The Novel α7β2-Nicotinic Acetylcholine Receptor Subtype Is Expressed in Mouse and Human Basal Forebrain: Biochemical and Pharmacological Characterization

Milena Moretti, Michele Zoli, Andrew A. George, Ronald J. Lukas, Francesco Pistillo, Uwe Maskos, Paul Whiteaker, and Cecilia Gotti

CNR Institute of Neuroscience, Biometra University of Milan, Milan, Italy (M.M., F.P., C.G.); Section of Physiology and Neurosciences, Department of Biomedical, Metabolic, and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy (M.Z.); Division of Neurobiology, Barrow Neurologic Institute, Phoenix, Arizona (A.A.G., R.J.L., P.W.); and Centre National de la Recherche Scientifique, Unité Neurobiologie Intégrative des Systèmes Cholinergiques, Institut Pasteur, Paris, France (U.M.)

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ABSTRACT

We examined αβ2-nicotinic acetylcholine receptor (αβ2-nAChR) expression in mammalian brain and compared pharmacological profiles of homomeric α7-nAChRs and αβ2-nAChRs. α-Bungarotoxin affinity purification or immunoprecipitation with anti-α7 subunit antibodies (Abs) was used to isolate nAChRs containing α7 subunits from mouse or human brain samples. αβ2-nAChRs were detected in forebrain, but not other tested regions, from both species, based on Western blot analysis of isolates using β2 subunit–specific Abs. Ab specificity was confirmed in control studies using subunit-null mutant mice or cell lines heterologously expressing specific human nAChR subtypes and subunits. Functional expression in Xenopus oocytes of concatenated pentameric (α7)2(β2)3, (α7)4(β2)1, and (α7)5(β2)1-nAChRs was confirmed using two-electrode voltage clamp recording of responses to nicotinic ligands. Importantly, pharmacological profiles were indistinguishable for concatenated (α7)2-nAChRs or for homomeric α7-nAChRs constituted from unlinked α7 subunits. Pharmacological profiles were similar for (α7)2-, (α7)4(β2)1, and (α7)5(β2)1-nAChRs except for diminished efficacy of nicotine (normalized to acetylcholine efficacy) at αβ2- versus α7-nAChRs. This study represents the first direct confirmation of αβ2-nAChR expression in human and mouse forebrain, supporting previous mouse studies that suggested relevance of αβ2-nAChRs in Alzheimer disease etiopathogenesis. These data also indicate that αβ2-nAChR subunit isoforms with different αβ2 subunit ratios have similar pharmacological profiles to each other and to α7 homopentameric nAChRs. This supports the hypothesis that αβ2-nAChR agonist activation predominantly or entirely reflects binding to α/α7 subunit interface sites.

Introduction

Several nicotinic acetylcholine receptor (nAChR) subtypes are expressed widely along the entire neuraxis and are involved in many of the physiologic functions of the central and peripheral nervous systems (Albuquerque et al., 2009; Hurst et al., 2013). nAChR activity controls important aspects of synaptic function and brain development, including the proliferation and differentiation of neural progenitors, neural migration, and neuronal maturation (Griguoli and Cherubini, 2012; Picciotto et al., 2012; Yakel, 2013). Furthermore, nAChR dysfunction may play an important role in a variety of neurologic diseases, including neurodegenerative and psychiatric diseases (Gotti and Clementi, 2004; Lewis and Picciotto, 2013).

αβ2- and homomeric α7-nAChRs are the most widely expressed subtypes in mammalian brain. The latter are thought to contain five identical agonist binding sites located at subunit interfaces in extracellular domains (Gotti and Clementi, 2004; Whiteaker et al., 2007). Pharmacological hallmarks of α7-nAChRs are their high sensitivity to antagonism by snake venom–derived polypeptide toxins such as α-bungarotoxin (α-Bgtx) and α-cobratoxin (α-Cbtx), and their sensitivity to choline [a product of acetylcholine (ACh) hydrolysis] as an agonist (Albuquerque et al., 1997, 2009). α7-nAChRs are highly expressed in the cortex, hippocampus, and subcortical limbic regions, and (at lower levels) in the thalamus and basal ganglia. α7-nAChRs that are...
located on or near nerve terminals are involved in control of neurotransmitter release, whereas α7-nAChRs on dendrites or soma are proposed to cholinergenic synaptic end play roles in classic neurotransmission. In both cases, the high calcium permeability of α7-nAChRs may also result in altered intracellular signaling and gene transcription (Dajas-Bailador and Wonnacott, 2004; Albuquerque et al., 2009). α7-nAChRs also may be associated with extrasynaptic volume transmission (Lendvai and Vizi, 2008).

Affinity purification of nAChRs using snake venom α-toxins has been performed from brain tissue of various species. Extracts from whole rat brain appear to be predominantly composed of homomeric α7-nAChRs (Drisdel and Green, 2000). However, homomeric α7- and α9-nAChRs (and heteromeric α7α8-nAChRs) have been identified in chick central nervous system extracts (Keyser et al., 1993; Gotti et al., 1994). Furthermore, studies using heterologous systems have shown that α7 subunits can form functional channels when combined with α3 (Girod et al., 1999), β2 (Khiroug et al., 2002), β3 (Palma et al., 1999), or β4 subunits (Criado et al., 2012). Fluorescently tagged nAChR α7 and β2 subunits were recently used to characterize the formation of α7β2-nAChRs, and functional differences between α7- and α7β2-nAChRs have been suggested (Murray et al., 2012). Coexpression of β2 and α7 subunits caused a significant decrease in agonist-evoked whole cell current amplitudes, but this decrease occurs without affecting the concentration-response characteristics of a range of common agonists and antagonists (Murray et al., 2012). Other studies have shown that α7 and β2 subunits are coexpressed in rat basolateral forebrain cholinergic neurons and appear to form heteromeric α7β2-nAChRs with subtly different biophysical and pharmacological properties from those of homomeric α7-nAChRs (Liu et al., 2009). In addition, interaction of these putative α7β2-nAChRs with oligomeric forms of amyloid-β (Aβ1–42) may be relevant in the etiology of Alzheimer disease (Liu et al., 2013).

These previous studies suggest that the function and pharmacology of α7*-nAChRs (where the asterisk denotes the known or possible presence of other nAChR subunits but α7; Lukas et al., 1999) may be more complex than previously thought, and that α7β2-nAChR expression may be restricted to forebrain areas. However, heteromeric α7*-nAChRs have not yet been directly detected biochemically, nor have they been definitively identified in human brain. We used the α7-nAChR-selective ligand, α-Bgtx, to affinity purify α7*-nAChRs from selected brain areas of humans or of wild-type (WT) or β2 subunit-null mutant (KO) mice. The subunit compositions of these isolated α7*-nAChRs were analyzed by Western blot analysis using subunit-specific anti-α7 or anti-β2 antibodies. The results show expression of α7β2-nAChRs in both WT mouse and human forebrain samples, but not in brains from β2 KO mice. Moreover, concatenemaric (linked subunit) constructs, the Xenopus oocyte system, and two-electrode voltage clamp (TEVC) recording were used to confirm functional expression of α7β2-nAChRs. This work defined α7 and β2 subunit stoichiometries that enable α7β2-nAChR function and showed similar pharmacological characteristics across α7- and α7β2-nAChR subtypes. The results confirm commonalities in expression of α7β2-nAChRs in humans and mice, and support hypotheses linking α7β2-nAChRs, cholinergic signaling loss, and roles for Aβ1–42 in etiopathogenesis of at least a subset of human dementias.

