Impact of Sustained Exposure to β-Amyloid on Calcium Homeostasis and Neuronal Integrity in Model Nerve Cell System Expressing α4β2 Nicotinic Acetylcholine Receptors*

Komal Arora, Naghum Alfulaij, Jason K. Higa, Jun Panee¹, and Robert A. Nichols²

From the Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96813

Background: The consequence of sustained exposure of nicotinic receptors to β-amyloid is unknown.
Results: Sustained exposure to β-amyloid potentiated nicotinic receptor function, promoting oxidative stress and cellular toxicity.
Conclusion: The presence of nicotinic receptors sensitizes cells to the toxic actions of β-amyloid.
Significance: The potent action of β-amyloid on nicotinic receptors may contribute to the cholinergic deficit found in Alzheimer disease.

Although the interaction between β-amyloid (Aβ) and nicotinic acetylcholine receptors has been widely studied, the impact of prolonged exposure to Aβ on nAChR expression and signaling is not known. In this study, we employed a neuronal culture model to better understand the impact of sustained exposure of Aβ on the regulation of cellular and synaptic function. The differentiated rodent neuroblastoma cell line NG108-15 expressing exogenous high-affinity α4β2 nAChRs was exposed to soluble oligomeric Aβ for several days. Ca²⁺ responses, expression levels of α4β2 nAChRs, rate of mitochondrial movement, mitochondrial fission, levels of reactive oxygen species, and nuclear integrity were compared between Aβ-treated and untreated cells, transfected or not (mock-transfected) with α4β2 nAChRs. Sustained exposure of Aβ1–42 to α4β2 nAChR-transfected cells for several days led to increased Ca²⁺ responses on subsequent acute stimulation with Aβ1–42 or nicotine, paralleled by increased expression levels of α4β2 nAChRs. The rate of mitochondrial movement was sharply reduced, whereas the mitochondrial fission protein pDrp-1 was increased in α4β2 nAChR-transfected cells treated with Aβ1–42. In addition, the presence of α4β2 nAChRs dramatically enhanced Aβ1–42-mediated increases in reactive oxygen species and nuclear fragmentation, eventually leading to apoptosis. Our data thus show disturbed calcium homeostasis coupled with mitochondrial dysfunction and loss of neuronal integrity on prolonged exposure of Aβ in cells transfected with α4β2 nAChRs. Together, the results suggest that the presence of nAChRs sensitizes neurons to the toxic actions of soluble oligomeric Aβ, perhaps contributing to the cholinergic deficit in Alzheimer disease.

Numerous studies using a wide range of preparations have shown that diffusible oligomeric β-amyloid (Aβ) assemblies are toxic (1), being specifically responsible for the development of synaptic impairment (2). The impact of soluble Aβ on synaptic function was found to be dependent upon concentration, with impairment typically occurring at micromolar levels of the peptide. In contrast, at picomolar concentrations Aβ enhanced synaptic plasticity, suggesting a neuromodulatory role under physiological conditions (3, 4). However, as Aβ accumulates in AD, its action will thus convert to synaptic (and cellular) disruption.

The synapse is likely a major site of action for Aβ, as evident from studies showing that the production and release of Aβ from the presynaptic terminal is regulated by synaptic activity (5, 6). The average “normal” concentrations of Aβ, i.e. concentrations found in nondemented adults, have been found to be in the high picomolar (~250 pm) range in brain (7), and the presynaptic terminal appears to be the dominant source of Aβ production (8) and undergoes synaptic regulation by Aβ (4). Aβ thus appears to be localized to the synapse at concentrations that regulate presynaptic dynamics.

Among an array of different targets at synapses to which Aβ might bind and exert downstream effects, those of notable significance are neuronal nicotinic acetylcholine receptors (nAChRs) and metabotropic glutamate receptors (9). The nAChRs play important modulatory roles in neuronal development and synaptic plasticity, participating in cognitive functions such as learning, memory, and attention (10). The most abundant high affinity nAChR in brain is comprised of α4 and β2 subunits, whereas the other major nAChR subtype in brain contains α7 subunits (11). Activation of α7-nAChRs produces a rapid, sharp increase in the intracellular Ca²⁺ signal, whereas α4β2-nAChRs cause a more delayed but long-lasting signal (11). Aβ activation of different nAChR subtypes will therefore

*This work was supported, in whole or in part, by National Institutes of Health Grant AG21586 from the NIA, American Health Assistance Foundation Grant AHAF-A2007-409, and Hawaii Community Foundation Grant 09ADV-C45413 (to R. A. N.).

¹Supported by IDEA Networks of Biomedical Research Excellence II Grant P20RR016467 from the National Center for Research Resources.

²To whom correspondence should be addressed: 651 Ilalo St., Honolulu, HI 96813. Tel: 808-692-1568; Fax: 808-692-1970; E-mail: robert.nichols@hawaii.edu.

© 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
have different impacts on intracellular Ca\(^{2+}\) and hence synaptic signaling.

Acute application of pico- to nanomolar A\(\beta\) evokes increases in Ca\(^{2+}\) and neurotransmitter release via presynaptic nAChRs (12–14). Mutation of a key tyrosine residue in the agonist-binding site of \(\alpha 7\) nAChRs eliminates the A\(\beta\) effect on presynaptic Ca\(^{2+}\) (15), directly confirming the agonist-like action of A\(\beta\). At picomolar concentrations, acute A\(\beta\) was also found to enhance long-term potentiation and contextual memory in a manner dependent upon presynaptic nAChRs (3, 4).

The high-affinity \(\alpha 4\beta 2\) nAChRs are significantly up-regulated in animals chronically treated with nicotine on a daily dose basis (16, 17). This nicotine-induced up-regulation has been further characterized in a variety of systems ranging from clonal cell lines to primary neurons in culture to mouse models to smokers’ brains (18–26). Up-regulation of \(\alpha 4\beta 2\) nAChRs may sensitize cellular targets to the action of nicotine.

Regulation of receptor expression also depends on steady-state control via endocytosis and membrane recycling, along with degradation via lysosomes. Changes in AMPA-type glutamate receptor expression in postsynaptic membranes in the context of synaptic plasticity, for example, have been shown to involve changes in receptor recycling. Rab proteins, members of the Ras family of small GTPases, have been shown to play a key role at various steps in endocytic, recycling, and degradative pathways. Specifically, Rab5 expression levels are higher in plasma membrane, recycling endosomes, and clathrin-coated pits (27, 28) and regulates early endosomal fusion and recycling. Rab11 is mainly found in the trans-Golgi network and recycling endosomes (29, 30) from where it regulates trafficking of proteins to the plasma membrane. The extent to which nAChRs are regulated by these processes in response to sustained agonist exposure remains to be determined.

