Nicotinic acetylcholine receptors (nAChRs) containing α7 subunits are thought to assemble as homomers. α7-nAChR function has been implicated in learning and memory, and alterations of α7-nAChR have been found in patients with Alzheimer’s disease (AD). Here we report findings consistent with a novel, naturally occurring nAChR subtype in rodent, basal forebrain cholinergic neurons. In these cells, α7 subunits are coexpressed, colocalized, and coassemble with β2 subunit(s). Compared with homomeric α7-nAChRs from ventral tegmental area neurons, functional, presumably heteromeric α7β2-nAChRs on cholinergic neurons freshly dissociated from medial septum/diagonal band (MS/DB) exhibit relatively slow kinetics of whole-cell current responses to nicotinic agonists and are more sensitive to the β2 subunit-containing nAChR-selective antagonist, dihydro-β-erythroidine (DHβE). Interestingly, presumed, heteromeric α7β2-nAChRs are highly sensitive to functional inhibition by pathologically relevant concentrations of oligomeric, but not monomeric or fibrillar, forms of amyloid β1-42 (Aβ1-42). Slow whole-cell current kinetics, sensitivity to DHβE, and specific antagonism by oligomeric Aβ1-42, also are characteristics of heteromeric α7β2-nAChRs, but not of homomeric α7-nAChRs, heterologously expressed in Xenopus oocytes. Moreover, choline-induced currents have faster kinetics and less sensitivity to Aβ when elicited from MS/DB neurons derived from α7β2-nAChR β2 subunit knock-out mice rather than from wild-type mice. The presence of novel, functional, heteromeric α7β2-nAChRs on basal forebrain cholinergic neurons and their high sensitivity to blockade by low concentrations of oligomeric Aβ1-42 suggests possible mechanisms for deficits in cholinergic signaling that could occur early in the etiopathogenesis of AD and might be targeted by disease therapies.

Key words: nicotinic receptor; basal forebrain; cholinergic neurons; patch clamp; amyloid β; Alzheimer’s disease

Introduction

Nicotinic acetylcholine receptors (nAChRs) in mammals exist as a diverse family of channels composed of different, pentameric combinations of subunits derived from at least 16 genes (Luksa et al., 1999; Jensen et al., 2005). Functional nAChRs can be assembled as either heteromers containing α and β subunits or as homomers containing only α subunits (Luksa et al., 1999; Jensen et al., 2005). In the mammalian brain, the most abundant forms of nAChRs are heteromeric αβ2-nAChRs and homomeric α7-nAChRs (Whiting et al., 1987; Flores et al., 1992; Gopalakrishnan et al., 1996; Lindstrom, 1996; Lindstrom et al., 1996). α7-nAChRs appear to play roles in the development, differentiation, and pathophysiology of the nervous system (Z. Liu et al., 2007; Mudo et al., 2007). nAChRs have been implicated in Alzheimer’s disease (AD), in part because significant losses in radioligand binding sites corresponding to nAChRs have been consistently observed at autopsy in a number of neocortical areas and in the hippocampi of patients with AD (Burghaus et al., 2000; Nordberg, 2001). Attenuation of cholinergic signaling is known to impair memory, and nicotine exposure improves cognitive function in AD patients (Levin and Rezvani, 2002). In addition, several studies have suggested that the activation of α7-nAChR function alleviates amyloid-β (Aβ) toxicity. For instance, stimulation of α7-nAChRs inhibits amyloid plaque formation in vitro and in vivo (Geerts, 2005), activates α-secretase cleavage of amyloid precursor protein (APP) (Lahiri et al., 2002), increases acetylcholine (ACh) release and facilitates Aβ internalization (Nagele et al., 2002), inhibits activity of the MAPK/NF-κB/c-myel signaling pathway (Q. Liu et al., 2007), and reduces Aβ production and attenuates tau phosphorylation (Sadot et al., 1996). These findings suggest that cholinergic signaling, mediated through α7-
Acutely dissociated neurons from the CNS and patch-clamp whole-cell current recordings

