Molecular Function of the Novel $\alpha_7\beta_2$ Nicotinic Receptor

Beatriz E. Nielsen¹, Teresa Minguez², Isabel Bermudez² and Cecilia Bouzat¹

¹Instituto de Investigaciones Bioquímicas de Bahía Blanca, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur-CONICET, Bahía Blanca 8000, Argentina.
²Department of Medical and Biological Sciences, Oxford Brookes University, Oxford OX3 0BP, United Kingdom.

Correspondence to: Dr. Cecilia Bouzat. Instituto de Investigaciones Bioquímicas de Bahía Blanca, UNS-CONICET, Bahía Blanca 8000, Argentina. Phone: 54-291-4861201, E-mail: inbouzat@criba.edu.ar

ORCID
Cecilia Bouzat. 0000-0003-0388-6129
Beatriz E. Nielsen. 0000-0001-9927-3639

Acknowledgements
This work was supported by grants from Universidad Nacional del Sur (UNS), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina and Bill and Melinda Gates Foundation (CB) and Nigel Groome and Brookes University Research Awards (TM and IB). NACHO and BOSC 23 cells were generously provided by Dr. Sine (Mayo Clinic).
Abstract

The α7 nicotinic receptor is a promising drug target for neurological and inflammatory disorders. Although it is the homomeric member of the family, a novel α7β2 heteromeric receptor has been discovered. To decipher the functional contribution of the β2 subunit, we generated heteromeric receptors with fixed stoichiometry by two different approaches comprising concatenated and unlinked subunits. Receptors containing up to three β2 subunits are functional. As the number of β2 subunits increases in the pentameric arrangement, the durations of channel openings and activation episodes increase progressively probably due to decreased desensitization. The prolonged activation episodes conform the kinetic signature of α7β2 and may have an impact on neuronal excitability. For activation of α7β2 receptors, an α7/α7 binding-site interface is required, thus indicating that the three β2 subunits are located consecutively in the pentameric arrangement. α7 positive allosteric modulators (PAMs) are emerging as novel therapeutic drugs. The presence of β2 in the pentamer affects neither type II PAM potentiation nor activation by an allosteric agonist whereas it impairs type I PAM potentiation. This first single-channel study provides fundamental basis required to decipher the role and function of the novel α7β2 receptor and opens doors to develop selective therapeutic drugs.

Key words: Cys-loop receptors, nicotinic receptor, single-channel recordings, patch-clamp.

Abbreviations:

nAChR: Nicotinic Acetylcholine Receptor.
ACh: Acetylcholine.
PAM: Positive Allosteric Modulator.
5-HI: 5-Hydroxyindole.
α7LC: α7 Low Conductance.
TM: Transmembrane Domain.
The \( \alpha_7 \) nicotinic acetylcholine receptor (nAChR) is one of the most abundant nAChRs in the brain. It is especially located in the hippocampus, thalamus, and cortex and contributes to cognition, attention, and working memory [1–3]. Decline or alterations of cholinergic signaling involving \( \alpha_7 \) have been implicated in neurological diseases, such as schizophrenia, epilepsy and Alzheimer disease [1, 4]. \( \alpha_7 \) is also expressed in non-neuronal cells where it plays a role in immunity, inflammation and neuroprotection [4, 5]. Enhancement of \( \alpha_7 \) activity is emerging as a potential therapeutic treatment for neurological, psychiatric, and inflammatory disorders [2–4, 6–9]. In particular, positive allosteric modulators (PAMs), which act only in the presence of the endogenous agonist, are emerging as novel tools to enhance \( \alpha_7 \) function [2, 3, 7, 10, 11]. Allosteric ligands have several advantages, including higher receptor subtype selectivity and conservation of the spatial and temporal pattern of the activation by the endogenous neurotransmitter [7]. \( \alpha_7 \) PAMs are classified as type I and type II based on their effects on macroscopic currents. Both types of \( \alpha_7 \) PAMs increase receptor sensitivity to agonists, current magnitudes and empirical Hill coefficients. The type I PAMs (for example 5-HI or NS-1738) do so with little or no effect on the onset and decay rates of the macroscopic responses to the agonist, while the type II PAMs (for example PNU-120596) markedly slow current decay rate and can also reactivate desensitized receptors [6, 10–13].

\( \alpha_7 \) was classically considered to be the homomeric member of the family. However, recent evidence has demonstrated the presence of heteromeric \( \alpha_7\beta_2 \) nAChR in rodent and human brain [14–17]. The physiological role of this receptor is not yet known, but it might be involved in therapeutic and pathological processes, such as anesthesia and Alzheimer’s disease [14, 15, 18, 19].

To date, the determination of functional properties of \( \alpha_7\beta_2 \) receptors has been restricted to the macroscopic level [14–18, 20, 21]. Currents from the heteromeric receptors have been recorded from neurons [14, 15, 18] and from heterologous expressing systems using unlinked subunits [17, 18, 20, 21] or concatenated \( \alpha_7 \) with one or two \( \beta_2 \) subunits [16].

Whether \( \alpha_7\beta_2 \) exhibits an altered pharmacological and functional profile compared to \( \alpha_7 \) is still unclear (Reviewed in [22]). To find a way to unequivocally establish the functional signature of \( \alpha_7\beta_2 \) we used two different approaches combined with patch clamp recording to monitor single-channel properties of receptors with defined stoichiometry. One strategy is based on the concatemeric technology, which allows control of stoichiometry and limits expression to exactly one receptor subtype [3, 23–25]. To confirm the functional equivalence of the concatemeric with the wild-type receptor [26], we also applied the electrical fingerprinting strategy with unlinked subunits [3, 27–30]. To this end, we used an \( \alpha_7 \) subunit carrying a triple mutation at the intracellular region as a reporter of subunit stoichiometry (\( \alpha_7\)LC for low conductance). Homomeric \( \alpha_7\)LC receptors are functional but single-channel openings cannot be detected due to their low amplitude. Co-expression of \( \alpha_7\)LC with \( \alpha_7 \) leads to multiple and discrete amplitude classes, each corresponding to receptors of a given stoichiometry [27–31]. In an analogous manner, we here combined \( \alpha_7\)LC with \( \beta_2 \), and inferred the possible stoichiometries of functional heteromeric receptors through the detected amplitude classes.

Our results, which include the first report of single \( \alpha_7\beta_2 \) channels, reveal the possible heteromeric arrangements, the contribution of \( \beta_2 \) subunit to channel kinetics and ion channel conductance, and the differences on PAM selectivity with \( \alpha_7 \). This information could be useful for identifying functional heteromeric receptors in native cells and for understanding their distinct roles; and opens doors for the development of specific ligands.
Materials and Methods

Drugs. Acetylcholine (ACh) and 5-Hydroxyindole (5-HI) were purchased from Sigma-Aldrich (St Louis, MO, USA). PNU-120596 (N-(5-Chloro-2,4-dimethoxyphenyl)-N’-(5-methyl-3-isoxazolyl)-urea) and 4BP-TQS (4-(4-Bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide) were obtained from Tocris Biosciences (Bristol, UK). NS-1738 (N-(5-Chloro-2-hydroxyphenyl)-N’-[2-chloro-5-(trifluoromethyl)phenyl]urea) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Site directed mutagenesis. Human $\alpha_7$ and $\beta_2$ subunits were used. Mutations were generated using the QuikChange® Site-Directed Mutagenesis kit (Agilent, UK). The low conductance form of $\alpha_7$ ($\alpha_7$LC) contained three mutations at the intracellular loop (Q428R, E432R, S436R, [29]).