An important downstream consequence of A\(\beta\) pathology is mitochondrial toxicity (31). As synapses are high energy demanding sites, mitochondria play critical roles in maintaining synaptic function, and mitochondria toxicity likely contributes to synaptic loss in AD (32). It has been reported that brains of AD patients show ultrastructural alterations in mitochondrial morphology, such as reduced number, increased size, and broken internal membrane cristae (33). A\(\beta\) may also affect mitochondrial dynamics (fission/fusion equilibrium) and distribution in the axon and synapse. Recent studies using an in vivo fly model overexpressing A\(\beta\) showed that depletion of presynaptic and axonal mitochondria was one of the earliest detectable deficits, preceding A\(\beta\)-induced presynaptic deficits in motor function (34, 35). A\(\beta\)-induced mitochondrial mislocalization was also confirmed in hippocampal neurons (36). Such mitochondrial trafficking disruption in AD could potentially compromise normal synaptic function. However, it is still not clear what role presynaptic receptors play in mitotoxicity induced by A\(\beta\) at the synapse.

Here, we addressed the impact of sustained exposure to A\(\beta\) on a model neuronal system expressing exogenous \(\alpha 4\beta 2\) nAChRs on [Ca\(^{2+}\)]\(_e\), receptor regulation, axonal mitochondrial dynamics, oxidative stress, nuclear and cellular integrity, and endocytosis/recycling. The results suggest that the presence of \(\alpha 4\beta 2\) nAChRs may sensitize neurons to the effects of sustained exposure to A\(\beta\).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NG108-15 hybrid neuroblastoma cells were plated on Cell-Tak-coated coverslips (Warner) in 35-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 15% fetal bovine serum (FBS) (37). The cells were induced to differentiate in media containing 1% FBS and 1 mM cyclic AMP for 48–72 h, elongating long neurites with varicosities. These varicosities display features found for presynaptic elements in the brain, like voltage-gated calcium channels (38), ER-based Ca\(^{2+}\) stores, vesicles, mitochondria, and synaptic proteins (39) and are capable of forming fully functional cholinergic synapses with target cells (40). As they do not express functional nAChRs themselves, mouse sequences for \(\alpha 4\) and \(\beta 2\) nAChR subunits housed in pcDNA3.1 expression vectors (courtesy of Dr. Jerry Stitziel, University of Colorado) were transiently transfected at a ratio of 1:4, respectively, into differentiated NG108-15 cells using FuGENE HD, a lipid-based transfection reagent, for 48 h. This ratio was found to yield maximal expression of functional receptors. Mock-transfected NG108-15 cells, containing only FuGENE HD and no plasmid DNA were used as controls.

**Live-cell Calcium Imaging**—To measure changes in Ca\(^{2+}\) levels in individual varicosities, the transfected, differentiated cells were loaded for 40 min at 37 °C with fluorescent Ca\(^{2+}\) indicator dye Fluo-4/AM (Invitrogen) at 5 \(\mu\)M in HEPES-buffered saline (HBS: containing (in mM) 10 HEPES, 142 NaCl, 2.4 KCl, 1.2 K\(_2\)PO\(_4\), 1 MgCl\(_2\), 1 CaCl\(_2\), 5 d-glucose, and 100 mM tetrodotoxin, pH 7.4, saturated with O\(_2\)) in preparation for confocal imaging, as described (41). Cells were mounted in a rapid exchange Warner open perfusion chamber for confocal imaging using a Zeiss LSM 5 Pascal imaging system (excitation: 488 nm; emission: 515–565 nm band-pass; \(\times 40/1.3\) NA, oil-immersion Plan-Neofluar objective). Imaging was started and after obtaining a baseline series of four images, reagents (agonists; antagonists) were applied by rapid switching via a Warner six-channel valve-controlled perfusion system (VC-66CS, Warner Instruments) using DN series constant flow reservoirs. Image series consisted of a time course of 200–400 s (typically to the peak of the response). For each run, a minimum of four to eight varicosities in each successive image were selected as regions of interest and their associated fluorescence intensities were determined for all 30 frames using ImageJ. Time series responses were normalized to baseline, corrected for photobleaching, and presented as F/F\(_o\).

**Immunocytochemistry**—Cell cultures were fixed with freshly prepared 4% paraformaldehyde in HBS at room temperature for 30 min and rinsed with phosphate-buffered saline (PBS) for 30 min. The cultures were then incubated in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and 1% normal goat serum for 30 min to block nonspecific binding. Affinity-purified antibodies (1:200) were then added to the cultures and incubated overnight at 4 °C. The cultures were then washed with 10% goat serum in TBS for 30 min and incubated with the FITC-conjugated secondary antibodies (IgGs, typically at 1:500) for 30 min at room temperature. The coverslips were
finally washed with 10% normal goat serum and TBS, plated onto glass microscope slides, and sealed for imaging via confocal microscopy. To detect the mitochondrial fission protein Drp-1 (dynamin-related protein-1) and the Rab proteins, cells were permeabilized before incubation with blocking buffer. For Drp-1, cells were then co-stained with rabbit anti-phospho-Drp-1 and mouse anti-Drp-1. Anti-rabbit Alexa-488 and antimouse Alexa-633 were used as secondary antibodies, respectively. The immunostained preparations were subsequently visualized using a Zeiss LSM 5 Pascal confocal imaging system on a Zeiss Axiovert 200M microscope with appropriate fluorescence filters via a ×40 Plan-Neofluar objective. Immunostaining for the α4 and β2 nAChR subunits was visualized on a Nikon Cameleon AOTF confocal microscopic system via a ×40 objective.

Reactive Oxygen Species (ROS)/Hoechst Staining—The Image iT live Reactive Oxygen Species Detection kit (Invitrogen) was used to determine the extent of oxidative stress in response to daily treatment with Aβ4–42 in NG108-15 cells transfected with α4β2-nAChRs as compared with mock-transfected (control) cells, exchanging the culture medium each day. After treatment, the cells were rinsed with warm HBS containing Ca2+ and Mg2+ and incubated with carboxy-H2DCFDA (component A) at 37 °C for 30 min. During the last 5 min of incubation, 2 μg/ml of Hoechst stain (component B) was added. The cells were washed twice with HBS and visualized using an Olympus IX71 epifluorescence microscope at excitation/emission of 495/529 nm (ROS) and 350/461 nm (Hoechst), respectively, linked to a Macrofire camera.

Mitochondrial Size and Movement Along the Neurite—Differentiated NG108-15 cells transfected with α4β2-nAChRs were treated daily with Aβ4–42 or not (control) for 3 days, exchanging the culture medium each day. On day 3, the cells were incubated with BacMam Mito-RFP reagent (Invitrogen) for another 16 h to label the mitochondria. The cells were then washed for 30 min and imaged for the live movement of individual RFP-labeled mitochondria in the axonal neurites. A total of 30 images were recorded over a period of 720 s using confocal imaging (see previous methods) under standardized settings. Using Image J, the x and y coordinates of each individual mitochondrion were measured to determine the relative distance traversed by that particular mitochondrion along a given neurite. In addition, the size of individual mitochondria was determined (diameter in pixels) across the 3-day time course. Mock-transfected cells were used as separate controls.

