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The nicotinic acetylcholine receptor as a molecular machine for neuromuscular transmission

Cecilia Bouzat[1] and Nuriya Mukhtasimova[2]

The nicotinic acetylcholine receptor (nAChR) has crucial functions at the neuromuscular junction (NMJ). It belongs to the superfamily of pentameric ligand-gated ion channels and has become the stereotype for probing fundamental structures and mechanisms. The nAChR operates as a molecular machine that transduces the binding of nerve-released ACh into an electrical signal that initiates the process of muscle contraction. Its molecular design has been tuned to function as a near perfect on–off switch that responds to ACh with the efficiency and speed required for proper muscle function. Biochemical, biophysical, electrophysiological and structural studies have allowed an integrated description of the muscle nAChR, providing information of its molecular function at the NMJ in health and disease states and guiding rational therapy.

Addresses
[1] Instituto de Investigaciones Bioquímicas de Bahía Blanca, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Bahía Blanca, Argentina
[2] Receptor Biology Laboratory, Department of Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Corresponding author: Bouzat, Cecilia (inbouzat@criba.edu.ar)

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Introduction: nAChR at the neuromuscular junction

The NMJ is a highly complex structure in which the three components — nerve terminal, muscle fiber and Schwann cells — are highly specialized to transduce the nerve stimulus into muscle contraction [1]. As with all chemical synapses, the NMJ has a presynaptic region comprising the nerve terminal, which is physically separated from the postsynaptic region, the muscle cell, by the synaptic cleft. Arrival of an action potential at the motor nerve terminal leads to opening of voltage-gated Ca^{2+}-channels, which results in an increase in intracellular calcium concentration and subsequent release of ACh-containing vesicles into the synaptic cleft. The muscle surface at the NMJ is formed by deep folders whose crests contain densely packed ($\sim 10^4/\mu m^2$) muscle nicotinic receptors (nAChRs) [1,2]. These receptors act as the main converters of the chemical signal, ACh, into the electrical response, mediated by influx of sodium after channel opening. Thus, activation of nAChRs evokes an endplate current (EPC) resulting in depolarization (the endplate potential, EPP) that triggers opening of voltage-dependent sodium channels, giving rise to a propagated action potential that spreads throughout the muscle cell and initiates muscle contraction. The response is limited because, due to the presence of acetylcholinesterase (AChE) and to additional ACh diffusion, ACh is removed from the synaptic cleft in less than a millisecond. Under normal circumstances, a molecule of ACh reacts with only one receptor before it is hydrolyzed into choline and acetate. Once triggered, the rise and exponential decline of the EPC are determined by the rates at which the nAChR switches among closed and open states [3]. Its exquisite design makes the nAChR a suitable molecular machine for neuromuscular transmission. Due to its key role in initiating muscle contraction, nAChR is the target of clinically-used neuromuscular blocking agents and of muscle diseases.

The muscle nAChR

The muscle nAChR became the reference neurotransmitter receptor due to its abundant quantities in the electric organ of electric fish (*Torpedo*) [4,5] and to the availability of the competitive cholinergic antagonist α-bungarotoxin from the snake *Bungarus multicinctus* [6,7], which favored the isolation, purification, and pharmacological characterization of the receptor.

The nAChR belongs to the Cys-loop receptor family that is included in the superfamily of pentameric ligand-gated ion channels (pLGIC), which are composed of five identical (homopentamers) or different (heteropentamers) polypeptide chains arranged around an axis perpendicular to the membrane. A wide number of subunits of the superfamily have been cloned (ligand-gated ion channel database, http://www.ebi.ac.uk/compneur-srv/LGICdb/cys-loop.php). nAChR subunits are classified in two types, α and non-α, with the α-type subunits containing a disulphide bridge in the agonist binding site.

