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Nicotinic acetylcholine receptors

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ABSTRACT

Nicotinic receptors are cation-permeable ion channels activated by the neurotransmitter acetylcholine

The muscle type receptor mediates all fast synaptic excitation on voluntary muscle. We review both the structure and the function of muscle/*Torpedo* receptor, and the function of several mutants. Recent developments in both knowledge of structure, and in analysis of single channel records, are beginning to throw light on the role of single amino acid residues in the molecular events (the binding of an agonist and the gating of the channel) that lead to channel opening.

In the nervous system, nicotinic channels mediate the majority of fast excitation only in autonomic ganglia, but are also present at presynaptic locations. Issues of receptor classification and subunit composition are particularly relevant for neuronal channels, because of the numerous subunit combinations possible and because relatively few selective competitive antagonists are available, a situation that may improve with the characterisation of alpha-conotoxins. Inherited mutations in nicotinic channels gives rise to rare congenital forms of human disease (myasthenia for muscle and epilepsy for neuronal receptors).

Introduction

Nicotinic acetylcholine (ACh) receptors are responsible for transmission of nerve impulses from motor nerves to muscle fibres (muscle types) and for synaptic transmission in autonomic ganglia (neuronal types). They are also present in the brain where they are presumed to be responsible for nicotine addiction, but little is known about their normal physiological function there. Nicotinic receptors form cation-selective ion channels. When a pulse of ACh is released at the nerve-muscle synapse, the channels in the postsynaptic membrane of the muscle cell open, and the initial electrochemical driving force is mainly for sodium ions to pass from the extracellular space into the interior of the cell. But as the membrane depolarises the driving force increases for potassium ions to go in the opposite direction. Nicotinic channels (particularly some of the neuronal ones) are also permeable to divalent cations, such as calcium.

Nicotinic receptors are the most intensively studied type of neurotransmitter-gated ion channel, and in this review we shall summarise what is known about their structure and function.

Structure and topology

Genes

All of the nicotinic receptors are oligomers that are composed of a ring of five subunits encircling a central pathway for the ions. The genes for the known subunit types are shown in Table 1

Table 1

	Chromosomal location (OMIM)	number of amino acids (including signal peptide)	Gene name	Swiss-Prot entry name and primary accession number	
Muscle subunits					
α1	2q24-q32	457*	CHRNA1	ACHA_HUMAN - P02708	*isoform 1
β1	17p12-p11	501	CHRNB	ACHB_HUMAN - P11230	
γ	2q33-q34	517	CHRNG	ACHG_HUMAN - P07510	embryonic
δ	2q33-q34	517	CHRND	ACHD_HUMAN - Q07001	
ε	17p13-p12	493	CHRNE	ACHE_HUMAN -	adult
Neuronal subunits					
α2	8p21	529	CHRNA2	ACH2_HUMAN - Q15822	
α3	15q24	503	CHRNA3	ACH3_HUMAN	

				- P32297	
α4	20q13.2-q13.3	627	CHRNA4	ACH4_HUMAN - P43681	
α5	15q24	468	CHRNA5	ACH5_HUMAN - P30532	
α6		494	CHRNA6	ACH6_HUMAN - Q15825	
α7	15q14	502	CHRNA7	ACH7_HUMAN - P36544	the human α7 gene is partially duplicated in the same chromosomal region
α9		479	CHRNA9	ACH9_HUMAN - P43144	
α10	11p15.5	450	CHRNA10	ACH10_HUMAN – Q9GZZ6	
β2	1p21	502	CHRNB2	ACHN_HUMAN - P17787	
β3	8p11.2	458	CHRNB3	ACHO_HUMAN - Q05901	
β4	15q24	498	CHRNB4	ACHP_HUMAN - P30926	

*A splice variant with an additional 25 amino acids is known (isoform 2): this does not form functional channels)

Structure of muscle type nicotinic receptors

Far more is known about the muscle-type nicotinic receptor than about neuronal receptors. One of the reasons for our greater understanding is that a receptor similar to the muscle-type nicotinic receptor is present in great abundance in the electric organ of the *Torpedo* ray. A large proportion of the surface in the modified muscle tissue in the electric organ is occupied by postsynaptic membrane that contains densely packed, partially crystalline arrays of nicotinic receptors. This has allowed purification (with the help of high-affinity ligands such as α-bungarotoxin), partial sequencing of the receptor subunits and hence cloning of the genes for these subunits. Tubular crystals of *Torpedo* receptors embedded in their native lipids can be grown from isolated postsynaptic membranes, and these have been used extensively in investigations of receptor structure and function by electron microscopy (see below).

