Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 502 (2004) 31–40

www.elsevier.com/locate/ejphar

Stable expression and characterisation of a human α7 nicotinic subunit chimera: a tool for functional high-throughput screening

Peter J. Craig[a,*], Suchira Bose[a], Ruud Zwart[a], Ruth E. Beattie[a], Elizabeth A. Folly[a], Laura R. Johnson[a], Emma Bell[a], Non M. Evans[a], Giovanni Benedetti[a], Kathy H. Pearson[a], Gordon I. McPhie[a], Stephen G. Volsen[a], Neil S. Millar[b], Emanuele Sher, Lisa M. Broad

[a]*Eli Lilly and Company Ltd., Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey GU20 6PH, UK*
[b]*Department of Pharmacology, University College London, Gower Street, London, WC1E 6BT, UK*

Received 29 March 2004; received in revised form 9 August 2004; accepted 19 August 2004
Available online 15 September 2004

Abstract

A chimera comprising the N-terminal region of the human α7 nicotinic acetylcholine receptor, fused to the transmembrane/C-terminal domains of the mouse serotonin 5-HT_3 receptor, was constructed. Injection of the chimera cDNA into *Xenopus* oocytes, or transient transfection in human embryonic kidney (HEK-293) cells, resulted in the expression of functional channels that were sensitive to nicotinic acetylcholine, but not serotonin receptor ligands. In both systems, the responses obtained from chimeric receptors inactivated more slowly than those recorded following activation of wild-type α7 receptors. A stable HEK-293 cell line expressing the human α7/mouse 5-HT_3 chimera was established, which showed that the chimera displayed a similar pharmacological profile to wild-type α7 receptors. Use of this chimera in high-throughput screening may enable the identification of novel pharmacological agents that will help to define further the role of α7 nicotinic receptors in physiology and disease.

Keywords: Nicotinic acetylcholine receptor; α7 subunit; α7/5-HT_3 chimera; HEK-293 cells; (Human)

1. Introduction

Neuronal nicotinic acetylcholine receptors are ligand-gated ion channels formed by the co-assembly of five constituent subunits. The subunits are classified into two families, α and β. Each subunit comprises four membrane-spanning regions and a large extracellular, ligand binding N-terminal domain. To date, nine neuronal α (α2 to α10) and three β (β2 to β4) subunits have been identified and much effort has been devoted to determine which combinations of subunits assemble to form functional receptors (for reviews see Karlin and Akabas, 1995; McGehee and Role, 1995; Lindstrom, 2000; Millar, 2003).

Neuronal nicotinic acetylcholine receptors are frequently sub-divided into two functionally and pharmacologically distinct classes: those that bind α-bungarotoxin and those that do not. Neuronal α-bungarotoxin binding nicotinic receptors, which have the unique properties of high Ca^{2+} permeability and very rapid desensitisation, are widely expressed throughout the nervous system (Seguela et al., 1993; Quik et al., 2000) and have been shown to contain the α7 subunit (Couturier et al., 1990; Seguela et al., 1993). Their abundance in the hippocampus suggests a possible function in learning and memory, whilst the observation that α7 receptors are spared, or even elevated, in Alzheimer's patients compared with normal controls make them a potential therapeutic target for pharmacological intervention in this disorder (for review see Court et al., 2001). Additionally, evidence for a role of α7 in psychosis is provided by the observation that genetic polymorphism in the α7 gene may be linked to the sensory deficits found in

* Corresponding author. Tel.: +44 1276 483503; fax: +44 1276 483525.
E-mail address: Peterc@lilly.com (P.J. Craig).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.08.042

schizophrenic patients and their relatives (Adler et al., 1998; Leonard et al., 2002). Use of the few selective α7 pharmacological tools currently available has confirmed a role for α7 nicotinic receptors in cognition (Kem, 2000) and in improving sensory deficits in mice models of schizophrenia (Simosky et al., 2001).

Heterologous expression of the α7 gene in *Xenopus* oocytes has shown that this subunit can assemble into homomeric receptors that bind α-bungarotoxin and which share the pharmacological characteristics of native α7-containing nicotinic receptors (Couturier et al., 1990; Seguela et al., 1993). However, relatively few reports of the successful heterologous expression of the α7 gene in mammalian cells have been published (Puchacz et al., 1994; Gopalakrishnan et al., 1995; Quik et al., 1996; Cooper and Millar, 1997; Peng et al., 1999; Sweileh et al., 2000), whilst many reports have detailed the failures and complications (Quik et al., 1996; Cooper and Millar, 1997; Rangwala et al., 1997; Kassner and Berg, 1997; Blumenthal et al., 1997; Rakhilin et al., 1999; Sweileh et al., 2000; Aztiria et al., 2000; Dineley and Patrick, 2000). A common finding has been that α7 receptor expression appears to be host-cell specific. Several studies have demonstrated the presence of α7 mRNA (Sweileh et al., 2000; Aztiria et al., 2000) or protein (Cooper and Millar, 1997) in a variety of cell types transfected with α7, however, few were capable of producing correctly folded and assembled α7 nicotinic receptors that bound α-bungarotoxin and which were transported to the cell surface.

Although stable cell lines expressing functional α7 nicotinic receptors have been generated, their pharmacological characterisation has often been complicated by the expression of other native nicotinic receptors in the host cells (Puchacz et al., 1994; Blumenthal et al., 1997), or by the low level of receptor expression at the cell surface (Sweileh et al., 2000, Schroeder et al., 2003). The lack of a well-characterised stable α7 cell line has limited the development of high-throughput techniques for screening chemical libraries against α7 receptors. Consequently, relatively few selective pharmacological tools are available to assess the role of this receptor in normal or disease states, or to determine the therapeutic value of such ligands.

Chimeric α7/5-hydroxytryptamine-3 (5-HT_3) receptors have been described which, by combining the pharmacology of the α7 nicotinic receptor with the favourable expression properties of 5-HT_3 receptors (Eisele et al., 1993; Cooper and Millar, 1998; Dineley and Patrick, 2000), provide one strategy to overcome the problems of expressing α7 in mammalian cells. Such chimeric α7/5-HT_3 receptors have been used as tools for investigating the role of various domains of the receptor (Corringer et al., 1995; Rakhilin et al., 1999; Dineley and Patrick, 2000) and have previously been based on either chick (Eisele et al., 1993) or rat (Cooper and Millar, 1998; Dineley and Patrick, 2000) α7 domains. The use of such chimeras is reported to avoid both the host-cell specific limitations associated with expression of wild-type α7 nicotinic receptors and to generate higher levels of cell surface binding (Rangwala et al., 1997; Cooper and Millar, 1998; Dineley and Patrick, 2000). This might predictably correlate with increased function and generate the robust signals necessary for use in assays, such as Ca^{2+}-based high-throughput methodologies, which are commonly used to support the identification and development of new pharmacological tools (Schroeder and Neagle, 1996; Evans et al., 2003). Here we report on the construction and pharmacological characterisation of a human α7/mouse 5-HT_3 (hα7/5-HT_3) chimera stably expressed in mammalian human embryonic kidney (HEK-293) cells.