Neuron dissociation and patch-clamp recordings were performed as described by Wu et al. (2002, 2004b). Briefly, each postnatal 2- to 4-week-old Wistar rat or mouse (wild-type C57BL/6 or nAChR β2 knockout mice) on a C57BL/6 background kindly provided by Dr. Marina Picciotto, Yale University, New Haven, CT) was anesthetized using isoflurane, and the brain was rapidly removed. Several 400 μm coronal slices, which contained the medial septum/diagonal band (MS/DB) or the ventral tegmental area (VTA), were cut using a vibratome (Vibratome 1000 plus; Jed Pella) in cold (2–4°C) artificial CSF (ACSF) and continuously bubbled with carbogen (95% O2–5% CO2). The slices were then incubated in a preincubation chamber (Warner Instruments) and allowed to recover for at least 1 h at room temperature (22 ± 1°C) in oxygenated ACSF. Thereafter, the slices were treated with Pronase (1 mg/ml) at 31°C for 30 min and subsequently treated with the same concentration of thermolytin for another 30 min. The MS/DB or VTA region was micropunched out from the slices using a well polished needle. Each punched piece was then dissociated mechanically by using several fire-polished micro-pipettes in a 35 mm culture dish filled with well oxygenated standard external solution [in mM: 150 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES; pH 7.4 (with Tris-base)]. The separated single cells usually adhered to the bottom of the dish within 30 min. Perforated-patch whole-cell recordings coupled with a U-tube or two-barrel drug application system were used (Wu et al., 2002). Perforated-patch recordings more closely maintain both intracellular divalent cation and cytosolic element composition (Horn and Marty, 1988). In particular, perforated-patch recording was used to maintain the intracellular ATP concentration at a physiological level. To prepare for perforated-patch whole-cell recording, glass microelectrodes (GC-1.5; Narishige) were fashioned on a two-stage vertical pipette puller (P-830; Narishige), and the resistance of the electrode was 3–5 MΩ when filled with the internal solution. A tight seal (≥2 GΩ) was formed between the electrode tip and the cell surface, which was followed by a transition from on-cell to whole-cell recording mode due to the partitioning of amphoterinic B into the membrane underlying the patch. After whole-cell formation, an access resistance lower than 60 MΩ was acceptable during perforated-patch recordings in current-clamp mode, and an access resistance lower than 30 MΩ was acceptable during voltage-clamp recordings. The series resistance was not compensated in the experiments using dissociated neurons. Under current-clamp configuration, membrane potentials were measured using a patch-clamp amplifier (200B; Axon Instruments). Data were filtered at 2 kHz, acquired at 11 kHz, and digitized on-line (Digidata 1322 series A/D board; Axon Instruments). All experiments were performed at room temperature (22 ± 1°C). The drugs used in the present study were GABA, glutamate, ACh, choline, methyllycaconitine (MLA), dihydロ-b-erythroidine (DHβE), muscarine (all purchased from Sigma-Aldrich), KJR-2403 (purchased from TocrisCookson), and Aβ(1–42) and scrambled Aβ(1–42) (purchased from Peptide).

Tissue RT-PCR

RT-PCR assays followed by Southern hybridization with nested oligonucleotides were done as previously described to identify nAChR subunit transcripts and to quantify levels of expression normalized both to housekeeping gene expression and levels of expression in whole brain (Wu et al., 2004), but using primers designed to detect rat nAChR subunits. The Southern hybridization technique coupled with quantitation using electronic isotope counting (Instant Image, Canberra Instruments) yielded results equivalent to those obtained using real-time PCR analysis.

Single-cell RT-PCR

Precautions were taken to ensure a ribonuclease-free environment and to avoid PCR product contamination during patch-clamp recording and single-cell collection before execution of RT-PCR. Single-cell RT-PCR was performed using the Superscript III CellDirect RT-PCR system (Invitrogen). Briefly, after whole-cell patch-clamp recording, single-cell content was harvested by suction into the pipette solution (~3 μl) and immediately transferred to an autoclaved 0.2 ml PCR tube containing 10 μl of cell resuspension buffer and 1 μl of lysis enhancer. Single cells were lysed by heating at 75°C for 10 min. Potential contaminating genomic DNA was removed by DNase I digestion at 25°C for 6 min. After heat-inactivation of DNase I at 70°C for 6 min in the presence of EDTA, reverse transcription (RT) was performed by adding reaction mix with oligo(dT)20 and random hexamers and SuperScriptIII enzyme mix and then incubating at 25°C for 10 min and 50°C for 50 min. The reaction was terminated by heating the sample to 85°C for 5 min. The PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and nAChR α3, α4, α7, β2, and β4 subunits were designed using the Primer 3 internet server (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and assuming an annealing temperature of ~60°C [nearest neighbor], PCR was performed with 20 μl of hot-start Platinum PCR Supermix (Invitrogen), 3 μl of cDNA template from the RT step, and 1 μl of gene specific primer pairs (5 pmol each) with the following thermocycling parameters: 95°C for 2 min; 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s × 70 cycles, 72°C for 1 min. PCR products were resolved on 1.5% TBE-agrose gels, and stained gels were used to visualize bands, using digital photography and a gel documentation system to capture images.
Tissue protein extraction, immunoprecipitation, and immunoblotting for confirmation of nAChR α7 and β2 subunit coassembly

Tissues were Dounce homogenized (10 strokes) in ice-cold lysis buffer [1% (v/v) Triton X-100, 150 mM EDTA, 10% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0] containing 1× general protease inhibitor cocktails (Sigma-Aldrich). The lysates were transferred to microcentrifuge tubes and further solubilized for 30 min at 4°C. The detergent extracts (super-natants) were collected by centrifugation at 15,000 × g for 15 min at 4°C, and protein concentration was determined for sample aliquots using bicinchoninic acid (BCA) protein assay reagents (Pierce Chemical). The detergent extracts of the following were mixed at a ratio of 1:10 of mixed α-subunits of protein A-Sepharose and protein G-Sepharose (1:1) (Amersham Biosciences) twice, each for 30 min at 4°C. For each immunoprecipitation, detergent extracts (1 mg) were mixed with 1 μg of rabbit anti-α7 anti-serum (H302) or rabbit IgG (as immunological control) (Santa Cruz sciences). The membranes were blocked with TBST buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (w/v) Tween 20] containing 2% (w/v) nonfat dry milk for at least 2 h and incubated with rat monoclonal antibodies (mAb270; Santa Cruz) or anti-α7 antiserum (H302), respectively, at 4°C overnight. After three washes in TBST, the membranes were incubated with goat anti-rat or goat anti-rabbit secondary antibodies (1:10,000) (Pierce Chemical) for 1 h and washed. The bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce Chemical).

