves for the compounds, except that the IC_{50} values were lower: 8 fM and 15 pM for $A\beta_{1-42}$, 1 pM and 2 nM for α-BTX, 4 pM and 7 nM for methyllycaconitine, and 0.3 nM and 82 nM for epibatidine (Fig. 4*F*). These results were not attributed to multiple forms of the ligands (*e.g.* aggregated forms) because SDS-stable oligomers were not detected for ^{125}I-$A\beta_{1-40}$ and $A\beta_{1-42}$ as revealed by nondenaturing polyacrylamide gel electrophoresis analyses. The reason for the existence of two saturable sites is unknown, but possible explanations include the presence of two conformational states or two different isoforms of the receptor, or two distinct binding sites on the receptor. Because the control peptides, including neuropeptide Y, $A\beta_{40-1}$, and α-conotoxin, at 10 μM did not inhibit the binding to the α7nAChR by either ligand, these results demonstrate that the α7nAChR is a high affinity and specific acceptor for Aβ.

Because Aβ peptides have been demonstrated to be neurotoxic (23, 24) and their interactions with scavenger receptors or RAGE generate free radicals that may lead to cell death (8–11), we evaluated if α7nAChR could mediate $A\beta_{1-42}$-induced cytotoxicity. The survival of wild type SK-N-MC cells and α7SK-N-MC cells maintained in growth medium supplemented with 0.1% fetal calf serum after treatment with $A\beta_{1-42}$ was measured by cell proliferation assay (Promega, Madison, WI). Only 5 ± 2% of the α7SK-N-MC cells treated with 100 nM $A\beta_{1-42}$ survived after 24 h, whereas >85% of the wild type SK-N-MC cells survived. Control peptides such as galanin and $A\beta_{40-1}$ did not cause significant (<15%) cell death at 10 μM. The addition of 100 μM nicotine or epibatidine prior to the addition of $A\beta_{1-42}$ promoted the survival of these $A\beta_{1-42}$-treated α7SK-N-MC cells to 48 ± 5% and 45 ± 2%, respectively (Fig. 5). These data suggest an important role for α7nAChR in mediating $A\beta_{1-42}$-induced cytotoxicity.

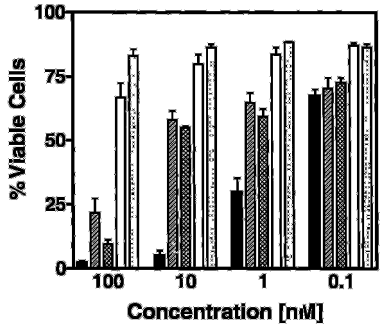

FIG. 5. **Serum-deprived α7SK-N-MC cells were killed readily by $A\beta_{1-42}$ (*closed bars*) but not $A\beta_{40-1}$ (*open bars*) or galanin (*dotted bars*).** The $A\beta_{1-42}$-induced cytotoxicity could be attenuated by pretreatment with 100 μM nicotine (*shaded bars*) or 100 μM epibatidine (*cross-checked bars*). Mean values from at least three measurements are provided; *error bars* indicate S.E.

DISCUSSION

In AD, elevated levels of $A\beta_{1-42}$ and cholinergic neuronal loss are two of the common pathological features observed. Although $A\beta_{1-42}$ is known to be neurotoxic (23, 24), the exact explanation of how it may cause a selective loss of cholinergic neurons, at least in the early stages of AD, is unclear. $A\beta_{1-42}$ is involved in the modulation of cholinergic neurotransmission such as high affinity choline uptake (25, 26) and release (25–27). It modulates choline acetytransferase activity and muscarinic receptor signaling and decreases glucose uptake in primary culture neurons (26). Our data provide, at least in part, a molecular mechanistic explanation that involves the cholinergic neuronal receptor, α7nAChR. The high levels of $A\beta_{1-42}$ in AD may promote interactions with those cholinergic neurons expressing α7nAChR. The high affinity binding of $A\beta_{1-42}$ to α7nAChR may result in the inhibition of ACh release (25–28) and altered Ca^{2+} homeostasis (29, 30) for that particular cholinergic neuron, thus affecting neuronal physiology. Such chronic physiological perturbations may lead to stress and even neurodegeneration. The detection of α7nAChR in neuritic plaques and the correlation of α7nAChR expression patterns in the brains to the occurrence of neuritic plaques provide further supporting evidence to such a hypothesis, which distinguish α7nAChR from other known receptors of $A\beta_{1-42}$.

Although the detailed biochemical mechanisms such as free radicals (13) and tau phosphorylation (31) which are involved in $A\beta_{1-42}$-induced neuronal death and which trigger elevated $A\beta_{1-42}$ levels remain to be elucidated, the high affinity α7nAChR-$A\beta_{1-42}$ interaction may be a critical step leading to AD pathology. It is especially intriguing when α7nAChR ligands such as nicotine and epibatidine were able to inhibit $A\beta_{1-42}$ binding to α7nAChR, which may account for the abilities of these compounds to protect against $A\beta_{1-42}$-induced cytotoxicity *in vitro*. This high affinity interaction between the two proteins results in the formation of a stable protein complex, which resists detergent treatment and remains detectable in the complex form by Western analyses. This α7nAChR·$A\beta_{1-42}$ complex can be detected in experimental systems employing human AD and normal brains, animal brains, human cells overexpressing α7nAChR, or rodent cells expressing APP, the protein precursor for $A\beta_{1-42}$, suggesting both the specificity of the protein-protein interaction and its possible natural occurrence in animals including human. This extraordinary stability exhibited by the α7nAChR·$A\beta_{1-42}$ complex may explain the observed accumulation of the two proteins in AD neurons (Figs. 1, *C–F*, and 2) and may also be related to its resistance to normal proteolytic clearance. Apparently, the sequence region 12–28 of $A\beta_{1-42}$ contains the binding motif to α7nAChR, as suggested in the co-immunoprecipitation peptide competition experiment. This was in part supported by the fact that rodent $A\beta_{1-42}$, which shares only three different amino acids in the NH_2-terminal 13-amino acid sequence region also forms the complex with α7nAChR.

In a series of immunohistochemical, biochemical, and pharmacological studies, we have demonstrated that (i) α7nAChR and $A\beta_{1-42}$ co-localize in neuritic plaques and in AD neurons; (ii) the two proteins form a stable complex as a result of high affinity interaction; and (iii) α7nAChR may mediate $A\beta_{1-42}$-induced cytotoxicity. In view of the severe physiological consequences of $A\beta_{1-42}$ binding to α7nAChR which are implicated in AD pathology, the Aβ sequence region 12–28 and the α7nAChR

could be important targets for the development of innovative therapeutics for AD.

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