The mammalian muscle nAChR exists in two developmentally regulated isoforms. Embryonic muscle expresses nAChRs composed of two α1 subunits, one β, one δ and

one γ subunit ($\alpha_2\beta\gamma\delta$). During maturation of the endplate the γ-subunit is replaced by the ε-subunit to yield adult nAChRs ($\alpha_2\beta\varepsilon\delta$). The γ-subunit is required for the proper maturation of the neuromuscular synapse; in the adult γ-containing nAChRs are found after denervation and in some congenital myasthenic syndromes and myogenic disorders [8,9]. The γ–ε switch changes the pharmacological, metabolical and electrophysiological properties of the nAChR and tunes receptor function to each developmental stage. The embryonic receptor has higher agonist affinity, smaller single-channel conductance, longer open-channel lifetime, lower Ca^{2+} permeability and lower probability of opening constitutively than the adult ε-containing nAChR [10–12].

The nAChR at the neuromuscular junction has been fine-tuned through evolution to transduce a chemical signal into an electrical signal with maximum efficiency and speed. In the absence of agonists, nAChRs rarely adopt the open state [13], although some mutations increase the degree of constitutive activity. Docking of ACh into the two neurotransmitter binding sites (binding process) enables the receptor to reach a stable open conformation with a higher rate and probability (gating). This open state has higher affinity for ACh than the closed state, and this difference contributes to the amount of favorable free energy required to stabilize the open state [14]. The very large shift in the equilibrium ratio of open/closed channels from neither binding site being occupied to both binding sites being occupied ensures that this protein functions as a near perfect on–off switch. The opening and closing events are fast, thus allowing rapid initiation and termination of the postsynaptic response, which is crucial for proper neuromuscular transmission. Prolonged exposure to agonist leads to the formation of one or more inactive desensitized conformations, which are characterized by a relatively high affinity for agonist. Desensitization is of little consequence for normal neuromuscular transmission, but it may have a role in some congenital myasthenic syndromes [15].

nAChR structure and the activation mechanism

Near atomic resolution structural insight of a pLGIC was first achieved by electron microscopy applied to the muscle *Torpedo* nAChR, initially yielding structures from 20 to 9 Å resolution [16,17], and culminating in a 4 Å structure [18]. Crystal structures of the ACh binding protein (AChBP), which is a water-soluble homo-pentamer that mirrors the nAChR extracellular region, of related pLGICs, and of the neuronal α4β2 nAChR have been reported (see review in Nemecz *et al.* [19]).

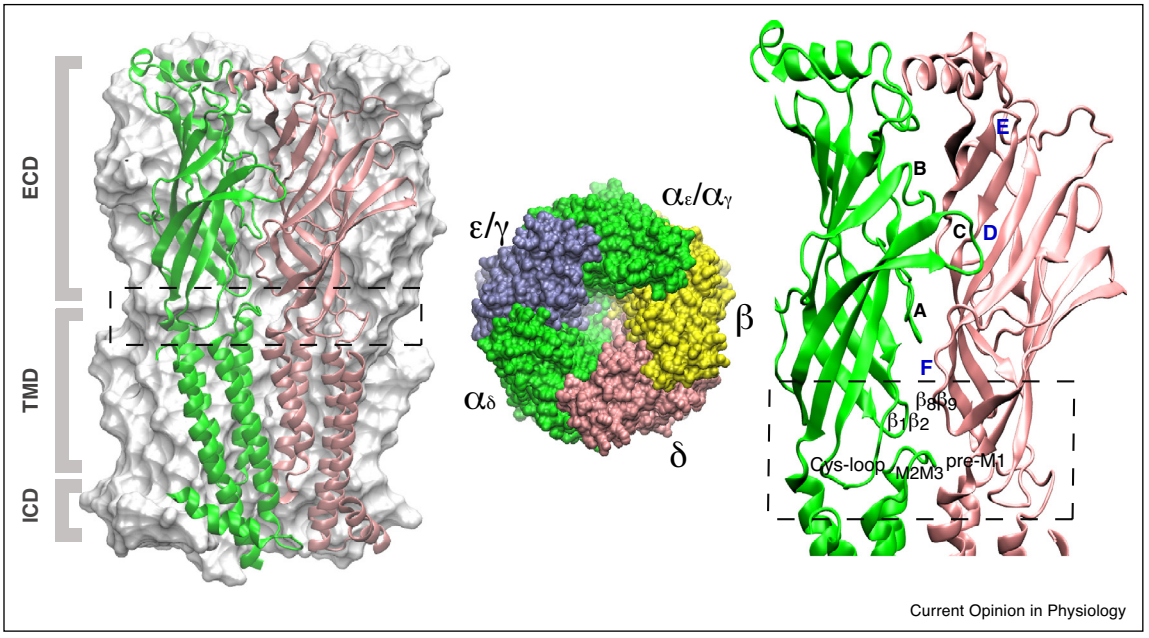
The five subunits are arranged pseudo-symmetrically around a central axis that functions as an ion channel and come together in a counterclockwise α–ε/γ–α–δ–β arrangement in the muscle nAChR (Figure 1). The receptor contains three distinct functional and structural domains: first, The N-terminal extracellular domain (ECD) that carries two orthosteric agonist binding sites. In each subunit, the ECD consists of 10 β-strands (β1–β10) forming two β-sheets that fold into a classic β-sandwich; second, the transmembrane domain (TMD) that forms the ion pore and contains the channel gate and is composed of four α-helices from each subunit (M1–M4), and; third, the intracellular domain (ICD) that links to the cytoskeleton, contributes to channel kinetics and contains sites for modulation [10,20].