### Materials and Methods

#### Animals and Materials

This study used 4- to 6-month-old male, pathogen-free, C57BL/6 WT, α2 KO, or β2 KO mice (Picciotto et al., 1995; Orr-Uri et al., 1997) obtained from Dr. U. Maskos (Pasteur Institute, Paris, France). All animal experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) of November 24, 1986. (±)-[3H]Epibatidine (Epi; specific activity, 660 Ci/mol) and [3H]-α-Bgtx (specific activity of 200–216 Ci/mmole) were purchased from Perkin Elmer (Waltham, MA). Nonradioactive α-Bgtx, Epi, and nicotine were purchased from Toecis Bioscience (Bristol, UK, or Minneapolis, MN), as were dihydro-β-erythroidine (DHβE), and methylbicyclonitine (MLA). Sazetidine-A (also known as AMOP-H-OH) was kindly supplied by Dr. Alan Kozikowski (University of Illinois, Chicago, IL, USA): 1,2-bis-N-cyctislylethene (CC4) also was used (Riganti et al., 2005). α-CBtx and all other reagents were sourced from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

#### Human Tissues

Human cerebellum was provided by the Newcastle Brain Tissue Resource on the basis of a collaboration with Dr. Jennifer Court (Newcastle University General Hospital, Newcastle upon Tyne, UK). Samples were all collected by the Newcastle Brain Tissue Resource with informed consent and appropriate ethical approval. Case details are shown in Table 1. The approvals and method for categorizing the subjects’ smoking status are outlined in the methods section of Court et al. (2005). Human basal forebrain tissue was provided by Dr. Emmanuelle Sher (Lilly Research Center, Windlesham, Surrey, UK), and was also collected with appropriate informed consent in accordance with all applicable laws and regulations.

#### Transfected Cells

Human α2, α7, β2, and β4-nAChR subunit clones in the mammalian expression vector pcDNA3 were gifts from Dr. Sergio Fucile (University of Rome, Rome, Italy). The human α7-nAChR subunit clone in pcDNA3 was a gift of Dr. Roberta Benfante (CNR Institute of Neuroscience, Milan, Italy). HEK293 and SH-SY5Y cells were transiently transfected using the CaPO4 (PO4) method or the Jet-PEI reagent (Polyplus, Euroclone, Italy) transfection. For the α2 plasmid, 1.5 × 10⁶ cells was transfected with 6 μg plasmid using the Jet-PEI. For each of the α2, α4, and β2 or β4 subunits, 20 μg plasmids for 1.5 × 10⁶ cells was used, with the CaPO4 method. nAChR expression by cells was analyzed 24 hours after transfection.

#### Antibody Production and Characterization

We used affinity-purified, subunit-specific polyclonal antibodies (Abs), produced in rabbit against peptides derived from the C-terminal (COOH) or intracytoplasmic loop (CYT) of human or mouse nAChR subunit sequences, as previously described (Gotti et al., 2006; Grady et al., 2009). The Ab against the COOH peptide (SAPNFVEAV85KDF) was used for α7 subunits in mouse and human tissues. Abs directed

### Table 1

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Cases</th>
<th>Age</th>
<th>Postmortem Delay</th>
<th>Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal forebrain</td>
<td>4</td>
<td>65.7 ± 9.4</td>
<td>2–8</td>
<td>3/1</td>
</tr>
<tr>
<td>Cerebellum smokers</td>
<td>4</td>
<td>73.0 ± 3.9</td>
<td>&gt;8</td>
<td>2/2</td>
</tr>
<tr>
<td>Cerebellum nonsmokers</td>
<td>4</td>
<td>68.7 ± 6.6</td>
<td>&gt;8</td>
<td>2/2</td>
</tr>
</tbody>
</table>

There were no significant differences between groups for age.
against the α2 mouse CYT peptide (PSGPDPLAKILEEVRVYANRFRC) or the human CYT peptide (QMKEADIGSYPNGQMKEADIGSYPNG) were used for mouse and human tissues, respectively. For the β2 subunit, we used antibodies directed against different cytoplasmic human β2 peptides: RFREREAGAFREFRAPGDSCITY (β2α1) and cglADHMRESDDQGQREWDKTV (β2β2).

The specificity of the affinity-purified Abs was tested by immuno-

precipitation studies using α2 WT or α2 KO hippocampus and β2 WT or β2 KO mouse cortex (the results are shown in Supplemental Fig. 1). The same Abs also were tested by means of Western blotting (Supplemental Fig. 1). To exclude any cross-reactivity between nACHR subunits, anti-β2/γ7 or anti-α7 human subunit Abs were also tested by means of immunopre-

cipitation studies and Western blotting in HEK293 cells transfected to express human αβγβ, αβγβ, αβγβ or αβγβ2 nACHR subtypes or in SH-SYSY cells transfected to express human α2-nACHRs (see above) (results are shown in Supplemental Fig. 2).

**Purification of α-Bgtx-Binding nACHRs**

For studies using mice, approximately 100 mg of basal forebrain or hippocampus tissue microdissected from either WT or subunit-null mice were pooled in every experiment. The tissue was homogenized in 10 ml of 50 mM Na phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfon fluoride (to covalently inactive serine protease activity), and the homogenates were diluted and centrifuged for 1.5 hours at 60,000g. The entire membrane homogenization, dilution, and centrifugation procedure were then repeated, and the resulting pellets were collected, rapidly rinsed with 50 mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, and 2 mM phenylmethylsulfon fluoride. The washed pellets were then resuspended in 2 ml of the same buffer, further supplemented with 20 μg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 4 hours in 2% Triton. The extracts were centrifuged for 1.5 hours at 60,000g, recovered, and an aliquot of the supernatants was collected for protein measurement using the BCA protein assay (Pierce Biotechnology, Rockford, IL), with bovine serum albumin (BSA) as the standard. Extracts (2 ml) were incubated with 200 μl Sepharose–α-Bgtx (concentration of coupled toxin 1 mg/ml of gel) and shaken overnight at 4°C. The following day, the beads were centrifuged, the supernatant was recovered, and the resins were washed 4–6 times by resuspension followed by centrifugation. After washing, the Sepharose–α-Bgtx beads with bound nACHRs (purified α-Bgtx–binding receptors) were incubated with one to two volumes of Laemmli sample buffer (125 mM Tris phosphate, 4% SDS, 20% glycerol, 0.2% bromphenol blue, and 10% 2-mercaptoethanol, pH 6.8) and boiled for 2 minutes. The supernatant was then recovered by centrifugation.

