

9 The Ionotropic Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptor (nAChR) is a glycoprotein present at nicotinic cholinergic synapses. The preparation that has been used most extensively to study the nicotinic receptor is the electric organ of the electric ray, *Torpedo* (*Torpedo* nAChR; **Figure 9.1a**), or of the electric eel. In part because this preparation is extremely rich in nicotinic receptors, and because snake venom α -toxins had been identified as highly

selective markers of nAChRs. In mammals, nAChRs have been mostly studied at the neuromuscular junction (muscle nAChR; **Figure 9.1b**) but also in the peripheral nervous system (synapses between pre- and post-ganglionic neurons of the autonomic nervous system), and more recently in the central nervous system where they are also present (neuronal nAChR; see Appendix 9.1).

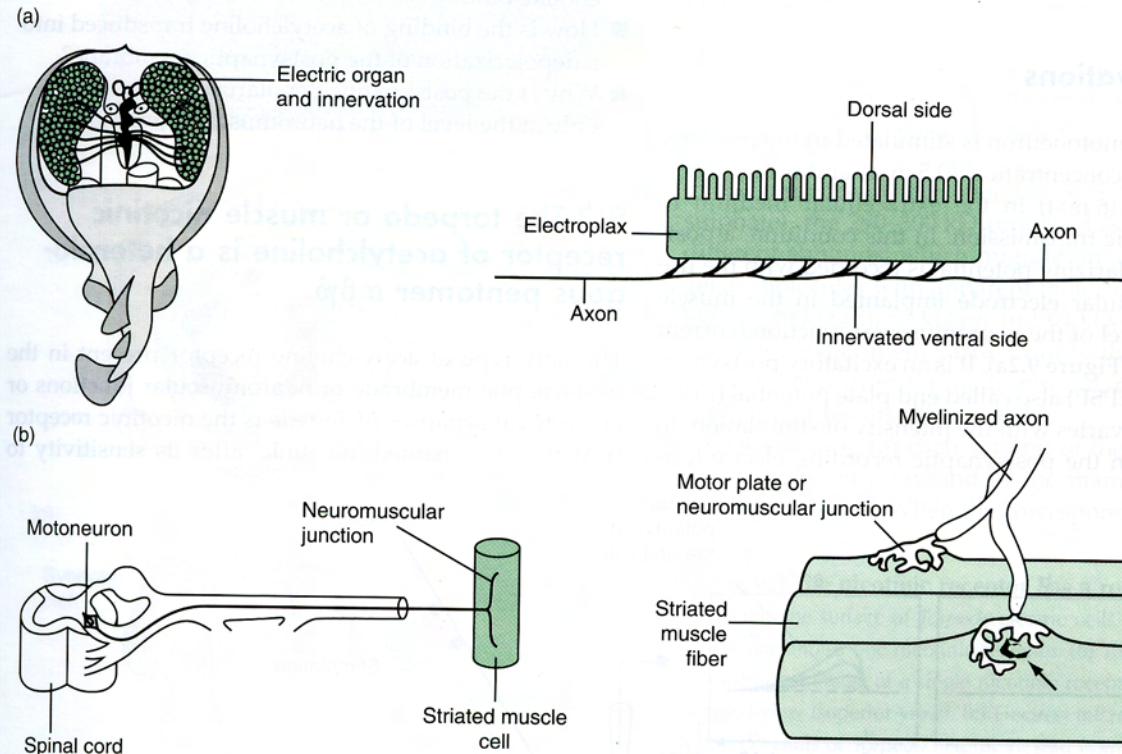


Figure 9.1 Examples of preparations in which nicotinic receptors have been extensively studied.

(a) The electric organ of the electric ray. On a dissected *Torpedo* (left) we can see the electric organs and their innervation. These organs constitute electroplax membranes (right) which are modified muscle cells that do not contract. Nicotinic receptors are present at the command neuron's synapse level, on the ventral side of the postsynaptic membrane of the electroplax. The electroplax are simultaneously activated and the summation of their electric discharges can be of the order of 500 V. (b) The neuromuscular junction. Striated muscle cells are innervated by motoneurons whose cell bodies are located in the ventral horn of the spinal cord (horizontal section, left). In mammals, each muscle cell is innervated by one nerve fibre. As the axon makes contact with the muscle cell, it loses its myelin sheath and divides into several branches that are covered by unmyelinated Schwann cells. The thick arrow (right) points to one terminal that has been lifted to show the postsynaptic folds where nicotinic receptors are located.

The nicotinic acetylcholine receptor is a pentamer composed of transmembrane subunits from a repertoire of 16 known different types referred to as $\alpha 1$ – $\alpha 9$, $\beta 1$ – $\beta 4$, γ , δ and ϵ . Each pentamer presents two acetylcholine receptor sites on its surface, contains the elements to form an ionic channel, and contains all the necessary structural elements for the required interactions between the different functional domains. The acetylcholine receptor sites and the ionic channel controlled by acetylcholine are, therefore, part of the same protein: the nicotinic receptor is a receptor channel (also called an ionotropic receptor).

The nicotinic receptors present in the *Torpedo* and neuromuscular synapses shown in **Figure 9.1a and b** have comparable structures and functions. For this reason they are presented simultaneously throughout this chapter. Acetylcholine also activates another type of receptor, the muscarinic receptors, also termed metabotropic cholinergic receptors (mAChR) which belong to the family of receptors linked to G-proteins. They will not be studied in this book.

9.1 Observations

The axon of a motoneuron is stimulated in the presence of a low Ca^{2+} concentration (0.5 mM) and a high Mg^{2+} concentration (6 mM) in the extracellular medium to reduce synaptic transmission. In this condition, a postsynaptic depolarizing potential is recorded with the use of an intracellular electrode implanted in the muscle fibre at the level of the neuromuscular junction (current clamp mode) (**Figure 9.2a**). It is an excitatory postsynaptic potential (EPSP) also called end plate potential (EPP). Its amplitude varies with the intensity of stimulation. In contrast, when the postsynaptic recording electrode is

far from the end plate, no response is recorded (**Figure 9.2b**).

The low Ca^{2+} and high Mg^{2+} concentrations in the extracellular medium is a necessary condition to avoid muscle fibre contraction during recording. The number of active zones per neuromuscular junction is in fact so high (around 300 to 1000) that a presynaptic axonal spike always triggers a very large EPP that in turn depolarizes the muscle membrane to the threshold potential for voltage-gated Na^+ channels opening and thus evokes a postsynaptic action potential (not shown) and muscle fibre contraction. The elimination of the muscle fibre action potential by lowering the release of acetylcholine from presynaptic terminals allows one to record the EPP in isolation.

Questions

- When released in the synaptic cleft, to which type(s) of postsynaptic receptor do the molecules of acetylcholine bind?
- How is the binding of acetylcholine transduced into a depolarization of the postsynaptic membrane?
- Why is the postsynaptic depolarization recorded only at the level of the neuromuscular junction?

9.2 The torpedo or muscle nicotinic receptor of acetylcholine is a heterologous pentamer $\alpha_2\beta\gamma\delta$

The only type of acetylcholine receptor present in the postsynaptic membrane of neuromuscular junctions or at electrical synapses of *Torpedo* is the nicotinic receptor (nAChR). It is named 'nicotinic' after its sensitivity to