2. Materials and methods

2.1. Construction of a human α7/mouse 5-HT_3 chimera

A chimera comprising the N-terminal domain of the human α7 nicotinic acetylcholine receptor and the transmembrane/C-terminal regions of the mouse 5-HT_3 (hα7/5-HT_3) receptor was constructed. The full-length human α7 cDNA, cloned between the *Bam*HI and *Xho*I sites of pcDNA3 (Elliot et al., 1996), was obtained from Merck Research Laboratories (La Jolla, CA, USA) and used as a template in PCR in order to amplify the extra-cellular coding region of the α7 sequence. The forward primer was designed to anneal to a region within the multiple cloning site of the plasmid (5′ AGGGAGACCCAAGCTTGGTACCGA 3′) whilst the reverse primer contained 22 nucleotides that were α7-specific together with an extended 5′ region containing the restriction site for *Bcl*I (5′ TGGTATTGATCACTGTGAAGGTGACATCGGG 3′). The reverse primer caused a *Bcl*I site to be introduced into the α7 sequence at a position corresponding to Val^{202} of the mature human α7 protein. The complete chimeric construct was generated by ligating pGEM-7Zf (Promega, Southampton, UK) containing the C-terminal domain of the mouse 5-HT_3 subunit cDNA (Maricq et al., 1991) with the PCR product of the modified N-terminal coding region of the α7 subunit via a common *Bcl*I site. The chimera was then sub-cloned between the *Bam*HI/*Xba*I sites of pcDNA3.1 and pcDNA3.1 Zeo (both from Invitrogen, Paisley, UK) for use in expression studies. The sequence of the construct was verified by automated sequencing (Cytomyx, Cambridge, UK) (Fig. 1).

2.2. Expression in Xenopus laevis oocytes and electrophysiological characterisation

Adult female *X. laevis* frogs were obtained from Blades Biological (Edenbridge, UK) and housed at Lilly Research Centre following the appropriate UK regulations governing the use of animals in scientific research.

Oocytes were defolliculated manually after treatment with collagenase type I (4 mg/ml in Ca^{2+}-free Barth's

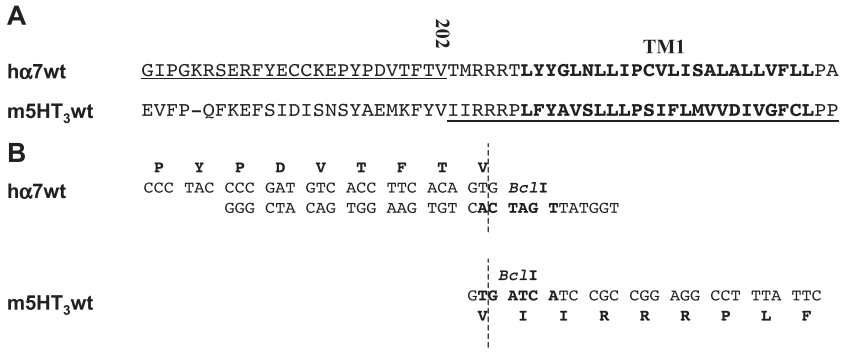

Fig. 1. Construction of human α7/5-HT_3 chimera. (A) Amino acid alignment between human α7 and mouse 5-HT_3 showing location of V202 and the first transmembrane domain (bold type). The amino acids that form the chimera are underlined. (B) Nucleotide sequences of human α7 and mouse 5-HT_3 in the region where the chimera was formed. The reverse PCR primer used to incorporate the *Bcl*I site into the α7 sequence and the intrinsic *Bcl*I site in 5-HT_3 are illustrated.

solution) for 1.5 h at room temperature. Plasmids containing the wild-type human α7 nicotinic receptor subunit (in pcDNA3) or hα7/5-HT_3 (in pcDNA3.1), were suspended in distilled water and injected into the nuclei of stages V and VI oocytes within 4 h after harvesting, using a Drummond (Broomall, PA, USA) variable volume microinjector. Approximately 2 ng of human α7, or 8 ng of hα7/5-HT_3 cDNA were injected into each oocyte in a total injection volume of 18.4 nl. After injection, oocytes were incubated at 18 °C in a modified Barth's solution (composition in mM, 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 50 mg/l neomycin), pH 7.6, 235 mOsm. Experiments were performed on oocytes 3–5 days after cDNA injection.

Oocytes were placed in a recording chamber (3 mm diameter), which was continuously perfused with a saline solution (composition in mM, 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES), pH 7.3, 235 mOsm, at a rate of ~10 ml/min. Drugs were prepared in external saline immediately before use. A BPS-8 solution exchange system (ALA Scientific, Westbury, NY, USA) was used to switch between control and drug-containing saline. Alternation between superfusion with agonist and agonist-free saline applications (5 min cycles for the chimera; 2 min cycles for α7) were made in order to allow the receptors to recover from desensitisation.

Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 MΩ) and voltage clamped using a Geneclamp 500B amplifier (Axon Instruments, Foster City, CA, USA). The external saline was clamped at ground potential by means of a virtual ground circuit using an Ag/AgCl reference electrode and a Pt/Ir current passing electrode. The membrane potential was held at −60 mV. The current required to keep the oocyte membrane at the holding potential was measured. Membrane currents were low-pass filtered (four-pole low-pass Bessel filter, −3 dB at 1 kHz), digitised (1000 samples/record), and stored on disk for off-line computer analysis. All experiments were performed at room temperature.

2.3. Cell culture and transient transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. HEK-293 cells were sub-cultured the day before transfection and plated to achieve ~60–70% confluency within 24 h. Cells were then transfected with 1μg of either the hα7/5-HT_3 in pcDNA3.1 or wild-type human α7 in pcDNA3 using 3μl of FuGENE 6 transfection reagent (Roche, Lewes, UK). Transfected cells were incubated for 48 h at 37 °C for hα7/5-HT_3 or 29°C for α7, before binding and function were assessed.

2.4. Fluorescent α-bungarotoxin binding assay

To assess cell-surface expression of hα7/5-HT_3 or wild-type α7 receptors, transfected cells were plated on glass coverslips then incubated with Alexa-fluor 488-conjugated α-bungarotoxin (Molecular Probes) at a final concentration of 0.002 $\mu g/\mu l$, for 30 min at 37 °C. Cells were washed extensively with Hanks Balanced Salt Solution to remove any unbound toxin and fluorescence then observed on an inverted microscope (Leica DMIRB). Cells were not permeabilised, so that only cell surface receptors were observed.

2.5. Stable cell line development and culture

Cells stably expressing hα7/5-HT_3 were selected by culturing hα7/5-HT_3 transfected HEK-293 cells in growth medium containing 2 mg/ml Geneticin G418. A total of 129 clones were isolated by limiting dilution then expanded and screened for functional expression using a Fluorometric Imaging Plate Reader (FLIPR). In an effort to boost the size of the functional response, the two clones that displayed the largest responses were re-transfected with hα7/5-HT_3 in pcDNA3.1-Zeo. A final re-transfected clone, which displayed large and stable Ca^{2+}responses on stimulation with nicotinic receptor agonists, was selected from a further 49

clones by culturing in the presence of Zeocin (160 μg/ml). This clone showed good growth characteristics over a 1-month selection period at which stage the concentration of Geneticin G418 and Zeocin in the growth medium was reduced to 500 and 40 μg/ml, respectively.