Expression of homomeric and heteromeric α7-containing nAChRs in Xenopus oocytes and two-electrode voltage-clamp recording

cDNAs encoding rat α7 and β2 subunits were amplified by PCR with pfuUltra DNA polymerase and subcloned into an oocyte expression vector, pGEMHE, with T7 orientation and confirmed by automated sequencing. cDNAs were synthesized by standard in vitro transcription with T7 RNA polymerase, confirmed by electrophoresis for their integrity, and quantified based on optical absorbance measurements using an Eppendorf Biophotometer.

Oocyte preparation and cRNA injection

Xenopus laevis (Xenopus 1) females were anesthetized using 0.2% MS-222. The ovarian lobes were surgically removed from the frogs and placed in an incubation solution consisting of (in mM): 82.5 NaCl, 2.5 KCl, 1 MgCl2, 1 CaCl2, 1 Na2HPO4, 0.6 theophylline, 2.5 sodium pyruvate, 5 HEPES, 50 mg/ml gentamycin, 50 U/ml penicillin, and 50 μg/ml streptomycin; pH 7.5. The frogs were then allowed to recover from surgery before being returned to the incubation tank. The frogs were cut into small pieces and digested with 0.08 Wunsch U/ml liberase blendzyme 3 (Roche Applied Science) with constant stirring at room temperature for 1.5–2 h. The dispersed oocytes were thoroughly rinsed with incubation solution. Stage VI oocytes were selected and incubated at 16°C before injection. Micropipettes used for injection were pulled from borosilicate glass (Drummond Scientific). cDNAs encoding α7 or β2 at proper dilution were injected into oocytes separately or in different ratios using a Nanoject microinjection system (Drummond Scientific) at a total volume of ~20–60 nl.

Two-electrode voltage-clamp recording

One to three days after injection, an oocyte was placed in a small-volume chamber and continuously perfused with oocyte Ringer’s solution (OR2), consisting of the following (in mM): 92.5 NaCl, 2.5 KCl, 1 CaCl2, 1 MgCl2, and 5 HEPES; pH 7.5. The chamber was grounded through an agarose bridge. The oocytes were voltage clamped at −70 mV to measure ACh (or choline)-induced currents using GenClamp 500B (Axon Instruments).

Immunocytochemical staining

Dissociated MS/DB neurons were fixed with 4% paraformaldehyde for 5 min, rinsed three times with PBS, and treated with saponin (1 mg/ml) for 5 min as a permeabilizing agent. After rinsing four times with PBS, the neurons were incubated at room temperature in anti-choline acetyltransferase (ChAT) primary antibody (AB305; Chemicon International) diluted 1:400 in HBBS (supplemented with 5% bovine serum albumin as a blocking agent) for 30 min. Following another three rinses with PBS, a secondary antibody (anti-mouse IgG; Sigma-Aldrich) was applied at room temperature for 30 min (diluted 1:100). After rinsing a final three times with PBS, the labeled cells were visualized using a Zeiss fluorescence microscope (Zeiss), and images were processed using Photoshop (Adobe Systems). For double immunolabeling of α7 and β2 subunits of nAChRs on single dissociated MS/DB neurons, we used the following antibodies: a rabbit antibody (AS-5631S, 1:400; R and D) against α7 subunit, a rat antibody against β2 subunit (Ab24698, 1:500; Abcam), Alexa Fluor 594-conjugated anti-rabbit IgG, and Alexa Fluor 488-conjugated anti-rat IgG (1:300; Invitrogen).

Aβ preparation and determination/monitoring of peptide forms

Aβ preparation

Amyloid β peptides (Aβ1–42, scrambled Aβ1–42) were purchased from rPeptide. As previously described (Wu et al., 2004a), some preparations involved reconstitution of Aβ peptides per vendor specifications in distilled water to a concentration of 100 μM, stored at −20°C, and used within 10 d of reconstitution. These thawed peptide stock solutions were used to create working dilutions (1–100 nM) in standard external solution before patch-clamp recording. Working dilutions were used within 4 h before being discarded. Atomic force microscopy (AFM) was used to define and analyze over time the morphology of prepared Aβ1–42. Aliquots of freshly prepared samples of Aβ1–42 diluted in standard external solution were spotted on freshly cleaved mica. After 2 min the mica was washed with 200 μl of deionized water, dried with compressed nitrogen, and completely air-dried under vacuum. Images were acquired in air using a multimode AFM nanoscope IIIA system (Veeco/Digital Instruments) operating in the tapping mode using silicon probes (Olympus).

Protocols to obtain different forms of Aβ1–42

Different conditions were used to specifically prepare monomeric, oligomeric, or fibrillar forms of Aβ1–42.

Monomers. Aβ1–42 was reconstituted in DMSO to a concentration of 100 μM and stored at −80°C. For each use, an aliquot of stock sample was freshly thawed and diluted into standard extracellular solution as above just before patch recordings and used for no more than 4 h. This protocol yielded a predominant, monomeric form [see supplemental Fig. 1C (left), available at www.jneurosci.org as supplemental material].

Oligomers. Aβ1–42, reconstituted in distilled water to a concentration of 100 μM and stored at −80°C was used within 7 d of reconstitution. Aliquots diluted in standard extracellular solution and used within 4 h yielded a predominantly oligomeric form [see supplemental Fig. 1C (middle), available at www.jneurosci.org as supplemental material].