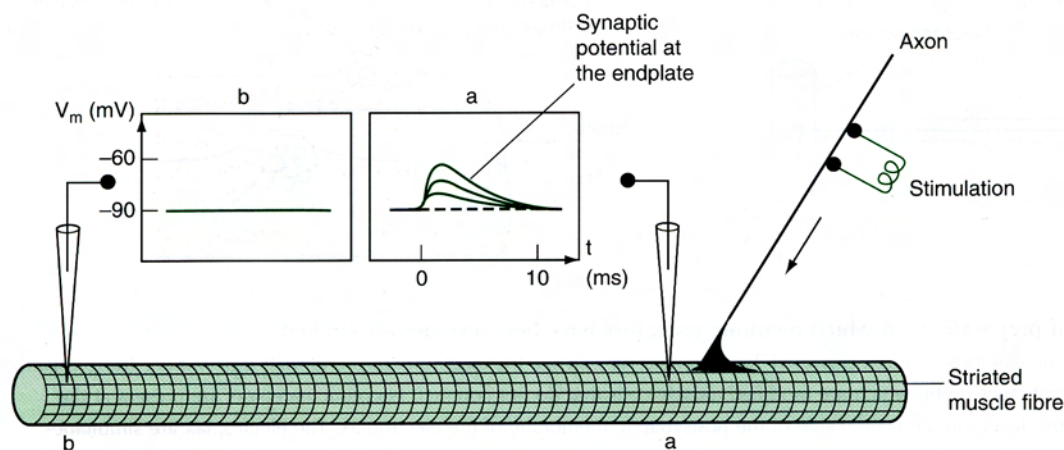


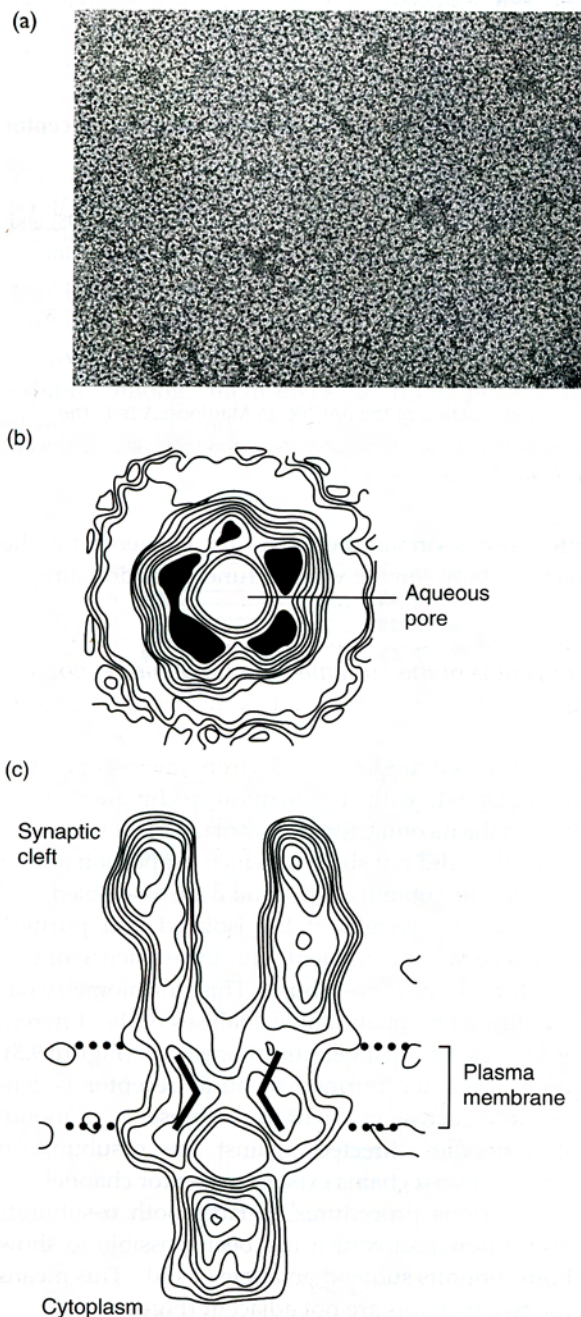
Figure 9.2 The endplate potential.

The axon of a motoneuron is stimulated and the postsynaptic response is recorded in the presence in the bath of a low Ca (0.5 mM) and a high Mg (6 mM) concentration. Two intracellular electrodes are implanted in the muscle fibre, one close to the neuromuscular junction (**a**) and the other more than 5 mm away (**b**). The evoked response is a transient depolarization called an endplate potential.

nicotine (nicotine is an agonist of nAChR).

9.2.1 Nicotinic receptors have a rosette shape with an aqueous pore in the centre

Under the electron microscope, the nicotinic receptor of the neuromuscular junction, located in the postsynaptic muscular membrane, has a rosette shape with an 8–9 nm diameter and a central depression of diameter 1.5–2.5 nm. This depression corresponds to the channel portion of the protein (**Figure 9.3**). Each rosette is made up of five regions of high electronic density arranged



around an axis perpendicular to the plane of the plasma membrane. In transverse section the rosette appears as a cylinder 11 nm long, extending beyond each side of the membrane (6 nm towards the synaptic cleft and 1.5 nm towards the cytoplasm).

9.2.2 The four subunits of the nicotinic receptor are assembled as a pentamer $\alpha_2\beta\gamma\delta$

The nicotinic receptor is normally purified from the electric organ of *Torpedo* or the electric eel. A 290–300 kD glycoprotein is obtained when this purification is performed on an affinity column using an agarose bound cholinergic ligand (**Figure 9.4**). When this glycoprotein is incorporated into a planar lipid bilayer or into lipid vesicles, it presents the same functional characteristics as the native receptor: if acetylcholine is present in the extracellular side at a concentration of 10^{-5} – 10^{-4} mol l $^{-1}$, it allows the passage of cations across the bilayer.

The nicotinic receptor is composed of four glycopolypeptide subunits $\alpha, \beta, \gamma, \delta$

In the presence of the detergent SDS (sodium dodecyl sulphate), the 290–300 kD protein dissociates into four different subunits, which migrate on a polyacrylamide gel as molecules with apparent molecular weights of 38 kD (α), 49 kD (β), 57 kD (γ) and 64 kD (δ) (**Figure 9.5a**). The same experiment carried out with nicotinic receptors obtained from the neuromuscular junction shows very similar results (**Figure 9.5b**).

Genes coding for each subunit of the nicotinic receptor of the electric ray and of the mammalian receptor have been cloned. When the corresponding mRNAs are

Figure 9.3 The nicotinic receptor has a rosette shape.

(a) Membrane surface of *Torpedo* electric cells (electroplax). Each rosette constitutes one nicotinic receptor. (b) This computer reconstructed image of a single nicotinic receptor provides a more detailed view (superior view). (c) Electron microscopic analysis of tubular crystals of *Torpedo* nAChR viewed from the side. Part (a) from Cartaud J, Benedetti EL, Sobel A, Chargeux JP (1978) A morphological study of the cholinergic receptor protein from *Torpedo narmorata* in its membrane environment and in its detergent-extracted purified form, *J. Cell Sci.* **29**, 313–337, with permission. Part (b) from Bon F *et al.* (1982) Orientation relative de deux oligomères constituant la forme lourde du récepteur de l'acétylcholine chez la torpille marbrée, *C. R. Acad. Sci.* **295**, 199, with permission. Part (c) from Unwin N (1993) The nicotinic acetylcholine receptor at 9 Å resolution, *J. Mol. Biol.* **229**, 1101–1124, with permission.

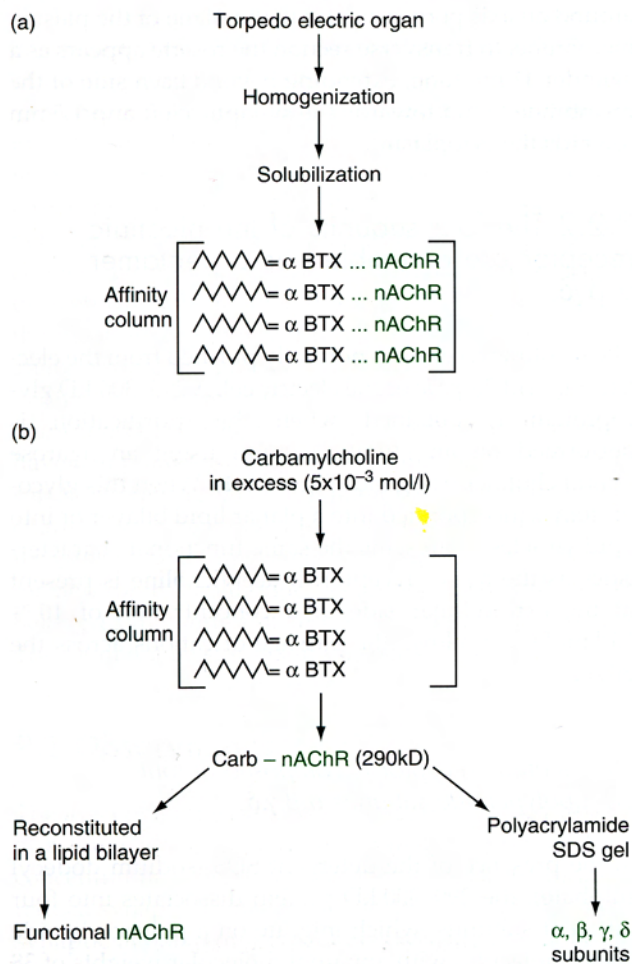


Figure 9.4 Stages of affinity column purification of the nicotinic receptor.

(a) The electric organ of the electric ray is homogenized and membrane proteins solubilized. The resulting extract is run through an affinity column, on to whose sepharose (^^^=) a nicotinic cholinergic ligand α -bungarotoxin (α -BTX) has been covalently bound. Owing to their affinity to α -BTX, the nicotinic receptors bind to it. (b) In order to recover the nicotinic receptors, another nicotinic ligand, carbamylcholine (Carb), is run in excess through the column to displace the binding of α -BTX to the receptor. Carbamylcholine-bound nicotinic receptor is obtained at the outflow of the column. Carbamylcholine is eliminated by dialysis and the nicotinic receptor is thus obtained in an isolated form. The nicotinic receptor can then be reincorporated into a lipid bilayer to study its functional characteristics. It may also be treated with a detergent (SDS) to dissociate its subunits.

injected into *Xenopus* oocytes, functional nicotinic receptors are synthesized and incorporated into the oocyte membrane. It has, therefore, been confirmed that the subunits α, β, γ and δ are sufficient to obtain a functional nicotinic receptor containing the acetylcholine receptor sites and the elements that form the ionic channel. The

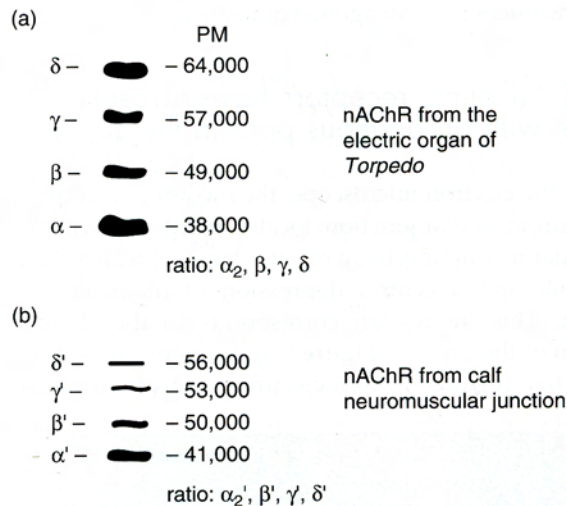


Figure 9.5 Separation of the different nicotinic receptor subunits on a gel.