2.6. Single-cell Ca^{2+} imaging

Cells plated on poly-D-lysine-coated, glass bottomed 35 mm micro-well dishes (MatTek, MA, USA) were loaded with 2 μM Fura-2-acetoxymethyl (AM) ester diluted in either growth medium, for 30 min at 37 °C, or in a HEPES-buffered saline solution (HBSS, composition in mM, 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 10 HEPES), pH 7.3, for 1 h at room temperature (21–24°C). Cells were then washed and incubated in HBSS for 30 min at room temperature. The culture dishes were viewed on an inverted epifluorescence microscope (Axiovert 100TV, Zeiss, Germany) using a 10× (air) or 40× (oil immersion) fluorescence objective. Cells were alternatively excited by light of 340 and 380 nm wavelength, provided by a polychrome II (from TILL Photonics, Planegg, Germany) housing a xenon lamp and a monochromator. Emitted light was captured by a SensiCam cooled CCD camera (PCO CCD Imaging, Kelheim, Germany) after passage through a dichroic mirror (400 nm) and high-pass barrier filter (480 nm). Digitised images were stored and processed using Axon Imaging Workbench software (Version 2.2 and 4.0, Axon Instruments) and Origin 6.1 software (OriginLab, MA, USA). Drugs were applied focally via an eight-channel valve-driven superfusion system (APS, Bad Homburg, Germany) and the cells were continuously superfused with HBSS between drug applications. All recordings were made at room temperature.

2.7. Cell population Ca^{2+} measurements using FLIPR

Confluent cells, re-suspended in the used growth medium, were plated at a density of 0.5×10^6 cells/ml directly into poly-D-lysine coated, black-walled, transparent bottomed 96-well plates (Becton Dickinson, Marathon Laboratories, London, UK) then incubated overnight at 37°C in an atmosphere of 95% air, 5% CO_2. Growth medium was removed before addition of 50 μl Tyrode's assay buffer (composition, in mM, 137 NaCl, 2.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 0.2 $NaHPO_4$, 5.5 glucose) containing 10 μM Fluo-3-AM/0.05% pluronic F-127 using a multi-drop pipettor (Lab Systems, Helsinki, Finland). Cells were incubated in the dark with the dye for 1 h at room temperature before the medium was removed and replaced with Tyrode's buffer without Fluo-3. The plates were transferred to the FLIPR where the cells were excited by light of 488 nm wavelength from a 4 W Argon-ion laser. After passing through a 510–570 nm bandpass inference filter, the emitted fluorescence was detected with a cooled CCD camera (Princeton Instruments). Fluorescence was recorded every 1 s for the first minute following drug addition, with additional readings every 6 s for a further 2 min. Parameters for drug addition to the cell plate were programmed using FLIPR system software on a Dell Optiplex GX110 computer. Drugs were prepared in a 96-well plate using a Biomek 2000 (Beckman Instruments, Fullerton, CA, USA) and compound delivery was automated through a 96-tip pipettor. Responses were measured as peak-minus basal-fluorescence intensity, and are expressed as a percentage of a maximal response obtained to the non-selective nicotinic receptor agonist epibatidine (3 μM). Data files were saved to the computer and stored for off-line analysis using FLIPR system software and Origin 6.1 (Origin-Lab).

Acetylcholine, nicotine, choline, cytisine, 5-hydroxytryptamine (5-HT), 5-hydroxyindole (5-HI), 1,1-dimethyl-4-phenylpiperazinium (DMPP), mecamylamine, D-tubocurarine, α-bungarotoxin and dihydro-β-erthroidine were purchased from Sigma-RBI (Poole, UK). Methyllycaconitine (MLA), epibatidine, *m*-chloropheylbiguanide (mCPBG) and tropanyl 3,5-dichlorobenzoate (MDL-72222) were from Tocris Cookson (Bristol, UK). Fura-2-AM, Fluo-3-AM, pluronic F-127 and Alexa-fluor-α-bungarotoxin were from Molecular Probes (Leiden, The Netherlands). All cell culture reagents were from (Sigma, Poole, Dorset), except for fetal calf serum, which was from Invitrogen.

3. Results

3.1. Expression of human α7/5-HT_3 chimera in oocytes

After construction of the hα7/5-HT_3 chimera its function and pharmacological properties were first assessed by expression in *Xenopus* oocytes. Selected nicotinic and serotonergic ligands were investigated and their effects on hα7/5-HT_3 and wild-type human α7 nicotinic receptors were compared.

Both hα7/5-HT_3 and human α7 wild-type nicotinic acetylcholine receptors were functional upon expression in *Xenopus* oocytes (Fig. 2A). The size and shape of the ion currents mediated by the hα7/5-HT_3 receptor were very similar to those previously reported for the chick α7/mouse 5-HT_3 chimeric receptor (Eisele et al., 1993). Superfusion of oocytes expressing hα7/5-HT_3 receptors with 1 mM acetylcholine evoked inward currents in 24.3% of injected oocytes (n=37) with peak amplitudes ranging between 6 nA and 1μA. This compared with 1 mM acetylcholine-induced current amplitudes ranging between 0.1 and 34.3 μA for oocytes expressing wild-type α7 nicotinic receptors. The onset of desensitisation, determined by fitting the decay phases of the responses to a single-exponential function of 1 mM acetylcholine-induced ion currents, was slower in oocytes expressing hα7/5-HT_3 compared with

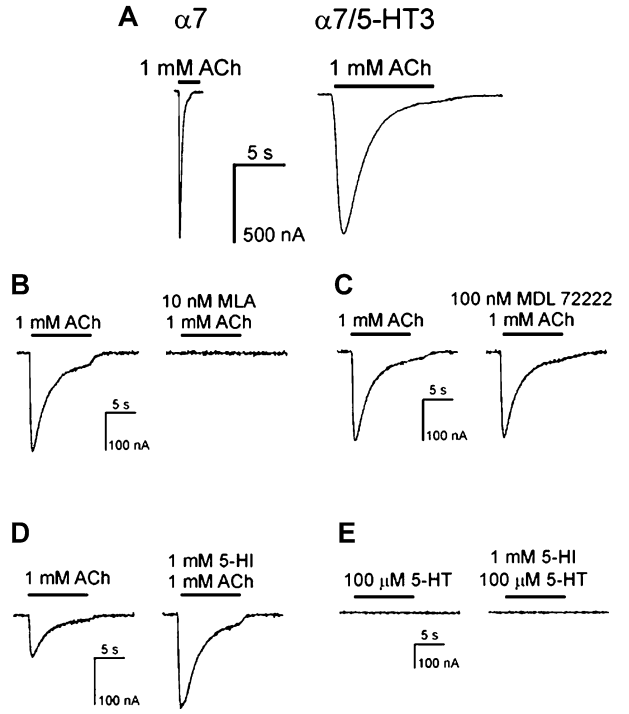

Fig. 2. Pharmacological properties of a human α7/5-HT_3 chimera expressed in *Xenopus* oocytes. Comparison of 1 mM acetylcholine-induced ion currents mediated by wild-type human α7 nicotinic receptors and a human α7/5-HT_3 chimera (A). α7 receptor-mediated ion currents desensitise within 1 s to baseline level, whereas the responses mediated by the chimera desensitise on a much slower timescale, as indicated. (B) 10 nM of a selective α7 nicotinic receptor antagonist MLA completely abolishes 1 mM acetylcholine-induced ion currents. (C) 100 nM of the selective 5-HT_3 receptor antagonist MDL 72222 does not inhibit 1 mM acetylcholine-induced ion currents. (D) 1 mM 5-hydroxyindole, a potentiator of α7 nicotinic and 5-HT_3 receptors, potentiates 1 mM acetylcholine-induced ion currents. (E) 100 μM 5-HT does not induce ion currents in oocytes expressing the human α7/5-HT_3 chimera. No responses were seen even in the presence of 1 mM of the potentiator 5-hydroxyindole.

oocytes expressing wild-type α7 nicotinic receptors. The time-constants obtained were 0.2±0.1 s (n=10) for wild-type α7 receptors, and 6.0±1.4 s (n=9) for hα7/5-HT_3 receptors.