Fibrils. Aliquots of Aβ1–42 stock solution (water dissolved to 100 μM) were thawed and incubated at 37°C for 4 h at low pH (pH = 6.0). Working stocks diluted in standard extracellular solution yielded a predominantly fibrillar form [see supplemental Fig. 1C (right), available at www.jneurosci.org as supplemental material].

Genotyping of the nAChR β2 subunit knock-out mice

Genomic DNA from mice newly born to heterozygotic, nAChR β2 subunit knock-out parents was extracted from mouse tail tips by using the QiAgen DNeasy Blood & Tissue Kit following the manufacture’s protocol. PCR amplification of the nAChR β2 subunit or lac-Z (an indicator for the knock-out) were performed using the purified genomic DNA as template and gene specific primer pairs (forward primer: CGG AGC ATT TGA ACT CTG AGC AGT GGG GTC GC; backward primer: CTC GCT GAC ACA AGG GCT GGC GAC; lac-Z forward primer: CAC TAC GTC TGA ACG TCG AAA ACC CG; backward primer: CGG GCA AAT AAT ACG GCA ACG GCA ACG GCA).
ATC GGT GGC CGT GG) with annealing at 55°C for 1 min and extension at 72°C for 1 min for 30 cycles with GoTaq DNA polymerase (Promega). PCR products were resolved on 1% agarose gels and stained for visualization before images were captured using digital photography.

**Results**

**Identification of cholinergic neurons dissociated from basal forebrain**

An initial series of experiments identified cholinergic neurons acutely dissociated from rat MS/DB (Fig. 1A). First, we identified the cholinergic phenotype of acutely dissociated neurons from the MS/DB (Fig. 1Ba–c) based on published criteria (Henderson et al., 2005; Thinschmidt et al., 2005). In current-clamp mode, MS/DB neurons exhibited spontaneous action potential firing at low frequency (2.3 ± 0.4 Hz, n = 25 from 21 rats). This spontaneous activity was insensitive to the muscarinic acetylcholine receptor agonist, muscarine (1 µM) (Fig. 1C). Depolarizing pulses induced adaptation of action potential firing (Fig. 1D), and hyperpolarizing pulses failed to induce “sag”-like membrane potential changes (Fig. 1E). In some cases, the fluorescent dye lucifer yellow (0.5 mg/ml) was delivered into recorded cells after patch-clamp recordings, and choline acetyltransferase (ChAT) immunocyto staining was used post hoc (Fig. 1F). The presence of ChAT immunoreactivity in recorded, dye-filled neurons confirmed that dissociated MS/DB neurons were cholinergic.

**Naturally occurring nAChrRs in rodent forebrain cholinergic neurons**

We next tested for the presence of functional nAChRs on MS/DB cholinergic neurons. Under voltage-clamp recording conditions, rapid application of 1 mM ACh induced inward current responses with relatively rapid activation and desensitization kinetics (Fig. 2A). These ACh-induced responses were mimicked by application of the selective α7- nAChr agonist choline, blocked by the relatively selective α7-nAChR antagonist methyllycaconitine (MLA), and insensitive to the relatively selective α4β2-nAChR agonist RJR-2403 (Fig. 2A). Thus, the inward current evoked in MS/DB neurons had features similar to receptors containing α7 subunits. In contrast, in acutely dissociated, dopaminergic (DAergic) neurons from the midbrain VTA, ACh-induced currents displayed a mixture of features that could be dissected pharmacologically and with regard to whole-cell current kinetics. Components of responses displaying slow kinetics and sustained, steady-state currents elicited by ACh were mimicked by RJR-2403, suggesting that they were mediated by α4β2-nAChRs, whereas choline only induced transient peak current responses with very fast kinetics that are characteristic of homomeric α7- nAChRs (Fig. 2B). Interestingly, choline-induced currents in MS/DB cholinergic neurons exhibited relatively slow macroscopic kinetics than observed in VTA DAergic neurons (Fig. 2C).
This impression was confirmed by quantitative analyses, which gave values for current rising time of 72.1 ± 9.1 ms (n = 8) for MS/DB neurons and 29.1 ± 2.9 ms (n = 12) for VTA neurons (p < 0.001) and decay constants (τ, rate of decay from peak to steady-state current) of 28.6 ± 2.8 ms (n = 8) for MS/DB neurons and 10.2 ± 1.5 ms (n = 12) for VTA neurons (p < 0.001). There were no significant differences between either peak current amplitudes or net charge movements for responses elicited by choline in MS/DB or VTA neurons (Fig. 2D). These results suggested that functional nAChRs naturally expressed on rat MS/DB cholinergic neurons with some features like α7-nAChRs had slower whole-cell current kinetics than found for α7-nAChR-like responses in VTA DAergic neurons.