Construction of pentameric concatamers. Concatenated subunit receptors were constructed as described before for $\alpha_4$$\beta_2$ receptors [23–25, 32]. Briefly, two consecutive PCR steps were used to prepare the subunits for concatenation. The first PCR step eliminated stop codons (for all constructs) and inserted the kozac sequence GCCACC immediately before the signal peptide of the first subunit. Half of the length of the linkers was added with the first PCR step upstream and downstream from the 5’ and 3’ coding regions of each subunit. The second PCR step introduced unique restriction sites upstream and downstream of the linkers to allow successive subcloning into a modified pcDNA3.1 Hygro (-) plasmid vector (Invitrogen, UK). This plasmid was also used to assemble the concatamers. To facilitate assembly and subcloning, Ascl, XbaI and AgeI restriction sites were inserted by oligonucleotide hybridization between the NheI and XhoI sites in the multiple cloning site of the plasmid. For all constructs, the enzyme restriction sites introduced were: 1st subunit Ascl/XbaI; 2nd subunit XbaI/AgeI; 3rd subunit AgeI/XhoI; 4th subunit XhoI/NotI; 5th subunit NotI/EcoRV.

The signal peptide was removed from all the subunits except in the first and the subunits were bridged by tripeptide alanine-glycine-serine linkers (AGS) of variable length to compensate differences in the length of the C-terminus of the $\alpha_7$ and $\beta_2$ subunits. The number of the AGS repeats was 10 for $\alpha_7$-$\alpha_7$, 9 for $\alpha_7$-$\beta_2$, and 8 for $\beta_2$-$\alpha_7$. The total number of residues from the C-terminal domain to the N-terminal domain of the following subunit was 44 ($\alpha_7$-$\alpha_7$), 41 ($\alpha_7$-$\beta_2$), and 48 ($\beta_2$-$\alpha_7$). Following assembly, pentameric concatamers were subcloned into the vector pCI (Promega, UK). The presence of a pentameric concatenated construct was verified by enzymatic digestion (EcoRV, XhoI) taking advantage of the restriction sites between subunits followed by electrophoresis in agarose gel (0.8%) to detect the fragments with specific lengths. Concatemeric receptors were first tested by functional assays in oocytes.

To engineer $\alpha_7$$\beta_2$ nAChR containing $\beta_2$ L9’T subunits, L9’T mutation was first introduced into the desired $\beta_2$ subunit subcloned into the modified pCI vector. The mutated subunit was then ligated into the concatemer using unique restriction enzyme sites. To confirm that the mutated subunit was incorporated into the concatemer, the subunit was cut from the concatemer using unique restriction enzyme sites and then its nucleotide sequence was verified by DNA sequencing (SourceBioscience, UK, Eurofins, UK). All concatemeric constructs were assayed for integrity using restriction enzyme digestion.

Expression of receptors in mammalian cells. Receptors were transiently expressed in BOSC 23 cells, which are modified HEK 293T cells (Provided by Dr. Sine, Mayo Clinic, USA). The cells were tested to discard mycoplasma contamination by 4,6-Diamidino-2-phenylindole (DAPI) staining and fluorescent microscopy. Cells were transfected by calcium phosphate precipitation with subunit or concatemeric cDNAs together with Ric-3 and/or NACHO cDNAs for cell surface expression [29, 33]. GFP cDNA (5 % of total cDNA amount) was incorporated during the transfection to allow identification of transfected cells. The ratio for unlinked subunit cDNAs was $\alpha_7$:$\beta_2$ 1:8 or 1:10, and the ratio for nAChR subunit and chaperone (Ric-3 or NACHO) cDNA was 1:4. For concatemers, the ratio was concatemer: Ric3 1:4 or concatemer: Ric3: NACHO 1:1:1. We did not observe significant differences in the expression among these conditions. All transfections were carried out for about 8-12 hours in DMEM with 10% FBS and were terminated by exchanging the medium. Cells were used...
for single-channel recordings 2 to 3 days after transfection at which the maximum expression levels are usually achieved [29–31, 33–36]

**Single-channel recordings.** Single-channel recordings were obtained in the cell-attached patch configuration [33]. Each patch corresponds to a different cell (n indicates the number of independent experiments). For each condition (different receptors or drugs), at least three different cell transfections from different days were used for the recordings. The bath and pipette solutions contained 142 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl₂, 1.7 mM MgCl₂ and 10 mM HEPES (pH 7.4). For potentiation, 2 mM 5-HI, 10 µM NS-1738 or 1 µM PNU-120596, were added to the pipette solution with ACh. Thus, single channels were recorded in the continuous presence of the drugs. Typical recordings lasted between 5 and 10 minutes. The final concentration of DMSO used to solubilize PAMs (PNU-120596 and NS-1738) was lower than 0.1% (v/v). This DMSO concentration does not affect α7 activation properties [29, 36]. ACh and 5-HI were solubilized directly in pipette solution [29]. For recordings in the absence of calcium, the pipette solution contained 80 mM KF, 20 mM KCl, 40 mM K-Aspartate, 2 mM MgCl₂, 1 mM EGTA and 10 mM HEPES (pH 7.4) [30]. Single-channel currents were digitized at 5-10 µs intervals, low-pass filtered at a cut-off frequency of 10 kHz using an Axopatch 200B patch-clamp amplifier (Molecular Devices Corp., CA) and analyzed using the program TAC (Bruxton Corporation, Seattle, WA, USA) with the Gaussian digital filter at 9 kHz (Final cut-off frequency 6.7 kHz). In the presence of PNU-120596 or 4BP-TQS the filter was 3 kHz as described before [29, 37].

Open-time histograms were fitted by the sum of exponential functions by maximum likelihood using the program TACFit (Bruxton Corporation, Seattle, WA, USA). Bursts of channel openings were identified as a series of closely separated openings preceded and followed by closings longer than a critical duration, which was taken as the point of intersection between components as described before [29, 33]. Critical durations were defined by the intersection between the first and second briefest components in the closed-time histogram for bursts of α7, (α7)₅ and (α7)₄β₂ (~200-400 µs) and second and third closed components for bursts of (α7)₃(β2)₂ and (α7)₄(β2)₃ (~2-6 ms), in the absence of PAMs.

In presence of potentiators, the critical time was defined for α7 and different concatemeric receptors between the second and third closed components in presence of 5-HI (~2-6 ms), between the third and fourth components in presence of NS-1738 (~10-20 ms) and between the third and fourth components in presence of PNU-120596 (~60-100 ms). The longest-duration closed components were not considered for the analysis since they depend on the expression level of α7 in each cell.

**Electrical fingerprinting strategy.** To define amplitude classes from receptors generated by co-expression of high and low conductance subunits, analysis was performed by tracking events regardless of current amplitude. Amplitude histograms were then constructed, and the different amplitude classes were distinguished. Open time histograms were then constructed for a given amplitude class by selecting only openings with amplitudes of ± SD pA of that of the mean of the class [27–29].

**Expression and electrophysiology in Xenopus oocytes.** Stage V and VI Xenopus oocytes were prepared as previously described [24], and then injected with 100 ng of α7, (α7)₅ or concatemeric α7β₂ receptor cRNA. Injected oocytes were incubated until use at 18°C in Barth’s solution: 88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES, supplemented with 0.1 mg/mL streptomycin, 1000 U/mL Penicillin and 100 µg/mL amikacin (pH 7.5, with 5 M NaOH).