To confirm that the hα7/5-HT_3 chimera displayed pharmacological characteristics comparable with α7 and not 5-HT_3 serotonin receptors, voltage-clamped oocytes expressing hα7/5-HT_3 were exposed to various nicotinic and serotonergic ligands (Fig. 2B,C). As expected, when superfused with 1 mM acetylcholine (but not 100 μM 5-HT) oocytes expressing hα7/5-HT_3 responded with a transient inward current. The acetylcholine-induced ion currents were completely abolished by 10 nM of the selective α7 nicotinic receptor antagonist methyllycaconitine (MLA), whereas 100 nM of a selective 5-HT_3 receptor antagonist, MDL-72222, had no effect.

To define further the pharmacological properties of hα7/5-HT_3 receptors, 5-hydroxyindole, a potentiator of both α7 nicotinic and 5-HT_3 serotonin receptors (Kooyman et al., 1993; Gurley et al., 2000; Zwart et al., 2002), was used. In oocytes expressing hα7/5-HT_3 receptors, 1 mM 5-hydroxyindole potentiated 1 mM acetylcholine-induced ion currents (Fig. 2D). In contrast, even in the presence of 1 mM 5-hydroxyindole, 100 μM 5-HT was unable to induce any ion current (Fig. 2E).

3.2. Expression of human α7/5-HT_3 chimera in HEK-293 cells

The cell surface expression, function and pharmacological properties of hα7/5-HT_3 and wild-type α7 nicotinic receptors were further assessed after transient transfection in mammalian HEK-293 cells.

Expression in mammalian cells was initially demonstrated by fluorescent α-bungarotoxin binding. Non-permeabilised HEK-293 cells transiently transfected with hα7/5-HT_3 displayed prominent α-bungarotoxin binding indicating abundant cell-surface receptor expression (Fig. 3B). Comparison of the same field under fluorescent and bright-field illumination indicated that >70% of the cells expressed detectable chimeric receptor. In contrast, HEK-293 cells transfected with wild-type α7 failed to display measurable fluorescence (data not shown). As nicotinic receptor expression (Cooper et al., 1999), and specifically α7 receptor expression (Schroeder et al., 2003), has been reported to be increased after incubation at low temperature, fluorescent α-bungarotoxin binding to HEK cells transiently transfected with wild-type human α7 nicotinic receptors was assessed after incubation at 29 °C for 48 h. Under these conditions a low level of fluorescence was detectable in a small proportion (<5%) of cells (data not shown).

In parallel studies, the functional properties of transiently transfected HEK-293 cells were measured using single cell Ca^{2+}imaging of Fura-2 loaded cells (Fig. 4). As expected from the absence of α-bungarotoxin binding, no function was detected in cells transiently transfected with wild-type α7 nicotinic receptors and incubated at 37°C (not shown). Despite evidence for low levels of specific α-bungarotoxin binding to α7-transfected cells grown at 29 °C, no

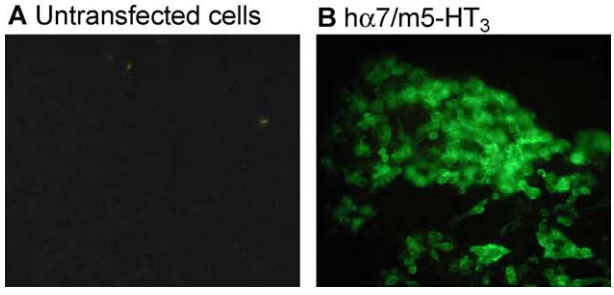

Fig. 3. Cell surface α-bungarotoxin binding in HEK cells. HEK-293 cells were either untransfected (A) or transiently transfected with the human α7/mouse 5-HT_3 pcDNA3.1 expression construct (B). Cell-surface receptor expression was detected by labelling non-permeabilised cells with Alexa-fluor 488-conjugated α-bungarotoxin.

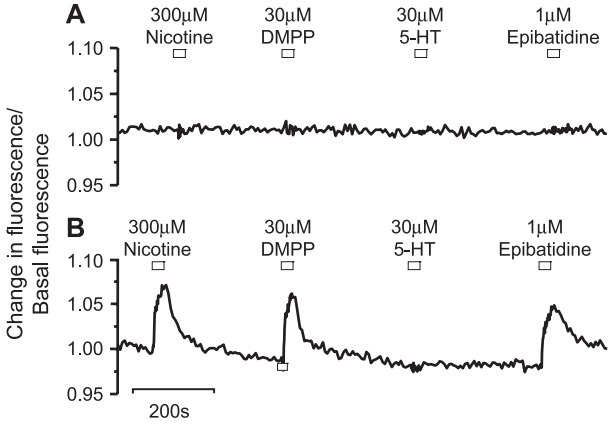

Fig. 4. Chimeric α7/5-HT_3 receptors form functional receptors after transient transfection in HEK-293 cells. Fura-2 loaded HEK-293 cells transiently transfected with either wild-type human α7 nicotinic receptors (A, incubated at 29 °C) or chimeric human α7/5-HT_3 receptors (B) were superfused with HBSS containing nicotinic or serotonergic agonists (30 s periods, as indicated). Results are the average of 10–12 cells from a single experiment, representative of three similar experiments.

functional responses to nicotinic receptor agonists were detected (Fig. 4A). In contrast, 30 s applications of 30 μM DMPP, 100 μM nicotine or 1 μM epibatidine to cells transiently expressing hα7/5-HT_3 receptors caused small, but reproducible increases in the intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$) in 10–30% of cells (Fig. 4B). Human α7/5-HT_3 receptors were not activated by the 5-HT_3 receptor agonist 5-HT (10 μM).

As was observed in oocytes, when expressed in mammalian cells the hα7/5-HT_3 chimera gave responses to a nicotinic agonist (DMPP, 30 μM) that could be potentiated by 5-hydroxyindole (1 mM). However, the 5-HT_3 receptor agonist mCPBG did not cause any increase in $[Ca^{2+}]_i$ even in the presence of 5-hydroxyindole (Fig. 5A). The response to DMPP and 5-hydroxyindole was fully and

