

## REVIEW ARTICLE

Nicotinic acetylcholine receptors at the  
single-channel level

**Correspondence** Cecilia Bouzat, Instituto de Investigaciones Bioquímicas de Bahía Blanca, INIBIBB (CONICET-UNS), Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina. E-mail: inbouzat@criba.edu.ar

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Cecilia Bouzat<sup>1</sup> and Steven M Sine<sup>2,3,4</sup>

<sup>1</sup>Instituto de Investigaciones Bioquímicas de Bahía Blanca, INIBIBB (CONICET-UNS), Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina, <sup>2</sup>Receptor Biology Laboratory, Department of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, MN 55905, USA, <sup>3</sup>Department of Neurology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA, and <sup>4</sup>Department of Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Over the past four decades, the patch clamp technique and nicotinic ACh (nACh) receptors have established an enduring partnership. Like all good partnerships, each partner has proven significant in its own right, while their union has spurred innumerable advances in life science research. A member and prototype of the superfamily of pentameric ligand-gated ion channels, the nACh receptor is a chemo-electric transducer, binding ACh released from nerves and rapidly opening its channel to cation flow to elicit cellular excitation. A subject of a Nobel Prize in Physiology or Medicine, the patch clamp technique provides unprecedented resolution of currents through single ion channels in their native cellular environments. Here, focusing on muscle and  $\alpha 7$  nACh receptors, we describe the extraordinary contribution of the patch clamp technique towards understanding how they activate in response to neurotransmitter, how subtle structural and mechanistic differences among nACh receptor subtypes translate into significant physiological differences, and how nACh receptors are being exploited as therapeutic drug targets.

**LINKED ARTICLES**

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**Abbreviations**

4BP-TQS, 4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide; 5-HI, 5-hydroxyindole; NAM, negative allosteric modulator; NS-1738, N-(5-chloro-2-hydroxyphenyl)-N'-[2-chloro-5-(trifluoromethyl)phenyl] urea; PAM, positive allosteric modulator; PNU-120596, N-(5-chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea; TMD, transmembrane domain

## Tables of Links

| TARGETS                          |
|----------------------------------|
| <b>Ligand-gated ion channels</b> |
| Nicotinic acetylcholine receptor |

| LIGANDS         |         |
|-----------------|---------|
| ACh             | NS-1738 |
| 5-hydroxyindole | 4BP-TQS |
| PNU-120596      |         |

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

## Introduction

Several years after the patch clamp technique was introduced, it became clear that it was more useful than anyone had expected (Sigworth, 1983, 1986). Not only could currents through single ion channels be registered but also currents could be registered in a wide variety of configurations, including unitary currents from isolated membrane patches and macroscopic currents from the whole cell. The nicotinic acetylcholine (nACh) receptor from skeletal muscle holds a special place in the history of application of the patch clamp, as it was the first biological ion channel from which unitary currents were registered with sub-millisecond resolution (Colquhoun and Sakmann, 1981). Now, in the fourth decade of patch clamp application, it is equally clear that the technique has become even more useful than was expected in the early years following its introduction. Major advances that complemented the patch clamp include molecular cloning technology, which allows structural modifications anywhere in an ion channel, and heterologous expression systems, which allow study of currents through virtually any ion channel. Here, we survey advances from application of the patch clamp technique to investigate the function and drug modulation of the nACh receptor, a prototypical neurotransmitter-gated ion channel (Alexander *et al.*, 2015).

## Patch clamp technique

The patch clamp technique essentially solved two problems simultaneously: a current capturing problem and a signal to noise problem. A fire-polished glass pipette filled with electrolyte solution is pressed against a cell, and following gentle suction, a giga-ohm seal is formed between the glass and the cell membrane (Hamill *et al.*, 1981). The high-resistance seal directs virtually all current across the membrane into the patch clamp electronics. Moreover, because background noise decreases as seal resistance increases, single channel currents stand out as rectangular pulses superimposed upon relatively small background fluctuations. Although background noise inherent to the patch clamp increases with increasing frequency, with present day technology, it is possible to detect sub-pico-ampere single channel currents at bandwidths of several hundred Hz, or currents of tens of pico-amperes at several thousand kHz. In the field of electrophysiology, such signal to noise capability was unheard of prior to 1980.

The patch clamp technique also spurred advances in mathematical descriptions of single channel dwell times based on discrete state kinetic schemes (Colquhoun and Hawkes, 1981), development of software to digitize and filter the long temporal sequences of current pulses, detection of transitions between closing and opening events (Colquhoun and Sigworth, 1983; Sigworth, 1983), and analysis of dwell time sequences according to kinetic schemes (Horn and Lange, 1983). In order to fit a kinetic scheme to the data, the dwell time omission problem had to be solved to account for the system dead time resulting from a finite recording bandwidth. This was done with both exact and approximate mathematical solutions (Roux and Sauve, 1985; Crouzy and Sigworth, 1990; Hawkes *et al.*, 1990; Qin *et al.*, 1996), or with Markov simulation followed by direct imposition of the dead time (Blatz and Magleby, 1986). Given present day technology, it is practical to fit a kinetic scheme with multiple closed and open states to single channel dwell time data, and estimate transition rate constants as rapid as  $100\,000\text{ s}^{-1}$ . In the case of the muscle nACh receptor, rate constants have been estimated for agonist association and dissociation, transition between successive closed states, closed and open states, and block and unblock of the channel (Mukhtasimova *et al.*, 2016). The ability to estimate rate constants, combined with changes in nACh receptor structure, will continue to offer a means to understand relationships between structure and function.

## nACh receptor structure

Current understanding of nACh receptor structure was achieved through application of a range of technologies. The first was in biochemistry where tissues naturally rich in nACh receptors from the motor endplate were subjected to affinity purification, and the constituent subunits resolved on polyacrylamide gels (Meunier *et al.*, 1974). The four subunits,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , were shown to form a pentameric assembly, with the  $\alpha$ -subunit present in two copies (Reynolds and Karlin, 1978; Lindstrom *et al.*, 1979). The second was in amino acid sequencing technology, which yielded the amino terminal partial sequences of isolated subunits (Raftery *et al.*, 1980). The third was molecular cloning where, given the amino terminal sequences, polynucleotide probes were generated to screen a cDNA library, and each subunit was ultimately cloned and sequenced (Noda *et al.*, 1983).