The subunits of the purified nicotinic receptor have been dissociated with the detergent sodium dodecyl sulphate (SDS) and separated on a polyacrylamide gel: subunits of (a) the nicotinic receptor of electric organ, and of (b) the calf neuromuscular junction. The four subunits obtained, $\alpha, \beta, \gamma, \delta$ and $\alpha', \beta', \gamma', \delta'$, have similar molecular weights. From Anholt R, Lindstrom J, Montal M (1984) The molecular basis of neurotransmission: structure and function of the nAChR. In Martinosi A (ed) *The Enzymes of Biological Membranes*, New York: Plenum Press, with permission.

receptor also contains the necessary elements for the interactions between the various functional domains.

Organization of the subunits: the nicotinic receptor model

Images observed under the electron microscope, and results obtained with biochemical techniques, have shown that the nicotinic receptor is made up of five units. However, they did not show in which proportion and in which order the subunits α, β, γ and δ are assembled.

The molecular weight of the isolated and purified nicotinic receptor is consistent with the existence of two α chains for each β, γ or δ -subunit. This stoichiometry has been confirmed by quantitative analyses of the different chains extracted from an electrophoresis gel (Figure 9.5). Likewise, when the purified nicotinic receptor is centrifuged in a sucrose gradient in the presence of monoclonal antibodies directed against the α -subunit, it appears that two α chains exist per receptor channel.

Using various procedures to label both α -subunits (notably α -bungarotoxin) it has been possible to show that both subunits subtend an angle of 150° . This means that the two α chains are not adjacent (Figure 9.6a).

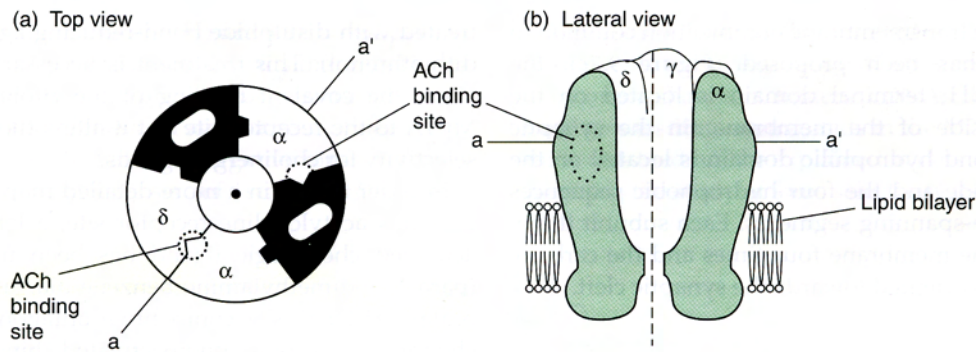


Figure 9.6 Nicotinic receptor (nAChR) model.

(a) Viewed from above, the nAChR has a rosette shape. Both α -subunits carry one acetylcholine (ACh) receptor site each. The subunits are attached to each other by non-covalent interactions. $\alpha\beta\alpha\gamma$ or $\alpha\gamma\alpha\beta$ are the two possible orders of the subunits around the symmetrical axis. (b) Profile view of the nAChR *in situ* (cut along the plane aa'). Adapted from Changeux JP, Revah F (1987) The acetylcholine receptor molecule: allosteric sites and the ion channel, *TINS* 10, 245–250, with permission.

9.2.3 Each subunit presents two main hydrophilic domains and four hydrophobic domains

The amino acid sequence of each subunit α , β , γ and δ has been deduced from the corresponding DNA nucleotide sequence. This has shown that the subunits exhibit among themselves a high percentage of sequence homology and a very similar organization (Figures 9.7a and b). For each subunit one finds:

- an NH_2 terminal region which forms a large hydrophilic domain of 210–224 amino acids and carries the glycosylation sites;
- it is followed towards the COOH end by three hydrophobic sequences (M1, M2 and M3) of 20–30 residues each with short connecting hydrophilic loops;
- a second large hydrophilic domain of about 150 residues containing functional phosphorylation sites;
- a fourth hydrophobic sequence and a short carboxy terminal tail.

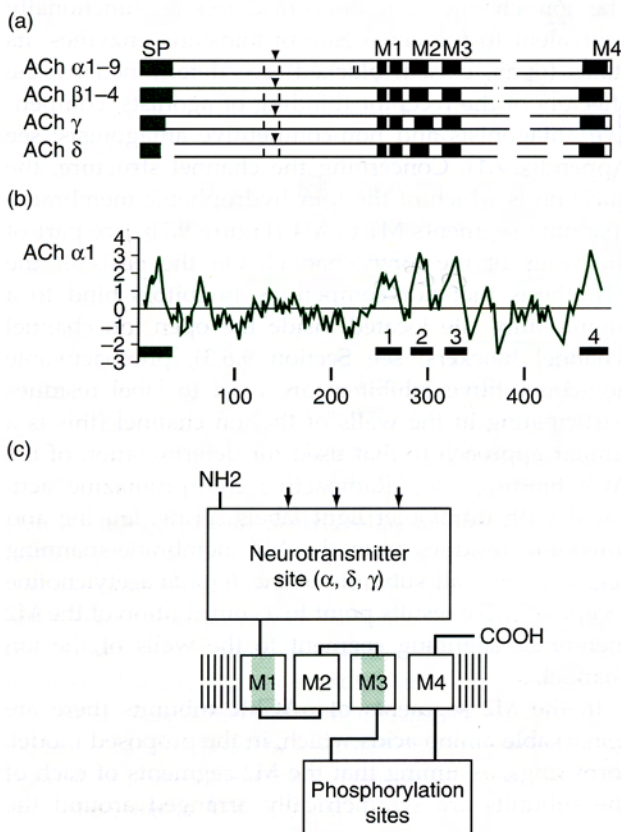


Figure 9.7 Diagrammatic representation of the primary structure of nicotinic acetylcholine receptor subunits from electric organ, muscle cells and neurons.

(a) The sequences are aligned to bring homologous regions into phase; gaps in sequences are indicated by blank spaces (polypeptide lengths are normalized), and cysteinyl residues by vertical bars. SP, signal peptide; α , β , γ and δ chains. One can distinguish two hydrophilic domains, the largest of which is located in the NH_2 terminal, and four hydrophobic segments M1, M2, M3 and M4. This observation has led to the assumption that all four subunits have identical transmembrane organizations. (b) Hydropathy plot of the α_1 -subunit from *Torpedo* electric organ receptor showing the four hydrophobic segments assumed to span the lipid bilayer, the large hydrophilic amino terminal domain and the hydrophilic domain separating M3 from M4. (c) Model of subunit transmembrane organization. The large hydrophilic amino-terminal domain of receptor subunits is exposed to the synaptic cleft and carries the neurotransmitter site and glycosylation sites (arrows). Each subunit spans the membrane four times (M1, M2, M3 and M4). The hydrophilic domain separating M3 from M4 faces the cytoplasm. It contains functional phosphorylation sites. Adapted from Changeux JP (1994) Functional architecture and dynamics of the nicotinic acetylcholine receptor: an allosteric ligand-gated ion channel: *Fidia Research Foundation Neuroscience Award Lecture*, New York: Raven Press, with permission.

A model of the transmembrane organization common to all subunits has been proposed (**Figure 9.7c**): the hydrophilic NH_2 terminal domain is located on the extracellular side of the membrane (in the synaptic cleft), the second hydrophilic domain is located on the cytoplasmic side and the four hydrophobic sequences are membrane-spanning segments. Each subunit therefore crosses the membrane four times and the carboxy terminal tail is oriented towards the synaptic cleft.

9.2.4 Each α -subunit contains one acetylcholine receptor site located in the hydrophilic NH_2 terminal domain

Before the structure of the nicotinic receptor was known, it had been demonstrated that two acetylcholine molecules had to be bound to the receptor in order to initiate an ionic flux (see Section 9.3). It seemed logical that these two sites had to be located on identical subunits; i.e. one on each α -subunit. Additionally, based on the organization of the hydrophilic sequences (**Figure 9.7b**), it was proposed that this site is located in the large hydrophilic NH_2 terminal domain which is exposed to the synaptic cleft.