Cell Surface Labeling and Recycling—Differentiated NG108-15 cells transfected with α4β2-nAChRs and treated (or not) with Aβ4–42 daily for 3 days, exchanging the culture medium each day, were surface labeled at 4 °C using a cleavable biotinylation reagent from the Pierce Cell Surface Protein Isolation kit (Thermo Scientific). After various treatment conditions, the cells were lysed using the lysis reagent and the biotinylated proteins were pulled down using NeutrAvidin-agarose beads. These were then mixed with SDS sample buffer and loaded onto 4–20% Tris-HCl gels, transferred onto a nitrocellulose membrane and probed for α4-nAChR subunits using a highly specific rat monoclonal (299) antibody.

Quantitative RT-PCR—Total RNA was isolated from mock-transfected and α4β2-nAChR-transfected cells treated daily for 3 days with Aβ4–42 or not (untreated), exchanging the culture medium each day, using the RNeasy Mini Kit (Qiagen). The total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer’s instructions, and quantitative real-time PCR was performed according to standard protocols. For data analysis, 18S rRNA was used as an endogenous control. Data are expressed as relative expression for each individual gene normalized to their corresponding controls. The following primer sequences were used for amplification of the mouse α4-nAChR cDNA (NM015730, GenBank™) at an annealing temperature of 61 °C; forward, 5′-ACTTTGCAGTCACCCACTTACACCAAA-3′; reverse, 5′-ATGACGACGAGAAGCGGTGAGAGAAA-3′. This yielded a 481-bp PCR product.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay—NG108-15 cells were seeded on coverslips in 24-well plates (6 × 104 cells/well). Cells were either mock- or α4β2-nAChR transfected as previously described (Cell Culture). After transfection, cells were treated daily with Aβ4–42 or Aβ42–1 (control peptide) for 5 days, exchanging the culture medium each day. The TUNEL assay was performed using Click-iT® TUNEL Alexa Fluor® Imaging Kit (Invitrogen) in accordance with the manufacturer’s protocol. In brief, cell cultures were fixed with freshly prepared 4% paraformaldehyde in PBS at room temperature for 20 min and permeabilized with Triton X-100 (0.25% in PBS) for another 20 min. The cultures were then washed twice and incubated with 50 μl of terminal deoxynucleotidyl transferase reaction buffer (Component A) for 10 min at room temperature. The buffer was removed and the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase was added, incubating the cultures in a humidified chamber at 37 °C for 60 min. After treatment, cells were washed three times with 3% BSA in PBS for 2 min each and then incubated with 50 μl of Click-iT reaction mixture (containing Alexa 488 azide) for 30 min at room temperature, while being protected from light exposure. The cells were then washed with 3% BSA in PBS and the cell nuclei were counterstained with Hoechst 33342 for 15 min at room temperature, protected from light. The coverslips were washed twice with PBS before mounting onto a slide with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Negative and positive controls were included in each experiment. For the negative control, cells were processed using a reaction mixture that did not contain terminal deoxynucleotidyl transferase. For the positive control, cells were incubated with DNase I (3 units/ml) for 30 min to induce DNA strand breaks. Labeled nuclei were detected by fluorescence microscopy on a Zeiss Pascal confocal imaging system on a Zeiss Axiovert 200 microscope, equipped with appropriate fluorescence filters and objectives. The TUNEL-positive cells were counted in eight different, random fields for each well.

Aβ—Aβ4–42 and the control reverse peptide Aβ42–1 were purchased from American Peptide. A stock solution of the peptides were prepared at 0.1 mM by dissolving solid peptide
in double deionized water (see Ref. 37). Aβ$_{1–42}$ (and Aβ$_{42–1}$) for each experiment was diluted from 1 μM to 100 nM from the 0.1 mM stock into oxygenated HBS and vortexed to assure full suspension. Under these conditions, the Aβ is largely present as stable oligomers, as assessed by SDS-PAGE and native gel analysis (36).

**Chemicals**—DMEM was from Mediatech, Inc. FBS was from Invitrogen. Cell-Tak was from BD Biosciences. Fluor 4/AM, Image-iT Live ROS Detection kit, and BacMam Mito-RFP were purchased from Invitrogen. BSA, nicotine tartrate citrate, and Rab5 antibody were purchased from Sigma. Nicotine (1 μM final) was freshly prepared by dissolving in oxygenated HBS before each experiment. Tetrodotoxin was from Calbiochem. 

**Results**

**Ca$^{2+}$ Responses Mediated by α4β2-nAChRs following Acute Treatment with Aβ**—To test the effect of Aβ$_{1–42}$ on α4β2-nAChRs, exogenous mouse α4-nAChR and β2-nAChR sequences in pcDNA3.1 vectors were transfected into differentiated NG108-15 cells, whereas mock-transfected cells were used as controls. Ca$^{2+}$ changes in the axonal varicosities of the NG108-15 cells, as relative changes in fluorescence intensities (F/F$_{0}$) were determined in cells loaded with the Ca$^{2+}$ indicator Fluo-4 (Fig. 1A) as test for synaptic regulation (37). As shown in Fig. 1B, left, α4β2-nAChR-transfected cells displayed apparent increases in [Ca$^{2+}$], on stimulation with 100 nM Aβ$_{1–42}$ ($p < 0.05$ relative to mock-transfected cells) and 1 μM nicotine ($p < 0.005$ relative to mock-transfected cells), whereas mock cells did not display significant Ca$^{2+}$ changes to Aβ$_{1–42}$ or nicotine over baseline. Ca$^{2+}$ changes were also apparent in select cell bodies. Ca$^{2+}$ responses to depolarization induced by elevated KCl (HK) were recorded after each drug/agonist treatment to assess the cell integrity. There were no significant differences in the responses in the varicosities to elevated KCl following various treatments as compared with control. These results indicate that Aβ$_{1–42}$ has an agonist-like effect on α4β2-nAChRs in axonal varicosities, which is consistent with the idea that Aβ$_{1–42}$ can serve as a physiological regulator of presynaptic nAChRs (3, 4, 37). Prior treatment with 1 μM dihydro-β-erythroidine (DHβE), an α4-selective antagonist, for 10 min attenuated Ca$^{2+}$ responses in the varicosities to acute stimulation with both Aβ ($p < 0.05$) and nicotine ($p < 0.05$) (Fig. 1B, right). These latter results confirm that the increased calcium levels elicited by Aβ are indeed mediated by α4β2-nAChRs.

To directly monitor the expression pattern of nicotinic receptors on transfected NG108-15 cells, immunostaining for α4β2 nAChRs was performed using specific antibodies for the α4 and β2 subunits. α4β2-nAChR-transfected cells and mock-transfected cells were compared. As shown in Fig. 1C, α4β2-nAChR transfected cells were strongly immunopositive for both α4 and β2 subunits when compared with mock-transfected cells immunostained for the α4 subunit. By comparison, the overall signal for mock cells is evenly weak, similar to the nonspecific binding of secondary antibody alone to the cell surface, whereas there is no clear signal on the varicosities. The immunostaining results provide direct evidence of efficient receptor expression, consistent with the functional responses.