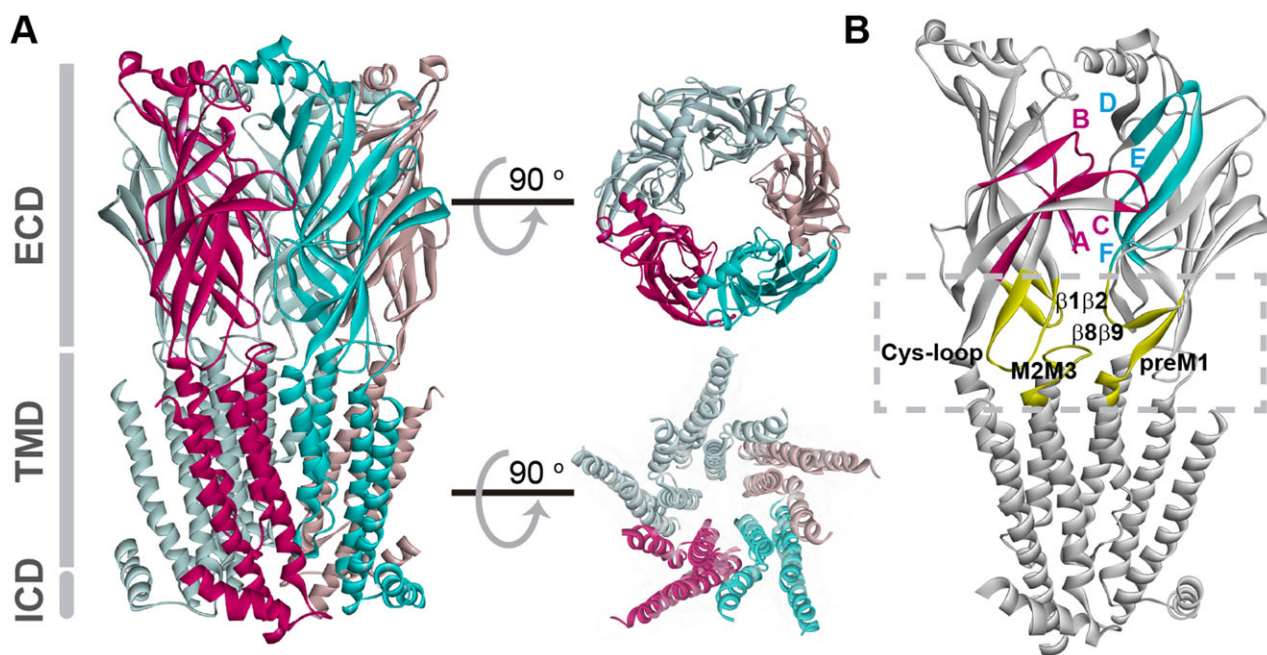
Analyses of the sequences established a receptor gene family and yielded models of how the subunit chains thread through the cell membrane. Genetic reconstitution of nACh receptor subunits in heterologous expression systems, combined with assessment of receptor function, gave ultimate proof of the composition of the nACh receptor (Methfessel *et al.*, 1986; Claudio *et al.*, 1987).

Near atomic resolution structural insight was achieved by electron microscopy applied to the *Torpedo* nACh receptor, initially yielding structures from 20 to 9 Å resolution (Toyoshima and Unwin, 1988; Mitra *et al.*, 1989; Unwin, 1993), and culminating in a 4 Å structure (Unwin, 2005). The first atomic resolution insight was achieved by the crystal structure of the ACh binding protein (AChBP), a water-soluble homo-pentamer that mirrored the nACh receptor extracellular region with some 24% sequence identity to the extracellular region of the homomeric  $\alpha 7$  nACh receptor (Brejc *et al.*, 2001). The structure confirmed previous mutagenesis and site-directed labelling studies, showing that the binding site is composed of multiple loops, each from separate sections of the primary sequence, which converge at the subunit interface. Over the subsequent decade, x-ray

crystallography yielded structures of bacterial homologues of the nACh receptor (Hilf and Dutzler, 2008, 2009; Bocquet *et al.*, 2009), then structures of homomeric eukaryotic relatives of the nACh receptor (Hibbs and Gouaux, 2011; Hassaine *et al.*, 2014; Miller and Aricescu, 2014; Du *et al.*, 2015; Haug *et al.*, 2015), and most recently, a heteropentameric neuronal nACh receptor (Morales-Perez *et al.*, 2016). The collective crystal structures depict, in vivid atomic detail, the extracellular and transmembrane domains (TMD), spatial relationships between the domains and between subunits, and residue-residue contacts within and between subunits (Figure 1).

## Activation of muscle nACh receptor

Development of kinetic schemes describing nACh receptor activation has been closely linked to advances in pharmacology. More than 50 years ago, del Castillo and Katz (1957) applied intracellular microelectrode recording to the frog motor endplate and found that full and partial agonists produced different sized depolarizing responses when



**Figure 1**

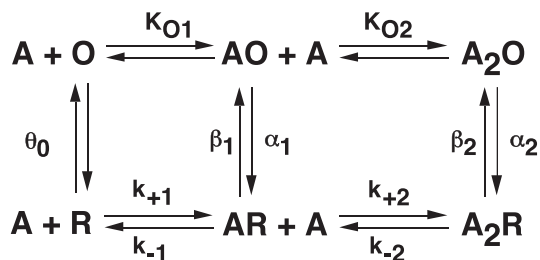
**Structural model of nACh receptor.** The structure corresponds to the  $\alpha 4\beta 2$  plus nicotine (PDB 5KXI) (Morales-Perez *et al.*, 2016). (A) View parallel to the plasma membrane with colouring to highlight  $\alpha$  (purple) and  $\beta$  subunits (cyan). Functional domains include the extracellular domain (ECD), which carries the binding sites at subunit interfaces; the TMD, which contains the ion pore and the gate and is formed by four  $\alpha$ -helices from each monomer (M1–M4); and the intracellular domain (ICD) between M3 and M4 domains, that contains determinants of channel conductance and sites for regulation. Most of the ICD is not shown since it was removed to obtain well-diffracting crystals (Morales-Perez *et al.*, 2016). Views of the ECD and the TMD perpendicular to the membrane are shown on the right, upper and lower parts respectively. The TMD contains intra-subunit and inter-subunit transmembrane cavities involved in allosteric modulation. (B) Adjacent subunits showing the loops contributing to the binding site and the coupling region (squared). Each nACh receptor ECD monomer consists of an N-terminal  $\alpha$ -helix and a core of ten  $\beta$ -strands that form a  $\beta$ -sandwich structure. Each agonist-binding site is found at an interface between two adjacent subunits. The principal face, provided by the  $\alpha$  subunit, contributes three loops that span  $\beta$  strands and harbour predominantly key aromatic residues, named as Loop A ( $\beta 4\beta 5$  loop), Loop B ( $\beta 7\beta 8$  loop) and Loop C ( $\beta 9\beta 10$  loop). The adjacent subunit, which forms the complementary face, contributes three  $\beta$  strands with residues clustered in segments called Loops D–F. The interface between the ECD and TMD, named as the coupling region, is important for coupling agonist binding to channel opening as well as for determining open channel lifetime and rate of desensitization (Bouzat *et al.*, 2004; Lee and Sine, 2005; Bouzat *et al.*, 2008; Bartos *et al.*, 2009; Yan *et al.*, 2015). The main loops are shown in yellow.

applied at saturating concentrations. They reasoned that binding of the agonist (A) to the receptor (R) initially produced an inactive complex (AR), which then isomerized to an active complex (AO):



Full and partial agonists thus differed in the efficiency with which the inactive agonist-receptor complex isomerized to the active complex.

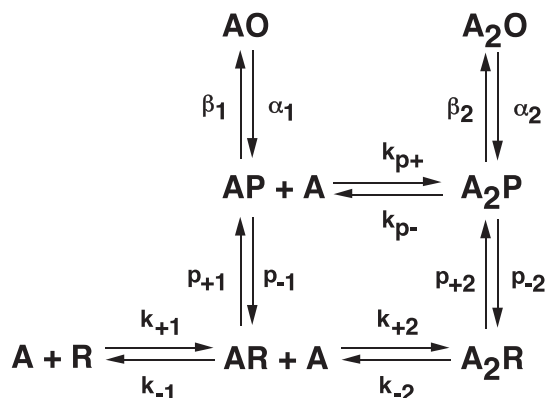
Over the succeeding years, to reconcile positive cooperativity in the dose–response relationship (Dionne *et al.*, 1978), a second agonist binding step was added ( $A_2R$ ), and to account for brief channel openings at low but not high agonist concentrations (Jackson, 1988), opening by singly occupied receptors was added. The observation that channels could open even in the absence of agonist (Jackson, 1986) completed a framework that mirrored the MWC model for an allosteric protein with two ligand binding sites (Monod *et al.*, 1965):



The subset of the MWC mechanism, shown with rate constants rather than equilibrium constants, has been fitted to sequences of single channel dwell times recorded over a range of agonist concentrations, yielding estimates for all the microscopic rate constants (Sine *et al.*, 1990; Ohno *et al.*, 1996). The agonist binding steps depended on the number of bound agonists, differing mainly in the rate constants for dissociation, which could be rationalized because the complementary subunits at the two binding sites differed ( $\gamma$  vs.  $\delta$ ). Moreover, measurements of opening and closing rate constants showed that a full agonist gated the channel efficiently, whereas a partial agonist gated inefficiently (Colquhoun and Sakmann, 1985; Marshall *et al.*, 1991; Grosman *et al.*, 2000). The origin of efficacy thus appeared to arise from a variable rate of channel opening with a nearly invariant rate of channel closing.