Subunit partnership for naturally occurring nAChRs in rodent basal forebrain cholinergic neurons

To test the hypothesis that the relatively slow kinetics of α7-nAChR-like responses in MS/DB cholinergic neurons were due to coassembly of α7 with other nAChR subunits, we performed relative quantitative RT-PCR analysis of nAChR subunit expression as messenger RNA in MS/DB compared with whole-brain and VTA tissues. The results demonstrated that nAChR α7 and β2 subunits were among those coexpressed regionally (Fig. 3A, B). These studies were extended to single-cell RT-PCR analysis of nAChR subunit expression in acutely dissociated neurons from the MS/DB used in patch-clamp recordings (Fig. 3C). Quantitative analysis indicated a high frequency of nAChR α7 and β2 subunit coexpression as message in recorded MS/DB neurons (Fig. 3D). Mindful of the current concerns about the specificity of all anti-nAChR subunit antibodies (Moser et al., 2007), we nevertheless showed qualitatively, based on dual-labeling immunofluorescent staining (Fig. 3D), that α7 and β2 subunits were colocalized in many MS/DB neurons subjected to patch-clamp recording. More direct evidence for coassembly of nAChR α7 and β2 subunit proteins came from coinmunoprecipitation studies using subunit-specific antibodies. Protein extracts from rat MS/DB or VTA tissues (collected from rats aged between 18 and 22 d) were subjected to immunoprecipitation (IP) (Fig. 3E, left) with a rabbit anti-nAChR α7 subunit antibody (H302) or with rabbit IgG (as an immunological control) followed by immunoblotting (IB) with a rat anti-nAChR β2 subunit monoclonal antibody (mAb270). As indicated, the β2 subunit was readily detected immunologically in anti-α7 immunoprecipitates from MS/DB but not from VTA regions under our experimental conditions (Fig. 3E, top left, lane 1 vs 2). Reprobing the same blot with the rabbit anti-α7 antibody (H302) verified that similar amounts of α7 subunits were precipitated from both MS/DB and VTA regions (Fig. 3E, bottom left, lanes 1 and 2). Thus, coprecipitation of nAChR α7 and β2 subunits appeared only in samples from the rat MS/DB but not from the VTA. Collectively, these results suggest that nAChR α7 and β2 subunits are most likely coassembled, perhaps to form a functional nAChR subtype, in rodent basal forebrain cholinergic neurons.

Pharmacological profiles of functional nAChRs in rat forebrain cholinergic neurons

Pharmacological approaches were used to compare features of functional nAChRs in MS/DB cholinergic or VTA DAergic neurons. The α7-nAChR-selective antagonist, MLA showed similar antagonist potency toward choline-induced currents in either MS/DB (Fig. 4Aa) or VTA (Fig. 4Ab) neurons. Analysis of concentration-inhibition curves (Fig. 4Ac) yielded IC_{50} values...
and Hill coefficients of 0.7 nM and 1.1, respectively, for MS/DB neurons (n/H110058) and 0.4 nM and 1.2, respectively, for VTA neurons (n/H110059, MS/DB vs VTA p/H11020.05). However, the nAChR-selective antagonist, DH/E was 500-fold less potent as an inhibitor of choline-induced current in MS/BD neurons (Fig. 4B) than in VTA neurons (Fig. 4Bb). IC_{50} values and Hill coefficients for DH/E-induced inhibition were 0.17 μM and 0.9, respectively, for MS/DB neurons (n = 8), and >100 μM and 0.3, respectively, for VTA neurons (n = 7; MS/DB vs VTA, p < 0.001) (Fig. 4Bc). These results are consistent with the hypothesis that functional α7*-nAChRs on MS/DB cholinergic neurons also contain DH/E-sensitive β2 subunits.

Figure 3. nAChR α7 and β2 subunits are coexpressed, colocalize, and coassemble in rat forebrain MS/DB neurons. RT-PCR products from whole brain and VTA and MS/DB regions (A) corresponding to the indicated nAChR subunits or to the housekeeping gene GAPDH were resolved on an agarose gel calibrated by the flanking 100 bp ladders (heavy band is 500 bp) and visualized using ethidium staining. Note that the representative gel shown for whole brain did not contain a sample for the nAChR α3 subunit RT-PCR product, which typically is similar in intensity to the sample on the gel for the VTA and MS/DB. B, Quantification of nAChR subunit mRNA levels for RT-PCR amplification followed by Southern hybridization with 32P-labeled, nested oligonucleotides normalized to the GAPDH internal control and to levels of each specific mRNA in whole rat brain (ordinate: SEM) for the indicated subunits. C, From 15 MS/DB neurons tested, after patch clamp recordings (Ca: representative whole-cell current trace), the cell content was harvested and single-cell RT-PCR was performed, and the results show that α7 and β2 were the two major nAChR subunits naturally expressed in MS/DB cholinergic neurons (Cb–Cd). Double immunofluorescence labeling of a MS/DB neuron with anti-α7 and anti-β2 subunit antibodies revealed that α7 and β2 subunit proteins colocalized, and similar results were obtained using 31 neurons from 12 rats (D). Protein extracts from rat MS/DB (lane 1) or rat VTA (lane 2) or from MS/DB from nAChR β2 subunit knock-out (lane 4) or wild-type mice (lane 5) were immunoprecipitated (IP) with a rabbit anti-α7 antibody (Santa Cruz H302; lanes 1, 2, 4, and 5) or rabbit IgG as a control (lane 3). The eluted proteins from the precipitates were analyzed by immunoblotting (IB) with rat monoclonal anti-β2 subunit antibody mAb270 (top) or rabbit anti-α7 antiserum H302 (bottom). The β2 and α7 bands are indicated by arrows (E). All these data suggested that nAChR α7 and β2 nAChR subunits are coassembled in MS/DB neurons.