**α4β2-induced Ca$^{2+}$ Responses Mediated by α4β2-nAChRs: Impact of Sustained Exposure to Aβ**—To determine the consequences of sustained exposure to Aβ$_{1–42}$ on subsequent acute stimulation by Aβ$_{1–42}$ or nicotine, calcium imaging was performed as previously described. Differentiated NG108-15 cells expressing α4β2-nAChRs were subjected to 100 nM Aβ$_{1–42}$ for 1, 2, and 3 days, exchanging the culture media every 24 h with media containing fresh oligomeric Aβ$_{1–42}$ On the day of calcium imaging, the culture medium containing Aβ$_{1–42}$ was removed and cells were washed with HBS for 15 min, followed by Fluo-4 loading. After an extensive second wash period, perfusion was started and Ca$^{2+}$ responses elicited by acute stimulation with Aβ$_{1–42}$ (100 nM) and nicotine (1 μM) were measured for control and Aβ$_{1–42}$-treated cells. As shown in Fig. 2, [Ca$^{2+}$]$_{i}$ responses in the varicosities were unchanged after 1 or 2 days but increased significantly after 3 days of Aβ$_{1–42}$ treatment in response to both Aβ$_{1–42}$ and nicotine as compared with control cells (Fig. 2, A–C; $p < 0.05$). There was no effect of sustained Aβ$_{1–42}$ treatment on mock-transfected cells (Fig. 2E). There was also no effect of sustained treatment with the control reverse peptide, Aβ$_{42–1}$ (Fig. 2F). These results are consistent with previous work demonstrating that chronic agonist exposure (nicotine) leads to an alteration in the expression and function of presynaptic α4β2-nAChRs (42). Similarly chronic nicotine exposure resulted in significantly increased levels of Ca$^{2+}$ in α4β2 nAChR-transfected cells in response to acute stimulation by Aβ$_{1–42}$ as well as nicotine (Fig. 2D; $p < 0.01$) as compared with control cells. As found for untreated NG108-15 cells expressing α4β2-nAChRs, the α4-selective antagonist DHβE fully attenuated Ca$^{2+}$ responses in the varicosities to acute stimulation with Aβ or nicotine in NG108-15 cultures treated with Aβ for 3 days (Fig. 2G). In addition, co-treatment with DHβE for 3 days fully attenuated the acute Aβ- or nicotine-induced increases in Ca$^{2+}$ responses in the varicosities found following sustained treatment with Aβ$_{1–42}$ for 3 days (Fig. 2H), confirming that the action of Aβ$_{1–42}$ was specifically through the nAChRs. Together, these results support a role for α4β2-nAChRs in mediating changes in calcium homeostasis on sustained exposure to Aβ.
Sustained Exposure to Aβ Caused Receptor Up-regulation in the Cell Bodies and Varicosities of Neurites of NG108-15 cells Expressing α4β2 nAChRs in a Manner Similar to Chronic Nicotine Treatment—The surface expression levels of α4β2-nAChRs in mock- and α4β2-nAChR transfected cells were compared with those in cells treated with Aβ1–42 and nicotine by immunostaining using a selective anti-α4-nAChR antibody (Fig. 3A). α4β2-nAChR-transfected cells treated with Aβ1–42 and nicotine for 3 days showed apparent increased expression of α4β2-nAChRs as compared with untreated cells (varicosities and cell bodies, Fig. 3B). Co-treatment with the antagonist DHβE blocked the apparent up-regulation of the α4-nAChRs, although the effect was only clear for the cell bodies. To demonstrate that this up-regulation was post-transcriptional, we
FIGURE 2. Prolonged exposure to Aβ led to augmented calcium responses on subsequent acute stimulation with Aβ or nicotine in varicosities of NG108-15 cells transfected with α4β2-nAChRs. [Ca^{2+}]_i responses were unchanged after 1 (A) or 2 days (B) but increased significantly after 3 days of daily Aβ1-42 treatment (C) in response to acute stimulation with either Aβ1-42 or nicotine (p < 0.05). Similarly, chronic nicotine exposure (D) resulted in significantly increased levels of Ca^{2+} in varicosities of α4β2-nAChR-transfected cells in response to acute stimulation by Aβ1-42 as well as nicotine. E, there was no effect of chronic Aβ1-42 treatment on mock-transfected cells. F, no change was observed in Ca^{2+} responses induced by 100 nM Aβ1-42 between untreated cells and cells treated with 100 nM Aβ42-1 (control reverse peptide) for 3 days. G, pre-treatment with 1 μM DHβE for 10 min blocked the agonist-evoked Ca^{2+} responses on acute application of Aβ1-42 and nicotine. H, DHβE also prevented the effect of 3 days treatment with Aβ1-42 to augment Ca^{2+} responses evoked by nicotine or Aβ1. I, summarized results for 3 independent experiments (n = 15–20 for each condition). **, p < 0.01; ***, p < 0.001 compared with control. HK, high K^+.
FIGURE 3. Up-regulation of α4β2-nAChRs in response to prolonged Aβ treatment. A, the expression levels of α4β2-nAChRs in mock- and α4β2-nAChR-transfected cells were compared with those in cells treated with 100 nm Aβ1–42 (with or without DHβE) or nicotine by immunostaining for the α4-nAChR subunit, counterstained with DAPI (nuclear stain). B, fluorescent signals from cell bodies and varicosities were selected and averaged to calculate the mean signal intensity, normalized to that obtained for mock-transfected cells (n = 5). α4β2-nAChR-transfected cells treated with Aβ1–42 or nicotine showed apparent increased expression of α4β2-nAChRs as compared with untreated cells. *, p < 0.05 when compared with controls. C, real-time PCR was performed to determine the changes in mRNA levels of α4-nAChRs on prolonged treatment with Aβ1–42. No difference was detected in transcript expression between control and 3-day Aβ1–42-treated cells transfected with α4β2-nAChRs (n = 4).
performed quantitative RT-PCR on α4-nAChR cDNA obtained following RNA isolation from control and 3-day Aβ₁₋₄₂-treated cells. There was no significant difference in transcript levels in Aβ₁₋₄₂-treated cells as compared with control cells (Fig. 3C).

**Altered Endocytosis-receptor Recycling on Sustained Exposure to Aβ: Rab5, and Rab11**—To consider the possibility that up-regulation of α4β₂-nAChRs in response to sustained exposure to Aβ results from an alteration in the endocytotic-receptor recycling pathway, we first tested for the endosomal marker Rab5 and the recycling marker Rab11. Relative changes in the expression levels of Rab proteins were detected using immunocytochemistry of differentiated NG108-15 cells treated or not with Aβ₁₋₄₂ for 3 days. α4β₂-nAChR-transfected cells showed increased levels of Rab5 as compared with that in mock-transfected cells (Fig. 4A, top series). Sustained exposure to Aβ₁₋₄₂ further increased Rab5 levels in α4β₂-nAChR-transfected cells. Similarly, intracellular Rab11 levels were also found to be increased in α4β₂-nAChR-transfected cells treated with Aβ to a significant extent as compared with that in mock-transfected cells (Fig. 4A, bottom series). The results from co-immunostaining with rabbit anti-α4-nAChR and rat anti-Rab5 or anti-Rab11 antibodies (Fig. 4C) and Western blots to detect the total levels of Rab5 and Rab11 (Fig. 4D) provided further support that the levels of endosomal/recycling markers are altered in a time-dependent manner in response to Aβ treatment. Rab5, in particular, was substantially increased in response to Aβ treatment at 2–3 days by over 3-fold, as assessed by immunoblot analysis (390 ± 25% of day 0; p < 0.01; n = 6).