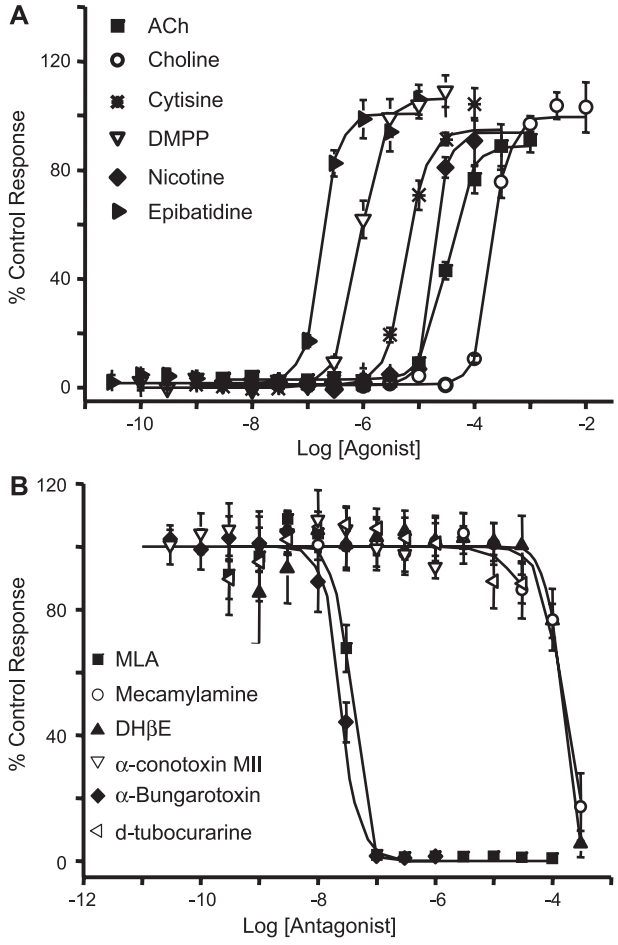

Fig. 6. Profile of nicotinic agonists and antagonists on chimeric α7/5-HT_3 receptors stably expressed in mammalian cells. Fluo-3 loaded HEKα7/5-HT_3 were challenged with nicotinic receptor agonists (A) or antagonists (B), as indicated. Agonist activity was measured in naïve cells, whilst antagonist activity was assessed after a 20-min pre-treatment of cells with the respective antagonists, followed by addition of epibatidine (1 μM). Concentration–response curves are mean values±S.E.M. from 4–22 experiments.

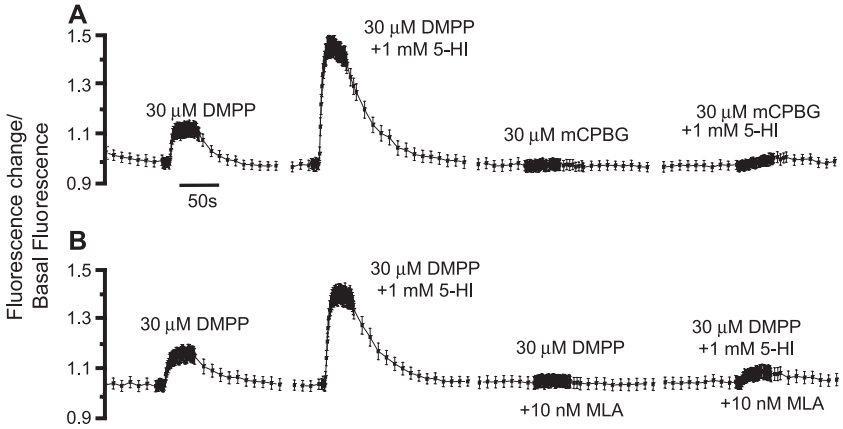

Fig. 5. Chimeric α7/5-HT_3 receptors transiently expressed in HEK-293 cells display nicotinic, not serotonergic pharmacology. Fura-2 loaded HEK-293 cells transiently transfected with chimeric human α7/5-HT_3 receptors were superfused with HBSS containing nicotinic or serotonergic agonists (30 s periods), as indicated. 5-hydroxyindole was co-applied with agonists, whilst MLA was pre-incubated for 5 min before co-application with agonist. Results are the average of 5–15 cells (mean values±S.E.M.) from a single experiment, representative of three similar experiments.

Table 1
Pharmacological profile of the human α7/5-HT_3 chimera stably expressed in HEK-293 cells

Agonist	EC_{50}μM	E_{max}	Antagonist	IC_{50}
Acetylcholine	32.6±1.9	89±2%	MLA	36±13.2 nM
Nicotine	19.1±7.3	94±27%	α-bungarotoxin	27.0±5.9 nM
Choline	208.5±17.2	100±3%	Mecamylamine	>100 μM
Epibatidine	0.18±0.02	101±4%	Dihydro-β-erithroidine	>100 μM
Cytisine	6.0±1.4	95±6%	D-tubocurarine	>100 μM
DMPP	0.86±0.16	106±6%	α-conotoxin MII	No block @ 1μM

Fluo-3-AM loaded cells stably expressing human α7/5-HT_3 chimeric receptors were challenged with nicotinic agonists or antagonists. For antagonist experiments cells were incubated with antagonist for 20 min, before addition of 1 μM epibatidine. Cell population Ca^{2+} measurements were recorded using a FLIPR. Each value represents the mean±S.E.M. from n=4–22 independent experiments.

reversibly blocked by a 5 min pre-incubation with 10 nM MLA, a selective α7 antagonist (Fig. 5B).

3.3. Pharmacological profile of the human α7/5-HT_3 chimera stably expressed in HEK-293 cells

As studies from *Xenopus* oocytes and transiently transfected HEK-293 cells confirmed both the function and basic nicotinic pharmacology of the hα7/5-HT_3 chimera, a stable HEK cell line expressing this construct was generated (HEKα7/5-HT_3). HEKα7/5-HT_3 cells displayed prominent cell surface fluorescent α-bungarotoxin binding, which was visible in almost all cells in the population when compared under fluorescent and bright-field illumination (data not shown). Fluo-3 loaded HEKα7/5-HT_3 cells responded to nicotinic agonists with large functional population Ca^{2+} responses, which could be monitored using a FLIPR. The level of α-bungarotoxin binding and size of Ca^{2+} responses in HEKα7/5-HT_3 cells analysed after incubation of cells (for up to 7 days) at 29 °C versus 37 °C was not altered. The amplitude of functional Ca^{2+} responses to maximal epibatidine stimulation was 4707±1040 and 6605±1339 fluorescent units at 29 and 37 °C, respectively (n=3).

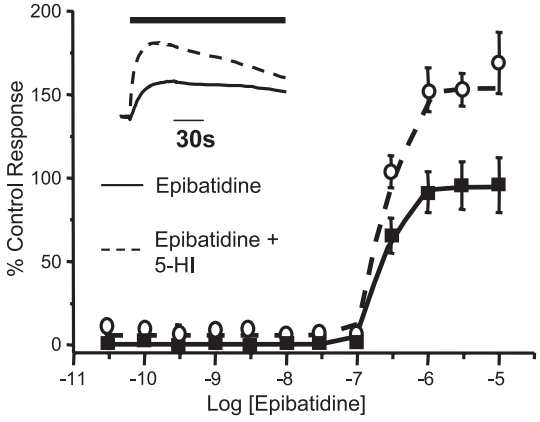

Fig. 7. Potentiation of chimeric α7/5-HT_3 receptors by 5-hydroxyindole. Fluo-3 loaded HEKα7/5-HT_3 cells were challenged with epibatidine in the presence or absence of 300 μM 5-hydroxyindole. In the presence of the potentiator, the E_{max} of the response was 158±8% of the epibatidine control. Cells were pre-incubated with 5-hydroxyindole for 5 min before agonist addition. Concentration–response curves are mean values±S.E.M. from at least three separate experiments. A typical example of the calcium response to 1μm epibatidine in the absence or presence of 5-hydroxyindole is shown as an insert.