and Hill coefficients of 0.7 nm and 1.1, respectively, for MS/DB neurons (n = 8) and 0.4 nm and 1.2, respectively, for VTA neurons (n = 9, MS/DB vs VTA p > 0.05). However, the β2*-nAChR-selective antagonist, DH/E was ~500-fold less potent as an inhibitor of choline-induced current in MS/BD neurons (Fig. 4Ba) than in VTA neurons (Fig. 4Bb). IC_{50} values and Hill coefficients for DH/E-induced inhibition were 0.17 μM and 0.9, respectively, for MS/DB neurons (n = 8), and >100 μM and 0.3, respectively, for VTA neurons (n = 7; MS/DB vs VTA, p < 0.001) (Fig. 4Bc). These results are consistent with the hypothesis that functional α7*-nAChRs on MS/DB cholinergic neurons also contain DH/E-sensitive β2 subunits.

Functional nAChRs on rat basal forebrain cholinergic neurons are inhibited by Aβ_{1–42}

Currently, it is not clear why basal forebrain cholinergic neurons are particularly sensitive to degeneration in AD. To test the hypothesis that novel α7β2-nAChRs on MS/DB cholinergic neurons are involved, we determined the effects of Aβ_{1–42} on these receptors. The experimental protocol involved repeated, acute challenges with 10 mM choline, and control studies in the absence of peptide demonstrated that there was no significant rundown of such responses when spaced at a minimum of 2 min intervals (Fig. 5Aa). During a continuous exposure to 1 mM Aβ_{1–42} starting just after an initial choline challenge and continuing for 10 min,
responses to choline challenges were progressively inhibited with time, although reversibly so as demonstrated by response recovery after 6 min of peptide washout (Fig. 5Ab). In contrast, exposure to 1 nM scrambled Aβ_{1–42} (as a control peptide) had no effect (Fig. 5Ac). Choline-induced currents in dissociated VTA DAergic neurons were not sensitive to 1 nM Aβ_{1–42} treatment (Fig. 5Ad). Quantitative analysis of several replicate experiments (Fig. 5B) confirmed that Aβ_{1–42}, even at 1 nM concentration, specifically inhibits putative α7β2-nAChR function on MS/DB cholinergic neurons but not function of homomeric α7-nAChRs on VTA DAergic neurons.

Concentration- and form-dependent inhibition by Aβ_{1–42} of α7β2-nAChR function on basal forebrain cholinergic neurons

Our previous studies indicated that α4β2-nAChRs were more sensitive to Aβ_{1–42} than homomeric α7-nAChRs (Wu et al., 2004a). Concentration dependence of effects of Aβ_{1–42} on choline-induced currents in MS/DB neurons was evident, with effects being negligible at 0.1 nM and effects at 1 nM being approximately half of those observed for 10 nM peptide (Fig. 6A). The magnitude of inhibition apparently had not yet reached maximum after 10 min of peptide exposure. We also asked which form(s) of Aβ_{1–42} showed the most potent inhibitory effect on choline-induced currents elicited in MS/DB neurons. Using different preparation protocols (see Materials and Methods), we produced Aβ_{1–42} monomers (peptide dissolved in DMSO), oligomers (peptide dissolved in water), or fibrils (peptides dissolved in water at low pH (pH = 6.0) and incubated at 37°C for 2 d). Peptide forms were defined and monitored using AFM (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material). At 1 nM, oligomeric Aβ_{1–42} exhibited the greatest suppression of choline-induced responses, fibrillar Aβ had weaker inhibitory effect, and monomeric Aβ_{1–42} failed to suppress choline-induced responses, indicating form-selective, Aβ_{1–42} inhibition of nAChRs in MS/DB cholinergic neurons. To test whether Aβ_{1–42} specifically inhibits nAChRs, we also examined the effects of 1 nM Aβ_{1–42} on GABA- or glutamate-induced currents in rat MS/DB cholinergic neurons, and the results demonstrated that both GABA_A receptors and ionotropic glutamate receptors were insensitive to inhibition by 1 nM Aβ_{1–42} even when peptide effects on ACh-induced current were evident (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Collectively, these results indicate that, under our experimental conditions, pathologically relevant, low nM concentrations of Aβ_{1–42}, especially in an oligomeric form, specifically inhibit function of apparently heteromeric α7β2-nAChRs, but peptides cannot inhibit function of homomeric α7-nAChRs, GABA_A, or glutamate receptors on MS/DB cholinergic neurons.

Heteromeric α7β2-nAChRs heterologously expressed in Xenopus oocytes display slower current kinetics and high sensitivity to Aβ_{1–42}

To further investigate features of presumed, novel α7β2-nAChRs as naturally expressed in basal forebrain cholinergic neurons, we introduced nAChR α7 subunits alone or in combination with β2 subunits into Xenopus oocytes. Compared with homomeric α7-nAChRs (Fig. 7Aa), heteromeric α7β2-nAChRs expressed in oocytes injected with rnaAChR α7 and β2 subunit cRNAs at a ratio of 1:1 exhibited smaller peak current responses to choline and slower current decay rates (Fig. 7Ab). These results are consistent with a previous report indicating that nAChR α7 and β2 subunits...
presence of β2 subunits with α7 subunits in rodent MS/DB neurons. We then tested the sensitivity of heterologously expressed α7β2-nAChRs in oocytes to Aβ. As was the case for presumed, native α7β2-nAChRs on MS/DB neurons, heterologously expressed heteromeric α7β2-nAChRs, but not homomeric α7-7nAChRs, demonstrated sensitivity to Aβ1–42 (10 nM) and insensitivity to 10 nM scrambled Aβ1–42 (Fig. 7B). These results obtained using heterologously expressed nAChRs again are consistent with the hypothesis that nAChR α7 and β2 subunits likely coassemble and form a unique α7β2-nAChR that enhances receptor sensitivity to pathologically relevant, low nM concentrations of Aβ1–42.