In the case of human tissue, α-Bgtx–binding sites were purified using the same procedure as that used for mouse tissue, starting from 600 mg of tissue (see Table 1 for subject details). **Binding Studies**

[125I]α-Bgtx. The binding of [125I]α-Bgtx to 2% Triton X-100 extracts of mouse tissues was determined by collection onto DEAE-

Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden). Triton extracts (250 μl) from each experimental group were incubated overnight with a saturating concentration (5 nM) of [125I]α-Bgtx at 20°C in the presence of 2 mg/ml BSA. Specific radioiodinated binding was defined as total binding minus the nonspecific binding determined in the presence of 1 μM unlabeled α-Bgtx. Nonspecific binding averaged 30–40% of total binding. Binding to α7-nACHRs could also be measured in an immunoprecipitation assay format. Receptor extracts were labeled with [125I]α-Bgtx (5 nM in the presence or absence of 1 μM unlabeled α-Bgtx to define total and nonspecific binding). The labeled extract could then be bound to protein A beads via anti-α7 subunit Abs (described later in the Materials and Methods). Similar amounts of specific binding were recorded in either assay format, and nonspecific binding was between 10 and 15% of total binding.

[3H]Epi. Binding of [3H]Epi to nACHRs in 2% Triton X-100 brain tissue extracts obtained was also assessed. [3H]Epi binds to multiple heteromeric nACHR subtypes with picomolar affinity and to α7-nACHR with nanomolar affinity. To ensure that the α2 nACHR did not contribute to [3H]Epi binding, in solubilized extracts, binding was performed in the presence of 1 μM α-Bgtx, which specifically binds to α7-nACHRs (and thus prevents [3H]Epi binding to these sites).

As for [125I]α-Bgtx binding assays, binding sites were captured using DEAE-Sepharose Fast Flow, after overnight incubation of 250-μl aliquots of the extracts with 1 nM [3H]Epi at 4°C. Nonspecific binding (averaging 5–10% of total binding) was determined in parallel samples containing 100 nM unlabeled Epi.

**Immunoprecipitation**

For immunoprecipitation studies of heteromeric receptors present in human tissues, we used Abs specific for α2, α3, α4, α5, β2, or β3 subunits directed against human subunit peptides as previously described (Gotti et al., 2006). For α4 and β3 subunits, we used Abs directed against peptides of mouse subunit sequences, also as previously characterized and described (Grady et al., 2009). The immuno-

precipitation capacities of the anti-human subunit Abs ranged from 90 to 100% of the [3H]Epi-labeled receptors (mean of three independent experiments). For immunoprecipitation experiments, affinity-

purified Abs were covalently immobilized on agarose-Protein A beads at a concentration of 4 mg/ml wet resin. Immunoprecipitation was then performed by adding 20 μl agarose-Protein A beads with bound, affinity-purified Abs to 200 μl of 1 nM [3H]Epi–labeled extracts. After overnight incubation, immunoprecipitates were recovered by centrifugation and washed three times with phosphate-buffered saline containing 0.1% Triton X-100.

**Immunoblotting and Densitometric Quantification of Western Blot Bands**

nACHR subunit contents of tissue extracts or of α-Bgtx–binding complexes were analyzed by Western blotting. For the extracts loaded before and after the purification, 10 μg of proteins were loaded, whereas for the α-Bgtx–purified receptors a constant volume (40 μl), that depending on the tissue, may represent 1/10 or 1/20 of the total recovered Laemmli sample buffer- eluted receptors was loaded onto a 9% acrylamide (Bio-Rad, Hercules, CA) gel and subjected to SDS-

PAGE. After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes with 0.45-mm diameter pores (Schleicher and Schuell, Dassel, Germany). The blots were blocked overnight in 5% nonfat milk in Tris-buffered saline, washed in a buffer containing 5% nonfat milk and 0.3% Tween 20 in Tris-buffered saline, incubated for 2 hours with the primary antibody (1–2.5 mg/ml), and then incubated with the appropriate peroxidase-conjugated secondary Abs (Sigma-Aldrich). After 10 washes, peroxidase was detected using a chemiluminescent substrate (Pierce Biotechnology). The signal intensity of the Western blot bands was measured using an Epson 4500 gel scanner. The developed films were scanned as a TIFF image in eight-bit grayscale format at a resolution setting of 300 dpi. All of the films obtained from the separate experiments were acquired in the same way and scanned in parallel with a calibrated optical density step tablet from Stouffer (Stouffer Graphics Arts, Mishawaka, IN).

The images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) (Schneider et al., 2012). The pixel values of the images were transformed to optical density values by the program using the calibration curve obtained by acquiring the calibrated tablet with the same parameters as those used for the images. The immunoreactive bands were quantified in four separate experiments for the mouse hippocampus and basal forebrain as previously described (Grady et al., 2009).
Concatemeric $\alpha_7^*$-nAChR Constructs

Fully pentameric nAChR concatemers were constructed from human nAChR subunit sequences. cDNAs encoding concatemers were created using the same subunit layout we have previously employed to encode high- and low-agonist-sensitivity $\alpha_2\beta_2\gamma_2\delta$-nAChR isoforms and $\alpha_2\beta_2\delta\gamma_2\delta$-[D/N]-nAChRs (George et al., 2012; Eaton et al., 2014). Subunits were arranged in the order $\alpha_7$-$\alpha_7$-$\alpha_7$-$\alpha_7$-$\alpha_7$ (homopentamer), $\alpha_7$-$\alpha_7$-$\beta_2$-$\alpha_7$-$\alpha_7$, or $\alpha_7$-$\beta_2$-$\gamma_2$-$\beta_2$. Kozak and signal peptide sequences were removed from all subunit sequences with the exception of subunits expressed in the first position of the concatemer. Subunits were linked by alanine-glycine-serine repeats designed to provide a complete linker length (including the C-terminal tail of the preceding subunit) of 40 ± 2 amino acids. At the nucleotide level, linker sequences were designed to contain unique restriction sites that allow easy removal and replacement of individual subunits. The protein sequences for the human nAChR subunits were encoded by synthetic nucleotide sequences optimized for expression systems (GeneArt; Life Technologies, Grand Island, NY). Optimization included minimization of high GC content sequence segments, improved codon usage, reduction of predicted RNA secondary structure formation, and removal of sequence repeats and possible alternative start and splice sites. Sequences of all subunits, together with their associated partial linkers, were confirmed by DNA sequencing (GeneArt). Each concatemer was subcloned into the pSGEM vector.