The two ligand binding cavities are located at 30 Å from the ECD-TMD interface between subunit interfaces (α/δ and α/γ or α/ε) (Figure 1). The α-subunits provides the principal face of each agonist site that is formed by Loop A (β4β5 loop), Loop B (β7β8 loop), and Loop C (β9β10 loop) and contribute to a nest of aromatic residues (Y190, W149, Y198, Y93) that stabilizes the ammonium of the agonist through cation–pi interactions and/or hydrogen bonding [21]. Loop B plays a principal role through W149 and Loop C closes to cap the agonist, and event associated with the triggering of channel opening [3,22–25]. The complementary face provided by the ε/γ or δ subunits is composed of Loops D–F from separate sections of three β-strands and carry the key W55 and εG57 amino acids involved in drug selectivity (Figure 1). Contiguous to the binding site and at subunit interfaces, a pair of intersubunit interacting residues is required for rapid and efficient gating of muscle nAChR [26].

In the TMD, the five M2 α-helices line the channel pore that is permeable to small monovalent cations and less to divalent cations. The order of permeability is for monovalent ions $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ and for divalent ions $Mg^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+}$ [27,28]. In M2, rings of highly conserved leucine (position 9′) and valine (position 13′) form the hydrophobic activating gate [5,19]. Rings of negatively charged side-chains are located strategically at the ends of the pore-lining helices, and on the helices forming the intracellular domain, to concentrate cations near the entrances of the narrow pore and contribute to ion conductance [29,30]. M1 and M3 from each subunit form a ring of α-helices that shield M2 from the membrane, and M4 segments are located on the periphery of each subunit and exposed to the lipid bilayer [5].

The interface between the ECD and TMD, also referred to as the coupling region, is a structural transition zone where β-sheets from the ECD merge with α-helices from the TMD (Figure 1). It is formed by a network of loops that by relaying structural changes from the binding site toward the pore have a key role in coupling agonist binding to channel opening [31–35]. It contains the conserved Cys-loop (link between β6 and β7 strands) that is the signature of the Cys-loop receptor family. Mutation analysis combined with single-channel currents of the muscle nAChR have revealed coupling between

Figure 1



nAChR structure. (Left) Side view of the model nAChR, corresponding to the structure of the α4β2 nAChR (PDB code: 5KXI) [94]. Two adjacent subunits are shown in color. The three main domains, extracellular (ECD), transmembrane (TMD) and intracellular (ICD), are marked. Most of the ICD is not shown since it was removed to obtain well-diffracting crystals [99••]. The square shows the location of the coupling region. (Center) View of the nAChR from the top. The disposition of muscle nAChR subunits is shown. (Right) Side view of the extracellular domain and coupling region. The principal face of the binding site is formed by loops A, B and C from the α-subunit (shown in green), and the complementary face is formed by loops D, E and F of ε/γ or δ subunits. Main loops of the coupling region include β1β2, Cys-loop (β6β7) and β8β9 loops, and terminus of β10 strand from the ECD and the pre-M1 and M2M3 linker from the TMD.

residues at different loops forming pathways that link ACh binding to opening of the pore [32,33,36,37,38•]. At this region, the structural rearrangements of the ECD elicited by agonist binding are transmitted to the TMD in each subunit by the covalent link between the C-terminus of β10 of the ECD and the N-terminus of M1 of the TMD (preM1), as well as by noncovalent connections between the β1β2 and β6β7 loops of the ECD and the M2M3 linker of the TMD, and between β1β2 and β8β9 loops from adjacent subunits.