Oocytes were impaled by two microelectrodes filled with 3M KCl (0.5–2.0 MΩ) and voltage-clamped at −60 mV using an Oocyte Clamp OC-725C amplifier (Warner Instruments, USA) and Labscribe software (Iworx, NH, USA). All experiments were carried out at room temperature. ACh concentration–response curves were obtained by normalizing ACh-induced responses to the control responses induced by 1mM ACh in the same oocyte (a near-maximum effective concentration at α7 as well as α7β₂ receptors). An interval of 5 min was allowed between agonist applications, as this was found to be sufficient to ensure reproducible recordings. Allosteric modulators (PNU-120596 or 5-HI) were co-applied with ACh EC₂₀ (30 µM) for the receptor under...
study and the peak current responses were normalized to the responses elicited by ACh EC\textsubscript{20} alone in the same oocyte.

Concentration–response curves for ACh or allosteric modulators were fitted by a non-linear least-squares algorithm according to the equation: 

\[ I = \frac{I_{\text{max}}}{1 + \left( \frac{EC_{50}}{x} \right)^n}, \]

in which \(I_{\text{max}}\) is the maximum obtainable peak current; \(EC_{50}\) is the concentration of the agonist that elicits 50% of the maximum obtainable peak current; \(x\) is the agonist or allosteric modulator concentration and \(n\) is the slope factor.

**Statistical analysis.** Data were presented as mean ± SD, or as mean ± SEM only when indicated. Data sets that passed the Shapiro-Wilk test for normality and the Levene Median test for equal variance were analysed using two-tailed Student's \(t\)-test for pairwise comparisons or OneWay ANOVA followed by Bonferroni's post-hoc tests for multiple comparisons. All the tests were performed with SigmaPlot 12.0 (Systat Software, Inc.). Statistically significance difference was established at \(p\)-values<0.05 (\(p<0.05^*, p<0.01^{**}, p<0.001^{***}\)). Concentration-response curves were determined by nonlinear regression fits to the Hill equation using Prism 5.0 (GraphPad, San Diego, CA).
Results

\(\alpha 7\) concatemeric receptors. The \(\alpha 7\) concatemeric receptor, \((\alpha 7)_{5}\), was constructed by linking five human \(\alpha 7\) subunits (Fig. 1a), expressed in BOSC 23 cells, and examined by single-channel recording.

In the presence of 100-500 \(\mu\text{M}\) ACh, \(\alpha 7\) exhibits infrequent and single brief openings \((-0.25\text{ ms})\) flanked by long closings, or less often, several openings in quick succession, known as bursts (Fig. 2a, Table 1, [29, 33]). Discarding the lower frequency of channel openings, there are no statistically significant differences in mean open and burst durations of \((\alpha 7)_{5}\) \((n=3)\) respect to \(\alpha 7\) \((n=4)\) \((p>0.05, \text{Fig. 2a, Table 1})\). For both receptors, channels show a broad amplitude distribution due to the lack of resolution of the brief events, being the maximal amplitude of \(~10\text{ pA}\) at \(-70\text{ mV}\) (Fig. 2a, [33]).

No single-channel activity elicited by ACh was detected from non-transfected cells \((n=8)\) or from cells transfected only with Ric-3 cDNA \((n=7)\) or Ric-3: NACHO \((1:1)\) \((n=5)\).

In the presence of 100 \(\mu\text{M}\) ACh, the type II PAM PNU-120596 gives rise to significantly prolonged openings of \(~10\text{ pA}\) \((-70\text{ mV})\). Openings separated by brief closings are grouped in bursts, which in turn coalesce into long activation periods, named clusters \((-1-3\text{ s})\) (Table 1, Fig. 2b, [35, 36]). The mean duration of the slowest open component and the mean cluster duration of potentiated \((\alpha 7)_{5}\) \((n=3)\) are indistinguishable from those of wild-type \(\alpha 7\) \((n=8)\) \((p>0.05, \text{Fig. 2b, Table 1})\).

In agreement with the single-channel results, the EC\(_{50}\) values for ACh as well as for two different types of PAMs, PNU-120596 (Type II PAM) and 5-HI (Type I PAM), determined from macroscopic currents in oocytes, are identical between \(\alpha 7\) \((n=10)\) and \((\alpha 7)_{5}\) \((n=10)\) \((p>0.05, \text{Fig. 3a-c, Table 2})\).

These findings led us to conclude that the concatemeric receptor \((\alpha 7)_{5}\) has comparable pharmacological signatures, in terms of activation by its endogenous agonist and potentiation by two types of PAMs, to those of wild-type \(\alpha 7\) receptors. It is an important control that shows that concatenation of \(\alpha 7\) subunits does not affect the functional properties of the receptor, and therefore, the concatemeric receptor is a valid model of the wild-type receptor. Therefore, we next constructed concatemers combining \(\alpha 7\) and \(\beta 2\) subunits in different stoichiometries to be used as models of native \(\alpha 7\beta 2\) receptors.

Kinetic signature of \(\alpha 7\beta 2\) concatemeric receptors. To determine how the number of \(\beta 2\) subunits contributes to function, we constructed concatemeric receptors containing one, two or three \(\beta 2\) subunits (Fig. 1b-e). All concatemeric receptors are functional in oocytes, and their EC\(_{50}\) values for ACh are similar to that of \(\alpha 7\) \((p>0.05, n=6-10\) for each receptor, Fig. 3a and Table 2).

When expressed in BOSC 23 cells, ACh-elicited single-channel currents of receptors comprising one \(\beta 2\) subunit in the second position of the linear sequence \((\alpha 7)_{4}\beta 2\) show a maximal mean amplitude of \(~10\text{ pA}\), similar to that of \(\alpha 7\) (Fig. 1b, Fig. 4b). The presence of one \(\beta 2\) subunit does not change significantly open-channel lifetime \((p>0.05, n=3)\). However, the burst duration increases \(~1.5\text{-fold}\) with respect to \(\alpha 7\) \((p<0.001, n=3, \text{Table 1, Figs. 4b and 5a})\).

Changing the position of the \(\beta 2\) subunit in the linear arrangement from the second to the third place (Fig. 1c), leads to channels with identical burst and open durations as those of \((\alpha 7)_{4}\beta 2\) with \(\beta 2\) in the second position (Fig. 4b).

Concatemeric receptors with two alternate \(\beta 2\) subunits are also functional (Fig. 1d). The second \(\beta 2\) subunit leads to an additional increase in mean open and burst durations, which are \(~1.6\text{-fold}\) \((p<0.01, n=3)\) and \(~3\text{-fold}\) \((p<0.001, n=3)\), respectively, longer than those of \(\alpha 7\) (Table 1, Fig. 4c and 5a). The maximal amplitude remains constant \((-10\text{ pA})\). Another construct containing two alternate \(\beta 2\) subunits but starting with \(\beta 2\) in the linear sequence did not show functional expression in BOSC 23 cells.