To address directly whether changes in the expression levels of nAChRs on prolonged exposure to Aβ resulted, at least in part, from a change in the endocytotic/recycling pathway, we

![Figure 4](image-url)

**FIGURE 4**. Prolonged exposure to Aβ alters the expression levels of endocytosis/recycling markers, Rab5 and Rab11, and the endocytotic-recycling pathway. A, immunostaining for the endosomal marker Rab5 (top) and the recycling marker Rab11 (bottom) after 3 days of 100 nM Aβ₁₋₄₂ treatment or not of α4β₂-nAChR-transfected cells as compared with mock-transfected cells, as summarized in B. Regulation of Rab5 and Rab11 in α4-nAChR-expressing cells in response to Aβ₁₋₄₂ treatment across the 3-day time course was verified in co-immunolabeling (C) and Western blots (D). E, after surface labeling with cleavable sulfo-NHS-SS-biotin, cells were allowed to undergo endocytosis at 37 °C followed by DTT treatment at 4 °C to remove biotin from the remaining surface protein, demonstrating an increased level of expression of the receptor following 3-day Aβ treatment, when probed with rat monoclonal anti-α4-nAChR antibody, as compared with untreated or mock-transfected cells (first to third lanes). (The molecular mass of the α4-nAChR band was confirmed relative to the protein standards by regression analysis as 72 kDa. The lower bands are nonspecific background also present in mock-transfected cells.) After the labeled cells were allowed to recycle the biotin-labeled receptors, the Aβ-treated cells displayed predominately DTT-sensitive, biotin-labeled α4-containing nAChRs, indicating substantial recycling of the receptor (lane 6 versus 9). *, p < 0.05; ***, p < 0.001 when compared with controls.
used surface labeling with biotin to follow the fate of the nAChRs. Following biotinylation at 4 °C, endocytosis of the surface proteins of \( \text{H9251/2-nAChR} \)-transfected cells was allowed to proceed by shifting the cells to 37 °C for 30 min, thereafter treating with DTT at 4 °C to remove biotin from the remaining surface proteins. As shown in Fig. 4 (lanes 1–3), the \( \text{H9251/2-nAChR} \)-transfected cells treated with \( \text{A/H9252} \) for 3 days showed a markedly increased surface expression of receptor as compared with untreated \( \text{H9251/2-nAChR} \)-transfected cells or mock-transfected cells, when probed with a rat monoclonal anti-\( \text{H9251/2-nAChR} \) antibody. The cells were then shifted back to 37 °C for 45 min to allow for receptor recycling. Reappearance of surface DTT-sensitive receptors was assessed as a measure of recycling.

The \( \text{A/H9252} \)-treated, \( \text{H9251/2-nAChR} \)-transfected cells had substantially more surface expression of the receptor as compared with controls. The results suggest that receptor up-regulation upon prolonged exposure to \( \text{A/H9252} \) is due, in part, to increased receptor recycling.

Altered Mitochondrial Dynamics in Axonal Neurites of NG108-15 Cells following Sustained \( \text{A/H9252} \) Treatment—\( \text{A/H9252} \) was previously found to alter mitochondrial dynamics (36, 43, 44). To examine the potential disruptive effect of sustained exposure to \( \text{A/H9252} \) on mitochondrial transport, differentiated NG108-15 cells were transfected with \( \text{H9251/2-nAChRs} \) and then treated with \( \text{A/H9252} \)-1–42 or not for 3 days. On the third day, the cells were incubated with BacMam Mito-RFP reagent for another 16 h to label the mitochondria. The \( \text{H9251/2-nAChR} \)-transfected cells treated with \( \text{A/H9252} \)-1–42 showed a significantly reduced rate of movement of mitochondria in the axonal neurites as compared with that found in untreated cells (Fig. 5, B–G) or mock cells.
Nicotinic Receptors Sensitize Neurons to β-Amyloid Toxicity

A

Mock-3d Aβ1-42
Mock-4d Aβ1-42
α4β2-3d Aβ1-42
α4β2-2d Aβ1-42

B

100nM Aβ1-42

Average size of mitochondria (pixels)

Mock-3d Aβ1-42
Mock-4d Aβ1-42
α4β2-3d Aβ1-42
α4β2-2d Aβ1-42

C

Phospho Drp1
Total Drp1
Merge

Mock-3d Aβ1-42
Mock-4d Aβ1-42
α4β2

D

pDrp-1/Total Drp-1

Mock-3d Aβ1-42
Mock-4d Aβ1-42
α4β2



E

Mock
α4β2

Aβ1-42
Time
0
3d
0
1d
2d
3d

pDrp-1
Actin

F

pDrp-1/Actin (%)

Mock
α4β2

Aβ1-42
Time
0
3d
0
1d
2d
3d
Nicotinic Receptors Sensitize Neurons to β-Amyloid Toxicity

Direct cell counting experiments showed that by day 4, a large portion of the α4β2-nAChR-transfected cells treated with Aβ1-42 had indeed died (Fig. 9), with the remaining gone by day 5. There was a very small effect of Aβ1-42 treatment on mock-transfected cells at day 5, but there was no significant effect of the control peptide Aβ2-1 over the same time course. Treatment of α4β2-nAChR-transfected cells or control cells with nicotine over the same time course had no significant impact on cell number (not shown).

**DISCUSSION**

Aβ has been shown to be released at synaptic sites in a highly dynamic fashion (5, 6), leading to fluctuations in local concentration in response to synaptic activity. This would, in turn, implicate Aβ in activity-coupled synaptic regulation through target receptors. In the absence of AD, Aβ appears to be present in the high picomolar range (7) and previous work has demonstrated a direct agonist-like action of soluble picomolar-nanomolar Aβ via nAChRs at presynaptic sites (12, 15, 37, 45, 46). Acute picomolar Aβ was also found to enhance synaptic plasticity in a nAChR-dependent fashion (3, 4). However, the interaction between nAChRs and long-term exposure to Aβ has not yet been clearly elucidated. The objective of the present study was to evaluate the extent to which nAChRs contribute to the effects mediated by sustained exposure to Aβ, addressing the impact of dynamic changes in the levels of soluble Aβ at cellular and presynaptic sites using an *in vitro* model. The primary advantages of the *in vitro* neuroblastoma model are that epigenetic expression of the receptors avoids transcriptional regulation and allows for a defined, reconstituted neuronal system (37).