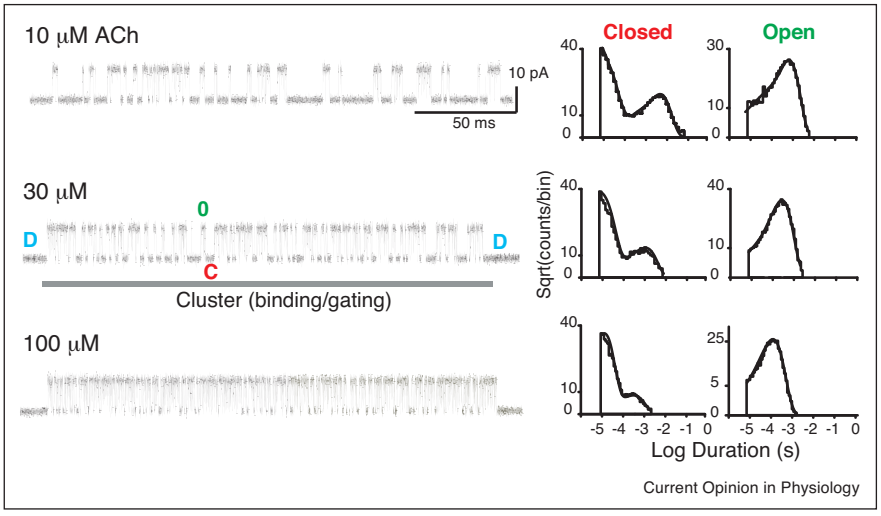
In an extensive series of rate-equilibrium free-energy relationship (REFER) analysis of the muscle nAChR using single-channel data but considering only a closed-to-open transition, it was suggested that gating occurs by means of a 'conformational wave' that proceeds from the extracellular domain to the region of the gate and a sequence of movements during channel activation was proposed [39,40]. It was then suggested that agonist binding leads to local rearrangements of the agonist site (catch and hold model), which generate short-lived intermediate states, that result in the low-to-high agonist affinity switch required for channel gating [41].

Insights into the structural basis of the activation mechanism of pLGICs have been provided by crystal structures of other pLGICs in different conformations and molecular dynamics simulations as well as cryo-EM of *Torpedo* nAChRs in native membranes and has been extensively discussed in the literature [19,23,42–44].

Single-nAChR channel activation

Activation of the muscle nAChRs has been thoroughly explored using cell-attached patch-clamp recordings that allowed identification of multiple closed, open and desensitized states. At a range of desensitized ACh concentrations (10–1000 μM), activation of muscle nAChR occurs in trains of several openings and closings from the same receptor molecule because the time for desensitization is long compared to the time it takes to close and reopen. These activation episodes (clusters) can be clearly identified from single-channel recordings and involve binding/gating transitions. Each cluster begins with the transition of a single receptor from the desensitized to the activatable state and terminates by returning to the desensitized state (Figure 2). With the increase of ACh concentration, the probability of being open within cluster increases, the cluster duration decreases, the closed interval durations within clusters decrease, and the open duration remains constant (at concentrations below blocking ones) [45,46,47••] (Figure 2). In the presence of very low-efficacy agonists, clusters are not distinguished, and,

Figure 2



Traces of nAChR single-channel currents as a function of ACh concentration. Single-channel currents were recorded from cell-attached patches at −120 mV membrane potential. Filter: 25 kHz. Each cluster includes the binding and gating activity of a single nAChR that adopts closed (C) or open (O) states. The silent periods between clusters of openings are periods when all nAChRs in the patch are desensitized (D). As the ACh concentration increases, the probability of channel opening increases, which is evidenced by decreased duration of the closings within clusters and tighter appearance of the clusters. The histograms of closed intervals within clusters show a main component that reflects the set of transitions between unliganded closed and diliganded open states. This component becomes progressively briefer with increasing ACh concentrations. In contrast, the duration of the main open component remains constant at all ACh concentrations (below the blocking ones). For clarity, the cluster as well as open and closed states have been marked for only one condition. Openings are shown as upward deflections.

instead, short bursts composed of few openings or isolated openings of reduced duration are detected [47••,48,49].