The muscle-type ACh receptor is a glycoprotein complex (~290kDa) which consists of five subunits arranged around a central membrane-spanning pore. Nicotinic subunits are similar in amino acid sequence and have the same topology (Figure 1): each subunit comprises a large extracellular amino-terminal domain, four predicted membrane-spanning segments (M1-M4) and a long cytoplasmic loop between M3 and M4.

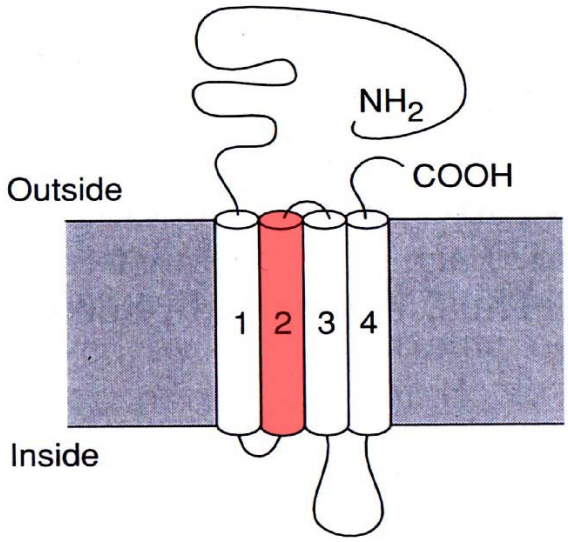

Figure 1
Diagram of topology of a single receptor subunit.
Each subunit is thought to cross the cell membrane four times

These characteristics are shared with subunits which form other ion channel/receptors and thus define a receptor superfamily, usually referred to as the nicotinic family. All members in this superfamily function as either cation- or anion-selective channels, thereby mediating fast excitatory or inhibitory synaptic transmission. In mammalian cells, the cation-selective members include nicotinic and $5HT_3$ receptors, while the anion-selective members include $GABA_A$, $GABA_C$ and glycine receptors. Invertebrates also contain . Anion-selective channels in this family are also found in invertebrates: these channels are gated by glutamate, 5-HT, histidine and acetylcholine anion-selective channels in this family, which are gated by glutamate, 5-HT, histidine and acetylcholine (Raymond & Sattelle, 2002).
The muscle-type receptor has the composition $(\alpha 1)_2, \beta, \gamma, \delta$ in embryonic (or denervated) muscle, but in the adult the γ subunit is replaced by an ε subunit. The adult receptor is found, at high density, only in the endplate region of the muscle fibre, but before innervation embryonic receptors are distributed over the whole muscle fibre. The electric organ contains only the embryonic, γ, form of the receptor. All the subunits share a high degree of homology (typically 31-41% pairwise identity to the α subunits, depending on the species).

The properties of, and interactions between individual subunits have been explored extensively by a range of biochemical, molecular genetic and electrophysiological techniques (for recent reviews, see Karlin, 2002; Corringer *et al.*, 2000). Their order around the pore is most likely to be $\alpha, \gamma, \alpha, \beta, \delta$ going in the clockwise sense and viewed from the direction of the synaptic cleft. Opening of the channel occurs upon binding of ACh to both α subunits (α_γ and α_δ) at sites that are at, or close to, the interfaces made with neighbouring γ and δ subunits (Karlin, 1993; Sine *et al.*, 1995a; Xie & Cohen, 2001). These sites are shaped by three separate regions of the polypeptide chain(Corringer *et al.*, 2000), and include the so-called C-loop (see below).

Molecular architecture

The tubular crystals from the *Torpedo* ray form the basis of almost all quantitative three-dimensional studies of the whole receptor (e.g. Kistler & Stroud, 1981; Miyazawa *et al.*, 1999). Tubes are built from tightly packed ribbons of receptor dimers, and intervening lipid molecules (Brisson & Unwin, 1984). They grow

naturally from the isolated postsynaptic membranes, retaining a curvature similar to that at the crests of the junctional folds. Apparently, there is a close structural correspondence between the tubes, which are simply elongated protein-lipid vesicles, and the receptor-rich membrane as it exists in vivo.