Sustained exposure of neuroblastoma cells expressing α4β2 nAChRs to nanomolar Aβ was found in the present study to induce an apparent up-regulation in agonist-triggered Ca2+ responses. Changes in axonal varicosities were monitored as an indication of presynaptic Ca2+ and hence synaptic regulation; however, the impact of Aβ was not restricted to the varicosities. Overall, the results are consistent with studies showing that functional responses of α4β2 nAChRs are increased on long-term exposure to agonist, such as nicotine (47, 48). A wide range of studies has shown that this functional up-regulation typically results from up-regulation of α4β2 nAChR expression (e.g. Ref. 49), although alternative mechanisms exist (50). Accordingly, our immunostaining results show an increased expression of α4β2 nAChRs in cell bodies and varicosities in cultures subjected to prolonged exposure to Aβ or nicotine. Although receptor expression was defined in this cell system using plasmids, our quantitative RT-PCR results for α4 transcripts rule out any effect on the plasmid promoters or the endogenous receptor genes. Moreover, previous work examining the impact of chronic nicotine demonstrated that up-regulation of α4β2 nAChRs occurs at

FIGURE 6. Sustained exposure to Aβ altered mitochondrial dynamics. A, changes in the size of labeled individual mitochondria on daily treatment with 100 nM Aβ1-42 (days 1–3) as compared with that observed in cells without Aβ treatment. Magnified regions show representative individual mitochondria (insets). B, average size (diameter) of mitochondria (n = 6). C, representative immunostaining for phospho- and total Drp-1. E, immunoblot analysis showing a time-dependent increase in the expression of phospho-Drp-1 on daily treatment with Aβ (n = 3). D and F, Aβ treatment for 3 days resulted in increased levels of phospho-Drp-1 relative to total Drp-1, when compared with controls (n = 5). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(datumt not shown) in live-cell imaging. In addition, the average size of mitochondria was significantly reduced when the cells were exposed to Aβ1-42 for various time points (days 1–3) as compared with that observed in untreated cells (Fig. 6, A and B). However, this reduction in size was observed starting at day 1, suggesting altered mitochondrial dynamics as an early consequence of Aβ toxicity. This effect seemed to be largely independent of α4β2-nAChR up-regulation, which was not evident until 3 days of treatment, in view of a similar effect on mitochondrial size in mock-transfected cells (Fig. 6B).

To further test whether the reduced size of mitochondria was a result of disturbed fission-fusion equilibrium, immunostaining and Western blot for the fission-inducing protein Drp-1 was performed using phospho-Drp-1 and total anti-Drp-1 antibodies. Phospho-Drp-1 was significantly increased in cells treated with Aβ for 3 days (Fig. 6, C–F), indicating an enhanced rate of mitochondrial fission.

**Sustained Exposure to Aβ Induced Increases in the Levels of ROS and Nuclear Disintegration in NG108-15 Cells Sensitized by the Presence of α4β2-nAChRs**—To investigate the impact of Aβ1-42 through α4β2 nAChRs on oxidative stress, mock-transfected and α4β2-nAChR-transfected cells were incubated with carboxy-H2DCFDA (ROS) and Hoechst (nuclear) stain (Fig. 7A). Exposure of α4β2-nAChR-transfected cells to 100 nM Aβ1-42 resulted in a time-dependent increase in the levels of ROS (p < 0.0001) as compared with mock-transfected cells exposed to the same concentration of the peptide (Fig. 7, C, black bars). In mock-transfected cells, there was only a slight increase in ROS staining on sustained exposure of nanomolar levels of Aβ1-42, whereas exposure to 1 μM Aβ1-42 did increase ROS levels to a significant extent (Fig. 7C, open bars), indicating that the presence of nAChRs sensitized the cells to the toxic action of Aβ.

A similar trend was seen for nuclear disintegration as assessed by Hoechst staining. Application of nanomolar Aβ1-42 to mock-transfected cells showed round, intact nuclei without any notable evidence of nuclear disruption, whereas the α4β2-nAChR-transfected cells treated with Aβ1-42 for 3 days had a significant (p < 0.0001) level of nuclear disintegration (Fig. 7, A and D), similar to that found with cells undergoing apoptosis.

**Sustained Exposure to Aβ Induced Apoptosis in NG108-15 Cells Sensitized by the Presence of α4β2-nAChRs**—The induction of apoptosis was evaluated by TUNEL assay to measure DNA fragmentation, performed on either mock-transfected or α4β2-nAChR-transfected cells exposed to Aβ1-42 (Fig. 8). The percentage of TUNEL-positive nuclei increased significantly in α4β2-nAChR-transfected cells (Fig. 8B; p < 0.001) as compared with that observed in mock-transfected cells treated with Aβ1-42 (Fig. 8A). In contrast, the control reverse peptide, Aβ2-1 (Fig. 8D), had no significant effect on either mock- or α4β2-nAChR-transfected cells across the 5-day time course.
**FIGURE 7.** A\(\beta\) induced substantially increased levels of ROS and nuclear disintegration in the presence of \(\alpha_4\beta_2\)-nAChRs. A, treatment with 100 nM A\(\beta_{1-42}\) for 3 days led to a significant level of nuclear disintegration (example noted by the yellow arrow in the inset) and ROS production in \(\alpha_4\beta_2\)-nAChR transfected cells as compared with treatment of mock-transfected cells, which retained round, intact nuclei without any notable evidence of nuclear disruption and had very weak staining for ROS. B, left panels, positive control using 100 \(\mu\)M t-butyldihydroperoxide for 30 min; right panels, negative (untreated) control. Quantification of ROS (\(n = 6\)) (C) and HOECHST (\(n = 20\)) (D) staining are presented as the mean fluorescent intensity and disintegrated nuclei/total nuclei, respectively, for all cells in a given field. Mock-transfected cells are represented by the open bars; \(\alpha_4\beta_2\)-nAChR-transfected cells are represented by the closed (black) bars. **, \(p < 0.01\); ***, \(p < 0.001\) when compared with control.
the post-translational level (51). Thus, sustained exposure to Aβ leads to up-regulation of nAChRs either at the level of receptor assembly, sorting, targeting, and transport or local endocytosis/recycling/degradation.

As there was no evidence for any detectable differences in up-regulation at the level of the cell somata as compared with the axonal varicosities, it would appear that assembly, sorting, targeting, and transport are likely not major contributors to the up-regulation observed here. Consequently, we focused on the endocytotic/recycling pathway. Our results for endocytotic and recycling markers indicate that an up-regulation of this pathway did occur, which was confirmed in a functional assay of nAChR recycling, suggesting that an increase in endocytosis/receptor recycling may account, in part, for the observed receptor up-regulation upon prolonged exposure to Aβ. How this up-regulation in recycling is induced will be the subject of future studies, focusing, in particular, on calcium-regulated pathways (52) and Aβ internalization (53). Nonetheless, it is proposed that up-regulation of the nAChRs with sustained, elevated levels of Aβ leads to a feed-forward dysregulation of calcium homeostasis.

As sustained exposure to high nanomolar-micromolar Aβ has consistently been found to be neurotoxic (e.g., Refs. 1 and 2), we next addressed the consequence of the Aβ-induced up-regulation of nAChRs on mitochondrial and neuronal integrity. Mitochondria are dynamic organelles and play significant roles in intracellular calcium homeostasis and reactive oxygen species production under conditions of oxidative stress. Mitochondrial dysfunction and oxidative damage occur in the earliest phase of AD, namely before significant plaque deposition.