This proposal has been confirmed by covalent binding studies of cholinergic agonists on α -subunits, isolated either from nicotinic receptor-rich membranes, or expressed in frog oocytes from the corresponding mRNA. Of the four subunit types α , β , γ and δ , the α -subunits have been shown to be the main contributors to cholinergic agonist binding.

The next step was to determine which amino acids are part of the acetylcholine receptor site. To this end, labelled cholinergic ligands are used. These ligands are able to bind covalently to the acetylcholine receptor sites. One of the most used is MBTA (4-(*N*-maleimido)benzyl trimethylammonium iodide) which binds covalently to α -subunit receptor sites after reduction of disulphide bridges. Once labelled, the α chain is sequenced and the labelled regions identified. In this way a region containing cysteines 192 and 193 was identified and proposed as one of the potential sites of interaction with cholinergic ligands (**Figure 9.8**).

Other data have provided additional evidence of the participation of cysteine residues 192 and 193 in the acetylcholine receptor site. In the first place, these cysteine residues are present only in the α -subunits. Furthermore, when frog oocytes are injected with mRNA coding for α -subunits that have been mutated at the level of cysteines 192 and 193 (serines replaced for cysteines), the α -subunits obtained are unable to bind cholinergic ligands.

However, all these results have the shortcoming of having been obtained from preparations previously

treated with disulphide bond-reducing agents (such as dithiothreitol). This treatment is necessary in order to allow the covalent binding of the cholinergic ligand MBTA to the receptor site but it alters the receptor site selectivity for cholinergic ligands.

In order to obtain a more detailed map of the native protein's acetylcholine receptor site, a labelled photoactivated cholinergic ligand has been used: ^3H -DDF (para-*N,N*-dimethylamino benzene diazonium fluoroborate). ^3H -DDF is a competitive antagonist of acetylcholine which, once photoactivated, binds covalently (irreversibly) to the acetylcholine receptor sites. This reaction is carried out on the whole nicotinic receptor channel and the α -subunits are then isolated, the segments labelled by ^3H -DDF are purified and their sequence analysed. This led to the demonstration that the residues tyr 93, trp 149, tyr 190, cys 192 and cys 193, all labelled by ^3H -DDF, are part of the acetylcholine receptor site. This labelling is in fact inhibited by other nicotinic agonists and competitive antagonists (**Figure 9.8b**). This result is valid for the nicotinic receptor of the electric organ as well as for that of the neuromuscular junction.

9.2.5 The pore of the ion channel is lined by the M2 transmembrane segments of each of the five subunits

The ion channel can be considered as functionally equivalent to the active site of allosteric enzymes: its states (open, closed, blocked) are determined by the effectors of the receptor (binding of agonists, competitive antagonists and non-competitive antagonists; see Appendix 7.1). Concerning the channel structure, the question is which of the four hydrophobic membrane-spanning segments M1 to M4 (**Figure 9.7b**) are part of the walls of the ionic channel. On the basis of the hypothesis that non-competitive inhibitors bind to a high-affinity site located inside the open ion channel (channel blockers; see Section 9.6.3), photoactivable non-competitive inhibitors are used to label residues participating in the walls of the ion channel (this is a similar approach to that used for determination of the ACh binding site). Radioactive chlorpromazine activated with ultraviolet light labels serine, leucine and threonine residues from the M2 membrane-spanning segment from all subunits of the *Torpedo* acetylcholine receptor. These results point to a contribution of the M2 membrane-spanning segment to the walls of the ion channel.

In the M2 segments of nAChR subunits there are remarkable amino acids which, in the proposed model, form rings, assuming that the M2 segments of each of the subunits are symmetrically arranged around the

central axis of the molecule (**Figure 9.9**): a cytoplasmic ring of negatively charged amino acids that repel negative ions, a hydrophobic ring of leucines, a ring of serines, a ring of threonines and again a ring of negatively charged amino acids that repel negative ions.

Site-directed mutagenesis of some amino acids

located in the M2 segment confirmed the contribution of M2 segments to the regulation of ion transport through the nicotinic channel. Chimeric cDNAs are constructed to add or substitute amino acids in the M2 segment. The results obtained will be explained in detail in the section describing the study of ionic selectivity (Section 9.3.2).

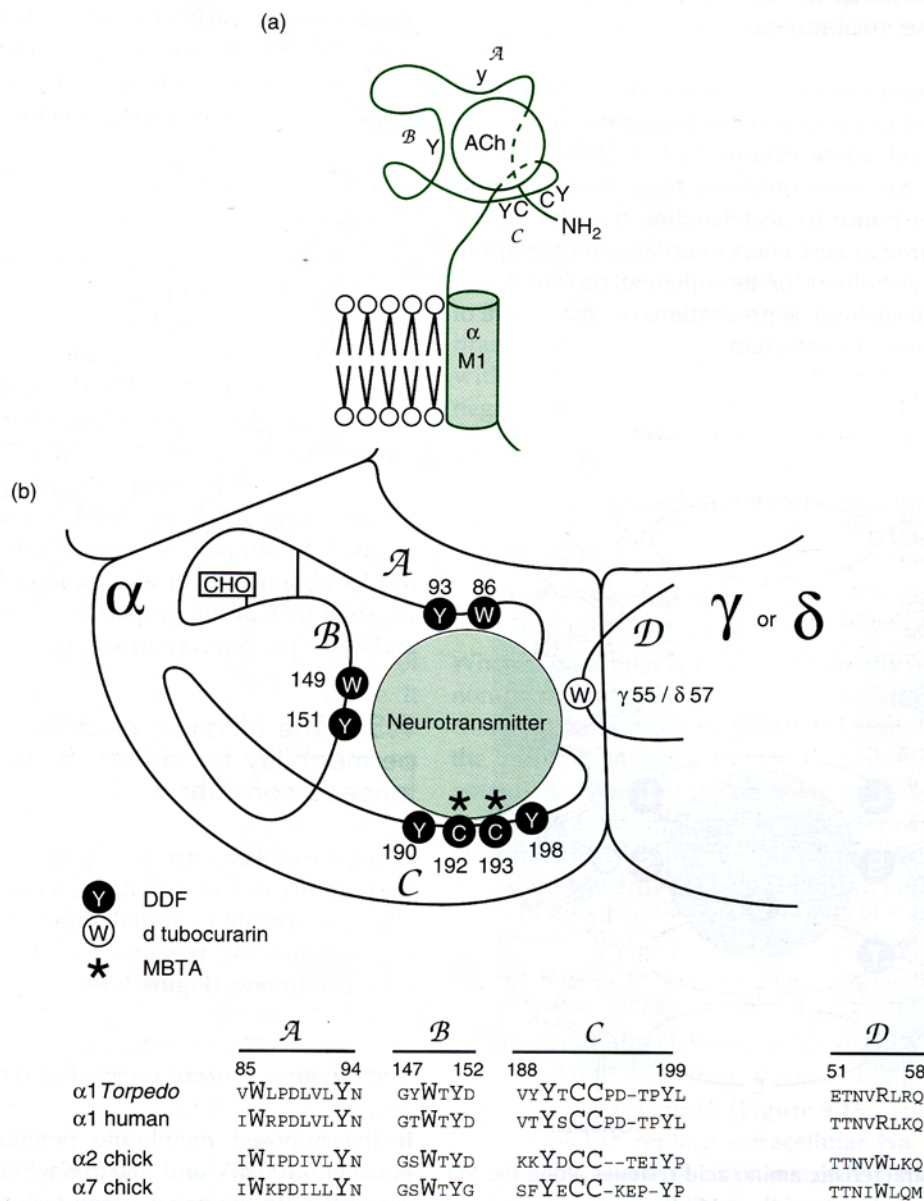


Figure 9.8 Model of the acetylcholine binding site.

(a) The amino acids labelled with the nicotinic antagonist ^3H -DDF are localized on different regions of the α -chain. In the three-dimensional structure of the protein, these regions fold into positions that are close to each other. (b) More precise view of the labelling of the receptor site. The acetylcholine binding site is located at the interface between α - and γ - or δ -subunits. The amino acids covalently labelled with ^3H -DDF are in the α -subunit: W86 and Y93 in loop A, W149 and Y151 in loop B, Y190, C192, C193 and Y198 in loop C. d-Tubocurarine, an antagonist of nAChR, labels one amino acid in loop D of the γ - or δ -subunit. C, cysteine; W, tryptophan, Y, tyrosine.

Drawing (a) adapted from Dennis M, Giraudat J, Kotzbya-Hibert F *et al.* (1988) Amino acids in the *Torpedo marmorata* acetylcholine receptor α subunit labelled by a photoaffinity ligand for the acetylcholine binding site, *Biochemistry* **27**, 2346–2357, with permission.