Ice-embedded tubes, imaged with the electron microscope, can be made to retain their circular cross-section and analysed as helical particles (Toyoshima & Unwin, 1990). At low resolution, using this approach, the receptor appears as a ~70Å diameter × 160 Å long cylinder composed of five similar rod-shaped subunits arranged around the central axis and aligned approximately normal to the membrane plane. The ion-conducting pathway, delineated by the symmetry axis, appears as a narrow (unresolved) pore across the membrane, bounded by two large (~20Å diameter) vestibules. Further development of this approach has lead to resolutions of 9Å (Unwin, 1993), and more recently, 4.6Å (Miyazawa *et al.*, 1999), being achieved.

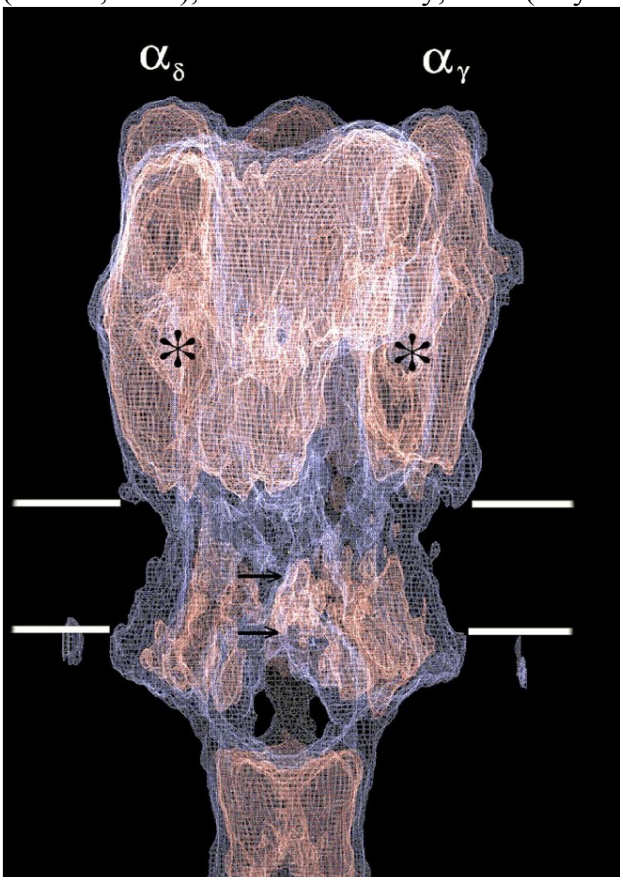

Figure 2
Architecture of whole receptor, emphasising the external surface and openings to the ion conducting pathway on the outer (extracellular) and inner (cytoplasmic) sides of the membrane. The positions of the two α subunits; the binding pockets (asterisks); gate of the closed channel (upper arrow); and the constricting part of the open channel (lower arrow) are indicated.

Figure 2 shows the appearance of the whole receptor at 4.6Å resolution. In the extracellular portion, the subunits form a pentagonal wall around the central axis and make the cylindrical outer vestibule of the channel. The outer vestibule is about 20 Å wide × 65Å long. About halfway up this portion are the ACh-binding regions in the two α subunits (asterisks). In the cytoplasmic portion, the subunits form an inverted pentagonal cone, which comes together on the central axis at the base of the receptor, so shaping a spherical inner vestibule of the channel. This is about 20Å in diameter. The only aqueous links between the inner vestibule and the cell interior are the narrow (< 8-9Å wide) 'windows' between the subunits lying directly under the membrane surface. The gate of the channel, made by the pore-lining segments, M2, is near the middle of the membrane (upper arrow), and the constriction zone (the narrowest part of the open channel) is at the cytoplasmic membrane surface (lower arrow).