Once again, however, advances in pharmacology led to deeper insight into the activation mechanism of nACh receptors. Focusing on very brief single channel dwell times, it was realized that for low concentrations of several agonists, openings from a single nACh receptor channel were interrupted by closings belonging to two rather than one kinetic class, at variance with the MWC mechanism (Lape *et al.*, 2008; Mukhtasimova *et al.*, 2009; reviewed in Sine, 2012). The class with briefest mean duration was common to openings regardless of agonist efficacy, whereas the succeeding class was variable both in its mean duration and

relative weight. Thus, schemes with a closed state intermediate between the resting (R) and open channel (O) states emerged, with the transition between the resting and intermediate states, called flipped or primed (P), depending on the efficacy of the agonist:



By implementing procedures that improved temporal resolution of dwell times, a recent study obtained estimates of all the rate constants in this scheme (Mukhtasimova *et al.*, 2016; see commentary by Sivilotti and Colquhoun, 2016). The estimates were determined with sufficient accuracy to assess relationships between rate and equilibrium constants, and how they depended on the number of bound agonists. The rate and equilibrium constants for the priming steps increased with successive agonist occupancy, and priming was more efficient for a full than a partial agonist. The priming rate relative to the priming equilibrium constant increased with successive agonist occupancy, and for a full agonist, the change in log rate constant relative to the change in log equilibrium constant was greater than one, suggesting agonist occupancy affected not only the stability of the ground states but also those of the transition states. Relationships between rate and equilibrium constants and how they are expected to depend on the extent of agonist occupancy are described by Fersht *et al.* (1986) and Edelstein *et al.* (1996) respectively. By contrast, for a partial agonist, the change in log rate constant relative to the change in log equilibrium constant approached unity, suggesting agonist occupancy solely affected the ground states. Thus, priming depends on both the degree of agonist occupancy and efficacy of the agonist. The rate and equilibrium constants for the gating step also increased with successive agonist occupancy, but unlike priming, the gating step was similar for a full and a partial agonist. The gating rate constant relative to the gating equilibrium constant increased with successive occupancy, but the change in log rate constant relative to the change in log equilibrium constant was less than one, suggesting agonist occupancy solely affected the stability of the ground states. Thus, whereas channel gating depends on the extent of agonist occupancy, it does not depend on agonist efficacy. Overall, single-channel kinetic analysis has added in-depth insight into the concept of activation and partial agonism in the Cys-loop receptor family (Burzomato *et al.*, 2004; Lape *et al.*, 2008;



Mukthasimova *et al.*, 2009; Corradi and Bouzat, 2014; Indurthi *et al.*, 2016).

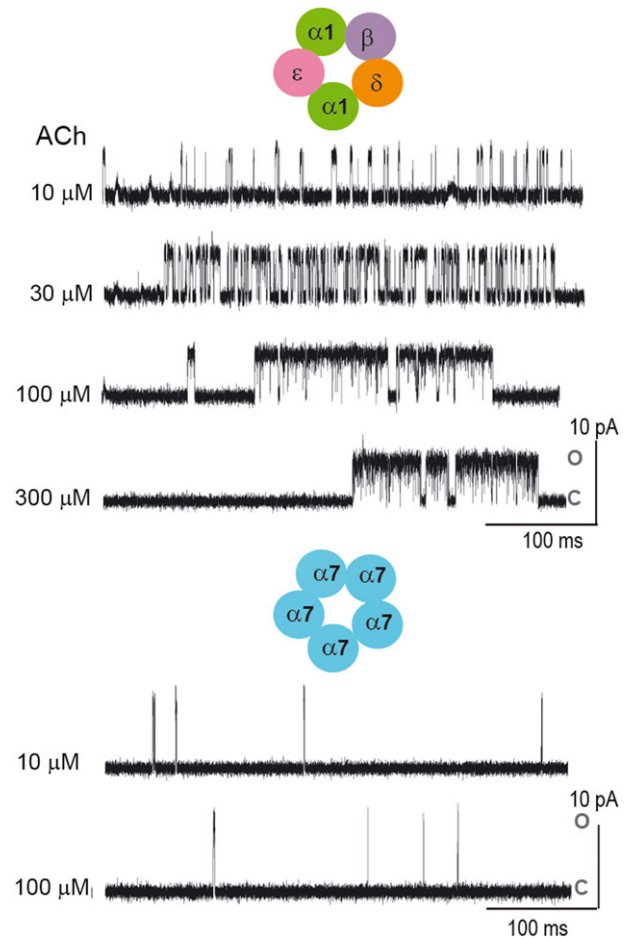
Although stable intermediates between closed and open states are not present in the MWC mechanism, the flip and primed mechanisms retains a key tenet of the MWC mechanism. In particular, the affinity of agonist for intermediate and open states is greater than affinity for the resting closed state, so that tighter binding of agonist drives entry to those states and thus promotes activation (Sine, 2012).

Before multiple closed states in advance of the open state were described, a comprehensive body of work showed that mutations of residues in the ligand binding domain affected a step that appeared to be channel opening, whereas mutations in the pore affected a step that appeared to be channel gating (Grosman *et al.*, 2000). In light of pre-open closed states, the correlation between structure and kinetics could be reconciled by a change in a residue's contribution to the priming step versus the gating step. Thus, residues in the binding domain may contribute mainly to priming, whereas those in the pore domain may contribute mainly to gating. In recent work by Auerbach and colleagues, a reaction mechanism with closed state intermediates was explicitly considered (Gupta *et al.*, 2017).

## Activation of homomeric $\alpha 7$ nACh receptors

In deciphering mechanisms of nACh receptor activation, an essential requirement is to identify sequences of channel openings and closings from the same receptor channel. In this way, desensitization has been pivotal in defining the activation mechanism of the muscle nACh receptor. When a patch of membrane containing muscle nACh receptors is exposed to high agonist concentrations, most of the time all receptors are desensitized and the current remains at baseline. On occasion, however, one receptor recovers from desensitization and then opens and closes repeatedly until it returns to the desensitized state (Sakmann *et al.*, 1980) (Figure 2). For the muscle nACh receptor, the time for desensitization onset is long compared to the time it takes to close and reopen, and trains of many openings and closings from the same receptor channel can be unambiguously identified; data from such trains enable global fitting just described for the muscle nACh receptor. By contrast, for the neuronal  $\alpha 7$  receptor, the time for desensitization onset approaches the open channel lifetime, so that desensitization determines the rate of channel closing as opposed to reversal of the channel opening step (Bouzat *et al.*, 2008) (Figure 2). As a result, trains of events from the same receptor channel are not observed, sequences of openings and closings show little dependence on the agonist concentration, and rate constants for kinetic steps that precede channel opening, such as agonist association and dissociation, cannot be estimated. Thus, whereas desensitization is a blessing for studying the kinetics of muscle nACh receptor activation, it is an obstacle to studying the kinetics of  $\alpha 7$  nACh receptor activation.