RNA Synthesis

Plasmids containing concatemeric $\alpha_7$-homopentameric or $\alpha_7\beta_2$-nAChR constructs, or individual $\alpha_7$-nAChR subunits, were linearized with NheI (2 hours at 37°C), and the reaction mix was treated with proteinase K (30 minutes at 50°C). cRNAs were transcribed using the mMessage mMachine T7 kit (Applied Biosystems/Ambion, Austin, TX). Reactions were treated with TURBO DNase (1 U for 15 minutes at 37°C) and cRNAs were purified using the Qiagen RNeasy Clean-Up kit (Qiagen, Valencia, CA). cRNA purity was confirmed on a 1% agarose gel and preparations were stored at −80°C.

Xenopus Oocytes and RNA injection

Xenopus oocytes were purchased from Ecocyte Bioscience US (Austin, TX) and incubated upon arrival at 13°C. The tips of pulled glass micropipettes were broken to achieve an outer diameter of approximately 40 µm (resistance of 2–6 MΩ), and pipettes were used to inject 20–60 nl containing 10 ng of cRNA/oocyte. To improve functional expression of $\alpha_7^*$-nAChRs, Ric-3 mRNA was also coinjected (Halevi et al., 2002). A ratio of 1:50 Ric-3/$\alpha_7$ subunit mRNA by mass was determined to be optimally effective in pilot experiments (data not shown).

TEVC Recording of $\alpha_7$- and $\alpha_7\beta_2$-nAChR Function

TEVC recordings were made at room temperature (20°C) in oocyte saline solution (containing 82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM CaCl₂·H₂O, and 1 mM MgCl₂·H₂O, pH 7.4). Seven to 14 days after injection, Xenopus oocytes expressing concatenated $\alpha_7^*$-nAChRs were voltage clamped at −70 mV with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA). Recordings were sampled at 10 kHz (low-pass Bessel filter, 40 Hz; high-pass filter, DC), and the resulting traces were saved to disk (Clampex v10.2; Molecular Devices). Data from oocytes with leak currents ($I_{\text{leak}}$) > 50 nA were excluded from recordings.

Nicotinic Receptor Pharmacology

Fresh stock drug solutions (agonists: ACh, choline, nicotine, sazetidine, and CC4; antagonists: DHβE, MLA, mecamylamine, and $\alpha$-Cbxtx) were made daily and diluted as required. Agonists and antagonists were applied using a 16-channel, gravity-fed perfusion system with automated valve control (AutoMate Scientific, Inc.; Berkeley, CA). All solutions were supplemented with atropine sulfate (1.5 µM) to ensure that muscarinic ACh receptor responses were blocked and thus not recorded. Oocytes expressing loose subunits and/or concatemeric $\alpha_7$- or $\alpha_7\beta_2$-nAChRs were perfused with nAChR agonists for 5 seconds with 60-second washout times between each subsequent application. Oocytes were preincubated with nAChR antagonists for 2 minutes prior to activation with ACh (10 nM; 5 seconds). For experiments using $\alpha$-Cbxtx, bath and drug solutions were supplemented with 0.1% BSA to reduce loss of this peptide ligand by adsorption to the TEVC apparatus.

Data Analysis

The expression of $[^3H]$Epi and $[^{125}I]$-α-Bgttx receptors and the subunit contents of the $[^3H]$Epi receptors expressed in the mouse and human samples were statistically compared using unpaired t tests. In human cerebellum samples from smokers and nonsmokers, results were compared using an unpaired t test. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA).

For TEVC data, EC50 and IC50 values were determined from nAChR-mediated peak currents through nonlinear least-squares curve fitting (GraphPad Prism 5.0) using unconstrained, monophasic logistic equations to fit all parameters, including Hill slopes. Desensitization/inactivation of $\alpha_7^*$-nAChR currents in the presence of 10 nM (maximally stimulating) ACh was also analyzed by nonlinear least-squares curve fitting in Graph Pad Prism 5.0. These data were best fit by a two-phase exponential decay equation. One-way analysis of variance was used to compare parameters between multiple groups in each case. The Tukey multiple comparison test was used for post hoc analysis to compare the means of three or more groups (GraphPad Prism 5.0).

Results

$\alpha$-Bgttx-Binding Sites in WT or $\beta_2$ KO Mice

In preliminary experiments, we analyzed nAChR expression in 2% Triton X-100 extracts obtained from the hippocampus or basal forebrain of WT or $\beta_2$ KO mice (Table 2). By two different approaches (immunoprecipitating $[^{125}I]$-α-Bgttx-labeled receptors using anti-$\alpha_7$ subunit Abs and by $[^{125}I]$-α-Bgttx binding to Triton extracts), we determined that the density of $\alpha_7^*$-nAChRs in the mouse hippocampus is more than two times higher than that in the murine basal forebrain. During $\alpha$-Bgttx binding site purification, we determined that 85–95% of those sites in Triton X-100 extracts were bound by α-Bgttx–Sepharose 4B affinity resin, whereas more than 95% of high-affinity $[^3H]$Epi binding was recovered in the flow-through.

The densities of $[^3H]$Epi–binding nAChRs were similar between the two regions (Table 2). Elimination of $\beta_2$ subunit expression dramatically reduced expression of $[^3H]$Epi–binding nAChRs in both regions, indicating that this binding is almost entirely due to $\beta_2^*$-nAChRs. By contrast, $[^{125}I]$-α-Bgttx ($\alpha_7^*$-nAChR) expression was not significantly different between WT and $\beta_2$ KO mice in either the hippocampus or basal forebrain.

In addition, Western blot analysis was performed on α-Bgttx binding sites affinity purified from the hippocampus of WT or $\beta_2$ KO mice and probed with anti-$\alpha_7$ (top) or anti-$\beta_2$ (bottom) subunit Abs (Fig. 1A). Confirming results from the binding studies, Western blots also showed no significant differences in presumed $\alpha_7^*$-nAChR levels (i.e., polypeptide labeled with
anti-α7 subunit Abs) in whole extracts from WT or β2 KO mouse hippocampus (lane 1 in top two panels). Moreover, affinity purification on α-Bgtx affinity resins isolated comparable levels of α7*-nAChRs from WT or β2 KO mouse hippocampus (compare lanes 1 and 3 of Fig. 1A top) but did not isolate nAChRs containing β2 subunits (compare lanes 1 and 3 of Fig. 1A, bottom left), which instead were found in the flow-through fraction (lane 2; Fig. 1A, bottom left). As expected, no β2*-nAChRs were found in extracts isolated on Bgtx resins or in the flow-through from that separation for tissue taken from β2 KO mice (Fig. 1A, lower right panel lanes 1–3).