FIGURE 8. Prolonged exposure to Aβ-induced apoptosis in α4β2-nAChR-transfected cells. A, to evaluate the induction of apoptosis, TUNEL was performed on either mock-transfected or α4β2-nAChR-transfected cells exposed to 100 nM Aβ1-42 for 0–5 days. The arrows indicate TUNEL-positive cells identified by HOECHST staining. B, quantification of the percentage of TUNEL-positive nuclei in mock-transfected and α4β2-nAChR-transfected cells exposed to 100 nM Aβ1-42 for 0–5 days. C, top panels, positive control using DNase I; bottom panels, negative (untreated) control. D, TUNEL staining on treatment with the control reverse peptide Aβ42-1.
Although these changes take years to develop in AD, because of attenuating if not mitigating processes in brain (e.g., clearance, antioxidative processes, competing neurotransmitter, and synaptic activities), the use of in vitro nerve cell models allows assessment of the impact of elevated Aβ in isolation. Exposure to micromolar Aβ was previously shown to alter mitochondrial dynamics and transport in cultured hippocampal neurons in a manner involving the microtubule-associating protein Tau (36). Here, we found that sustained exposure to nanomolar Aβ also results in altered mitochondrial dynamics and transport. Of particular note is the increased expression of phospho-Drp-1, which is a key player in coupling Aβ to mitochondrial fission via nitric oxide (55, 56), consistent with our findings of decreased mitochondria size following Aβ treatment. As for mitochondrial transport, Tau has been found to contribute to Aβ-induced reductions in general axonal transport (57), and thus it will also be important to consider the role of Tau in the effects of Aβ in the presence of nAChRs. We also found significant production of ROS as well as nuclear fragmentation, followed by apoptosis, all of which were greatly enhanced by the presence of the α4β2 nAChRs, with nuclear fragmentation and apoptosis following receptor up-regulation. Taken together, the results indicate that the presence of nAChRs sensitizes neurons to Aβ such that sustained picomolar to nanomolar levels of the peptide can now disrupt calcium regulation and neuronal integrity. As mitochondrial function is essential for cellular and synaptic integrity, the neurotoxic effect of accumulating Aβ will thus be magnified for cholino-
Nicotinic Receptors Sensitize Neurons to β-Amyloid Toxicity

J. Neurochem. 109, 1452–1458

15. Tong, M., Arora, K., White, M. M., and Nichols, R. A. (2011) Role of key aromatic residues in the ligand-binding domain of α7 nicotinic receptors in the agonist action of β-amyloid. J. Biol. Chem. 286, 34373–34381

16. Flores, C. M., Rogers, S. W., Pabreza, L. A., Wolfe, B. B., and Kellar, K. J. (1992) A subtype of the nicotinic cholinergic receptor in rat brain is composed of α4 and β2 subunits and is up-regulated by chronic nicotine treatment. Mol. Pharmacol. 41, 31–37

17. Zhang, X., Gong, Z.-H., and Nordberg, A. (1994) Effects of chronic treatment with (+)- and (−)-nicotine on nicotinic acetylcholine receptors and N-methyl-d-aspartate receptors in rat brain. Brain Res. 644, 32–39

18. Peng, X., Gerzanich, V., Anand, R., Whiting, P. J., and Lindstrom, J. (1994) Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. Mol. Pharmacol. 46, 523–530

19. Bencherif, M., Fowler, K., Lukas, R. J., and Lippiello, P. M. (1995) Mechanisms of up-regulation of neuronal nicotinic acetylcholine receptors in clonal cell lines and primary cultures of fetal rat brain. J. Pharmacol. Exp. Ther. 275, 987–994

Acknowledgments—We thank Dr. Jerry Stitzel, University of Colorado, for kindly providing the mouse α4 and β2 nAChR subunit sequences. We also thank Drs. Ghous Khan and Mei Tong for help with preliminary experiments examining expression of α4β2-nAChRs in differentiated NG108-15 cells. We thank Dr. Joe Ramos for advice on the endocytosis/recycling experiments and for providing anti-Rab5 antibody for preliminary experiments. We thank Dr. Marla Berry for valuable input. We also thank Drs. Peter Hofmann and Frederick Bellingier for insightful comments on the manuscript. Additional support for the microscopy core at the University of Hawaii was provided by Research Center for Minority Institutions grants from the National Center for Research Resources (G12RR030961) and the National Institute on Minority Health and Health Disparities (G12MD007501), with additional funding from COBRE Grant 2P20RR016453.

REFERENCES

1. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Kraft, G. A., and Klein, W. L. (1998) Diffusible, nonfibrillar ligands derived from Aβ42 are potent central nervous system neurotoxins. Proc. Natl. Acad. Sci. U.S.A. 95, 6448–6453

2. Walsh, D. M., and Selkoe, D. J. (2007) Aβ oligomers. A decade of discovery. J. Neurochem. 101, 1172–1184

3. Puzio, D., Privitera, L., Leznik, E., Fa, M., Staniszewski, A., Palmeri, A., and Arancio, O. (2008) Picomolar amyloid-β positively modulates synaptic plasticity and memory in hippocampus. J. Neurosci. 28, 14537–14545

4. Puzio, D., Privitera, L., Fa, M., Staniszewski, A., Hashimoto, G., Aziz, F., Sakurai, M., Ribe, E. M., Troy, C. M., Mercken, M., Jung, S. S., Palmeri, A., and Arancio, O. (2011) Endogenous amyloid-β is necessary for hippocampal synaptic plasticity and memory. Ann. Neurol. 69, 819–830

5. Cirrito, J. R., Yamada, K. A., Finn, M. B., Skoviter, R. S., Bales and R. K., May, P. C., Schoep, D. D., Paul, S. M., Mennerick, S., and Holtzman, D. M. (2005) Synaptic activity regulates interstitial fluid amyloid-β levels in vivo. Neuron 48, 913–922

6. Cirrito, J. R., Kang, J. E., Lee, J., Stewart, F. R., Verges, D. K., Silverio, L. M., Bu, G., Mennerick, S., and Holtzman, D. M. (2008) Endocytosis is required for synaptic activity-dependent release of amyloid-β in vivo. Neuron 58, 42–51

7. Religa, D., Laudon, H., Styczynska, M., Winblad, B., Nåslund, J., and Haroutunian, V. (2003) Amyloid β pathology in Alzheimer’s disease and schizophrenia. Am. J. Psychiatry 160, 867–872

8. Lazarow, O., Lee, M., Peterson, D. A., and Sisodia, S. S. (2002) Evidence that synaptically released β-amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. J. Neurosci. 22, 9785–9793

9. Patel, A. N., and Jhamandas, J. H. (2012) Neuronal receptors as targets for the action of amyloid-β protein (Aβ) in the brain. Expert Rev. Mol. Med. 14, e2