Control of open channel lifetime by desensitization may be important for inter-response latency at a synapse. Desensitized  $\alpha 7$  receptors expressed in HEK cells recover with a time constant of  $\sim 1$  s (Bouzat *et al.*, 2008). Thus, for  $\alpha 7$



**Figure 2**

**Single-channel recordings as a function of ACh concentration for muscle and  $\alpha 7$  nACh receptors.** For the muscle nACh receptor, single-channel activity appears as bursts separated from each other by relatively long silent periods in which the receptor is desensitized. Bursts are composed of successive opening events corresponding to the same individual channel. The main closed time within a burst reflects the transitions between unliganded closed and diliganded open states and becomes progressively briefer with increasing ACh concentration. In contrast, the temporal pattern of  $\alpha 7$  channel currents does not show any concentration dependence due to the fact that desensitization determines the rate of channel closing. Channel activity appears as brief ( $\sim 0.1$  ms) isolated events. Openings are shown as upward deflections. C and O correspond to closed and open states respectively. Membrane potential:  $-70$  mV. Filter: 9 kHz.

receptors, after a brief response, a latency of several seconds would be required to generate another response of full amplitude. Because the  $\alpha 7$  receptor is highly permeable to calcium, a brief open duration may avoid cell toxicity during overstimulation.

## Subunit type and stoichiometry

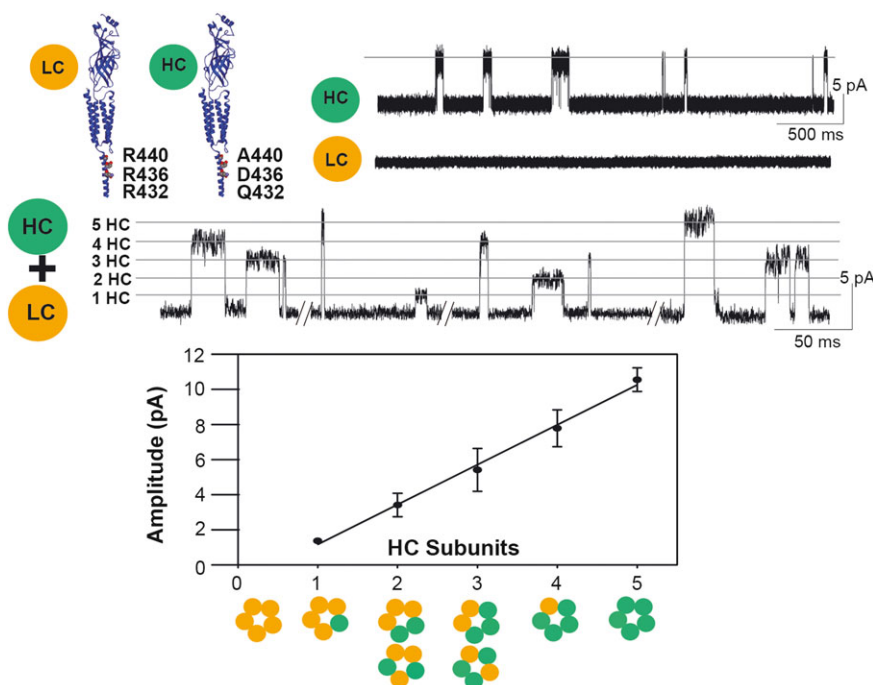
The many types of nACh receptor subunits assemble in various combinations, enabling a wide diversity of functional and pharmacological profiles (Alexander *et al.*, 2015). In a

classical confluence of molecular biology and patch clamp, the  $\gamma$  subunit of the muscle nACh receptor was shown to confer low conductance and long open time, mimicking nACh receptor from embryonic and denervated muscle, whereas the  $\varepsilon$  subunit increased conductance and shortened the open time, mimicking nACh receptors from adult innervated muscle (Mishina *et al.*, 1986). Combining the patch clamp with site-directed mutant or chimeric subunits revealed that the conductance difference arose from differences in charged residues at the extracellular end of the M2 TMD (Imoto *et al.*, 1988; Herlitze *et al.*, 1996), whereas the mean open time difference arose from differences in residues in the cytoplasmic (M3-M4) and M4 domains (Bouzat *et al.*, 1994). Thus, differences in functional signatures between the two subunits could be traced to amino acid differences in distinct structural domains.

The ability to vary subunit stoichiometry within the receptor pentamer further increases the diversity in functional and pharmacological profiles. In neuronal heteromeric  $\alpha 4\beta 2$  nACh receptors, changes in the stoichiometry of  $\alpha$  to  $\beta$  subunits not only alter the ACh sensitivity, but also the selectivity for different agonists and antagonists (Nelson *et al.*, 2003; Moroni *et al.*, 2006). The stoichiometry-dependent differences are likely to arise from changes in the number of agonist binding sites, as well as changes in the interactions between subunits. Two general

approaches have been developed to study how changes in binding site number or inter-subunit interactions affect receptor function. In the first, receptors are generated by covalently linking the subunits so that the stoichiometry and positioning of the subunits are predetermined (Zhou *et al.*, 2003; Carbone *et al.*, 2009). These receptor concatemers have been largely studied through measurements of macroscopic currents, though recently, single channel currents from pentameric concatemers composed of  $\alpha 4$  and  $\beta 2$  subunits showed that subunit stoichiometry determined conductance, kinetics and susceptibility to drug potentiation (Mazzaferro *et al.*, 2017). In the second, called electrical fingerprinting, receptors are generated using combinations of unlinked subunits where one subunit of the combination alters the receptor's electrical signature (Rayes *et al.*, 2009; Andersen *et al.*, 2011, 2013).

In electrical fingerprinting, a mutant and a wild type subunit are co-expressed to yield a population of receptors with a range of subunit stoichiometry. To determine the stoichiometry of an individual receptor, one of the subunits is tagged with a mutation that alters the single-channel current amplitude (Figure 3). A good site for a conductance mutation is the intracellular domain between M3 and M4, which forms portals through which permeating cations pass; if the portals are lined with arginine residues, the contribution of that subunit to the single channel current



### Figure 3

**Electrical fingerprinting strategy to determine receptor stoichiometry.** To determine receptor stoichiometry, a subunit with a reporter mutation that alters unitary conductance is generated, and mutant and non-mutant subunits are co-expressed. Although receptors with a range of different subunit compositions are produced, patch clamp recordings reveal that the amplitude of each single channel opening event reports the number of mutant subunits in the receptor that originated that event. Due to its low conductance, single-channel currents of the  $\alpha 7$ -5HT<sub>3</sub>A chimeric receptor (LC) are not resolved from cell-attached patches. The triple mutation increases the conductance (HC) and single-channel openings appear as a uniform population of 10 pA at  $-120$  mV. Recordings from cells expressing HC and LC subunits show discrete classes of channel amplitudes, each one corresponding to a receptor containing a given number of HC subunits. Thus, the amplitude of the opening event is the signature of the stoichiometry of the receptor that originated the event.

amplitude approaches zero (Kelley *et al.*, 2003). In an experiment in which high and low conductance subunits are co-expressed, single channel current recordings reveal five distinct current amplitudes spaced at a constant increment of the unitary current. Inspection of individual channel openings reveals the stoichiometry of the receptor that elicited each opening. Segregation of the channel openings according to current amplitude then allows determination of the mean open or burst duration for each subunit stoichiometry. A crucial control is to show that the different current amplitudes are kinetically indistinguishable, and thus the conductance mutations are kinetically silent.