**Basal forebrain nAChRs in nAChR β2 subunit-null mice do not show coimmunoprecipitation of nAChR α7 and β2 subunits, exhibit fast whole-cell current kinetics, and show low sensitivity to Aβ1–42.**

As another test of our hypothesis that basal forebrain cholinergic neurons express novel α7β2-nAChRs, we used wild-type and nAChR β2 subunit knock-out (β2−/−) mice. PCR genotyping was used to identify wild-type or β2−/− mice (Fig. 8A, B). Using the immunoprecipitation protocol previously described and protein extracts from the MS/DB, nAChR β2 subunits were found to coprecipitate with nAChR α7 subunits only for samples from wild-type but not from β2−/− mice (Fig. 3E, right). Choline-induced currents in MS/DB cholinergic neurons dissociated from β2−/− mice exhibited higher current amplitude, faster kinetics (Fig. 8C), and lower sensitivity to DHβE (Fig. 8D–E) than responses in cholinergic neurons dissociated from wild-type mice. As expected, 1 nM Aβ1–42 failed to suppress choline-induced currents in MS/DB neurons from β2−/− mice but did suppress choline-induced currents in MS/DB neurons from wild-type mice (Fig. 8E). These results again strongly support the hypothesis that heteromeric, functional α7β2-nAChRs on basal forebrain MS/DB cholinergic neurons are highly sensitive to a pathologically relevant concentrations of Aβ1–42.

**Discussion**

nAChRs in basal forebrain participate in cholinergic transmission and cognitive processes associated with learning and memory (Levin and Rezvani, 2002; Mansvelder et al., 2006). During the early stages of AD, decreases in nAChR-like radioligand binding sites have been observed (Burghaus et al., 2000; Nordberg, 2001), suggesting that nAChR dysfunction could be involved in AD pathogenesis and cholinergic deficiencies (Nordberg, 2001). Evidence indicates that enhancement of α7-nAChR function protects neurons against Aβ toxicity.
through any or some combination of a number of different mechanisms, as outlined previously (Sadot et al., 1996; Lahiri et al., 2002; Nagele et al., 2002; Geerts, 2005; Q. Liu et al., 2007). On the other hand, pharmacological interventions or diminished nAChR expression produces learning and memory deficits (Levin and Rezvani, 2002).

The current findings are consistent with the natural expression of a novel, heteromeric, functional α7β2-nAChR subtype on forebrain cholinergic neurons that is particularly sensitive to functional inhibition by a pathologically relevant concentration (1 nM) of Aβ1–42. Some previous studies investigating the acute loss of Aβ1–42 on nAChRs examined receptors on neurons from regions other than the basal forebrain or that were heterologously expressed (Liu et al., 2001; Pettit et al., 2001; Grassi et al., 2003; Wu et al., 2004a; Lamb et al., 2005; Pym et al., 2005) and/or used Aβ peptides at concentrations (between 100 nM and 10 μM) that greatly exceed Aβ concentrations found in AD brain (Kuo et al., 2000; Mehta et al., 2000). Other studies identified α7-nAChR-like, ACh-induced currents in MS/DB cholinergic neurons by using slice-patch recordings (Henderson et al., 2005; Thinschmidt et al., 2005) and characterized functional, non-α7-nAChRs by using acutely dissociated forebrain neurons (Fu and Jhamandas, 2003). Our present study combined whole-cell current recordings from acutely dissociated neurons and investigation of MS/DB cholinergic neuronal nAChRs to identify functional nAChRs that have some features of receptors containing α7 subunits, but we also found high sensitivity of these nAChRs to low concentrations of Aβ1–42.

Heterologous expression of functional α7β2-nAChRs with a unique pharmacological profile also has been reported using the Xenopus oocyte expression system (Khiroug et al., 2002). Our results agree with these previous findings and also indicate that functional α7β2-nAChRs can be heterologously expressed in oocytes. Histological studies have demonstrated coexpression of nAChR α7 and β2 subunits in most forebrain cholinergic neurons (Azam et al., 2003). Our results also are consistent with those observations and show cell-specific, coexpression of nAChR α7 and β2 subunits at both message and protein levels. There are other reports (Yu and Role, 1998; El-Hajj et al., 2007) that nAChR α7*-nAChRs. Our present findings are consistent with those observations.