10. Dani, J. A., and Bertrand, D. (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 47, 699–729

11. McGehee, D. S., and Role, L. W. (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu. Rev. Physiol. 57, 521–546

12. Dougerty, J. J., Wu, J., and Nichols, R. A. (2003) β-Amyloid regulation of presynaptic nicotinic receptors in rat hippocampus and neocortex. J. Neurosci. 23, 6740–6747

13. Wu, J., Khan, G. M., and Nichols, R. A. (2007) Dopamine release in prefrontal cortex in response to β-amyloid activation of α7 nicotinic receptors. Brain Res. 1182, 82–89

14. Mehta, T. K., Dougerty, J. J., Wu, J., Choi, C. H., Khan, G. M., and Nichols, R. A. (2009) Defining pre-synaptic nicotinic receptors regulated by β amyloid in mouse cortex and hippocampus with receptor null mutants.
Nicotinic Receptors Sensitize Neurons to β-Amyloid Toxicity

disease. *J. Neurosci.* **21**, 3017–3023
34. Iijima-Ando, K., Hearn, S. A., Shenton, C., Gatt, A., and Zhao, L. (2009) Mitochondrial mislocalization underlies Aβ42-induced neuronal dysfunction in a *Drosophila* model of Alzheimer’s disease. *PLoS One* **4**, e8310
35. Zhao, X. L., Wang, W. A., Tan, J. X., Huang, J. K., Zhang, X., Zhang, B. Z., Wang, Y. H., Yang, Cheng, H. Y., Zhu, H. L., Sun, X. J., and Huang, F. D. (2010) Expression of β-amyloid induced age-dependent presynaptic and axonal changes in *Drosophila*. *J. Neurosci.* **30**, 1512–1522
36. Zempel, H., Thies, E., Mandelkow, E., and Mandelkow, E. M. (2010) Aβ oligomers cause localized Ca²⁺ elevation, missorting of endogenous tau into dendrites, tau phosphorylation, and destruction of microtubules and spines. *J. Neurosci.* **30**, 11938–11950
37. Khan, G. M., Tong, M., Jhun, M., Arora, K., and Nichols, R. A. (2010) β-Amyloid activates presynaptic α7 nicotinic acetylcholine receptors reconstituted into a model nerve cell system. Involvement of lipid rafts. *Eur. J. Neurosci.* **31**, 788–796
38. Lukyanetz, E. A. (1998) Diversity and properties of calcium channel types in NG108015 hybrid cells. *Neuroscience* **87**, 265–274
39. Fried, G., and Han, H. Q. (1995) Increase in synaptic vesicle proteins in synapsin-transfected NG108-15 cells. A subcellular fractionation study. *Synapse* **20**, 44–53
40. Nelson, P., Christian, C., Nirenberg, M. (1976) Synapse formation between clonal neuroblastoma X glioma hybrid cells and striated muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* **73**, 123–127
41. Nichols, R. A., Dengler, A. F., Nakagawa, E. M., Bashkin, M., Paul, B. T., Wu, J., and Khan, G. M. (2007) A constitutive, transient receptor potential-like Ca²⁺ influx pathway in presynaptic nerve endings independent of voltage-gated Ca²⁺ channels and Na⁺/Ca²⁺ exchange. *J. Biol. Chem.* **282**, 36102–36111
42. Dougherty, J. J., Wu, J., Mehta, T. K., Brown, B., and Nichols, R. A. (2008) Chronic nicotine alters nicotinic receptor-induced presynaptic Ca²⁺ responses in isolated nerve terminals. *Neurochem. Res.* **33**, 1106–1112
43. Reddy, P. H. (2009) Amyloid β, mitochondrial structural and functional dynamics in Alzheimer’s disease. *Exp. Neurol.* **218**, 286–292
44. Wang, X., Su, B., Zheng, L., Perry, G., Smith, M. A., and Zhu, X. (2009) The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer’s disease. *J. Neurochem.* **109**, 153–159
45. Fu, W., and Jhamandas, J. H. (2003) β-Amyloid peptide activates non-α7 nicotinic acetylcholine receptors in rat basal forebrain neurons. *J. Neurophysiol.* **90**, 3130–3136
46. Chin, J. H., Ma, L., MacTavish, D, and Jhamandas, J. H. (2007) Amyloid β protein modulates glutamate-mediated neurotransmission in the rat basal forebrain. Involvement of presynaptic neuronal nicotinic acetylcholine and metabotropic glutamate receptors. *J. Neurosci.* **27**, 9262–9269
47. Buisson, B., and Bertrand, D. (2002) Nicotine addiction. The possible role of functional up-regulation. *Trends Pharmacol. Sci.* **23**, 130–136
48. Nashmi, R., Dickinson, M. E., McKinney, S., Jareb, M., Labarca, C., Fraser, S. E., Lester, H. A. (2003) Assembly of Aβ42 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits. Effects of localization, trafficking, and nicotine-induced up-regulation in clonal mammalian cells and in cultured midbrain neurons. *J. Neurosci.* **23**, 11554–11567
49. Perry, D. C., Dávila-García, M. I., Stockmeier, C. A., and Kellar, K. I. (1999) Increased nicotinic receptors in brains from smokers. Membrane binding and autoradiography studies. *J. Pharmacol. Exp. Ther.* **289**, 1545–1552
50. Vallejo, Y. F., Buisson, B., Bertrand, D., and Green, W. N. (2005) Chronic nicotine exposure up-regulates nicotinic receptors by a novel mechanism. *J. Neurosci.* **25**, 5563–5572
51. Marks, M. J., Pauly, J. R., Gross, S. D., Deneris, E. S., Hermans-Borgmeyer, I., Heinemann, S. F., and Collins, A. C. (1992) Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J. Neurosci.* **12**, 2765–2784
52. Martinez-Pena y Valenzuela, I., Mouslim, C., and Akaaboune, M. (2010) Calcium/calcmodulin kinase II-dependent acetylcholine receptor cycling at the mammalian neuromuscular junction in vivo. *J. Neurosci.* **30**, 12455–12465
53. Mohamed, A., and Posse de Chaves, E. (2011) Aβ internalization by neurons and glia. *Int. J. Alzheimers Dis.* **2011**, 127984
54. Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., Jones, P. K., Ghanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C. S., Petersen, R. B., and Smith, M. A. (2001) Oxidative damage is the earliest event in Alzheimer’s disease. *J. Neuropathol. Exp. Neurol.* **60**, 759–767
55. Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., and Mihara, K. (2007) Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission in *Drosophila*. *Exp. Biol. Chem.* **282**, 11521–11529
56. Cho, D. H., Nakamura, T., Fang, J., Cieplak, P., Godzik, A., Gu, Z., and Lipton, S. A. (2009) S-Nitrosylation of Drp1 mediates β-amyloid-related mitochondrial fission and neuronal injury. *Science* **324**, 102–105
57. Vossel, K. A., Zhang, K., Brodbeck, J., Daub, A. C., Sharma, P., Finkbeiner, S., Cui, B., and Mucke, L. (2010) Tau reduction prevents Aβ-induced defects in axonal transport. *Science* **330**, 198