Electrical fingerprinting was applied to the homomeric  $\alpha 7$ -5HT<sub>3</sub>A chimeric receptor in which the extracellular domain contained the  $\alpha 7$  sequence and the pore and cytoplasmic domains contained the 5-HT<sub>3</sub>A sequence (Rayes *et al.*, 2009). Given that homomeric receptors contain five identical binding sites, the study aimed to determine the number of agonist binding sites required to maximize the open duration. To vary the number of binding sites, one of the subunits contained a mutation that prevented agonist binding, and either that subunit or the non-mutant subunit included arginine substitutions within the intracellular portal. The results revealed that maximal open duration was achieved with three functional binding sites, two of which were consecutive within the pentamer. In receptors with two non-consecutive binding sites, the open duration was reduced to half of the maximum, and in receptors with a single site to about a tenth. Thus, binding of agonist to each of the first three sites on the  $\alpha 7$ -5HT<sub>3</sub>A receptor contributes an equal increment of free energy to stabilize the open state, but binding of agonist to a fourth or fifth site provides no further stabilization.

Subsequently, electrical fingerprinting was applied to study the agonist stoichiometry question for native  $\alpha 7$  receptors (Andersen *et al.*, 2013), but the conclusion diverged from that for the  $\alpha 7$ -5HT<sub>3</sub>A receptor. Because only a small fraction of channel openings from native  $\alpha 7$  receptors last long enough to reach full amplitude, the experiments were done in the presence of the potentiator 5-hydroxyindole (5-HI) to prolong the openings, or with an  $\alpha 7$  mutant with prolonged open time. Both methods showed that agonist occupancy of a single site produced maximal open duration. To corroborate this conclusion,  $\alpha 7$  subunits were co-expressed with a threefold excess of a subunit with an inactivated agonist binding-site. Channel open-time was the same as that obtained for  $\alpha 7$  alone, even though the vast majority of receptors contained one or two functional binding sites. Thus in native  $\alpha 7$  receptors, occupancy of a single site is transduced into the maximally stable open state structure, whereas in the chimeric receptor, structural mismatches impair transduction and reduce the stabilization provided by the occupancy of fewer than three binding sites.

In an alternative approach to the agonist stoichiometry question, the stoichiometry of  $\alpha$ -bungarotoxin binding required to prevent channel opening was determined using electrical fingerprinting (daCosta *et al.*, 2015). The first step was to generate an  $\alpha$ -bungarotoxin-resistant  $\alpha 7$  subunit by replacing two residues at the binding site with equivalent

residues from the toxin-insensitive  $\alpha 4$  subunit (Sine *et al.*, 2013). The toxin-resistant subunit was then co-expressed with the native  $\alpha 7$  subunit, and one of the subunits was tagged with intracellular arginine substitutions. Before  $\alpha$ -bungarotoxin application, recordings revealed multiple amplitude classes of channel openings reflecting receptors with multiple subunit compositions. However, after  $\alpha$ -bungarotoxin application, only one class of openings was observed corresponding to receptors with five high conductance,  $\alpha$ -bungarotoxin insensitive subunits. Thus, occupancy of a single site by  $\alpha$ -bungarotoxin was enough to prevent channel opening. Given that occupancy of one site by agonist is enough to open the channel in the absence of the toxin, the findings suggested binding of one  $\alpha$ -bungarotoxin molecule immobilizes the four remaining sites so they either cannot bind agonist or cannot transduce agonist binding into channel opening. These findings show that whereas  $\alpha$ -bungarotoxin competes with ACh for the binding sites, functional antagonism is achieved by a conformational arrest mechanism.

## Allosteric modulation of nACh receptor

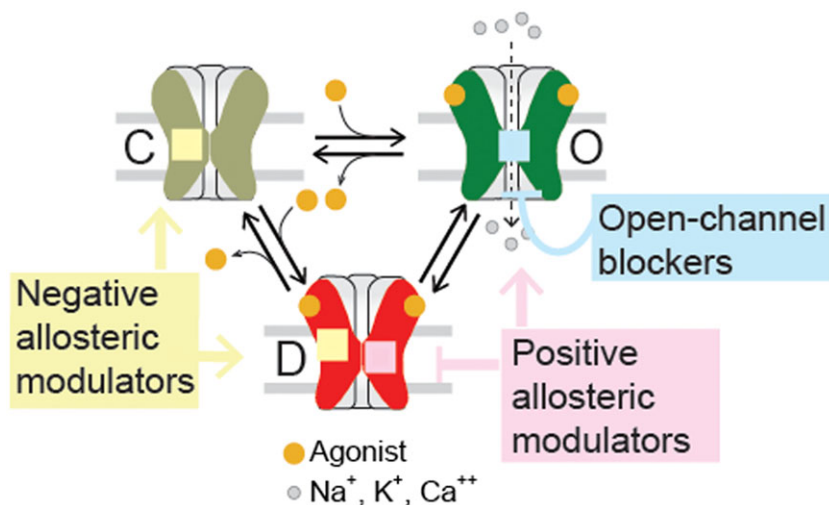
The ACh-elicited response can be modulated by a broad spectrum of structurally diverse molecules that bind to sites distinct from the orthosteric site. These allosteric ligands are likely to alter energy barriers for transitions between conformational states, and therefore potentiate or inhibit receptor function (Figure 4). nACh receptors are emerging as therapeutic targets for pain, epilepsy, Alzheimer's disease, Parkinson's disease, Tourette's syndrome, schizophrenia, anxiety, depression and inflammatory processes (Changeux and Taly, 2008; Egea *et al.*, 2015; Zanetti *et al.*, 2016). Therefore, understanding the basis of allosteric modulation is essential for rational design of novel therapeutic agents.

From a functional perspective, allosteric ligands are classified into four groups: (i) negative allosteric modulators (NAMs); (ii) positive allosteric modulators (PAMs), which potentiate agonist-elicited responses; (iii) allosteric agonists, which activate receptors from non-orthosteric sites; and (iv) silent allosteric modulators (SAMs), which have no effect on orthosteric agonist responses but block allosteric modulation (Figure 4). Single-channel recordings have been invaluable in probing the mechanistic bases of these actions.

## Mechanistic actions and sites of NAMs

NAMs comprise a wide range of structurally different compounds that inhibit receptor function. The negative modulation probably occurs via several different molecular mechanisms and sites.