A pentameric arrangement with three consecutive \(\beta 2\) subunits \((\alpha 7)_{2}(\beta 2)_{3}\) (Fig. 1e) also forms functional channels. For this arrangement, open channel lifetime and burst durations are even more prolonged than in \((\alpha 7)_{3}(\beta 2)_{2}\) receptors, and are \(2\text{-fold}\) \((p<0.001, n=5)\) and \(7\text{-fold}\) \((p<0.05, n=5)\).
openings and clusters detected in 
1738) were recorded. From the slope of the curve, we determined that each β2 subunit contributes to 0.10 ± 0.03 ms. Interestingly, the burst duration increases exponentially with the number of β2 subunits, indicating that it is more sensitive to the presence of this subunit (Fig. 5a, Inset to Fig. 5a).

The amplitude histograms constructed only for events longer than 0.3 ms to allow full amplitude resolution show no statistically significant differences (p>0.05) between α7 (9.65 ± 0.41 pA, n=4) and (α7)2(β2)3 (9.70 ± 0.21 pA, n=3) (Fig. 5b).

α7β2 concatemeric receptors carrying pore-lining mutations. The ring of hydrophobic residues in TM2 at 9’ position forms the channel gate [38]. Replacement by polar residues significantly increases open probability [20, 39]. As another means to confirm the assembly of β2 with α7 and to determine the functional contribution of the hydrophobic residues at TM2 9’, we incorporated the L9’T mutation in the β2 subunit of the concatemeric receptors and compared channel activity of the mutant heteromeric receptors with that of α7L9’T receptors. The single-channel pattern of α7L9’T shows, instead of the typical isolated brief openings of α7 wild-type receptors, long-duration bursts (~200 ms), and, occasionally, super long-duration clusters (~1 s) containing openings of ~10 ms (Fig. 6a). The activity patterns of concatemeric receptors containing wild-type α7 together with one, two or three β2 subunits carrying the L9’T mutation (Fig. 1f-h) are also strikingly different from their respective controls (Fig. 6). For receptors with one mutant β2 subunit, open channel lifetime (0.61 ± 0.06 ms, n=5) and mean burst duration (3.31 ± 1.22 ms, n=5) are longer than the corresponding control. Occasionally, even longer openings (~3-4 ms) forming clusters of ~100-200 ms are detected (Fig. 6b). Activity patterns of concatemeric receptors with two and three mutant β2 subunits exhibit a similar variable behavior but even more prolonged openings and bursts respect to their controls (Fig 6c-d). Receptors with three β2L9’T subunits show several long-duration open components (0.72 ± 0.04 ms, 2.28 ± 0.20 ms, 8.28 ± 2.00 ms, n=4). Occasionally, very long-duration clusters (1459 ± 156 ms) containing even more prolonged openings (43.90 ± 7.40 ms) are observed (Fig. 6d). Thus, we conclude that β2 is incorporated into the pentamer and that it contributes to channel gating and to the stability of the open channel.

Positive allosteric modulation of α7β2 concatemeric receptors. PAMs have been developed as selective allosteric ligands for α7 homomeric receptors. However, the presence of α7β2 heteromeric receptors led us to explore if PAMs can also act at α7β2 instead of being selective for α7. To determine the effects of α7 PAMs on α7β2 receptors, single-channel currents elicited by ACh in the presence of either type II PAMs (PNU-120596) or type I PAMs (5-HI or NS-1738) were recorded.

In the presence of 100 μM ACh and 1 μM PNU-120596 (type II PAM), the typical long openings and clusters detected in α7 are also observed for (α7)4β2, (α7)3(β2)2 and (α7)2(β2)3 (Fig. 7a, Table 1). As determined from Table 1, there are no statistically significant differences in the mean open and cluster durations with respect to those of α7 (p>0.05, Fig. 7b, Table 1). Also, macroscopic current recordings in oocytes show no differences in EC50 values for PNU-120596 between α7 and heteromeric receptors (Fig. 3b, Table 2). Thus, we conclude that PNU-120596 does not select between homomeric and heteromeric receptors. For all pentameric arrangements, the mean channel amplitude is similar to that of α7 (9.87 ± 0.20 pA, 10.21 ± 0.72 pA, and 10.20 ± 0.26 pA for receptors with one, two or three β2 subunits, respectively, p>0.05, n=4 for each condition).

For α7, single channel activity in the presence of ACh and 2 mM 5-HI (Type I PAM) appears in bursts composed of successive openings of prolonged duration [29, 36] (Table 1). For the heteromeric receptors, the increase in open and burst durations due to the presence of 5-HI decreases with the number of β2 subunits (Table 1 and Fig. 8a-b). Although (α7)4β2 and (α7)3(β2)2 have longer durations than α7 in the absence of 5-HI, these durations are significantly briefer than those of α7 in the presence of 5-HI (n=4 for each receptor, p<0.01 for (α7)3(β2)2 and
decreased potentiation as a function of the number of current-voltage relationships. We found no differences in the conductance between receptors. We took advantage of PNU-120596, which by leading to frequent and prolonged potentiated episodes of similar durations between both receptor types.

show higher selectivity for durations in the presence of 5-HI to its respective control value in the absence of 5-HI reveals conductance values are: 155.2 ± 7.0 pS for \((\alpha 7)2(\beta 2)3\) (p<0.001), is significantly lower than that of \(\alpha 7\) (n=4) (Fig. 8c-d, Table 1). Thus, 5-HI and NS-1738 show higher selectivity for \(\alpha 7\) than for heteromeric receptors, whereas PNU-120596 leads to potentiated episodes of similar durations between both receptor types.

In the presence of all PAMs, channel amplitude remains constant among all heteromeric receptors. We took advantage of PNU-120596, which by leading to frequent and prolonged openings allows accurate measurement of single-channel amplitude, to determine the conductance from current-voltage relationships. We found no differences in the conductance between \(\alpha 7\) and \((\alpha 7)2(\beta 2)3\) in the presence or absence of calcium (n=3 for each receptor and condition). The mean conductance values are: 155.2 ± 7.0 pS for \(\alpha 7\) and 159.8 ± 4.4 pS for \((\alpha 7)2(\beta 2)3\) in 1.8 mM Ca\(^{2+}\), and 175.2 ± 5.8 pS for \(\alpha 7\) and 184.0 ± 10.7 pS for \((\alpha 7)2(\beta 2)3\) in the absence of Ca\(^{2+}\) (Fig. 7c).

**Activation of \((\alpha 7)2(\beta 2)2\) by an \(\alpha 7\) allosteric agonist.** \(\alpha 7\) is activated by the allosteric agonist 4BP-TQS probably through an intrasubunit transmembrane cavity [40, 41]. In \(\alpha 7\), this agonist elicits prolonged openings (55.4 ± 40 ms, n=4), which are grouped in very long duration clusters (1834 ± 970 ms, n=4) (Fig. 9). We found that \((\alpha 7)2(\beta 2)2\) receptors can be also activated by 4BP-TQS. The activation pattern as well as mean open (71.7 ± 38 ms, n=6) and cluster durations (1396 ± 539 ms, n=6) are similar to those of \(\alpha 7\) (p>0.05, Fig. 9).

**Co-assembly of unlinked \(\alpha 7\) and \(\beta 2\) subunits.** To confirm that co-assembly of \(\beta 2\) and \(\alpha 7\) subunits takes place with unlinked subunits and to establish the stoichiometry of the functional arrangements, we applied the electrical fingerprinting strategy. The strategy is based on the use of an \(\alpha 7\) subunit that contains three arginine substitutions at the intracellular TM3-TM4 loop region (\(\alpha 7\)LC). Although the receptors are functional as evidenced by macroscopic current recordings, single channels cannot be detected because the amplitude is reduced to undetectable levels [27–31] (Fig 10a). Due to the brief duration of \(\alpha 7\) openings, the strategy has to be performed in the presence of a modulator that increases open channel lifetime to accurately measure channel amplitude [29, 42]. We here used PNU-120596 since it well potentiates all heteromeric receptors.