The profile of a number of nicotinic receptor agonists (epibatidine, cytisine, nicotine, DMPP and choline) and antagonists (MLA, α-bungarotoxin, mecamylamine, dihydro-β-erthroidine and D-tubocurarine) was assessed (Fig. 6, Table 1). Agonist activity was measured in naïve cells, whilst antagonist activity was assessed after a 20 min pre-treatment of cells with the respective antagonists followed by addition of the broad nicotinic receptor agonist epibatidine (1 μM). As expected, the α7 selective agonist choline activated hα7/5-HT_3 receptors. Agonist potencies followed a rank order of epibatidine>DMPP>cytisine>nicotine>acetylcholine>choline at hα7/5-HT_3 receptors. Similar to wild-type α7 nicotinic receptors, hα7/5-HT_3 receptors displayed high sensitivity to blockade by two known α7 antagonists, α-bungarotoxin and MLA, with antagonists displaying a rank order of potency for blockade of α-bungarotoxin=MLA≫mecamylamine=dihydro-β-erthroidine>D-tubocurarine.

Modulation of agonist-induced responses by potentiators was also assessed in HEKα7/5-HT_3 cells. Potentiation of epibatidine-induced responses was seen in cells pre-incubated for 5 min with 5-hydroxyindole (Fig. 7).

4. Discussion

Functional heterologous expression of the human α7 nicotinic receptor in a mammalian cell line has proved elusive. In the current study a chimeric receptor comprising the extracellular, N-terminal domain of the human α7 subunit joined with the transmembrane and C-terminal regions of the mouse 5-HT_3 receptor subunit has been stably expressed in HEK-293 cells where it displays similar pharmacological properties to wild-type human α7 nicotinic receptors.

Analysis of HEK-293 cells transiently transfected with the hα7/5-HT_3 chimera demonstrated the presence of α-bungarotoxin binding sites that appeared to be cell-surface associated. This observation indicates that the chimera protein is being expressed and assembled into α7-like receptors that are inserted into the plasma membrane.

Functional expression studies in both *Xenopus* oocytes and HEK-293 cells showed that the hα7/5-HT_3 chimera has a similar pharmacology to a nicotinic α7 receptor. However, as has been reported previously for a chick α7/mouse 5-HT_3 chimera (Eisele et al., 1993), hα7/5-HT_3 displayed the characteristic slow desensitisation of the 5-HT_3 receptor. Selective nicotinic receptor ligands were active on the chimera, with α7 selective agonists (choline) and antagonists (MLA and α-bungarotoxin) showing potencies expected for wild-type α7 receptors. In contrast, 5-HT_3 selective ligands were inactive on the chimera. Potentiation of agonist responses at the chimeric receptor was demonstrated with a non-selective potentiator, 5-hydroxyindole (Kooyman et al., 1993; Gurley et al., 2000; Zwart et al., 2002), confirming that the chimera is sensitive to allosteric modulators. The chimera will, however, only detect α7 modulators that act on the N-terminal domain, as this is the only region of the α7 nicotinic receptor present. Interestingly, a similar chick α7/mouse 5-HT_3 chimera was recently used to localise the activity of β-amyloid to the N-terminal of α7 receptors (Lui et al., 2001).

Although the presence of α-bungarotoxin binding sites was also demonstrated in a small proportion of HEK-293 cells transfected with wild-type α7 nicotinic receptors and grown at lowered temperature, function was not detected. The facilitatory role of decreased temperature in surface expression of α7 nicotinic receptors has recently been reported in SHEP-1 cells, where it was found to increase both surface α-bungarotoxin binding and functional whole-cell current responses to nicotinic agonists (Schroeder et al., 2003). Cooper et al. (1999) have also previously described a clear effect of temperature on wild-type α4β2 nicotinic receptors, with cell surface receptor expression being dramatically increased when temperature was lowered. In the same study, the authors also reported that the expression of chimeric α4/5-HT_3 β2/5-HT_3 receptors was not upregulated by decreased temperature. The latter finding is in agreement with the present study, where the cell surface expression and function of hα7/5-HT_3 receptors was not enhanced to a detectable level by low temperature. These combined findings support a receptor-sequence specific, as well as a cell-type specific, regulation of receptor expression (Cooper and Millar, 1997: Cooper and Millar, 1998; Sweileh et al., 2000; Dineley and Patrick, 2000).

The results of the present study support previous reports that in mammalian cells α7/5-HT_3 chimeric receptors are more readily expressed than wild-type α7 nicotinic receptors. This would suggest that the transmembrane and C-terminal portions of the α7 nicotinic receptor can influence receptor assembly and function (Cooper and Millar, 1998; Rakhilin et al., 1999; Sweileh et al., 2000). Interestingly, the functional activity of the hα7/5-HT_3 receptor was much lower than that of wild-type mouse 5-HT_3 receptors after similar transient transfection in HEK cells (author's unpublished observation). Additionally, although greater than 70% of HEK cells transiently transfected with hα7/5-HT_3 displayed strong α-bungarotoxin binding at the cell surface, only 10–30% of them showed detectable function. These observations indicate that, compared with the native 5-HT_3 receptor, the hα7/5-HT_3 receptor either has a significantly reduced opening probability or is less able to assemble correctly.

Several studies have highlighted the importance of individual amino acids, amino acid motifs and complete subunit domains in the trafficking and assembly of nicotinic receptors. For example, a highly conserved arginine residue adjacent to the M1 transmembrane domain is reported to be essential for the transport of assembled α7 nicotinic receptors to the cell surface (Vicente-Agullo et al., 2001). Other reports have focused on the large M3–M4 cytoplasmic loop. A pair of adjacent basic amino acids in this region has been shown to be involved in endoplasmic reticulum sequestration of the muscle α1 subunit (Keller et al., 2001), whilst replacement of the α7 cytoplasmic loop with that of α3 or α5 causes differential localisation of the expressed receptor within cultured ciliary ganglion neurons (Williams et al., 1998; Temburni et al., 2000). In another study, various chimeras and point mutants composed of rat α7 and alternative regions of the mouse 5-HT_3 sequence to those described in the present study, have helped to identify some domains and key amino acids downstream of V201 (equivalent to V202 of the human receptor) that are required for the assembly of functional α7 receptors (Dineley and Patrick, 2000).

Other factors essential for functional expression of nicotinic receptors include post-translational modifications and chaperone proteins, which have been proposed to influence protein folding and assembly (Dineley and Patrick, 2000; Keller et al., 2001; Vicente-Agullo et al., 2001; Drisdel and Green, 2000; Drisdel and Green, 2002; Schroeder et al., 2003). A recent study on the expression of α7 and α7/5-HT_3 receptors in HEK-293 and PC12 cells (Drisdel and Green, 2002) showed that the palmitoylation state of the receptors differed in the two cell-types. In PC12 cells both α7 and chimeric chick α7/5-HT3 receptors were highly palmitoylated, whereas in HEK-293 cells, α7/5-HT3 receptors were palmitoylated but α7 receptors were not. These observations demonstrate that both cell- and receptor-specific factors are important in obtaining functional expression.