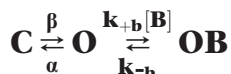
Electrophysiology, mutagenesis and photoaffinity labelling have shown that the ion channel is one of the sites for receptor inhibition. Several compounds, called open-channel blockers, bind within the channel when the receptor is in the open state, thereby physically blocking ion permeation and inhibiting the receptor non-competitively. They can bind between the  $-2'$  and  $20'$  positions of the M2



**Figure 4**

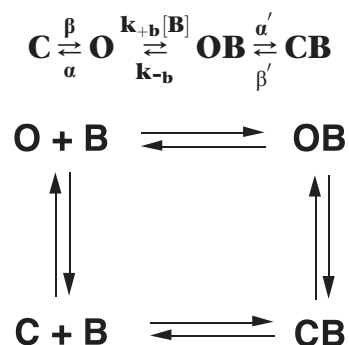
**Schematic allosteric scheme for activation and drug modulation.** The resting (C), open (O) and desensitized (D) states can be positively or negatively modulated by allosteric compounds. Open-channel blockers bind to the ion channel and inhibit the flux of ions. NAMs stabilize resting or desensitized states. PAMs stabilize open states or decrease desensitization.

domains (Leonard *et al.*, 1991). By studying the action of local anaesthetics on nACh receptors, Neher and Steinbach (1978) were the first to show the remarkable power of single-channel recording in elucidating kinetic mechanisms for drug action (Jackson, 2010). They analysed the data using a sequential model for a pure open channel blocker (B), from which they calculated rate constants for blocking and unblocking ( $k_{+b}$  and  $k_{-b}$ ):



Open-channel block of single nACh receptor channels has been observed for a wide spectrum of compounds, including, among others, local anaesthetics (Neher and Steinbach (1978), antiepileptic drugs (Vallés *et al.*, 2007), and pyrantel (Raves *et al.*, 2001). In general, there is a relationship between rate constants in the sequential mechanism and the appearance of channel opening events: openings can appear as sequences of incompletely resolved spikes or as resolved but brief pulses, depending on the concentration of the drug and the blocking and unblocking rate constants (Dilger *et al.*, 1991; Figure 5). At high concentrations, ACh and other agonists also act as open-channel blockers (Sine and Steinbach, 1984; Zhang *et al.*, 1995; Raves *et al.*, 2007). Most agonists act as low-affinity blockers, binding and unbinding very quickly and leading to unresolved openings and closings that reduce the apparent current amplitude.

Some compounds are not pure open-channel blockers, but instead allow the channel to close while still bound, or block the receptor channel in the resting state. Their actions can be described by an expanded linear or cyclic scheme:

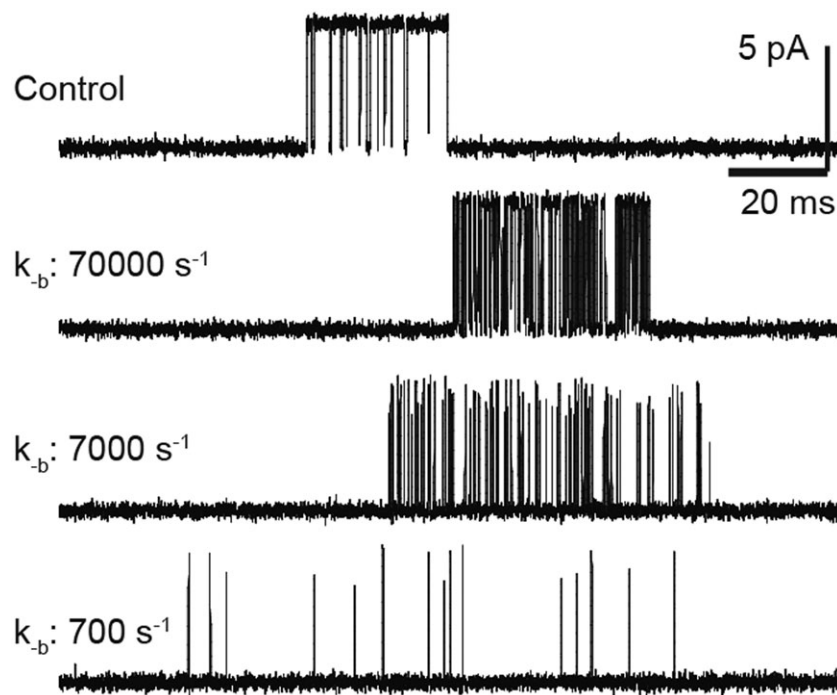


These schemes describe the actions of many channel blockers, including isoflurane and general anaesthetics (Dilger *et al.*, 1991), chlorpromazine and phencyclidine (Changeux *et al.*, 1986), ephedrine (Milone and Engel, 1996; Bouzat, 1996), MK-801 (Amador and Dani, 1991), and amphetamine (Spitzmaul *et al.*, 1999). The ability of blockers of nACh receptors to become trapped by closure of the channel has been described for the first time for methonium compounds (Gurney and Rang, 1984) and chlorisondamine (Neely and Lingle, 1986).

For some cases, NAMs can be therapeutically useful. For example, long-lived open-channel blockers, such as quinidine and fluoxetine, which do not allow rapid unblocking, are therapeutically effective in shortening abnormally prolonged channel openings of the muscle nACh receptor in slow-channel congenital myasthenic syndromes (Fukudome *et al.*, 1998; Harper *et al.*, 2003; Engel, 2007).

In addition to the channel block mechanism, NAMs may inhibit receptor function by preferentially stabilizing the nACh receptor in a non-conducting state (resting or desensitized state), or by increasing the rate and extent of desensitization (Figure 4). For example, tricyclic





**Figure 5**

**Effects of open-channel blockers on nACh receptor activity as a function of the unblocking rate.** Single-channel currents for muscle nACh receptor activated by 100  $\mu\text{M}$  ACh were simulated on the basis of the linear open-channel blocking scheme, including a closed (C), an open (O) and a blocked state (OB). A desensitized state was also included to allow the termination of the bursts in the simulations. Rate constants are  $\beta = 50\,000\text{ s}^{-1}$  (opening rate),  $d_+ = 100\text{ s}^{-1}$  (desensitization rate),  $d_- = 1\text{ s}^{-1}$  (recovery from desensitization),  $k_{+b} = 1 \times 10^7\text{ M}^{-1}\text{ s}^{-1}$ ,  $[B] = 5\text{ mM}$ ,  $k_b = 700, 7000\text{ or }70\,000\text{ s}^{-1}$ .

antidepressants and adiphenine produce a concentration-dependent decrease in the number of openings per activation episode without changing open and closed durations, indicating acceleration of desensitization onset (Gumilar *et al.*, 2003; Spitzmaul *et al.*, 2009).

Given its transmembrane nature, the nACh receptor establishes close physical contact with lipids, which modulate function (Bouzat *et al.*, 1998, 2000; Antollini and Barrantes, 1998; Tamamizu *et al.*, 2000; Baenziger *et al.*, 2015; Barrantes, 2015). The annular (surrounding the perimeter of the receptor) and non-annular lipid domains (between transmembrane helices) are sites for a great variety of hydrophobic NAMs that by different mechanisms inhibit function. These compounds include, among others, quinacrine, which decreases the frequency and duration of nACh receptor activation episodes due to increased desensitization (Spitzmaul *et al.*, 2001), fatty acids, which reduce open duration and open probability (Bouzat and Barrantes, 1993; Antollini and Barrantes, 2016), and steroids, which produce slow channel blockade (Bouzat and Barrantes, 1996).