When \(\alpha 7\) is co-expressed with \(\alpha 7\)LC, instead of the homogenous amplitude population detected for \(\alpha 7\) alone, different amplitude populations can be well distinguished from the histograms (Fig. 10a). Our previous works show that the different populations report the number of low conductance subunits in each pentameric arrangement [27–29]. Thus, populations of ~2, 4, 6, 8 and 10 pA channels correspond to arrangements containing four, three, two, one and zero \(\alpha 7\)LC subunits, respectively [29–31].

We first used this strategy as an important control of \((\alpha 7)5\). When we co-expressed \(\alpha 7\)LC with \((\alpha 7)5\), only one amplitude population corresponding to that of wild-type \(\alpha 7\) was detected (~10 pA), in contrast to the results with \(\alpha 7\)LC and unlinked \(\alpha 7\) (n=4). This result confirms that the \(\alpha 7\) concatemeric receptor remains intact and does not yield \(\alpha 7\) individual subunits as degradation products (Fig 10a).

Although single-channel openings in the presence of ACh and 1 μM PNU-120596 are not detected from cells transfected with \(\alpha 7\)LC cDNA alone, they are detected when the \(\beta 2\) subunit cDNA is added during transfection (1:8-1:10 subunit ratio). This result indicates that \(\beta 2\) assembles with \(\alpha 7\)LC. The frequency of the active patches is significantly lower, indicating that the functional expression of heteromeric receptors is lower than that of homomeric receptors. Instead of the five amplitude classes detected for \(\alpha 7\) and \(\alpha 7\)LC, only the three of lower amplitude are detected for \(\alpha 7\)LC and \(\beta 2\) (Fig. 10a).
Our concatemeric receptors show that β2 subunits do not affect channel amplitude. β2 does not contain the arginine residues at the portal region shown to govern the low conductance of 5-HT₃A and α7LC receptors, instead it contains negatively charged residues as α7 (Fig. 10b). Thus, we infer that the contribution of β2 to α7β2 channel conductance is mainly governed by these portal residues and that each β2 subunit contributes approximately equally (~2 pA) to the single-channel conductance (Fig 10c). In consequence, similarly to the α7:α7LC results (this work and [29]), amplitude classes of ~2, 4 and 6 pA would correspond to receptors containing one, two and three β2 subunits, respectively. In line with this, we do not detect the ~8 and 10 pA classes, which would correspond to receptors containing four and five β2 subunits, respectively (Fig. 10c).

We conclude that wild-type α7 can assemble with β2 into pentameric arrangements containing one, two or three β2 subunits. As shown for the concatemeric receptors, the open and cluster durations of the amplitude classes in the presence of PNU-120596 are not statistically significantly different from those of α7 (p>0.05, Table 3).

To determine if the β2 subunit contributes to the complementary face of the binding site, we co-expressed β2 with an α7LC subunit containing the W55T mutation at Loop D of the complementary face that completely inactivates ACh activation in α7 receptors [27], and recorded single channels in the presence of ACh and 1 μM PNU-120596. The hypothesis is that if activation takes place through the α7/β2 interface, where β2 provides the complementary face of the ACh binding site, single-channel activity should be restored in α7LCW55T/β2 receptors. In a total of 19 patches from three different cell transfections no channels were detected (Fig. 10d). This result indicates that the α7 complementary face is required for activation and, consequently, that activation of α7β2 occurs mainly through the α7/α7 binding-site interface (Fig. 10d).
Discussion

The discovery of the novel α7β2 receptor inevitably led to key questions regarding its distinct role and location. To find the answers, it is first required to establish the molecular functional differences between α7 and α7β2 receptors that will help distinguish each functional receptor in native cells. However, functional studies using macroscopic current recordings have shown that the impact of the β2 subunit on the pharmacology of the α7β2 receptors, compared to that of α7 homomeric receptors, appears not sufficient to distinguish unequivocally the two receptor subtypes [16, 17, 21, 22]. The fact that α7β2 can assemble into different pentameric arrangements introduces additional complexity. Therefore, there is an urgent need to decipher the features of activation and potentiation contributed by the β2 subunit to the different α7-containing pentameric arrangements.

We here report the first single-channel study of heteromeric α7β2 receptors. By combining single-channel recordings with two different approaches -concatemeric receptor technology and electrical fingerprinting strategy- we reveal the stoichiometry of receptors and the kinetic signature of each pentameric arrangement.

Concatenation of subunits is used as a strategy to express channels with fixed stoichiometry [26]. However, the resulting information should be verified with unlinked subunits to discard that the concatenation allows assembly of subunits that cannot occur in native systems. On the other hand, our established electrical fingerprinting strategy for α7 [27–29, 31], which uses unlinked subunits, can only be applied in the presence of a potentiator that by increasing open duration allows accurate measurement of channel amplitude [29]. Thus, each strategy has proven significant by itself while their combination has provided a complete picture of α7β2 activation and modulation.

We show that α7 co-assembles with one, two or three β2 subunits and that the α7/α7 interface is required for activation [20]. This scenario clearly differs from that in which β2 is combined with α4, where only two receptor stoichiometries, comprising two or three β2 subunits, are functional [43, 44]. It also differs by the fact that in both α4β2 receptor stoichiometries each β2 subunit contributes to the complementary face of an agonist binding site and that the additional α4/α4 binding site present in the (α4)3β22 receptor cannot per se drive efficacious activation [24]. Thus, among β2-containing heteromeric receptors, the presence of the α7 subunit provides a unique functional behavior. This discovery opens doors to explore why α7β2 binding site interfaces cannot mediate efficacious activation. The conclusion that only one α7/α7 interface is enough to activate (α7)2β2 receptors is in line with our previous findings showing that only one functional ACh binding site is sufficient for α7 activation [29]. It also agrees with previous results from macroscopic current recordings of α7β2 receptors formed by unlinked subunits [20]. Moreover, the fact that the 8 pA-amplitude class is not detected in recordings from cells co-expressing α7LC and β2 indicates that receptors with four β2 subunits, which should contain one α7β2 binding site interface, are not functional.

When compared to α7, heteromeric α7β2 receptors show increased open and burst durations. The open duration increases linearly with the number of β2 subunits whereas the burst duration is more sensitive since it shows an exponential increase. Increased burst duration is probably a consequence of decreased desensitization, indicating that the kinetics of activation and desensitization differs between heteromeric and α7 receptors. This result explains previous observations from macroscopic current recordings showing reduced decay rates for α7β2 [16, 17, 21]. It also explains the variable results regarding its pharmacological properties because in a mixed population of homomeric and different heteromeric arrangements the changes may be too subtle to be resolved at the macroscopic level [22]. The prolonged bursts, never detected in α7, can be used as the signature of the presence of α7β2 receptors. Prolonged activation and reduced desensitization may have an important impact on calcium-dependent intracellular signaling and neuronal excitability.