Kinetic analysis of single-channel currents has added in-depth insight into the activation mechanism and partial agonism of the muscle nAChR and other pLGICs. Kinetic analysis can dissect the binding from the gating steps, and can provide information about the agonist affinity, how many agonist molecules must bind to activate it maximally, the number of conformational changes that separate the binding events from channel opening as well as rate constants for each activation step [50,51,52•]. As of the first two-step model (binding and gating steps) proposed by del Castillo and Katz [53], different models, including the allosteric Monod–Wyman–Changeux model and subsets, have been followed for describing muscle nAChR activation [3,45,52•,54–58]. The most recent models include a conformational change of the receptor with bound agonist to an activatable state while the pore is still closed. This intermediate step, called flip if it is concerted or priming if the two binding sites change conformation independently, dictates the efficacy of an agonist [25,51,59,60]. High-resolution single-channel recordings, which detected brief single channel currents at 8 μs resolution, allowed to distinguish priming from gating steps and revealed how they depend on agonist occupancy [47••]. It was shown that 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 whereas the gating step was similar for a full and a partial agonist [47••,51,52•].

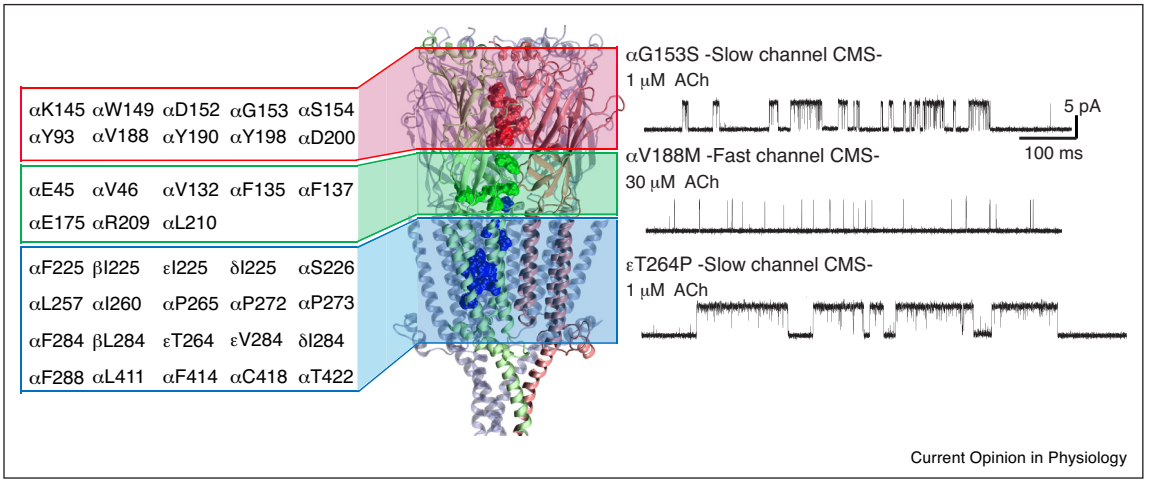
Amino acids involved in receptor function

Studies combining site-directed mutagenesis with electrophysiological recordings have provided in-depth information regarding the mechanistic and structural basis of channel function. Amino acid determinants of nAChR function are located at the binding site, subunit interfaces, transmembrane domains, intracellular sites, and particularly, the coupling region. Mutations to different side chains have identified the structural basis of their contributions, kinetic analysis have dissected the kinetic step in which they are involved (Binding, priming, gating or desensitization), and double-mutant cycle analyses have revealed functional coupling between residues. Figure 3 shows some relevant residues located at different domains whose mutations profoundly affect function and whose kinetic contributions have been identified [26,33,36,38•,45,48,61–64].