In further agreement with the findings of the binding studies, Western blot analysis showed that basal forebrain extracts contained fewer α7*-nAChRs (i.e., immunoreactive α7 protein) than hippocampal extracts, whereas amounts of immunoreactive β2 subunits were very similar across brain regions. Analysis of the Western blots probed using anti-β2 subunit Abs showed clearly detectable β2 subunit presence in α7*-nAChRs isolated on Bgtx resins from tissue derived from basal forebrain, but not from the hippocampus (compare lanes 3 of the bottom-left panels of Fig. 1, A and B). The percentage of β2 subunit present in the α-Bgtx purified receptor was determined by western blotting by loading on the same gel 10 μg of 2% Triton X-100 extracts with Sepharose 4B covalently bound with α-Bgtx. The bound receptors were recovered from the beads using Laemmli sample buffer. Western blot analysis of 10 μg 2% Triton X-100 extracts of the hippocampus before (lane 1) and after α-Bgtx purification (lane 2; supernatant), and 1/20 of the corresponding α-Bgtx purified receptors (lane 3; recovered from beads). The percentage of β2 subunit present in the α-Bgtx purified receptor was determined by western blotting by loading on the same gel 10 μg of 2% Triton X-100 extracts with Sepharose 4B covalently bound with α-Bgtx. The bound receptors were recovered from the beads using Laemmli sample buffer. Western blot analysis of 10 μg 2% Triton X-100 extracts of the hippocampus before (lane 1) and after α-Bgtx purification (lane 2; supernatant), and 1/20 of the corresponding α-Bgtx purified receptors (lane 3; recovered from beads).

The level of [3H]Epi–binding nAChRs in cerebellum depended on smoking status. As shown in Table 3, the density of non-α7*-nAChRs measured by means of [3H]Epi binding was higher in smokers than in nonsmokers (P = 0.02). Based on immunoprecipitation using subunit-specific Abs, in both tissues the large majority of [3H]Epi–binding sites contained the β2 subunit associated with the α4 subunit (α4β2*-nAChR: 75% in basal forebrain and 60 and 67% in cerebellum of smokers and nonsmokers, respectively). An additional 14% of [3H]Epi–binding sites in the basal forebrain were αβ2*-nAChRs, whereas this subtype accounted for only 7% of cerebellar [3H]Epi–binding sites.

### TABLE 2

Levels of [3H]Epi and [125I]α-Bgtx binding to 2% Triton X-100 extracts in two different brain areas of WT and β2 KO mice

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>[3H]Epi fmol/mg protein</th>
<th>[125I]α-Bgtx fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2 WT Hippocampus</td>
<td>36.3 ± 2.3</td>
<td>37.7 ± 2.3</td>
</tr>
<tr>
<td>β2 KO Hippocampus</td>
<td>1.0 ± 0.3*</td>
<td>39.9 ± 1.0</td>
</tr>
<tr>
<td>β2 WT Basal forebrain</td>
<td>44.5 ± 3.5</td>
<td>15.2 ± 2.5</td>
</tr>
<tr>
<td>β2 KO Basal forebrain</td>
<td>0.5 ± 0.2*</td>
<td>15.1 ± 2.6</td>
</tr>
</tbody>
</table>

*P < 0.001 (significantly different from β2*+* by t test).
Human $\alpha_7\beta_2$-nAChR Human Expression and Functional Pharmacology

Functional Expression of Concatemeric $\alpha_7$-nAChRs from Human Subunits

Heterologous expression has shown assembly of functional $\alpha_7\beta_2$-nAChRs (see the Introduction), but the way(s) in which $\alpha_7$ and $\beta_2$ subunits might combine from individual, unlinked, subunits could not be defined. Accordingly, we used a linked-subunit approach to produce $\alpha_7$-nAChRs with defined subunit ratios and assembly orders. Each of the three concatemeric constructs [(2)$\alpha_7\beta_2$-nAChR homopentamer, (2)$\alpha_7]\beta_2$-nAChR, and (2)$\alpha_7\beta_2\beta_2$-nAChR] showed concentration-dependent ACh-evoked function (representative traces shown in Fig. 3, A–D). This function, although smaller than that measured in Xenopus oocytes expressing homeric $\alpha_7$-nAChRs from unlinked human $\alpha_7$ subunits (typically $>1 \mu$A at 7 days after mRNA injection) was easily measurable (approximately 100–300 nA peak current response, depending on the construct). The time course of desensitization/inactivation after a peak response stimulated by 10 mM ACh (maximally stimulating concentration) was also measured for each construct. For each construct, desensitization/inactivation was best fit by a double-exponential decay model. As detailed in the legend to Fig. 3, no significant differences were seen between the fast desensitization/inactivation time constants calculated for each group. This is not surprising since the apparent time constants will likely reflect the relatively slow kinetics of agonist application in the apparatus, rather than the much faster kinetics of $\alpha_7$-nAChR desensitization (Papke, 2010). Indeed, the apparent $\tau_{slow}$ values are very similar to those measured for solution exchange in our apparatus (Eaton et al., 2014). However, the $\tau_{fast}$ value calculated for the (2)$\alpha_7\beta_2\beta_2$ construct was significantly slower than those associated with the other groups. Thus, despite the admitted disadvantages of measuring kinetic parameters in the Xenopus oocyte expression system, there is some evidence that $\alpha_7\beta_2$-nAChR desensitization may be slower than that of homomeric $\alpha_7$-nAChRs.

Agonist and Antagonist Pharmacology of Concatemeric Human $\alpha_7$-nAChRs

Pharmacological parameters of selected ligands were determined at concatenated $\alpha_7$-nAChRs. Compounds chosen included the prototypical agonists, ACh and nicotine, choline (which is a relatively selective agonist of $\alpha_7$-nAChR; Alkondon et al., 1997), and two further agonists with established selectivity for other $\beta_2$-nAChR subtypes (sazetidine-A and CC4; Xiao et al., 2006; Kozikowski et al., 2009; Sala et al., 2013). Agonist pharmacological profiles for (2)$\alpha_7\beta_2$, (2)$\alpha_7\beta_2\beta_2$, and (2)$\alpha_7\beta_2\beta_2\beta_2$-nAChR subtypes were largely indistinguishable from each other, and from that for nonconcatemeric (loose-subunit), homomeric $\alpha_7$-nAChRs (Fig. 4; Table 4). The only exception is that nicotine has significantly lower efficacy (normalized to that of ACh) at both (2)$\alpha_7\beta_2$-nAChR subtypes than at concatemeric (2)$\alpha_7$-nAChRs or unlinked $\alpha_7$-nAChRs (which are statistically indistinguishable on this measure). There was also a trend toward lower choline potency across all concatemeric $\alpha_7$-nAChR constructs, but this did not reach statistical significance (see Table 4). The observed slight trend toward lower choline efficacy, although not significant, is suggestive of the previous observation of 50–70% efficacy of choline versus ACh at putative $\alpha_7\beta_2$-nAChRs expressed from nonlinked subunits (Khiroug et al., 2002; Zwart et al., 2014). Strikingly, both sazetidine-A and CC4 were very weak agonists (<10% efficacy normalized to that of ACh) at all $\alpha_7$-nAChR subtypes tested, including both $\alpha_7\beta_2$-nAChRs, making it impossible to reliably calculate EC$_{50}$ or Hill slope values from the resulting concentration-response data.