The difficulty in heterologously expressing functional nicotinic receptors in mammalian cells appears to be a common, although not exclusive, feature of subunits capable of forming homomeric channels. Whilst α8 and α9 nicotinic receptors have been shown to generate functional homomeric receptors in *Xenopus* ooctyes (Gerzanich et al., 1994; Elgoyhen et al., 1994), in mammalian cells there is only one report of functional expression of α8 nicotinic receptors (Ragozzino et al., 1997, but see Cooper and Millar, 1998) and functional expression of wild-type α9 nicotinic receptors has yet to be demonstrated. Once again a chimeric approach, using α8/5-HT_3 and α9/5-HT_3 receptor

constructs, has led to successful cell surface receptor expression in mammalian cells, although function was not reported (Cooper and Millar, 1998; Baker et al., 2004). Similar approaches have also been successful in alleviating the problems of expressing heteromeric nicotinic receptors. Chimeric α4/5-HT_3 and β2/5-HT_3 subunits have been co-expressed in order to boost the expression of α4β2 containing nicotinic receptors in mammalian cells (Cooper et al., 1999). Additionally, a chimera containing the N-terminal, extracellular region of α6 linked with the trans-membrane and C-terminal domains of the α4 nicotinic subunit has also been shown to form functional receptors when co-expressed with β4 not only in oocytes (Kuryatov et al., 2000), but also in mammalian cells (Evans et al., 2003).

In summary, although stable cell lines expressing functional wild-type α7 nicotinic receptors have been generated, their use in screening is complicated by either the expression of native nicotinic receptors (Puchacz et al., 1994; Blumenthal et al., 1997), poor exogenous α7 receptor expression levels (Sweileh et al., 2000; Schroeder et al., 2003) or the presence of other channels (Quik et al., 1997). For example, although GH4 cells have been used to study functional wild-type α7 nicotinic receptor pharmacology (Quik et al., 1997, Virginio et al., 2002; Zwart et al., 2002), the Ca^{2+} responses in these cells are small and in part made up from Ca^{2+} flux through voltage-gated Ca^{2+} channels (Quik et al., 1997). For these reasons, the HEKα7/5-HT_3 receptor cell-line described here may be more compatible with the high throughput Ca^{2+} assays commonly used to screen for novel α7 selective ligands. Thus the generation of stable cell-lines containing such chimeras may provide a general tool for the identification of novel pharmacological agents acting on receptors that in their native form are difficult to express functionally.

Acknowledgements

Ella Saunders and Annabel Martin are gratefully acknowledged for providing cell culture support. We also thank Dr. David Julius for generously supplying the mouse 5-HT_3 receptor cDNA used in this work.

References

Adler, L.E., Olincy, A., Waldo, M., Harris, J.G., Griffith, J., Stevens, K., Flach, K., Nagamoto, H., Bickford, P., Leonard, S., Freedman, R., 1998. Schizophrenia, sensory gating and nicotinic receptors. Schizophr. Bull. 24 (2), 189–202.

Aztiria, E.M., Sogayar, M.C., Barrantes, F.J., 2000. Expression of a neuronal nicotinic acetylcholine receptor in insect and mammalian host cell systems. Neurochem. Res. 25 (1), 171–180.

Baker, E.R., Zwart, R., Sher, E., Millar, N.S., 2004. Pharmacological properties of α9α10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. Mol. Pharmacol. 65 (2), 453–460.

Blumenthal, E.M., Conroy, W.G., Romano, S.J., Kassner, P.D., Berg, D., 1997. Detection of functional nicotinic receptors blocked by α-bungarotoxin on PC12 cells and dependence of their expression on post-translational events. J. Neurosci. 17 (16), 6094–6104.

Cooper, S.T., Millar, N.S., 1997. Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor α7 subunit. J. Neurochem. 68 (5), 2140–2151.

Cooper, S.T., Millar, N.S., 1998. Host cell-specific folding of the neuronal nicotinic receptor α8 ubunit. J. Neurochem. 70 (6), 2585–2593.

Cooper, S.T., Harkness, P.C., Baker, E.R., Millar, N.S., 1999. Up-regulation of cell surface α4β2 neuronal nicotinic receptors by lower temperature and expression of chimeric subunits. J. Biol. Chem. 274 (38), 27145–27152.

Corringer, P.J., Galzi, J.L., Eisele, J.L., Bertrand, S., Changeaux, J.P., Bertrand, D., 1995. Identification of a new component of the agonist binding site of the nicotinic α7 homooligermeric receptor. J. Biol. Chem. 270 (20), 11749–11752.

Court, J., Martin-Ruiz, C., Piggott, M., Spurden, D., Griffiths, M., Perry, E., 2001. Nicotinic receptor abnormalities in Alzheimer's disease. Biol. Psychiatry 49, 175–184.

Couturier, S., Bertrand, D., Mattler, J.–M., Hernandez, M.–C., Bertrand, S., Millar, N., Valera, S., Barkas, T., Ballivet, M., 1990. A neuronal nicotinic acetylcholine receptor subunit α7 is developmentally regulated and forms homo-oligomeric channels blocked by α-Btx. Neuron 5, 847–856.

Dineley, K.T., Patrick, J.W., 2000. Amino acid determinants of alpha 7 nicotinic receptor surface expression. J. Biol. Chem. 275, 13974–13985.

Drisdel, R.C., Green, W.N., 2000. Neuronal α-bungarotoxin receptors are α7 subunit homomers. J. Neurosci. 20 (1), 133–139.

Drisdel, R.C., Green, W.N., 2002. The role of palmitoylation in neuronal nicotinic acetylcholine alpha 7 subunit expression. Soc.-Abstr. Neurosci. (Abstract No. 537.6).

Eisele, J.-L., Bertrand, S., Galzi, J.-L., Devillers-Thlery, A., Changeux, J.–P., Bertrand, D., 1993. Chimaeric nicotinic–serotonergic receptor combines distinct ligand binding and channel specificities. Nature 366, 479–483.

Elgoyhen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E., Heinemann, S., 1994. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79, 705–715.

Elliot, K.J., Ellis, S.B., Berckhan, K.J., Urrutia, A., Chavez-Noriega, L.E., Johnson, E.C., Velicelebi, G., Harpold, M.M., 1996. Comparative structure of human neuronal α2–α7 and α2–α4 nicotinic acetylcholine receptor subunits and functional expression of the α2, α3, α4, α7, β2, and β4 subunits. J. Mol. Neurosci. 7, 217–228.

Evans, N.M., Bose, S., Benedetti, G., Zwart, R., Pearson, K.H., McPhie, G.I., Craig, P.J., Benton, J.P., Volsen, S.G., Sher, E., Broad, L.M., 2003. Expression and functional characterisation of a human chimeric nicotinic receptor with alpha6beta4 properties. Eur. J. Pharmacol. 466, 31–39.

Gerzanich, V., Anand, R., Lindstrom, J., 1994. Homomers of alpha8 and alpha7 subunits of nicotinic receptors exhibit similar channels but contrasting binding site properties. Mol. Pharmacol. 45, 212–220.

Gopalakrishnan, M., Buisson, B., Touma, E., Giordano, T., Campbell, J.E., Hu, I.C., Donnelly-Roberts, D., Arneric, S.P., Bertrand, D., Sullivan, J.P., 1995. Stable expression and pharmacological properties of the human α7 nicotinic acetylcholine receptor. Eur. J. Pharmacol. 290, 237–246.

Gurley, D.A., Harris, E.W., Li, C., Johnson, E.C., Lanthorn, T.H., 2000. 5-Hydroxyindole potentiates the nicotinic acetylcholine receptor alpha 7 subtype. Abstr. - Soc. Neurosci. 26 (1–2) (Abstract No. 716.15).