nAChR as target of diseases

Myasthenia gravis is the most common type of myasthenia involving the nAChR as the main protagonist. It is an autoimmune disease whose main antigen is the nAChR, although some patients have auto-antibodies directed

Figure 3



Amino acids involved in receptor function. (Right) Model of the *Torpedo* nAChR (PDB code 2BG9) with amino acids at different domains (ECD, TMD and coupling region) that have been shown to have a key role in receptor function. The combination of site-directed mutagenesis of the residues with single-channel recordings and kinetic analysis of the mutant receptors heterologously expressed has allowed identification of their mechanistic contribution by dissecting the kinetic step in which they are involved. (Left) Traces of single-channel currents corresponding to mutant receptors found in patients with fast and slow channel CMS. In the two examples of slow channel CMS, the mutation αG153S at the binding site enhances agonist affinity [100] and the mutation εT264P at the M2 domain increases open probability and spontaneous opening [101]. In the example of the fast channel CMS, the mutation αV188M, located at Loop C, decreases the apparent channel opening rate and gating efficiency [102]. Membrane potential: −70 mV, Filter: 10 kHz.

against other NMJ components [65,66]. Antibodies bind to the ECD of nAChR subunits, called the main immunogenic region (MIR) [67]. The reduced number of nAChRs correlates with disease severity, and antibodies against the α subunit are more pathogenic than those against the β subunit [65].

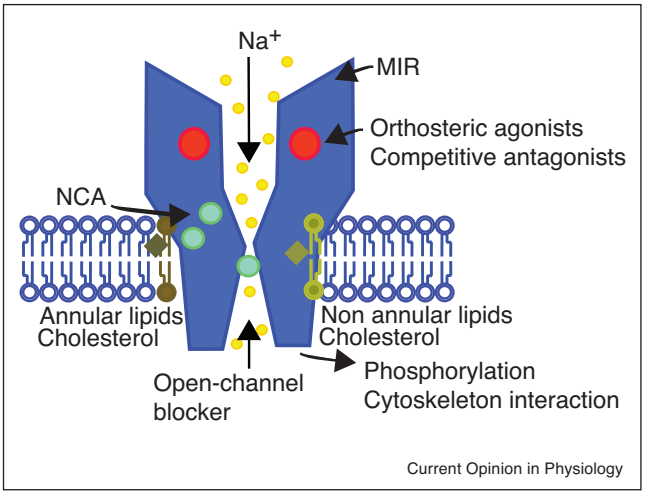
Congenital myasthenic syndromes (CMSs) represent a heterogeneous group of disorders in which the safety margin of neuromuscular transmission is compromised by one or more specific mechanisms. They are caused by genetic defects in presynaptic, synaptic or postsynaptic proteins, glycosylation, or endplate development (reviewed in Engel *et al.* [68•,69]). The nAChR is one of the targets for these diseases. Defects in nAChR subunits may decrease or eliminate expression of nAChR or alter the kinetics of nAChR activation. Syndromes mediated by changes in the kinetics of activation divide further into slow channel CMSs, that show prolonged ACh-mediated postsynaptic responses due to gain-of-function mutations, and fast channel CMSs, that show decreased responses due to loss-of-function mutations. To date, a great number of mutations located at the agonist binding site, the coupling region, the transmembrane region and the cytoplasmic loop of different subunits were identified in patients with slow and fast CMS [68•,70]. Mutations change responses by affecting binding and/or gating steps of the activation process. In general, mutations in slow-channel patients increase agonist affinity, the probability of opening in the absence of agonist, and the opening rate, and/or decrease the closing rate whereas in fast-channel patients, impair opening, increase the closing rate or decrease agonist affinity (Figure 3). A novel congenital myasthenic syndrome due to decreased ion-channel conductance was also reported. This low conductance phenotype resulted from the deletion of a single amino acid within the M2 transmembrane domain [71].

Kinetic analyses of single channel currents of the patient receptors expressed in heterologous systems have been especially powerful to give insight into structure–function relationships and the mechanistic consequences of the mutations, as well as to guide rational therapy. In fact, the treatment of slow and fast channel CMS needs to be achieved by drugs with opposing mechanisms of action.