**Fig. 2.** Western blot analysis of $\alpha$-Bgtx-purified nAChRs prepared from human basal forebrain and cerebellum. $\alpha$-Bgtx-binding nAChRs were purified from the same volume of 2% Triton X-100 extracts of basal forebrain and cerebellum by incubating them with Sepharose 4B covalently bound with $\alpha$-Bgtx. The bound receptors were eluted using sample buffer and an identical volume of purified receptors was loaded on the gel. The Western blots were probed with anti-$\alpha_7$ Ab (top) or anti-$\beta_2$ Ab (bottom).
Concentration-response relationships were also explored for archetypal α7 antagonists (MLA and the snake venom α-toxin, α-Cbtx), together with the β2-selective antagonist DHβE and the noncompetitive antagonist mecamylamine (Fig. 5). The resulting pharmacological parameters are summarized in Table 5. Similar to the agonist pharmacology, antagonist responses were statistically indistinguishable between the α7* subtypes (including between α7-only nAChRs expressed from either unlinked subunits, or from the concatenated α7 homopentameric construct).

Discussion

This study provides the first direct evidence that αβ2-nAChRs are expressed in the mammalian central nervous system. This is demonstrated by isolation of Bgtx-binding or α7 subunit-containing complexes also shown to contain β2 subunits from human or mouse forebrain samples. In addition, we have demonstrated for the first time that multiple human αβ2-nAChR isoforms of defined subunit composition have pharmacological profiles similar to each other and to homopentameric α7-nAChRs.

Our findings indicate that αβ2-nAChRs are found in post mortem, human basal forebrain but not in the cerebellum. Note that total amounts of α7*-nAChRs are <2-fold different in the two brain regions. Specificity of the anti-α7 or anti-β2 Abs used in Western blot analysis of these nAChRs is demonstrated by control studies using cell lines transfected with specific nAChR subunits, and by studies using WT and subunit-null mice. We also found αβ2-ACh expression in mouse basal forebrain but not hippocampus. Our results agree with earlier findings of αβ2-nAChR expression in mouse basal forebrain (Liu et al., 2009) but not with the same investigators’ study in mouse hippocampus (Liu et al., 2012). There could be several explanations for these seemingly discrepant observations. nAChR α7 and β2 subunit mRNAs are coexpressed in both basal forebrain and hippocampal cholinergic neurons (Azam et al., 2003). However, fewer than 3% of β2*-nAChRs in WT mouse basal forebrain extracts (this study) were associated with the α7 subunit. This indicates that the large majority of α-Bgtx–binding sites are homomorphic α7-nAChRs. Accordingly, we feel that the most likely explanation for the lack of an immunocytochemically detectable αβ2-nAChR in mouse hippocampus is that it is even less prevalent than in basal forebrain. The previous electrophysiology experiments (Liu et al., 2012) used brain slices from very young mice, whereas our work used tissue from 4- to 6-month-old mice. Therefore, it is also possible that mouse hippocampal αβ2-nAChR expression levels fall from early life into adulthood. Multiple examples of developmental modulation of nAChR subunit expression (including of α7) have previously been seen (Zoli et al., 1995; Conroy and Berg, 1998; Zhang et al., 1998; Balestra et al., 2000; Flora et al., 2000).

The use of a linked-subunit approach allowed us, for the first time, to directly assess the effects of defined β2-nAChR subunit incorporation on α7*-nAChR function. Of critical importance, no significant differences in EC50 values or efficacy relative to ACh were seen between concatenated or unlinked-subunit homomeric α7-nAChRs. This indicates that, as has previously been shown for αβ3*-nAChRs (George et al., 2012; Stokes and Papke, 2012), αβ2-nAChR (Zhou et al., 2003; Carbone et al., 2009; Mazzaferro et al., 2011; Eaton...
containing a gain-of-function mutation (L9)

2014). If concatemer fragments were contributing to the func-

be used by us and others (Carbone et al., 2009; Eaton et al.,

transmembrane domain. This additional control has previously

can be performed without altering nAChR functional phar-

opposite of the situation in which loose

was seen.

No such effect

catemeric construct as opposed to unlinked

ACh (10^{-5.5} to 10^{-2}; n = 6) (A),

choline (10^{-5.5} to 10^{-2}; n = 3) (B), nicotine (10^{-5.5} to 10^{-3}; n = 3) (C), sazetidine-A (10^{-7.5} to 10^{-4}; n = 3) (D), or CC4 (10^{-6.5} to 10^{-3}; n = 3) (E). All

responses within each group were normalized to an initial control stimulation with 10 mM ACh. Data points represent the mean ± S.E.M. Drug potency

efficiency parameters were calculated by nonlinear least-squares curve fitting to the Hill equation (see Materials and Methods). The resulting

pharmacological parameters and statistical analyses are summarized in Table 4.

expression of loose \( \beta_3 \) and \( \beta_4 \) subunits alone, but incorporation of the \( \alpha_5 \) subunit into a concatemeric construct actually

increased observed function of an \( \alpha_5 \beta_2 \)-nAChR pentameric
concatemer (George et al., 2012). As in the previous publication,

we suspect that uncontrolled assembly of an unlinked

additional subunit (in this case \( \beta_2 \)) may be deleterious, but
directed assembly may result in greater functional expression of

the new nAChR subtype. Certainly, this study provides
direct evidence that \( \beta_2 \) subunit incorporation into \( \alpha_7 \)-nAChRs

is compatible with agonist-induced function.

The pharmacological profiles of \( \alpha_7 \beta_2 \)-nAChRs were very

similar to those of homopentameric \( \alpha_7 \)-nAChRs. Even agonists

(sazetidine-A, CC4) and an antagonist (DHβE) previously

shown to have significant \( \beta_2 \)-nAChR selectivity had indistin-

guishable pharmacology across homomeric \( \alpha_7 \)-nAChRs and the
two different \( \alpha_7 \beta_2 \)-nAChR isoforms. Each of these findings

matches those very recently published using *Xenopus* oocytes

expressing \( \alpha_7 \) and \( \beta_2 \) subunits at a 1:10 ratio (Zwart et al.,

2014). The only statistically significant difference in this study

was a diminution of nicotine’s efficacy relative to that of ACh in

the two \( \alpha_7 \beta_2 \)-nAChR isoforms (also seen by Zwart et al.,

2014). This nicotine partial agonism further confirms that \( \beta_2 \)

was incorporated into \( \alpha_7 \beta_2 \)-nAChR concatemers as planned and

may represent a pharmacological marker for the presence of

\( \alpha_7 \beta_2 \)-nAChRs. The same may be true of the slower
desensitization kinetics measured for the \((\alpha_7 \beta_2 \beta_2 \beta_2)\) (Fig. 3), although

it is important to note the limitations of measuring receptor

kinetics in a *Xenopus* oocyte system (Papke, 2010). We note

that the similar \( \alpha_7 \)-nAChR versus \( \alpha_7 \beta_2 \)-nAChR potency of

DHβE observed by us and by Zwart et al. (2014) does not match

the observations made in two previous studies (Liu et al., 2009;