The two different arrangements of α4β2 receptors - (α4)3β2 and (α4)2β2 - show different single-channel amplitude [25]. Unexpectedly, we found that all α7β2 receptors show similar amplitudes to that of α7 and the conductance of (α7)3β2 is in the presence of PNU-120596 is not statistically different to that of α7. As described before for α7 [2, 33, 35, 36], α7β2 receptors show a homogenous amplitude population in the presence of PAMs whose mean amplitude is the same as
that of the longest-duration openings in the absence of PAMs (which can be fully resolved). Therefore, we can infer that \( \beta_2 \) contributes mainly to channel kinetics. The determinants governing the different amplitudes of \( \alpha 4\beta_2 \) arrangements have not been determined to date. However, for \( \alpha 7 \)-containing receptors our electrical fingerprinting strategy shows that portal residues at the intracellular TM3-TM4 domain [45], previously reported as responsible for the low conductance of 5-HT3A [46, 47], are the main determinants of channel amplitude. When combined with \( \alpha 7 \), the contribution of \( \beta_2 \) to channel amplitude is mainly governed by these portal residues, and each \( \beta_2 \) subunit contributes approximately equally and similarly as \( \alpha 7 \) to the single-channel amplitude of \( \alpha 7\beta_2 \) receptors. It is important to note that although the single channel conductance in high K\(^+\) solution is similar between \( \alpha 7 \) and \((\alpha 7)_2(\beta_2)_3\), our results cannot discard differences in calcium selectivity that due to technical reasons could not be determined.

\( \alpha 7 \) PAMs are emerging as novel therapeutic drugs for neurological and inflammatory disorders. They are promising drugs because they maintain the temporal and spatial characteristics of endogenous activation, are more selective than agonists, and reduce tolerance due to desensitization [2, 7]. PAMs have been classified based on their macroscopic effects on \( \alpha 7 \), and the determination of their selectivity has been performed under the premise that \( \alpha 7 \) is a homomeric receptor. It is therefore required to establish if they also act at \( \alpha 7\beta_2 \). We here show that PNU-120596, the prototype type II PAM, which has been typically considered as highly selective for \( \alpha 7 \) [48], cannot select between \( \alpha 7 \) and \( \alpha 7\beta_2 \) receptors. Potentiation of \( \alpha 7\beta_2 \) by this PAM has been suggested in previous macroscopic current studies [17]. Thus, it should be kept in mind that in \textit{in vivo} situations both receptor types will be potentiated and their kinetic differences will be probably unmasked. On the other hand, the tested type I PAMs appear to be more specific for \( \alpha 7 \) than for \( \alpha 7\beta_2 \), and their exposure will make \( \alpha 7 \) activity prevail over that of \( \alpha 7\beta_2 \). Indeed, 5-HI potentiation of \((\alpha 7)_2(\beta_2)_3\) is negligible. Thus, we propose that the characterization of novel PAMs should include their actions at \( \alpha 7\beta_2 \).

The most plausible explanation for the different actions of PAMs is that they interact at different sites which, in turn, are differently conserved between \( \alpha 7 \) and \( \beta_2 \). To date there is no structural evidence unequivocally showing the PAM binding site(s) for \( \alpha 7 \). One of the key \( \alpha 7 \) residues for PNU-120596 potentiation or 4BP-TQS allosteric activation, M254, is a leucine in \( \beta_2 \), and the \( \alpha 7\)M254L mutant is insensitive to both compounds [35, 41, 49]. Thus, it could be possible that in \( \alpha 7\beta_2 \), \( \alpha 7 \) is the only subunit involved in the actions of these two compounds. NS-1738 (type I) and PNU-120596 (type II) share structural determinants for potentiation [35, 36, 49]. Since the effects of these two PAMs are different on \( \alpha 7 \) and \( \alpha 7\beta_2 \), it could be possible that the allosteric mechanism of potentiation differs between homo and heteromeric receptors or between type I and type II PAMs. Thus, our study opens doors to explore new aspects of \( \alpha 7 \) potentiation and shows that it is possible to selectively potentiate one of the two receptors. This information will help in the design of more specific ligands.

The unique fast kinetics of \( \alpha 7 \) -extremely rapid desensitization and very brief open duration-indicates that this receptor harbors a built-in filtering mechanism against excessive stimulation. Because the incorporation of \( \beta_2 \) slows receptor kinetics and reduces desensitization, it is possible that the action of the two receptors occurs at different temporal scales. Our study is focused on deciphering the kinetic differences of the ionotropic responses. However, \( \alpha 7 \) has been shown to act as a dual ionotropic/metabotropic receptor [2, 3, 50–52]. Thus, further studies would be required to determine how the metabotropic activity differs between homomeric and heteromeric \( \alpha 7 \)-containing receptors.

The identification of the kinetic signature by which \( \alpha 7\beta_2 \) can be distinguished from \( \alpha 7 \) provides tools for the elucidation of its physiological role and functional location in native tissues, which is emerging as a new field of research.
References


47. Corradi J, Gumilar F, Bouzat C (2009) Single-channel kinetic analysis for activation and


Figure legends

**Fig. 1** Schematic diagram of pentameric concatenated constructs α7 and β2 subunits are shown in blue and red, respectively. Subunits containing TM2 L9T mutation are shown in green. The linkers of AGS are represented with black lines in linear constructs and dotted lines bridging the subunits in the assembled concatemers.

**Fig. 2** Comparison between single-channel profiles of α7 and (α7)_5 Left: Typical single-channel traces from recordings in the continuous presence of 100 μM-1 mM ACh (a) or 100 μM ACh + 1 μM PNU-120596 (b). Right: Typical open and burst/cluster duration histograms are shown. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 9 kHz (a) and 3 kHz (b).

**Fig. 3** Pharmacological properties of human α7, (α7)_5 and α7β2 concatameric receptors expressed in Xenopus oocytes. Concentration dependent effects of ACh (α), PNU-120596 (b) or 5-HI (c) were fitted by the Hill equation, as described in Materials and Methods. Dose-response curves were obtained from macroscopic currents elicited by ACh at different concentrations (α), or by 30 μM ACh, which corresponds to the EC_{50} value, in the presence of different concentrations of the PAMS (b and c). Data points are mean values ± SEM of α7 (n=10), (α7)_5 (n=10), (α7)_4β_2 (n=8), (α7)_3β_2 (n=8) and (α7)_2β_3 (n=6). n corresponds to the number of independent experiments (oocytes) for each condition. EC_{50} and percentage of maximal potentiation values are shown in Table 2.

**Fig. 4** Kinetic properties of concatemeric receptors with increasing number of β2 subunits

Left: Typical traces from single-channel recordings of concatemeric receptors in the continuous presence of 1 mM ACh. Right: Representative open and burst duration histograms. Blue dotted lines indicate the mean open and burst durations for (α7)_5 showing their increase with the increase in the number of β2 subunits. Membrane potential: -70 mV. Filter: 9 kHz. Channel openings are shown as upward deflections.

**Fig. 5** Single-channel properties of α7β2 concatameric receptors (a) Plot of mean open (τ_{open}) and burst (τ_{burst}) durations as a function of the number of β2 subunits in the receptor. Data are plotted as mean ± SEM for zero β2 (n=3), one β2 (n=3), two β2 (n=3) and three β2 subunits (n=5). n corresponds to the number of independent experiments, each from different cell patches (See Table 1). Solid lines represent the fitted curves. For open, the curve was obtained by linear regression. For bursts, the exponential function parameters were obtained from the linear regression of the ln(τ_{burst}) vs number of β2 subunits (inset). (b) Amplitude histograms for α7 and (α7)_2β_2 constructed with opening events longer than 0.3 ms.