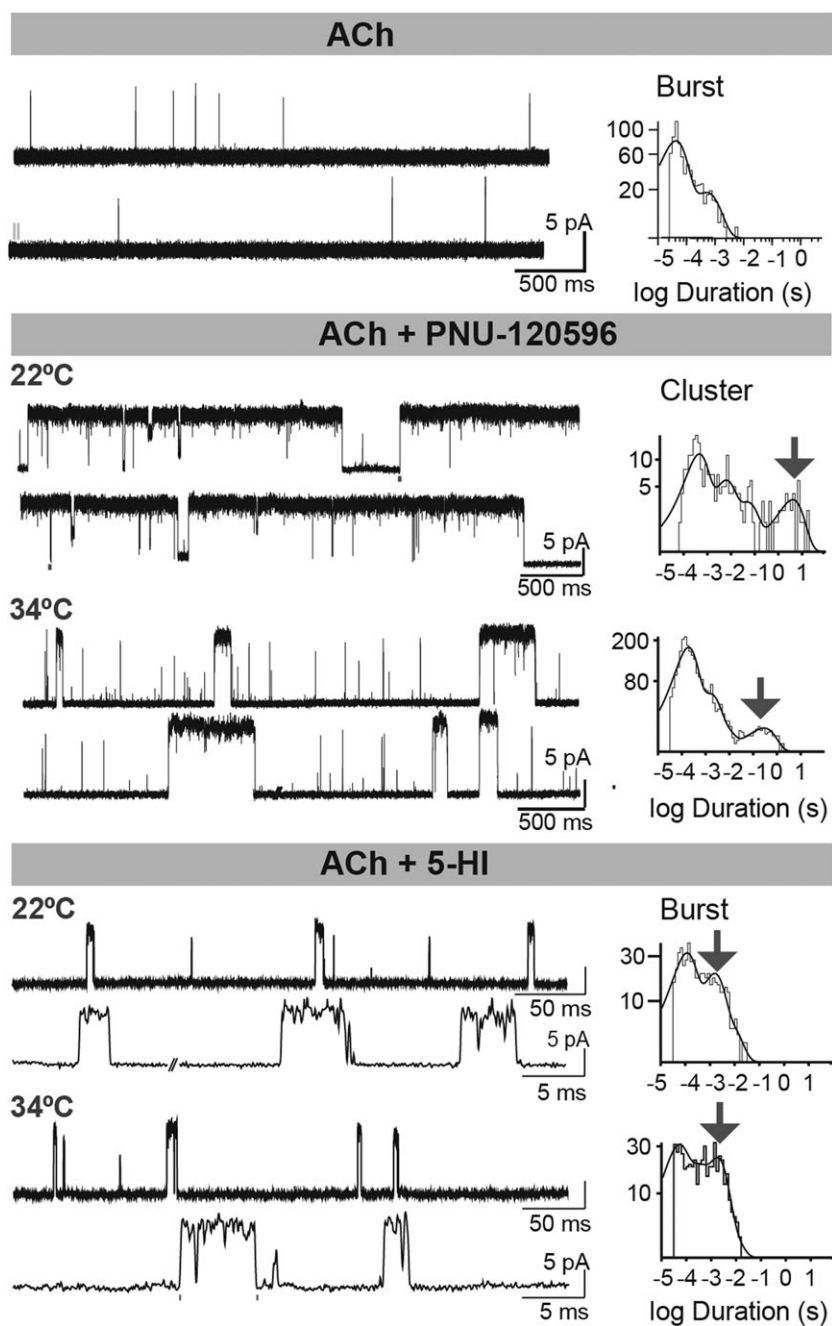
## Positive allosteric modulators of $\alpha 7$ nACh receptors

PAMs neither activate nACh receptors nor compete with ACh binding, but instead potentiate agonist-evoked responses.

PAMs of  $\alpha 7$  receptors are emerging as novel therapeutic agents for neurological and inflammatory disorders. When compared with exogenous agonists, PAMs are promising therapeutics because they maintain the temporal and spatial characteristics of endogenous activation processes, are more target selective, and reduce tolerance due to desensitization (Uteshev, 2014; Corradi and Bouzat, 2016).

$\alpha 7$  PAMs have been classified as type I or II on the basis of their effects on macroscopic currents. Type I PAMs, which include ivermectin (Krause *et al.*, 1998), genistein (Grønlien *et al.*, 2007), N-(5-chloro-2-hydroxyphenyl)-N'-[2-chloro-5-(trifluoromethyl)phenyl]urea (NS-1738) (Bertrand *et al.*, 2008) and 5-HI (Thinschmidt *et al.*, 2008), enhance agonist-induced peak currents without significantly affecting current decay, whereas type II PAMs slow the onset of desensitization and reactivate desensitized receptors (Bertrand and Gopalakrishnan, 2007; Williams *et al.*, 2011; Corradi and Bouzat, 2016).

The most efficacious  $\alpha 7$  PAM to date is N-(5-chloro-2,4-dimethoxyphenyl)-N'-(5-methyl-3-isoxazolyl)-urea (PNU-120596), a type II PAM (Hurst *et al.*, 2005). When analysed at the single-channel level, PNU-120596 prolongs channel openings approximately a thousand-fold, and the openings coalesce into long clusters lasting several seconds (daCosta *et al.*, 2011; Pałczyńska *et al.*, 2012; Andersen *et al.*, 2016) (Figure 6). Type II PAMs may increase the energetic barrier for desensitization onset, accounting for the long clusters (daCosta *et al.*, 2011), and reduce the barrier for



## Figure 6

**Potentiation of human  $\alpha 7$  receptors by type I and type II PAMs as a function of temperature.** Single-channel currents of human  $\alpha 7$  receptors activated by 100  $\mu\text{M}$  ACh in the absence and presence of potentiators. The increase of temperature reduces the long clusters induced by PNU-120596 but does not affect significantly the duration of bursts induced by 5-HI. Channel openings are shown as upward deflections. Membrane potential:  $-70$  mV. ACh: 100  $\mu\text{M}$ , PNU-120596: 1  $\mu\text{M}$ , 5-HI: 2 mM.

desensitization recovery, which allows reversal of agonist-induced desensitization (Williams *et al.*, 2011). Alternatively, they may induce conformations for which channel opening is energetically favoured (Szabo *et al.*, 2014). Based on the macroscopic observations that type I PAMs only increase the peak of ACh-elicited currents, it has been postulated that these PAMs act by decreasing the energetic barrier for opening (Williams *et al.*, 2011; Hurst *et al.*, 2013). However,

single-channel recordings show that type I PAMs prolong  $\alpha 7$  receptor openings and activation episodes, which reveals that they do affect the closing and opening rates of  $\alpha 7$  receptors (Andersen *et al.*, 2016) (Figure 6). Alternatively, as seen with type II PAMs, the increase in the open duration could be due to changes in desensitization rate, which in turn, could be too slight to be detected from whole-cell macroscopic currents. However, this may not be the case because for some

type II PAMs, the increase in open and burst durations is similar to that mediated by type I PAMs, but the effects on the decay of macroscopic currents are different between both types (Andersen *et al.*, 2016). Thus, PAMs potentiate  $\alpha 7$  receptors through multiple mechanisms.

Potentiation of  $\alpha 7$  receptors by PAMs depends strongly on temperature and type of PAM. In the presence of PNU-120596, the enhancement of macroscopic peak currents (Williams *et al.*, 2012), and of cluster duration (Andersen *et al.*, 2016) is markedly reduced as the temperature increases from 22 to 34°C. In contrast, the increase in burst duration elicited by 5-HI does not change significantly over the same temperature range, although the proportion of potentiated bursts at 34°C decreases with respect to that at room temperature (Andersen *et al.*, 2016) (Figure 6). Previous studies have demonstrated that the desensitization rate increases at higher temperatures in the absence or presence of PNU-120596 (Gupta and Auerbach, 2011; Sitzia *et al.*, 2011; Jindrichova *et al.*, 2012). Thus, if the effect of temperature were mainly on desensitization, increasing temperature would affect mainly type II PAM modulation. This could explain the reduction in the duration of the longest bursts or clusters in the presence of type II PAMs, while the maximal duration of bursts elicited by 5-HI remains more constant when the temperature is increased. These differences suggest that bursts/clusters have a different mechanistic origin in type I (5-HI) or type II PAMs. Nonetheless, more PAMs should be tested to confirm this hypothesis. Alternatively, it could be possible that dissociation of PAMs from their binding sites responds differently to temperature depending on both the PAM and the binding-site structures.