Murray et al., 2012). The reason for this discrepancy between

Fig. 4. Agonist concentration-response profiles for \( \alpha_7 \)- and \( \alpha_7 \beta_2 \)-nAChRs. Oocytes were injected with mRNA encoding unlinked \( \alpha_7 \) subunits (○),

concatenated \( \alpha_7 \) homopentamers (●), or concatenated \( \alpha_7 \beta_2 \) pentameric concatemers (□) indicates \( \alpha_5 \beta_2 \)-nAChR with the \( \beta_2 \) subunit in position 3; ■ indicates \( \alpha_7 \beta_2 \)-nAChR

with the \( \beta_2 \) subunit in positions 2 and 4. Oocytes were perfused with the following nAChR agonists: ACh (10^{-5.5} to 10^{-2}; n = 6) (A),

choline (10^{-5.5} to 10^{-2}; n = 3) (B), nicotine (10^{-5.5} to 10^{-3}; n = 3) (C), sazetidine-A (10^{-7.5} to 10^{-4}; n = 3) (D), or CC4 (10^{-6.5} to 10^{-3}; n = 3) (E). All

responses within each group were normalized to an initial control stimulation with 10 mM ACh. Data points represent the mean ± S.E.M. Drug potency

efficiency parameters were calculated by nonlinear least-squares curve fitting to the Hill equation (see Materials and Methods). The resulting

pharmacological parameters and statistical analyses are summarized in Table 4.

et al., 2014), and \( \alpha_6 \beta_2^{+} \)-nAChR (Kuryatov and Lindstrom,

2011) subtypes, introduction of appropriately sized linkers

can be performed without altering nAChR functional phar-

mcology. Several of these previous studies also showed that

concatemeric constructs were assembled correctly. To further

confirm correct that concatemers were being assembled cor-

rectly and not fragmenting and rearranging into unanticipat-

ated functional forms, we also co-injected unlinked \( \beta_2 \) subunits

containing a gain-of-function mutation (L9S) in the second

transmembrane domain. This additional control has previously

been used by us and others (Carbone et al., 2009; Eaton et al.,

2014). If concatemer fragments were contributing to the func-

tional nAChR population, the \( \beta_2 \)-gain-of-function subunit would

assemble into resulting \( \alpha_7 \)-nAChR as previously shown (Khiroug

et al., 2002; Murray et al., 2012; Zwart et al., 2014). Therefore,

if fragments containing \( \alpha_7 \) were present, this would result in

appearance of a novel \( \alpha_7 \beta_2 \)-gain-of-function population with

distinctive (more agonist-sensitive) properties. No such effect

was seen.

It is noted, however, that overall function was reduced

when \( \alpha_7 \)-nAChR homopentamers were expressed from a con-

catemeric construct as opposed to unlinked subunits. This

relative diminution in function of concatenated nAChR

constructs has been noted in the previous publications cited

above and appears to be a regular feature of using

concatemeric nAChR constructs. Importantly, both \((\alpha_7 \gamma_2)(\beta_2)\) and

\((\alpha_7 \gamma_2)(\beta_2)\)-nAChR concatemeric constructs expressed

more function than did the \((\alpha_7 \gamma_2)\)-nAChR concatemer. This is

the opposite of the situation in which loose \( \beta_2 \)-nAChR subunits

are coexpressed with \( \alpha_7 \) subunits (Murray et al., 2012) and

replicates an earlier finding in which coexpression of unlinked

\( \alpha_5 \), \( \alpha_3 \), and \( \beta_4 \) nAChR subunits reduced function compared with

expression of loose \( \beta_3 \) and \( \beta_4 \) subunits alone, but incorporation

of the \( \alpha_5 \) subunit into a concatemeric construct actually
the pairs of studies is not clear, but two possible explanations occur. First, the differences previously measured are relatively subtle, so they may be hard to reproduce. Related to this point, we note that the Hill slopes of the αβ2−nAChR DH2E concentration response curves (Fig. 5A) are shallower than those measured for other competitive antagonists (≤ 1, as opposed to significantly > 1 for MLA and α-Ctx). This would tend to obscure fine differences in IC50 values. Second, other α2 and β2 subunit associations are possible, in addition to those used in the αβ2−nAChR concatemers deployed in this study. It is possible that an αβ2−nAChR population expressed from unlinked subunits may assemble differently, giving rise to the slightly different DH2E sensitivity previously measured. This would match the previous experience in which αβ2−nAChR pharmacology perfectly matched between concatenated and unlinked-subunit nAChRs, but that of loose-subunit αβ2-only nAChRs was close, but not identical, between loose-subunit and concatemeric constructs (George et al., 2012). Further work may be needed to understand the (admittedly subtle) pharmacological differences between alternative αβ2−nAChR subunit stoichiometries and association orders.

Overall, however, the functional pharmacology of α7−nAChR and αβ2−nAChR subtypes is remarkably similar. This observation indirectly supports the concept that activation of αβ2−nAChRs may be predominantly or exclusively mediated only through agonist binding sites at α7/α7 (not αβ2 interfaces) (Murray et al., 2012). If this is true, it seems unlikely that any competitive agonist could exhibit a significantly different potency between the α7−nAChR and αβ2−nAChR. However, antagonists capable of disrupting the allosteric transitions required for nAChR activation (Celic et al., 2005), and of selectively binding to α7/β interfaces, could be valuable in this regard as could other noncompetitive ligands. In the concatemeric (α7)4(β2)1−nAChR construct (subunit order α7−α7−β2−α7−α7), only three α7/α7 subunit interfaces will be retained (between the first two subunits, the last two subunits, and between the first and last subunits that will assemble together to complete the pentameric nAChR structure). In the (α7)3(β2)2−nAChR construct, only the α7/α7 interface formed between the first and last subunits will be retained. At first glance, it may seem remarkable that an α7−nAChR containing such a diminished complement of putative agonist binding sites could be effectively activated. However, elegant recent work indicates that the nAChR, including α7−nAChR, can be activated effectively by as few as one agonist binding site (Rayes et al., 2009; Williams et al., 2011; Andersen et al., 2013).