Drawing (b) adapted from Galzi JL, Changeux JP (1994) Neurotransmitter-gated ion channels as unconventional allosteric proteins, *Curr. Opin. Struct. Biol.* **4**, 554–565, with permission.

9.3 Binding of two acetylcholine molecules favours conformational change of the protein towards the open state of the cationic channel

9.3.1 Demonstration of the binding of two acetylcholine molecules

It has been demonstrated that two acetylcholine molecules must bind to the receptor to trigger the opening of the channel and allow cations to flow through. The proof of this has been obtained from dose-response curves. The response to acetylcholine (i.e. the flux of cations measured at very short intervals after the application of acetylcholine), or the opening probability of the channel (see below), is proportional to the square of the acetylcholine concentration:



where R is the nicotinic receptor in its closed configuration; R* is the nicotinic receptor in its open configuration; and A is acetylcholine.

The rate of isomerization between R and R* lies in the microsecond to millisecond timescale. The passage of cations through the open channel is the result of the conformational change ($A_2R \rightleftharpoons A_2R^*$). Electrophysiological techniques (patch clamp recordings of the unitary cationic current flowing through a single channel) can be used to study this flow of cations. Based on the results obtained with electrophysiological techniques, we shall look at the properties of the nicotinic channel and of the protein conformational changes.

9.3.2 The nicotinic channel has a selective permeability to cations: its unitary conductance is constant

When the unitary current crossing a nicotinic channel in the presence of acetylcholine is recorded in patch clamp, all the preparations tested show an inward current at negative holding potentials (and under physiological ionic conditions) (Figure 9.10).

The nicotinic current reverses at 0 mV

If the imposed membrane potential (V_m) is varied between -100 mV and +80 mV while recording unitary currents with the patch clamp technique (i_{ACh}), one can trace an i_{ACh}/V curve (Figure 9.10). This curve is approximately linear between -80 and +80 mV. The measured current is inward for negative voltages and outward for positive voltages.

The i_{ACh}/V curve crosses the voltage axis at a value where the current is zero. This value is called the *reversal potential of the nicotinic response*, or E_{ACh} . The value of this reversal potential is close to 0 mV in the experimental conditions of Figure 9.11 but may vary slightly towards negative voltages depending on the preparation.

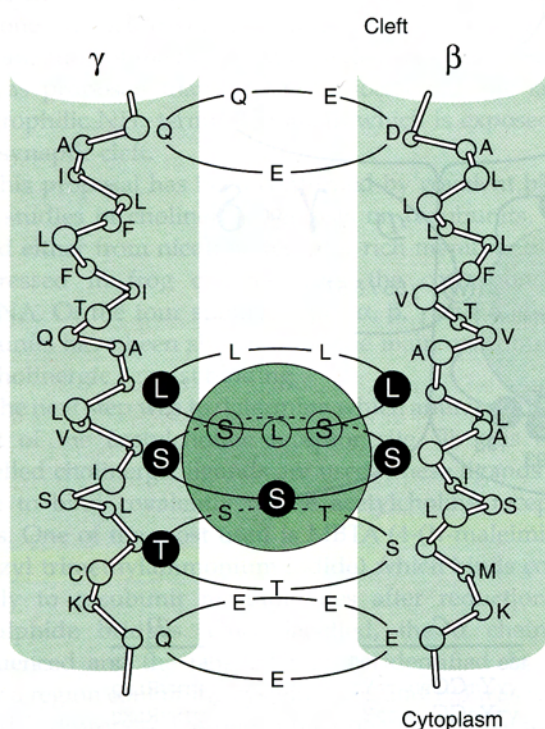


Figure 9.9 Characteristic amino acid residues along the M2 segment of the subunits of the nAChR.

The M2 membrane-spanning segments are symmetrically arranged around the central axis of the molecule (two of them are represented). The relative position of the α -carbons of the amino acids is shown as one-letter code. E, glutamic acid, S, serine, T, threonine, L, leucine, Q, glutamine. Adapted from Revah F, Galzi JL, Giraudat J *et al.* (1990) The noncompetitive blocker [^3H]-chlorpromazine labels three amino acids of the acetylcholine receptor γ subunit: implications for the α -helical organization of the M2 segments and the structure of the ion channel, *Proc. Natl Acad. Sci. USA* **87**, 4675–4679, with permission.

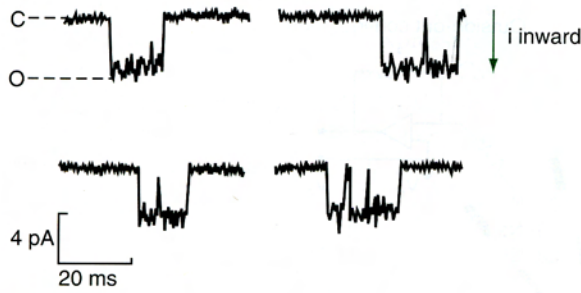


Figure 9.10 Patch-clamp recording of nicotinic receptor activity in rat sympathetic neurons.

Channel activity is recorded in the attached-cell configuration. While the membrane is kept at a negative potential an inward current is recorded in the presence of acetylcholine under physiological conditions. C, closed channel; O, open channel. Adapted from Colquhoun D, Ogden DC, Mathie A (1987) Nicotinic acetylcholine receptors of nerve and muscle: functional aspects, *TIPS* 8, 465–472, with permission.

The unitary conductance is constant

The linear i_{ACh}/V relationship observed in **Figure 9.11b** (between -80 and $+80$ mV) is described by the equation $i_{\text{ACh}} = \gamma_{\text{ACh}}(V_m - E_{\text{ACh}})$, where V_m is the membrane potential, E_{ACh} is the reversal potential of the nicotinic response, and γ_{ACh} is the conductance of a single nicotinic channel, or its unitary conductance. The value of γ_{ACh} is given by the slope of the linear i_{ACh}/V curve. It has a constant value at any given membrane potential. This value varies between 35 and 55 pS depending on the preparation and is a fundamental property of a nicotinic channel.

The nicotinic channel is a cationic channel

The reversal potential of the nicotinic response ($E_{\text{ACh}} = 0$ mV) does not correspond to the equilibrium potentials of any of the ions in solution (**Figure 9.11**). It is not an Na^+ channel because, in the experimental conditions of **Figure 9.11**, $E_{\text{Na}} = 58 \log(160/3) = +100$ mV. It is not a K^+ channel either, since $E_{\text{K}} = 58 \log(3/160) = -100$ mV. And it is not a Cl^- channel because, if the chloride ions are replaced by large anions that cannot cross the channel, such as SO_4^{2-} , no reversal potential change of the nicotinic response is observed.

By performing extracellular ionic substitution experiments, it has been shown that the nicotinic channel is permeable to Na^+ , K^+ , Ca^{2+} and Mg^{2+} ions. However, Ca^{2+} and Mg^{2+} ions contribute only a small fraction to the nicotinic current, which is essentially due to the flux of Na^+ and K^+ ions through the open channel. If different cations cross the same channel and have similar permeabilities, we define:

$$E_{\text{cations}} = 58 \log ([\text{cations}]_e / [\text{cations}]_i),$$

where $[\text{cations}] = [\text{Na}^+] + [\text{K}^+]$. In our case we obtain $E_{\text{cations}} = 0$ mV. In other words, $E_{\text{cations}} = E_{\text{ACh}} = 0$ mV.

In what direction do Na^+ and K^+ ions cross the open nicotinic channel at different membrane potentials?

When the membrane is at a voltage of -80 mV, the Na^+ ion driving force is inward and equal to -180 mV, while the K^+ ion driving force is outward and equal to $+20$ mV (**Figure 9.12**). If channels open, more Na^+ will enter the cell than K^+ ions will leave it. The net flux of positively charged ions is, thus, inward: an inward current is recorded (**Figures 9.10** and **9.11a**).

If the same reasoning is followed for different membrane potential values, the same result is obtained as with the i_{ACh}/V curve: the unitary current is inward for negative membrane potentials (net flux of positive charges is inward), and the unitary current is outward for positive membrane potentials (net flux of positive charges is outward) (**Figures 9.11a** and **9.13**).

Effect of a decrease in $[\text{Na}^+]_e$

When extracellular Na^+ ions are partially replaced with a non-permeant substance such as sucrose (without changing the osmotic pressure), the reversal potential of the nicotinic response shifts towards more negative potentials (**Figure 9.14**). This is explained by the fact that, at this point, extracellular Na^+ ions contribute less to the nicotinic current, and the nicotinic reversal potential E_{ACh} shifts towards the K^+ equilibrium potential (E_{K}).

Substitution of K^+ ions for extracellular Na^+ ions

When almost all extracellular Na^+ ions are replaced by extracellular K^+ ions, the I/V curve obtained superposes on the control I/V curve (**Figure 9.15**). Thus, extracellular K^+ ions can replace extracellular Na^+ ions; i.e. the nicotinic channel does not distinguish between Na^+ and K^+ ions. In other words, it presents similar permeabilities for both ions. For this reason the reversal potential of the nicotinic response is independent of the relative concentrations of extracellular Na^+ and K^+ ions. It depends solely on the sum of these concentrations.