**Fig. 6** Single-channel recordings from α7β2 concatameric receptors containing β2 subunits carrying the L9′T mutation in the TM2 segment Left: Typical traces from single-channel recordings of in the continuous presence of 1 mM ACh are shown at two different time scales. For comparison, single-channel activity of α7L9′T is also shown (a). The recordings show the longest duration clusters detected. Right: representative duration histograms are shown. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 9 kHz.

**Fig. 7** Potentiation of concatemeric α7β2 receptors by PNU-120596 (a) Single-channel traces of recordings in the continuous presence of 100 μM ACh + 1 μM PNU-120596. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 3 kHz. (b) Mean open (τ_{open}) and cluster (τ_{cluster}) durations in presence of PNU-120596. Data are plotted as mean ± SD for zero β2 (n=4), one β2 (n=5), two β2 (n=5) and three β2 subunits (n=4). n corresponds to the number of independent experiments, each from different cell patches (See Table 1). τ_{open} and τ_{cluster} for β2-containing concatemers in presence of PNU-120596 compared with homomeric α7 do not show significance differences (p>0.05 by two-tailed Student’s t-test). (c) Curves showing the relationship between the single-channel current amplitude and the holding potential for α7 and (α7)_2β_3 potentiated by PNU-120596 in the absence or presence of 1.8 mM Ca^{2+} in the pipette solution. Non-statistically significant differences in amplitude at each holding potential are detected between α7 and (α7)_2β_3 for each condition (Two-tailed Student’s t-test, p>0.05, n=3 independent experiments from different cell patches for each condition).

**Fig. 8** Potentiation of concatenated α7β2 receptors by 5-HI and NS-1738 Single-channel traces of recordings in the continuous presence of 500 μM ACh and 2 mM 5-HI (a) or 10 μM NS-1738 (c).
Membrane potential: -70 mV. Filter: 9 kHz. Channel openings are shown as upward deflections. (b and d, left) Mean open ($\tau_{\text{open}}$) and burst ($\tau_{\text{burst}}$) durations in presence of PAMs. Data are plotted as mean ± SD. For 5-HI n=4 for each condition, for NS-1738 n=4 for $\alpha_7$ and n=5 for ($\alpha_7)_2(\beta_2)_3$. n corresponds to the number of independent experiments, each from different cell patches (See Table 1). Statistical significance was determined by comparing the durations of $\beta_2$-containing receptors with respect to $\alpha_7$ in the presence of PAMs, by two-tailed Student’s t-test (p<0.05*, p<0.01**, p<0.001***). (b and d, right) Mean open and burst durations in the presence of PAMs (shown in panels b and d left) were normalized to the values of their respective control receptors in the absence of PAMs. Statistical significance was determined by comparing the degree of potentiation between $\beta_2$-containing receptors respect to $\alpha_7$, by two-tailed Student’s t-test (p<0.05*, p<0.01**, p<0.001***).

**Fig. 9 Allosteric activation of concatenated $\alpha_7\beta_2$ receptors by 4BP-TQS** Left: Single-channel traces of $\alpha_7$ and ($\alpha_7)_2(\beta_2)_3$ from recordings in the continuous presence of 10 $\mu$M 4BP-TQS. Right: Typical open and burst/cluster duration histograms are shown. Channel openings are shown as upward deflections. Membrane potential: -70 mV. Filter: 3 kHz.

**Fig. 10 Electrical fingerprinting strategy** (a, left) Single-channel currents activated by 100 $\mu$M ACh + 1 $\mu$M PNU-120596 from $\alpha_7$LC, $\alpha_7$LC + $\alpha_7$, $\alpha_7$LC + ($\alpha_7)_2$ and $\alpha_7$LC + $\beta_2$. The traces for the mixed subunits are excerpts from the same recording in the continuous presence of ACh and PNU-120596. Amplitude histograms constructed with events longer than 0.3 ms are shown (right). Membrane potential: -70 mV. Filter: 3 kHz. Channel openings are shown as upward deflections. (b) Alignment of amino acid sequences of human 5HT$_3$A, $\alpha_7$LC, $\alpha_7$ and $\beta_2$ subunits highlighting the amino acids that determine single-channel conductance. (c) Plot of mean current amplitude against the number of $\beta_2$ subunits. The fitted slope by least-squares method is 1.91 ± 0.02 pA/ $\beta_2$ subunit. Data are plotted as mean ± SD of n=4 for amplitude classes of 2 pA and 4 pA, and n=5 for the amplitude class of 6 pA. n corresponds to the number of independent experiments, each from different cell patches. (d) Representative single-channel recording in the presence of 100 $\mu$M ACh + 1 $\mu$M PNU-120596 showing lack of single channel-activity from cells expressing $\alpha_7$LC carrying the W55T mutation in Loop D (complementary face (-)) and $\beta_2$ subunits. Membrane potential: -70 mV. Filter: 3 kHz. Schematic diagrams of ($\alpha_7)_2(\beta_2)_3$ concatemeric receptor showing possible binding sites at $\alpha_7/\alpha_7$ or $\alpha_7/\beta_2$ interfaces are shown. The presence of an $\alpha_7/\alpha_7$ interface allows activation (grey arrow).
Fig 1
Fig 4
ACh

a

b

(cT)β2

(cT)β2

(cT)β2

(cT)β2
Fig 5

[Graph showing duration (ms) vs. number of G subunits, with inset showing ln(C) vs. number of G subunits, and histogram of amplitudes (pA).]
Fig 6
ACh
a  α7 L9'T
b  (α7)β2 L9'T
c  (α7),(β2 L9')
d  (α7),(β2 L9')
Fig 7
ACh + PNU-120596

(a) 

(b) 

(c) 

Amplitude (pA) vs. Holding potential (mV) graph showing the relationship between the number of subunits and the amplitude of the response. The graph includes data for 

- 
- 
- 

1. 
2. 
3. 
4. 

The data points are represented by circles, with error bars indicating variability. The graph illustrates a linear relationship between the number of subunits and the amplitude, with slopes labeled for different conditions: 

- **-Ca**
- **+Ca**

The bars in (b) represent the mean and standard deviation for each condition, with the number of subunits increasing from 0 to 3. The graph in (c) shows the holding potential on the x-axis and amplitude on the y-axis, with different lines for each condition.
Fig 8

a - ACh + 5-HI

(b) Open  Burst

(c) ACh + NS-1738

(d) Open  Burst

Number of β2 subunits
Fig 10

a - ACh + PNU-120596

\[ \alpha 7 \text{LC} \]

\[ \alpha 7 \text{LC} + \alpha 7 \]

\[ \alpha 7 \text{LC} + (\alpha 7)_5 \]

\[ \alpha 7 \text{LC} + \beta 2 \]

b

- h 5-HT, A: ELSSIRQFKKDEKBEVAVDQMMLRGGV
- h \alpha 7LC: EVRYIANRFRCCDSEAVCGKFAACV
- h \alpha 7: EVRYIANRFRCCDSEAVCGKFAACV
- h \beta 2: GVRFIADHRSEDDQGAVSWNKTVVAVV

d - ACh + PNU-120596

\[ \alpha 7 \text{W55T LC} + \beta 2 \]

\[ 5 \text{ pA} \]