The notion that the Aβ1–42-sensitive, functional nAChR subtype in MS/DB neurons displaying some features of nAChRs containing α7 subunits, but distinctive from homomeric α7-nAChRs, is composed of α7 and β2 subunits, is supported by the loss of Aβ sensitivity and the conversion of functional nAChR properties to those like homomeric α7-nAChRs in nAChR β2 subunit knock-out animals. It has been reported that there are two isoforms (α7-1 and α7-2) of α7-nAChR transcript in homomeric α7-nAChRs. The α7-2 transcript that contains a novel exon is widely expressed in the brain and showed very slow cur-
rent kinetics (Saragoza et al., 2003; Severance and Cuevas, 2004; Severance et al., 2004). However, we contend that the hetero-
meric, presumed /H92517/H92522-nAChR described in the present study
and expressed in MS/DB neurons is not a homomeric nAChR
composed of or containing the /H92517 transcript for three reasons:
(1) in /H92522 mice, /H92517-nAChR-like whole-cell current responses
to choline acquire fast kinetic characteristics like those of /H92517-
nAChR responses in VTA neurons, (2) immunoprecipitation–
Western blot analyses show coassembly of /H92517 and /H92522 subunits
from the MS/DB but not from the VTA, nor from the MS/DB of
/H92522 mice, and (3) pharmacologically, apparently heteromeric
/H92517 /H92522-nAChRs were sensitive not only to MLA, but also to
DH/H9252E.

A recent study suggested that levels of oligomeric forms of
A/H92521–42, rather than monomers or A/H9252 fibrils, most closely correlate
with cognitive dysfunction in animal models of AD (Haas and Selkoe, 2007). Our current findings also suggest that A/
oligomers have the most profound effects on nAChR function,
thus extending our earlier studies of A/H9252-nAChR interactions
(Wu et al., 2004a) and perhaps illuminating why there have been
apparent discrepancies in some of the earlier work concerning
A/H9252-nAChR interactions.

Alzheimer’s disease (AD) is a dementing, neurodegenerative
disorder characterized by accumulation of amyloid β (Aβ)
peptide-containing neuritic plaques, degeneration of basal fore-
brain cholinergic neurons, and gradually impaired learning and memory (Selkoe, 1999). The extent of learning and memory deficits in AD is proportional to the degree of forebrain cholinergic neuronal degeneration, and the extent of Aβ deposition is used to characterize disease severity (Selkoe, 1999). Processes such as impairment of neurotrophic support and disorders in glucose metabolism have been implicated in cholinergic neuronal loss and AD (Dolezal and Kasparova, 2003). However, clear neurotoxic effects of Aβ across a range of in vivo and in vitro models suggest that Aβ plays potentially causal roles in cholinergic neuronal degeneration and consequent learning and memory deficits (Selkoe, 1999).

Although the “cholinergic hypothesis” of AD etiopathogenesis
was established in the 1990s, the exact mechanism(s) by which
Aβ accumulation harms cholinergic neurons and results in learning
and memory deficits is largely unclear. Seemingly contradictory
findings about the effects of Aβ on function of nAChRs (Liu
et al., 2001; Pettit et al., 2001; Dineley et al., 2002b; Dougherty et
al., 2003; Fu and Jhamandas, 2003; Wu et al., 2004a) have been
hard to reconcile, although they may be explained by the use of
different experimental preparations and different or non-
pathologically relevant concentrations or forms of Aβ. Other
studies indicate that α7-nAChR expression is increased in both
an AD animal model and in human AD (Dineley et al., 2002a;
Counts et al., 2007), suggesting that pathologically relevant con-

Figure 8. Kinetics, pharmacology, and Aβ sensitivity of α7-containing- nAChRs in nAChR β2 subunit knock-out mice. Genotype analyses demonstrated that nAChR β2 subunits are not expressed in nAChR β2 knock-out mice (A), whereas Lac-Z (as a marker for the knock-out) was absent in wild-type (WT) mice (B). Kinetic analyses showed that whole-cell current kinetics and amplitudes differed for MS/DB neurons from WT compared with nAChR β2 subunit knock-out homozygote mice (Ca, CB). VH, Holding potential. Compared with MS/DB neurons from WT mice (Da), choline-induced currents in MS/DB (Db) neurons from β2 knock-outs were insensitive to DH/E but retained sensitivity to MLA (Dc). Aβ1–42 (1 nM) suppressed choline-induced currents in MS/DB neurons from WT (Ea) but not from β2 knock-out (Eb) mice (Ec). “Control” responses (Ed) were choline-induced currents in neurons from WT mice without exposure to Aβ1–42. *p < 0.05, **p < 0.01.
centrations of Aβ_{42} upregulate α7β2- nAChRs, potentially leading to cholinergic neuronal dysfunction due to abnormal accumulation of intracellular Ca^{2+}. Although this all might occur as a consequence of acute suppression of α7β2-nAChR function by Aβ, the present studies did not assess this possibility.

Based on the current findings, we suggest that selective, high-affinity effects of oligomeric Aβ_{42} on basal forebrain, cholinergic neuronal α7β2-nAChRs could acutely contribute to disruption of cholinergic signaling and diminished learning and memory abilities (Yan and Feng, 2004). Moreover, to the extent that basal forebrain cholinergic neuronal health requires activity of α7β2-nAChRs, inhibition of α7β2-nAChR function by oligomeric Aβ_{42} could lead to losses of trophic support for those neurons and/or their targets, and cross-catalyzed spirals of receptor functional loss and neuronal degeneration also could contribute to the progression of AD. Drugs targeting α7β2-nAChRs to protect them against Aβ effects or restoration of α7β2-nAChR function in cholinergic forebrain neurons could be viable tertiary or even primary therapies for AD.

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