Thus, to better mimic the *in vivo* situation, *in vitro* studies should also be assessed at physiological temperatures. That potentiation is reduced at physiological temperatures may be beneficial in attenuating potential toxicity due to calcium influx in the case of very efficacious PAMs (Hu *et al.*, 2009; Guerra-Álvarez *et al.*, 2015; Uteshev, 2016).

## Modulatory sites for nACh receptor PAMs

Electrophysiological studies on  $\alpha 7$  receptors have identified amino acids within the transmembrane  $\alpha$ -helices of a single subunit that, when mutated individually, significantly reduced potentiation of macroscopic  $\alpha 7$  receptor responses by PNU-120596, LY2087101 and ivermectin (Young *et al.*, 2008; Collins and Millar, 2010). Particularly, two individual point mutations (A226D in M1 and M254L in M2, numbering of human  $\alpha 7$ ) reduced PNU-120596 potentiation of macroscopic currents by 90%. Given the sensitivity of single-channel recordings, potentiated clusters of the single mutants were still evident. However, the simultaneous mutation of these two residues with three other ones, one of which was also previously identified by Young *et al.* (2008) (S223 at  $\alpha 7M1$ ), abolished the long ACh-elicited clusters typical of PNU-120596 potentiation, thus defining structural determinants of potentiation (daCosta *et al.*, 2011).

Reference to homology models of  $\alpha 7$  receptors based on the 4 Å structure of the *Torpedo* nACh receptor suggested that

the amino acids involved in PNU-120596 potentiation may be part of an intra-subunit transmembrane cavity (Young *et al.*, 2008). A cavity in the upper part of the TMD between the four transmembrane helices of each subunit was also identified from structural studies on the prokaryotic homologue GLIC as the binding site of general anaesthetics (Nury *et al.*, 2011; Sauguet *et al.*, 2013, 2014) and from photolabeling studies on *Torpedo* nACh receptor as the site of propofol (Jayakar *et al.*, 2013). Although it is tempting to speculate that this cavity is also the site for PNU-120596 in  $\alpha 7$  receptors, it should be taken into account that mutagenesis experiments cannot rule out indirect allosteric effects, and that docking studies were carried out using a homology model. Thus, the unequivocal localization of the PNU-120596 binding site to an intra-subunit cavity requires structural studies of the complex with  $\alpha 7$  subunits.

The quintuple mutant subunit that prevents PNU-120596 potentiation was used to determine the stoichiometry required for potentiation. To this end, the electrical fingerprinting strategy was applied by generating  $\alpha 7$  receptors composed of normal and PNU-resistant subunits, one of which contained arginine substitutions of the intracellular portal, and patch clamp recording monitored PNU-120596 potentiation of  $\alpha 7$  receptors with defined stoichiometry (daCosta and Sine, 2013). Potentiation was found to depend steeply on the number of PNU-120596-resistant subunits in the pentamer, and at least four and probably five subunits must be sensitive to PNU-120596 for  $\alpha 7$  potentiation to occur.

The quintuple mutant receptor insensitive to PNU-120596 is also insensitive to another type II PAM, PAM-2, and to the type I PAM, NS-1738, indicating common structural determinants for their actions (Andersen *et al.*, 2016). In contrast, 5-HI potentiates the PNU-insensitive mutant  $\alpha 7$  receptors when evaluated at the single-channel level, thus indicating that not all PAMs bind to the same site, interact with the same residues, or require the same determinants for transduction.

In  $\alpha 7$  receptors, the intrasubunit transmembrane cavity has also been proposed as the binding site for the allosteric agonist, 4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide (4BP-TQS), which elicits long clusters of channel openings in the absence of ACh (Gill *et al.*, 2011, 2012; Pałczyńska *et al.*, 2012). It was proposed that, in addition to occupancy of this transmembrane site, allosteric activation by the active isomer of 4BP-TQS requires simultaneous occupancy of an extracellular binding site (Horenstein *et al.*, 2016), which is analogous to a vestibular binding pocket previously reported from structural studies in an  $\alpha 7$ -AChBP chimeric receptor (Spurny *et al.*, 2015).

In addition to being the site of PAMs and allosteric agonists, the intrasubunit transmembrane site has been proposed to be the site for some NAMs and silent allosteric modulators of  $\alpha 7$  receptors (Gill *et al.*, 2013; Gill-Thind *et al.*, 2015).

An inter-subunit transmembrane site between transmembrane helices of adjacent subunits has been identified as a modulatory site for ethanol, general anaesthetics and ivermectin in pentameric ligand-gated ion channels (Hibbs and Gouaux, 2011; Lynagh and Lynch, 2012; Sauguet *et al.*,

2013, 2015). Photoreactive anaesthetics label homologous residues of the  $\alpha/\gamma$  interface in *Torpedo* nACh receptors (Husain *et al.*, 2006; Nirthanan *et al.*, 2008; Forman *et al.*, 2015). This site has been proposed for binding of short chain alcohols that positively modulate *Torpedo* and neuronal nACh receptors by stabilizing the open state (Nagata *et al.*, 1996; Forman and Zhou, 1999; Zuo *et al.*, 2001).

Other PAMs act through different sites and by different mechanisms. Galantamine, an inhibitor of AChE, acts as a low-efficacy agonist of the muscle nACh receptor and a potentiator of  $\alpha 7$  receptors by binding to a site at subunit interfaces close to the ACh-binding site (Akk and Steinbach, 2005; Hansen and Taylor, 2007; Ludwig *et al.*, 2010). 17- $\beta$ -estradiol increases open probability of neuronal  $\alpha 4\beta 2$  receptors; a site at the C-terminal tail of the  $\alpha 4$  subunit is required for the potentiation (Paradiso *et al.*, 2001; Curtis *et al.*, 2002; Jin and Steinbach, 2011). Three different allosteric sites in the extracellular domain of an  $\alpha 7$ -AChBP chimera were also identified by an innovative fragment-library screening in combination with X-ray (Spurny *et al.*, 2015). Although all the allosteric binders tested behaved on human  $\alpha 7$  receptors as NAMs, it was proposed that their chemical modification could lead to a change in functional activity.

In conclusion, multiple binding sites and domains may be involved in the conformational changes associated with potentiation of  $\alpha 7$  receptors. This is in line with structural studies in prokaryotic pLGICs proposing that the different allosteric binding sites present in Cys-loop receptors form an almost continuous path stretching from top to bottom of the receptor (Nys *et al.*, 2016).

## Concluding remarks

Investigators remain as stunned by the extraordinary power of patch clamp as when Neher and Sakmann first observed currents through single ion channels. Single-channel recording has provided molecular insights that are unattainable from macroscopic measurements, and has made invaluable contributions to understanding nACh receptor function, the molecular bases of human diseases and the mechanisms of drug modulation. Kinetic analysis has provided deep insight into activation of ligand-gated channels by describing how the receptor moves through different conformations in response to the neurotransmitter. Still, there is a long way to go. New challenges include improvement of patch clamp temporal resolution, although we are approaching the limit of present technology. The combination of high-resolution crystal structures at defined conformational states with single-channel electrophysiological characterization of drug action on mutant nACh receptors emerges as a means towards defining sites and mechanisms of drug action essential to rational drug design for disorders involving nACh receptors.

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## Conflict of interest

The authors declare no conflicts of interest.

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