Karlin, A., Akabas, M.H., 1995. Towards a structural basis for the function of nicotinic acetylcholine receptors and their cousins. Neuron 15, 1231–1244.

Kassner, P.D., Berg, D.K., 1997. Differences in the fate of neuronal acetylcholine receptor protein expressed in neurons and stably transfected cells. J. Neurobiol. 33, 968–982.

Keller, S.H., Lindstrom, J., Ellisman, M., Taylor, P., 2001. Adjacent basic amino acid residues recognised by the COP I complex and ubiquitination govern endoplasmic reticulum to cell surface trafficking of the nicotinic acetylcholine receptor α-subunit. J. Biol. Chem. 276 (21), 18384–18391.

Kem, W.R., 2000. The brain alpha7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). Behav. Brain Res. 113, 169–181.

Kooyman, A.R., Van Hooft, J.A., Vijverberg, H.P.M., 1993. 5-Hydroxyindole slows desensitization of 5-HT3 receptor-mediated ion current in N1E-115 neuroblastoma cells. Br. J. Pharmacol. 108, 287–289.

Kuryatov, A., Olale, F., Cooper, J., Choi, C., Lindstrom, J., 2000. Human α6 AchR subtypes: subunit composition, assembly, and pharmacological responses. Neuropharmacology 39, 2570–2590.

Leonard, S., Gault, J., Hopkins, J., Logel, J., Vianzon, R., Short, M., Drebing, C., Berger, R., Venn, D., Sirota, P., Zerbe, G., Olincy, A., Ross, R., Adler, L.E., Freedman, R., 2002. Association of promoter variants in the α7 nicotinic acetylcholine receptor subunit gene with an inhibitory deficit found in schizophrenia. Arch. Gen. Psychiatry 59, 1085–1096.

Lindstrom, J., 2000. The structures of neuronal nicotinic receptors. In: Clementi, F., Fornasari, D., Gotti, C. (Eds.), Handbook of Experimental Pharmacology, Neuronal Nicotinic Receptors vol. 144. Springer-Verlag, New York, pp. 101–162.

Lui, Q.S., Kawai, H., Berg, D.K., 2001. β-Amyloid peptide blocks the response of α7-containing nicotinic receptors on hippocampal neurons. Proc. Natl. Acad. Sci. 98 (8), 4734–4739.

Maricq, A.V., Petersen, A.S., Brake, A.J., Myers, R.M., Julius, D., 1991. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. Science 254, 432–437.

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

Millar, N.S., 2003. Assembly and subunit diversity of nicotinic acetylcholine receptors. Biochem. Soc. Trans. 31, 869–874.

Peng, J.H., Lucero, L., Fryer, J., Herl, J., Leonard, S.S., Lukas, R.J., 1999. Inducible, heterologous expression of human α7-nicotinic acetylcholine receptors in a native nicotinic receptor-null human clonal line. Brain Res. 825, 172–179.

Puchacz, E., Buisson, B., Bertrand, D., Lukas, R.J., 1994. Functional expression of nicotinic acetylcholine receptors containing rat α7 subunits in human Sh-SY5Y neuroblastoma cells. FEBS Lett. 354, 155–159.

Quik, M., Choremis, J., Komourain, J., Lukas, R.J., Puchacz, E., 1996. Similarity between rat brain nicotinic α-bungarotoxin receptors and stably expressed α-bungarotoxin binding sites. J. Neurochem. 67, 145–154.

Quik, M., Philie, J., Choremis, J., 1997. Modulation of α7 nicotinic receptor-mediated Ca^{2+} influx by nicotinic agonists. Mol. Pharm. 51, 499–506.

Quik, M., Polonskaya, P., Gillespie, A., Jakowec, M., Lloyd, G.K., Langston, J.W., 2000. Localisation of nicotinic receptor subunit mRNAs in monkey brain by in situ hybridisation. J. Comp. Neurol. 425, 58–69.

Ragozzino, D., Fucile, S., Giovannelli, A., Grassi, F., Mileo, A.M., Ballivet, M., Alema, S., Eusebi, F., 1997. Functional properties of neuronal nicotinic acetylcholine receptor channels expressed in transfected human cells. Eur. J. Neurosci. 9, 480–488.

Rakhilin, S., Drisdel, R.C., Sagher, D., McGehee, D.S., Vallejo, Y., Green, W.N., 1999. α-Bungarotoxin receptors contain α7 subunits in two different disulphide-bonded conformations. J. Cell Biol. 146 (1), 203–217.

Rangwala, F., Drisdel, R.C., Rakhilin, S., Ko, E., Atluri, P., Harkins, A.B., Fox, A.P., Salman, S.B., Green, W.N, 1997. Neuronal α-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors. J. Neurosci. 17 (21), 8201–8212.

Schroeder, K.S., Neagle, B.D., 1996. FLIPR: A new instrument for accurate, high throughout optical screening. J. Biomol. Screen. 1, 75–80.

Schroeder, K.M., Wu, J., Zhao, L., Lukas, R.J., 2003. Regulation by cycloheximide and lowered temperature of cell-surface α7-nicotinic acetylcholine receptor expression on transfected SH-EP1 cells. J. Neurochem. 85, 581–591.

Seguela, P., Wadiche, J., Dinley-Miller, K., Dani, J.A., Patrick, J.W., 1993. Molecular cloning, functional properties, and distribution of rat brain α7: a nicotinic cation channel highly permeable to Ca^{2+}. J. Neurosci. 13, 596–604.

Simosky, J.K., Stevens, K.E., Kem, W.R., Freedman, R., 2001. Intragastric DMXB-A, an α7 nicotinic agonist, improves deficient sensory inhibition in DBA/2 mice. Biol. Psychiatry 50, 493–500.

Sweileh, W., Wenberg, K., Xu, J., Forsayeth, J., Hardy, S., Loring, R.H., 2000. Multistep expression and assembly of neuronal nicotinic receptors is both host-cell and receptor-subtype dependent. Brain Res. Mol. Brain Res. 75, 293–302.

Temburni, M.K., Blitzbau, R.C., Jacob, M.H., 2000. Receptor targeting and heterogeneity at interneuronal nicotinic cholinergic synapses in vivo. J. Physiol. 252 (1), 21–29.

Vicente-Agullo, F., Rovira, J.C., Sala, S., Rodriguez-Ferrer, C., Campos-Caro, A., Criado, M., Ballesta, J.J., 2001. Multiple roles of the conserved key residue arginine 209 in neuronal nicotinic receptors. Biochemistry 40, 8300–8306.

Virginio, C., Giacometti, A., Aldegheri, L., Rimland, J.M., Terstappen, G.C., 2002. Pharmacological properties of rat α7 nicotinic receptors expressed in native and recombinant cell systems. Eur. J. Pharmacol. 445 (3), 153–161.

Williams, B.M., Temburni, M.K., Levey, M.S., Bertrand, S., Bertrand, D., Jacob, M.H., 1998. The long internal loop of the α3 subunit targets nAChRs to subdomains within individual synapses on neurons in vivo. Nat. Neurosci. 1 (7), 557–562.

Zwart, R., DeFilippi, G., Broad, L.M., McPhie, G.I., Pearson, K.H., Baldwinson, T., Sher, E., 2002. 5-Hydroxyindole potentiates human α7 nicotinic receptor-mediated responses and enhances acetylcholine-induced glutamate release in cerebellar slices. Neuropharmacology 43, 374–384.