Mutations in the M2 membrane-spanning segment can convert ion selectivity from cationic to anionic

The question was: do substitutions and/or additions of

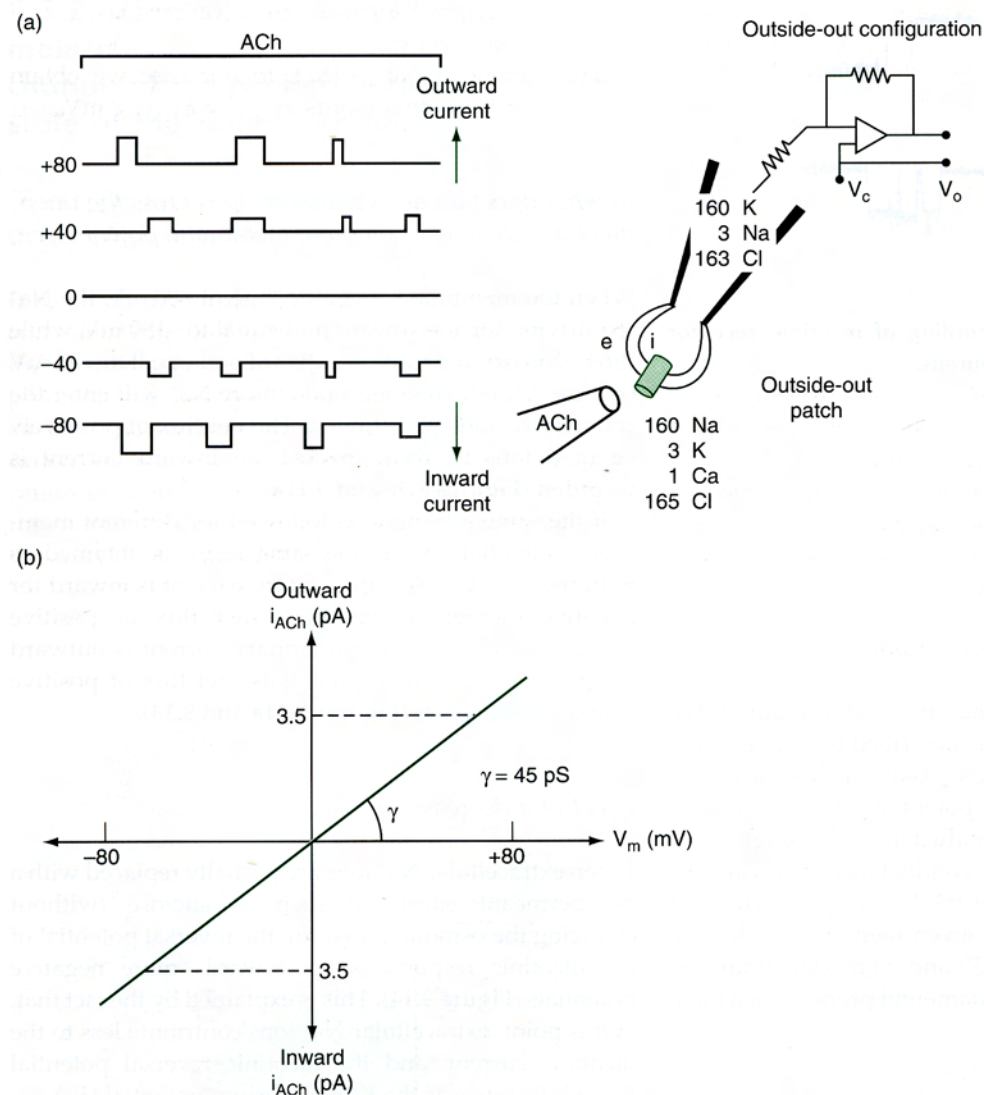


Figure 9.11 Nicotinic unitary current recorded in patch clamp (outside-out configuration) at various membrane potentials (from -80 mV to +80 mV).

(a) Nicotinic unitary current recorded at different membrane potentials in response to the application of acetylcholine (ACh). A downward deflection indicates an inward current and an upward deflection indicates an outward current. (b) i_{ACh}/V curve obtained from the average values of i_{ACh} at each membrane potential V_m . The curve reverses at 0 mV and the slope corresponds to the unitary conductance γ .

amino acids within (or near) the M2 segment from a nicotinic α -subunit (here neuronal α_7 -subunit) with homologous amino acids of the glycine receptor suffice to convert α subunit ion-channel selectivity from cationic to anionic? (The glycine receptor is a receptor channel selectively permeable to anions, Cl^- ions). The M2 sequences of α -subunits of a cationic channel (nAChR) and anionic channels (GlyR and GABA_AR) show similarities at the level of the threonine (244) and leucine (247) rings and differences at the level of rings of negative amino acids, Glu 237 and Glu 258 (Figure 9.16a). A chimeric cDNA encoding the α_7 -subunit of neuronal nAChR is constructed in which in the M2 segment, a pro-

line residue, is added at position 236 bis, and amino acids at positions 237, 240, 251, 254, 255 and 258 are exchanged with those found in the M2 segment of the glycine receptor α -subunit (nAChR α_7^* ; Figure 9.16a). Interestingly, glutamates (E) 237 and 258 which form negative rings in the nAChR (repelling negative ions) are exchanged with alanine (A) and asparagine (N) residues, respectively. The chimeric cDNA is injected into oocytes that express homomeric mutated nAChR* (formed by five identical mutated α_7 -subunits). The current recorded with double-electrode voltage clamp (see Appendix 5.2) is compared with that recorded in oocytes expressing wild-type (non-mutated) homomeric AChR.

Ionic currents recorded in response to ACh application ($100\ \mu\text{M}$, 2 s duration) from oocytes expressing wild-type homomeric $\alpha_7\text{nAChR}$ (in the presence of a Ca^{2+} chelator inside the oocyte) reverses around $+3\text{ mV}$. Substitution of 90% of the external chloride ions did not change the value of the reversal potential. These data thus support the con-

clusion that wild-type homomeric αnAChR , like native nAChR, is selective for cations.

Ionic currents recorded in response to ACh application ($100\ \mu\text{M}$, 2 s duration) from oocytes expressing mutated homomeric $\alpha^*\text{nAChR}$ reverses around -20 mV . Substitution of 90% external chloride ions by isethionate (an impermeant anion) shifts the reversal potential towards positive voltage (around $+30\text{ mV}$) (**Figure 9.16b**, left). This shift is well described by the Goldman-Hodgkin relationship for chloride specific channels (**Figure 9.16b** right). This indicates that ACh-activated currents are almost entirely carried by chloride ions in oocytes expressing mutated homomeric nAChR*. Then, introducing appropriate amino acid residues from the putative channel domain of a chloride-selective GlyR α -subunit into that of a cation selective nAChR α -subunit allows the design of an ACh-gated channel now selective for chloride. This confirms that the M2 segment forms the walls of the channel and strongly suggests that the exchanged residues face the lumen of the channel.

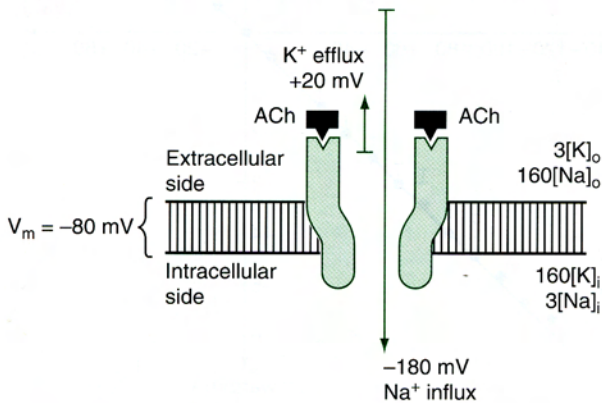


Figure 9.12 Determination and vectorial representation of the Na^+ ion driving force ($V_m - E_{\text{Na}}$) and K^+ ion driving force ($V_m - E_{\text{K}}$) for a membrane potential of -80 mV .

Observe that 90% of the current is due to Na^+ ions. The net flux of positive charges is inward. This explains why ACh induces an inward current at $V_m = -80\text{ mV}$.