500 ms
Table 1. Mean open and burst or cluster durations of \(\alpha7\) and \(\alpha7\beta2\) concatemeric receptors in the absence or presence of PAMs.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist</th>
<th>PAM</th>
<th>(\tau_{\text{open}}) (ms)</th>
<th>(\tau_{\text{burst}}) or (\tau_{\text{cluster}}) (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha7)</td>
<td>100 (\mu)M-1 mM ACh</td>
<td>-</td>
<td>0.25 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>(\alpha7)</td>
<td>100 (\mu)M ACh</td>
<td>1 (\mu)M PNU-120596</td>
<td>221 ± 72***</td>
<td>1988 ± 718***</td>
<td>8</td>
</tr>
<tr>
<td>(\alpha7)</td>
<td>500 (\mu)M ACh</td>
<td>2 mM 5-HI</td>
<td>2.26 ± 0.33***</td>
<td>5.20 ± 0.28***</td>
<td>4</td>
</tr>
<tr>
<td>(\alpha7)</td>
<td>500 (\mu)M ACh</td>
<td>10 (\mu)M NS-1738</td>
<td>2.91 ± 0.66*</td>
<td>18.42 ± 3.00***</td>
<td>4</td>
</tr>
<tr>
<td>((\alpha7)_{5})</td>
<td>100 (\mu)M-1 mM ACh</td>
<td>-</td>
<td>0.24 ± 0.02</td>
<td>0.33 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>((\alpha7)_{5})</td>
<td>100 (\mu)M ACh</td>
<td>1 (\mu)M PNU-120596</td>
<td>205 ± 44***</td>
<td>1867 ± 937***</td>
<td>3</td>
</tr>
<tr>
<td>((\alpha7)_{4}\beta2)</td>
<td>100 (\mu)M-1 mM ACh</td>
<td>-</td>
<td>0.22 ± 0.10</td>
<td>0.49 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>((\alpha7)_{4}\beta2)</td>
<td>100 (\mu)M ACh</td>
<td>1 (\mu)M PNU-120596</td>
<td>256 ± 79**</td>
<td>2229 ± 442***</td>
<td>5</td>
</tr>
<tr>
<td>((\alpha7)_{4}\beta2)</td>
<td>500 (\mu)M ACh</td>
<td>2 mM 5-HI</td>
<td>1.64 ± 0.64**</td>
<td>6.24 ± 0.86***</td>
<td>4</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{2})</td>
<td>100 (\mu)M-1 mM ACh</td>
<td>-</td>
<td>0.41 ± 0.03</td>
<td>0.98 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{2})</td>
<td>100 (\mu)M ACh</td>
<td>1 (\mu)M PNU-120596</td>
<td>234 ± 57***</td>
<td>2382 ± 540***</td>
<td>5</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{2})</td>
<td>500 (\mu)M ACh</td>
<td>2 mM 5-HI</td>
<td>0.93 ± 0.14**</td>
<td>4.14 ± 0.10***</td>
<td>4</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{3})</td>
<td>100 (\mu)M-1 mM ACh</td>
<td>-</td>
<td>0.52 ± 0.06</td>
<td>2.42 ± 0.79</td>
<td>5</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{3})</td>
<td>100 (\mu)M ACh</td>
<td>1 (\mu)M PNU-120596</td>
<td>178 ± 45***</td>
<td>2081 ± 587***</td>
<td>4</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{3})</td>
<td>500 (\mu)M ACh</td>
<td>2 mM 5-HI</td>
<td>0.74 ± 0.01***</td>
<td>2.54 ± 0.20</td>
<td>4</td>
</tr>
<tr>
<td>((\alpha7)(\beta2)_{3})</td>
<td>500 (\mu)M ACh</td>
<td>10 (\mu)M NS-1738</td>
<td>2.28 ± 0.57***</td>
<td>10.46 ± 3.22***</td>
<td>5</td>
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</tbody>
</table>

\(\tau_{\text{open}}\) and \(\tau_{\text{burst}}\) or \(\tau_{\text{cluster}}\) correspond to the slowest open components of the corresponding histograms. Single-channel currents were recorded in the continuous presence of agonist alone or combined with PAM. Values are mean ± SD. n: number of independent experiments, each from different cell patches. Statistical significance was determined by comparing the mean value in the presence of the PAM respect to the absence of the PAM by two-tailed Student's t-test (p<0.05*, p<0.01**, p<0.001***).
Table 2. Concentration effects of ACh, PNU-120596 and 5-HI on α7, (α7)5 and α7β2 concatemeric receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ACh</th>
<th>PNU-120596</th>
<th>5-HI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>% Potentiation</td>
</tr>
<tr>
<td>α7</td>
<td>108 ± 5</td>
<td>1.5±0.2</td>
<td>3513± 33</td>
</tr>
<tr>
<td>(α7)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>93 ± 5</td>
<td>1.5±0.4</td>
<td>3492 ± 85</td>
</tr>
<tr>
<td>(α7)&lt;sub&gt;4&lt;/sub&gt;β2</td>
<td>95 ± 4</td>
<td>1.6±0.2</td>
<td>3338 ± 76</td>
</tr>
<tr>
<td>(α7)&lt;sub&gt;3&lt;/sub&gt;(β2)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>97 ± 6</td>
<td>1.4±0.3</td>
<td>3300 ± 50</td>
</tr>
<tr>
<td>(α7)&lt;sub&gt;2&lt;/sub&gt;(β2)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>104 ± 12</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The concentration-dependent effects of PNU-120569 or 5-HI were determined on ACh responses elicited by EC<sub>20</sub> ACh (30 µM) in the same oocyte. The data points were used to generate concentration response curves from which EC<sub>50</sub> was estimated, as described in Materials and Methods. The percentage of maximal potentiation was calculated as \((I_{ACh \ EC_{20}}+PAM) / I_{ACh \ EC_{20}}\). Values represent the mean ± SEM. Statistical analysis on ACh and PAMs was conducted using OneWay ANOVA followed by Bonferroni’s post-hoc tests for multiple comparisons (p<0.05*, p<0.01**, p<0.001***, ND: not determined). N is the number of batches of oocytes used for experiments and n corresponds to the number of independent experiments carried out.
Table 3. Mean open and cluster duration of channels corresponding to each amplitude class detected from cells expressing α7LC and β2 in the continuous presence of 100 μM ACh + 1 μM PNU-120596.

<table>
<thead>
<tr>
<th>Amplitude classes (pA)</th>
<th>$\tau_{\text{open}}$ (ms)</th>
<th>$\tau_{\text{cluster}}$ (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.15 ± 0.26</td>
<td>108 ± 27</td>
<td>1451 ± 1322</td>
<td>4</td>
</tr>
<tr>
<td>4.10 ± 0.32</td>
<td>142 ± 55</td>
<td>1907 ± 752</td>
<td>4</td>
</tr>
<tr>
<td>5.97 ± 0.26</td>
<td>179 ± 55</td>
<td>2897 ± 743</td>
<td>5</td>
</tr>
</tbody>
</table>

$\tau_{\text{open}}$ and $\tau_{\text{cluster}}$ correspond to the slowest component of the open and burst or cluster duration histograms, respectively. Values are mean ± SD. n corresponds to the number of independent experiments, each from different cell patches. There are not statistically significant differences in $\tau_{\text{open}}$ and $\tau_{\text{cluster}}$ between the distinct amplitude classes and respect to α7, p>0.05 by two-tailed Student’s t-test.