Table 4

<table>
<thead>
<tr>
<th>Subtype</th>
<th>nAChR expressed in</th>
<th>EC50 (μM)</th>
<th>nH</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβ2−nAChR</td>
<td>Oocytes from unlinked subunits</td>
<td>6.32 ± 0.1</td>
<td>1.66</td>
<td>0.2</td>
</tr>
<tr>
<td>αβ2−nAChR</td>
<td>Oocytes from concatenated subunits</td>
<td>6.32 ± 0.1</td>
<td>1.66</td>
<td>0.2</td>
</tr>
<tr>
<td>αβ2−nAChR</td>
<td>Oocytes from concatenated subunits</td>
<td>6.32 ± 0.1</td>
<td>1.66</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Agonist logEC50, Hill slope (nH), and efficacy values relative to the functional pharmacology of Oocytes from unlinked subunits were used as a control group, to which the functional properties of Oocytes from concatenated subunits were compared (unlinked subunit order is shown). Antagonist logEC50, Hill slope (nH), and efficacy values relative to the functional pharmacology of Oocytes from unlinked subunits were used as a control group, to which the functional properties of Oocytes from concatenated subunits were compared (unlinked subunit order is shown). Antagonist logEC50, Hill slope (nH), and efficacy values relative to the functional pharmacology of Oocytes from unlinked subunits were used as a control group, to which the functional properties of Oocytes from concatenated subunits were compared (unlinked subunit order is shown).
and cognitive processes associated with learning and memory (Voytko et al., 1994; Hernandez et al., 2010). One of the most marked pathologic changes in the brain in Alzheimer disease is the degeneration of this cholinergic projection and the consequent reduction in the number of nAChRs (Dumas and Newhouse, 2011; Pinto et al., 2011). A number of studies have found that the β-amyloid (Aβ) peptide (a hallmark of Alzheimer disease) plays a critical role in neuronal degeneration and subsequent memory deficits (Price et al., 1985; Holtzman et al., 1992; Wenk, 1993; Fraser et al., 1997; Hernandez et al., 2010). One of the elements that is difficult to recapitulate in animal models alone (McCullumsmith et al., 2014). This study’s definitive evidence that αββ2- nAChRs are found in human as well as mouse basal forebrain provides valuable support for the concept that this subtype may be relevant to the study and etiology of Alzheimer disease. The similarities in human- and mouse-brain basal forebrain αββ2*- nAChR expression are also supportive of the use of mouse models in this context.

**TABLE 5**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>DHβE IC50 (M)</th>
<th>MLA IC50 (M)</th>
<th>Mecamylamine IC50 (M)</th>
<th>α-Ctx IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α7 (unlinked)</td>
<td>-5.2 ± 0.05</td>
<td>-1.0 ± 0.1</td>
<td>-1.6 ± 0.2</td>
<td>-5.6 ± 0.2</td>
</tr>
<tr>
<td>α7-α7-α7-α7-α7</td>
<td>-5.3 ± 0.07</td>
<td>-0.8 ± 0.1</td>
<td>-1.6 ± 0.2</td>
<td>-6.0 ± 0.2</td>
</tr>
<tr>
<td>α7-β2-α7-α7-β2-α7</td>
<td>-5.4 ± 0.10</td>
<td>-0.6 ± 0.1</td>
<td>-1.7 ± 0.1</td>
<td>-6.0 ± 0.1</td>
</tr>
<tr>
<td>α7-β2-α7-β2-β2-α7</td>
<td>-5.4 ± 0.10</td>
<td>-0.7 ± 0.1</td>
<td>-1.8 ± 0.1</td>
<td>-6.0 ± 0.2</td>
</tr>
</tbody>
</table>

Antagonist log IC50 and Hill slope values were derived by nonlinear least-squares curve fitting of the data shown in Fig. 5 to the Hill model. Pharmacological parameters obtained for each agonist were statistically indistinguishable between all four groups of oocytes according to analysis with one-way analysis of variance.

Post mortem tissue is an underused substrate for genetic and/or preclinical studies, and provides a translational element that is difficult to recapitulate in animal models alone (McCullumsmith et al., 2014). This study’s definitive evidence that αββ2- nAChRs are found in human as well as mouse basal forebrain provides valuable support for the concept that this subtype may be relevant to the study and etiology of Alzheimer disease. The similarities in human- and mouse-brain basal forebrain αββ2*- nAChR expression are also supportive of the use of mouse models in this context.


Supplemental data

α-Bgtx TITLE: The novel α7β2-nicotinic acetylcholine receptor subtype is expressed in mouse and human basal forebrain: Biochemical and pharmacological characterisation

Milena Moretti, Michele Zoli, Andrew A George, Ronald J Lukas, Francesco Pistillo, Uve Maskos, Paul Whiteaker, and Cecilia Gotti

Supplementary figure 1: Specificity of the subunit-specific polyclonal antibodies tested in 2% TritonX-100 extracts of mouse brain tissue: Specificity was tested by immunoprecipitation in extracts of α7+/+ and α7−/− mouse hippocampus of α7 mice, and β2+/+ and β2−/− mouse cortex, as described in Materials and Methods. The extracts were labelled with 5 nM [125I]-α-Bungarotoxin (α-Bgtx), or with 1 nM [3H]-Epibatidine (Epi).

% of [125I]–α-Bgtx labeled receptors immunoprecipitated

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>α7+/+ hippocampus</th>
<th>α7−/− hippocampus</th>
<th>β2+/+ cortex</th>
<th>β2−/− cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>α7 mouse cyt</td>
<td>95 ± 5 %</td>
<td>3 ± 2</td>
<td>91 ± 3</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>α7 COOH rat</td>
<td>90 ± 2</td>
<td>2 ± 2</td>
<td>92 ± 2</td>
<td>90 ± 2</td>
</tr>
</tbody>
</table>

% of [3H]–Epi labeled receptors immunoprecipitated

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>α7+/+ hippocampus</th>
<th>α7−/− hippocampus</th>
<th>β2+/+ cortex</th>
<th>β2−/− cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2 human cyt (1)</td>
<td>95 ± 5 %</td>
<td>92 ± 2</td>
<td>91 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>β2 COOH Rat</td>
<td>90 ± 2</td>
<td>85 ± 3</td>
<td>92 ± 2</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

The reported values are expressed as % of specific immunoprecipitation calculated from binding to the extract, and are the mean ± SEM of three determinations.

The same extracts were also analyzed by western blotting with the indicated Abs: β2 (top) and α7 (bottom) On the left the standard molecular weight is expressed in kDa.
Supplementary figure 2: Specificity of the subunit-specific polyclonal antibodies tested in 2% TritonX-100 extracts of nAChR-expressing cell lines: Specificity was tested by immunoprecipitation of nAChR from extracts of HEK cells transfected with the α2β4, α4β2, α3β4, or SH-SY5Y cells transfected with α7 subunits as described in Materials and Methods. The extracts were labelled with 2nM [3H]-Epi. The reported values are expressed as % of specific immunoprecipitation, and are the mean ± SEM of three determinations.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>α2β4</th>
<th>α4β2</th>
<th>α3β4</th>
<th>α7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α2 human</td>
<td>90 ± 2 %</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Anti-α3 human</td>
<td>2 ± 2</td>
<td>0</td>
<td>92 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Anti-α4 human</td>
<td>2</td>
<td>95 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-β2 human</td>
<td>2 ± 1</td>
<td>93 ± 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-β4 human</td>
<td>85 ± 4</td>
<td>2</td>
<td>92 ± 3</td>
<td>0</td>
</tr>
</tbody>
</table>

The same extracts were also analyzed by Western blotting and probed with the indicated Abs anti-α7 (top), anti-β2 (middle) anti-β4 (bottom). On the left the standard molecular weight is expressed in kDa.