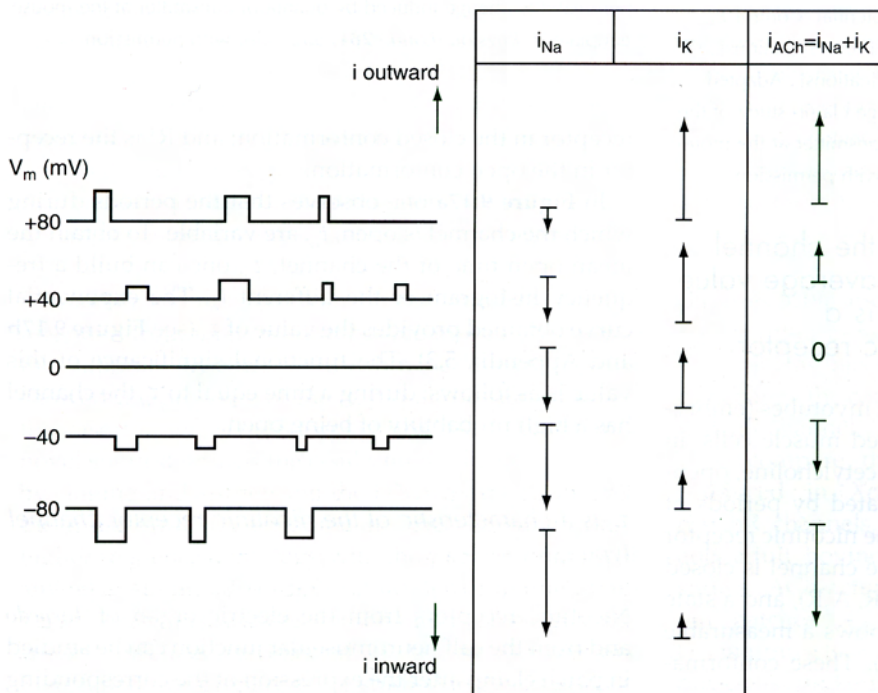


Figure 9.13 Evolution of sodium and potassium currents (i_{Na} and i_{K}) through the nicotinic channel as a function of membrane potential V_m .

The current induced by acetylcholine i_{ACh} corresponds to the sum of two currents: $i_{\text{ACh}} = i_{\text{Na}} + i_{\text{K}}$. When $i_{\text{Na}} = -i_{\text{K}}$ the current is zero. This occurs at the reversal potential of the nicotinic response ($V_m = E_{\text{ACh}}$). A deflection of the traces (left) or an arrow of the chart (right) in the downward direction represents an inward current, and in the upward direction an outward current.

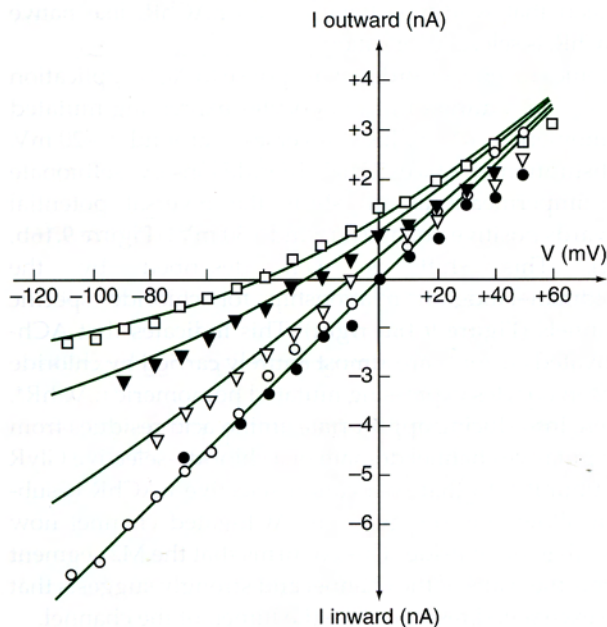
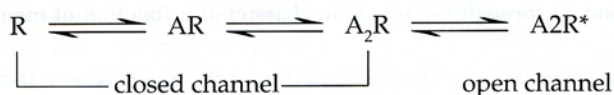


Figure 9.14 Effect of lowering the external Na^+ concentration on the I_{ACh}/V curve.

The composition of the external environment is: 5 mM of K^+ ; 0.1 mM of Ca^{2+} ; and 21 ($\square$), 46 ($\blacktriangledown$), 96 (∇) or 146 ($\bullet$ and $\circ$) mM of Na^+ . Each point represents the average of 25 measurements of I_{ACh} . When $[\text{Na}^+]_e$ decreases, the reversal potential of the nicotinic response shifts towards the K^+ equilibrium potential. Control E_{ACh} : ($\bullet$, $\circ$) $58 \log (146 + 5)/[\text{cations}]_i$; (∇) $58 \log (96 + 5)/[\text{cations}]_i$; ($\blacktriangledown$) $58 \log (46 + 5)/[\text{cations}]_i$; ($\square$) $58 \log (21 + 5)/[\text{cations}]_i$. Adapted from Linder TM, Quastel DMJ (1978) A voltage clamp study of the permeability change induced by quanta of transmitter at the mouse end plate, *J. Physiol. (Lond.)* **281**, 535–556, with permission.

9.3.3 The time during which the channel stays open varies around an average value τ_o , the mean open time, and is a characteristic of each nicotinic receptor

When recording in patch clamp from myotubes (embryonic muscle cells) or from denervated muscle cells, in the presence of very small doses of acetylcholine, openings of the nicotinic channels separated by periods of silence are observed (Figure 9.17). The nicotinic receptor switches between states in which the channel is closed and the unitary current is zero (R , AR , A_2R), and a state in which the channel is open and shows a measurable unitary current (A_2R^*) (Figure 9.18). These conformational changes can be modelled as:



where A is acetylcholine or any nicotinic agonist; R is the

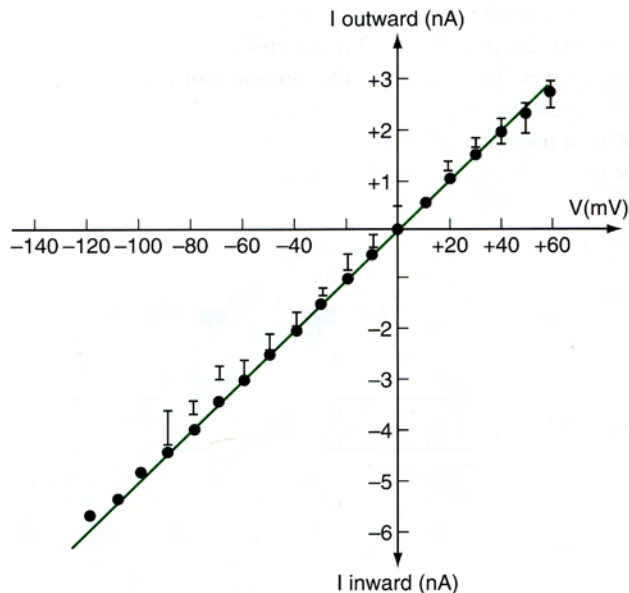


Figure 9.15 Replacing external K^+ for Na^+ ions has no effect.

Control I/V curve ($\bullet$) 146 mM of Na^+ and 5 mM of K^+ , and (∇) 2 mM of Na^+ and 149 mM of K^+ . Observe that K^+ ions can replace Na^+ ions without affecting the I/V curve. The nicotinic channel does not distinguish between these two cations. Adapted from Linder TM, Quastel DMJ (1978) A voltage clamp study of the permeability change induced by quanta of transmitter at the mouse endplate, *J. Physiol. (Lond.)* **281**, 535–556, with permission.

receptor in the closed conformation; and R^* is the receptor in the open conformation.

In Figure 9.17a one observes that the periods during which the channel is open, t_o , are variable. To obtain the mean open time of the channel, τ_o , one can build a frequency histogram of the different t_o . The exponential curve obtained provides the value of τ_o (see Figure 9.17b and Appendix 5.3). The functional significance of this value is as follows: during a time equal to τ_o the channel has a high probability of being open.

τ_o is a characteristic of the nicotinic receptor channel type

Nicotinic receptors from the electric organ of *Torpedo* and from the calf neuromuscular junction can be studied in patch clamp after the expression of the corresponding mRNA injected into *Xenopus* oocytes (outside-out configuration). Recording of such channels shows that electric organ and neuromuscular junction channels present very similar conductances (40 and 42 pS) but very different mean open times ($\tau_o = 0.6$ and 7.6 ms, respectively).

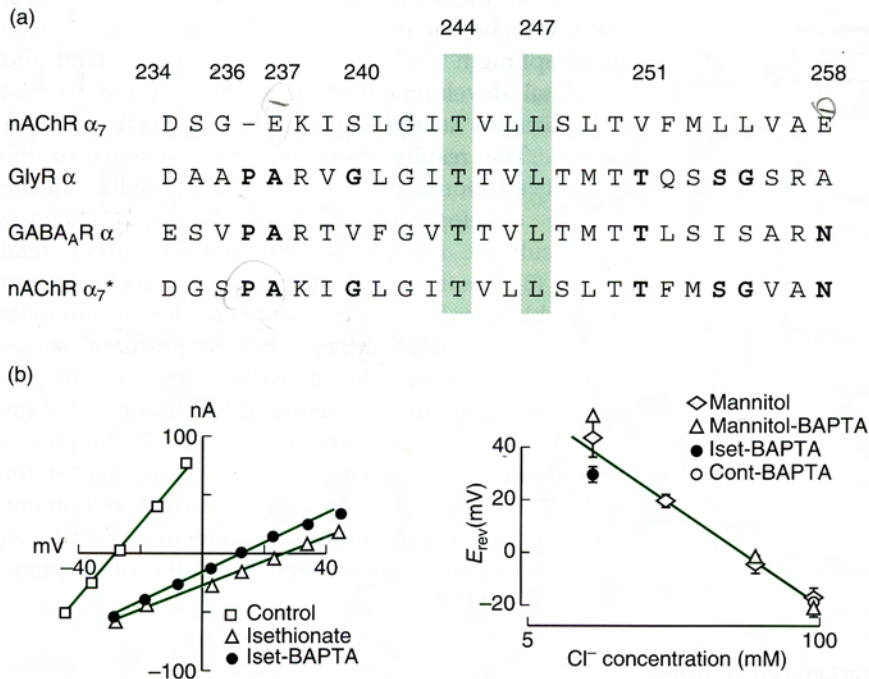


Figure 9.16 Mutations in the M2 segment of an α -subunit of the nAChR convert ion selectivity of the homomeric nAChR from cationic to anionic.