Pharmacology and modulators of muscle nAChR function

Several compounds can activate, inhibit or modulate nAChR function [72,73] (Figure 4). Apart from ACh, other compounds act as agonists of different efficacies of the muscle nAChR, including nicotine, serum choline, which may be of importance in gain-of-function nAChRs associated to slow-channel CMS [74], carbamycholine, Anatoxin A, succinylcholine, which is used to cause short-term paralysis as part of general anesthesia. Muscle nAChR is allosterically activated by Galantamine, an inhibitor of AChE, which acts as a low-efficacy agonist [75]. The prototype competitive antagonists include

Figure 4



Cartoon of nAChR with sites of modulation. Orthosteric agonists (partial or full) and competitive antagonists bind to the binding pockets at subunit interfaces in the ECD. Non-competitive antagonists (NCA) may bind to the M2 domain (open channel blockers), allosteric sites, and to annual or non-annular lipid domains; lipids located at annular or non-annular sites (phospholipids and cholesterol) modulate function; auto-antibodies associated with myasthenia gravis react with the MIR at the ECD; the ICD is a site for phosphorylation and interaction with cytoskeleton.

α-bungarotoxin, which binds pseudo-irreversible, and d-tubocurarine and pancuronium that are non-depolarizing muscle relaxants.

Muscle nAChRs are modulated by a great variety of different compounds, which may mediate physiological, therapeutic or adverse effects.

A great variety of compounds act as non-competitive antagonists (NCA) that by different molecular mechanisms and sites inhibit muscle nAChR function (see review in Bouzat and Sine [52•]). 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. While many blockers bind and unbind from the open state, others can either allow the blocked channel to close or block closed channels. Channel blockers include compounds such as the neurotransmitters ACh and choline at high concentrations [76], anesthetics [77,78], anthelmintic drugs [79], antipsychotic drugs [80], ephedrine [81], amphetamine [82]. NCAs may also inhibit receptor function by preferentially stabilizing the nAChR in a non-conducting state (resting or desensitized state), or by increasing the rate and extent of desensitization, such as tricyclic antidepressants and adiphenine [83,84]. Mechanistic actions of NCAs evaluated at the single-channel level have been described in Bouzat and Sine [52•]. While most of these actions may involve secondary effects, NCAs can be of therapeutic benefit. 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 nAChR in slow-channel CMS [85,86]. In this regard, by using a transgenic mouse model of a slow-channel CMS, it was shown that ephedrine enhanced neuromuscular transmission, suggesting that it may be an appropriate additional therapy for this syndrome [87].

The annular (surrounding the perimeter of the receptor) and non-annular lipid domains (between transmembrane helices and subunits) are sites for a great variety of hydrophobic NCAs that by different mechanisms inhibit function. These compounds include, among others, free fatty acids and a great variety of steroids [88–90].

Given its transmembrane nature, the nAChR establishes close physical contact with lipids, which have a modulatory functional role [91]. Lipids/membranes influence nAChR function by both conformational selection and kinetic mechanisms [92]. The M4 transmembrane domain may act as a lipid sensor [7,92] and mutations in lipid exposed amino acids at this domain alter channel kinetics [45,93].

The intracellular M3M4 loop contains tyrosines whose phosphorylation has been shown to have a role in desensitization [94] and receptor stability [95]. It is involved in stabilization at the NMJ via a cytoskeleton-dependent mechanism mainly through rapsyn, which is a 43-kD protein that associates with nAChR subunits and whose deletion abolishes nAChR clustering and leads to diseases [20,96–98].

Concluding remarks

The nAChR plays a key role in the functioning of the neuromuscular synapse. Its fundamental mechanism of operation has been thoroughly studied over the last decades by a wide range of experimental approaches. Single-channel recordings and kinetic analysis have given insight into structure-function relationships, have provided information about how the receptor moves through different states in response to the neurotransmitter, and have revealed the molecular bases of human diseases and the mechanisms of drug modulation. In the near future, the combination of high resolution 3D structures of muscle wild-type and mutant nAChRs at defined conformational states with single-channel kinetic analysis will pave the way for rational drug design directed to targeting a specific conformational state. The detection of novel briefer intermediate states, achieved by improving patch clamp temporal resolution, will constitute an essential step toward deciphering the highly complex receptor activation mechanism.

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Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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