(a) Comparison of M2 sequences from subunits of the cation selective nicotinic α_7 receptor subunit with those of the anion-selective glycine α_1 , GABA $_A$ α_1 and β_1 and mutated nicotinic α_7^* receptor subunits. (b, left) I/V relationship of the α_7^* mutant receptor is first determined in control conditions (control) with 2 s ACh (100 μ M) applications (outside-out patch clamp recordings of a patch of membrane containing a large number of nicotinic receptors called macropatch). Then, 90% of chloride ions of the extracellular medium were replaced by the non-permeant anion isethionate and the I/V curve determined (isethionate). This last experiment was also performed in the presence of a chelator of Ca^{2+} ions (BAPTA) injected inside the cell (iset-BAPTA) in order to reduce secondary currents that could be triggered by the entry of Ca^{2+} ions through the nicotinic receptors. (b, right) Reversal potential values as a function of the logarithm of external chloride concentration (92, 50.5, 19.75 and 9.5 μ M external chloride after substitution of NaCl by mannitol or isethionate). The solid line corresponds to the theoretical Nernst relation. From Galzi JL, Devillers-Thiéry A, Hussy N (1992) Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic, *Nature* 359, 500–505, with permission.

Another example is given by the study of nicotinic receptors from fetal or adult bovine muscle. A study of the subunit structure of the bovine muscle nAChR showed the presence of the α , β , γ and δ -subunits as in the case for *Torpedo* electroplax nAChR. In addition, a novel subunit termed the ϵ -subunit has been discovered by cloning and sequencing the DNA complementary to the muscle mRNA encoding it. The ϵ -subunit shows higher sequence homology with the γ -subunit than with any other subunit. In order to study the properties of the γ and ϵ -subunits, various combinations of the subunit-specific mRNAs are injected in *Xenopus* oocytes and their functional properties are studied in the presence of acetylcholine.

Figure 9.19a shows recordings of ACh-activated single channels from outside-out patches isolated from oocytes injected with the α , β , γ and δ -subunit-specific mRNAs (left) or with the α , β , ϵ and δ -subunit-specific mRNAs (right). The conductance and mean open time τ_o

(Figures 9.19c and d) of the channels formed in a given oocyte differ in relation to the mRNA combination with which it was injected. This suggests that a single subunit can change the conductance and gating properties of the nAChR channel.

To compare the two classes of nAChR channels produced in *Xenopus* oocytes with native bovine nAChR channels, the ACh-activated channels of fetal and adult bovine muscle are recorded. Figure 9.19b shows ACh-activated single currents from outside out patches of native fetal (left) and adult (right) bovine muscle. nAChR single-channel current in fetal muscle is similar to that of nAChR γ whereas the nAChR single-channel current in adult muscle is similar to that of nAChR ϵ (compare Figures 9.19a and b). This suggests that the nAChR channel in fetal muscle is assembled from α , β , γ and δ -subunits whereas the endplate channel in adult muscle is assembled from the α , β , ϵ and δ -subunits. To study this developmental

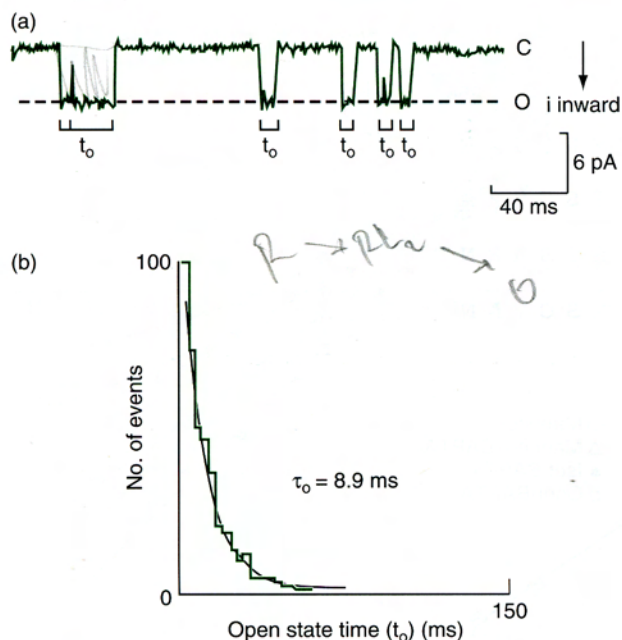


Figure 9.17 Patch clamp recording (attached-cell configuration) of myotube nicotinic receptor channel activity ($V_m = -170$ mV).

(a) Myotubes (embryonic muscle cells) are recorded in the presence of a low concentration of acetylcholine (200 nM). At this concentration, the channels open during periods t_0 . This recording does not correspond to a single nAChR because one finds approximately 100 000 nAChR per patch. The repeated openings (downward deflections) correspond, therefore, to the opening of different nAChR. However, all the nAChR being identical, it seems as though the activity of the same nAChR was recorded from. (b) The mean open time τ_0 can thus be calculated. C, closed channel; unitary current is zero. O, open channel; inward unitary current (downward deflections).

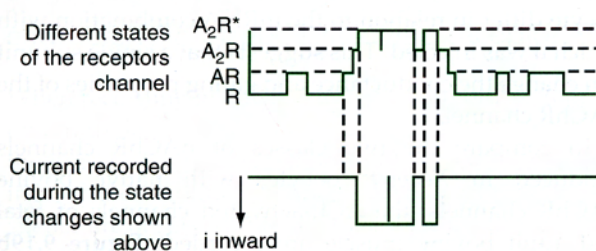


Figure 9.18 Correlation between the nicotinic current and the states of the channel.

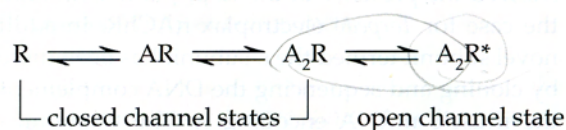
The channel opens only (inward current, lower trace) when the protein is in the A_2R^* state. The rapid fluctuations between states A_2R^* and A_2R correspond to short-lived closures. When the receptor channel loses one or two of its acetylcholine molecules, the closures last longer. Adapted from Colquhoun D, Ogden DC, Mathie A (1987) Nicotinic acetylcholine receptors of nerve and muscle: functional aspects, *TIPS* 8, 465–472, with permission.

change in the contents of the five nAChR-subunit mRNAs in bovine muscle, total RNA is extracted from the diaphragm muscle at various stages of fetal and postnatal development. It is then subjected to blot hybridization analysis using the respective cDNA probes. The results show that the contents of the γ - and ϵ -subunit mRNAs varies markedly during muscle development showing reciprocal changes: the γ -subunit mRNA is abundant at earlier fetal stages (3–5 months' gestation), but is hardly or not detectable after birth; conversely, considerable amounts of ϵ -subunit mRNA appear only at postnatal stages and is not detectable at earlier fetal stages (3–4 months' gestation). Therefore, the replacement of the γ -subunit by the ϵ -subunit in the nAChR complex is responsible for the changes in the properties of the nAChR channel that occur during muscle development. This phenomenon of subunit replacement during development cannot be generalized to all the other mammalian nAChR.

9.4 The nicotinic receptor desensitizes

During the recording of a nicotinic receptor channel with the patch clamp technique (whole-cell configuration) in the presence of a high and constant concentration of acetylcholine, there is a progressive diminution of the total current I_{ACh} (Figure 9.20a). This decrease in current corresponds to the progressive desensitization of the nicotinic receptor present in the membrane.

When recording unitary nicotinic currents (outside-out or cell-attached configuration) in the presence of a strong concentration of acetylcholine, there are repeated openings separated by long periods of silence (Figure 9.20b). These sequences of openings are known as *unitary current bursts*. Within a burst, the protein rapidly fluctuates between the closed and open states, symbolized as follows:



The long silent periods correspond to desensitization of the receptor in the presence of acetylcholine. In the desensitized state, the nicotinic receptor is refractory to activation. Consequently, the channel does not open despite the fact that two molecules of ACh are bound to the receptor.

In summary, desensitization is a phenomenon that renders the nicotinic receptor incapable of being activated by its agonists. The desensitized nicotinic receptor presents two main characteristics: (i) a high affinity for