The vestibules

One likely physiological role of the vestibules is to serve as pre-selectivity filters for ions, making use of charged groups at their mouths and on their inner walls to concentrate the ions they select for (cations), while screening out the ions they discriminate against (anions). In this way, the ionic environment would be modified close to the narrow membrane-spanning pore, increasing the efficiency of transport of the permeant ions, and enhancing the selectivity arising from their direct interaction with residues and/or backbone groups lining the constriction zone. A more direct means of increasing the cation conductance may be achieved by rings of negative charge located at the mouth of the pore. These rings (at positions 4′, 1′ and 20′ of M2, using the numbering system for M2 residues defined in Figure 3) are significant have been shown to influence channel conductance (Imoto *et al.*, 1988).

Consistent with a screening role, the cylindrical shape and ~10Å radius of the outer (extracellular) vestibule provides a route that is narrow enough for charged groups on the inner wall to influence ions at the centre, but not too narrow to restrict their diffusion. The design of this portion of the receptor might therefore have some parallels with the fast-acting enzyme, acetylcholinesterase, where the whole protein surface plays a role in producing an electrostatic field that guides the positively charged ACh substrate to the active site (Ripoll *et al.*, 1993). The inner (cytoplasmic) vestibule is architecturally distinct from the outer vestibule, yet presumably plays a similar functional role in concentrating the cations, since electrophysiological experiments on the muscle-type receptor have shown that there is no marked preference for cations to go in one direction across the membrane (i.e. rectification). Negatively charged groups framing the windows would have a strong local effect, since the windows are not significantly wider than the diameter of an ion including its first hydration shell.

The large proportion of mass (c. 70%) extending outside the membrane and shaping these vestibules is also needed for other purposes, such as making the (complex) ACh-binding pockets, and providing sites of attachment for regulatory molecules and other proteins (such as rapsyn) that are concentrated at the synapse.

Membrane-spanning pore

The membrane-spanning portion of the receptor has not yet been completely resolved by direct structural methods, although the pore-lining segments are partially visible as a ring of five rod-shaped densities, consistent with an α-helical configuration. This helix is the part of the structure closest to the axis of the receptor and therefore must correspond to M2, the stretch of sequence shown by chemical labelling (Hucho *et al.*, 1986; Giraudat *et al.*, 1986) and by site-directed mutagenesis/electrophysiology experiments (Imoto *et al.*, 1986; Imoto *et al.*, 1988; Leonard *et al.*, 1988) to be lining the pore. In the shut-channel form of the receptor, this helix is bent inwards, towards the central axis, making the lumen of the pore narrowest near the middle of the membrane. This is the most constricted region of the whole ion pathway and therefore presumably corresponds to the gate of the channel.

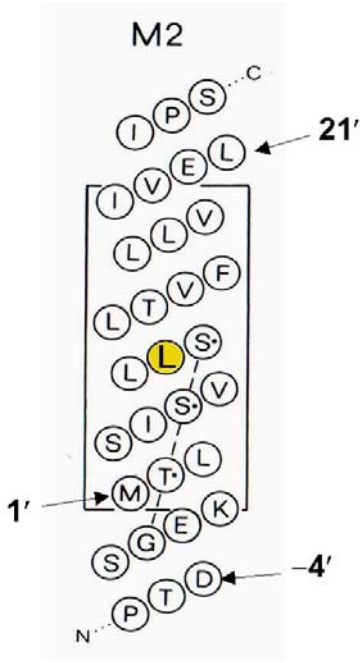

Figure 3
Helical net plot of the amino acid sequence around the membrane-spanning segment, M2 (*Torpedo* α subunit); the leucine residue near the middle of the membrane (yellow) is the conserved leucine, L251 (at the 9' position), which may be involved in forming the gate of the channel; the dots denote other residues that have been shown to affect the binding affinity of an open channel blocker (Leonard *et al.*, 1988; Charnet *et al.*, 1990), and ion flow through the open pore (Villarroel *et al.*, 1991). The numbers shown refer to the numbering scheme for M2 residues that is used in the text.

A tentative alignment can be made between the three-dimensional densities and the amino acid sequence of M2 (Figure 3; Unwin, 1993). This alignment places the charged groups at the ends of M2 symmetrically on either side of the lipid bilayer, and a highly conserved leucine residue (*Torpedo* αLeu251) at the level of the bend. It seems likely that the leucine side-chains, by side-to-side interactions with neighbouring M2 segments, are involved in making the gate of the channel. Site-directed mutagenesis experiments, combined with electrophysiological study of function, have highlighted the uniqueness of the conserved leucine residue in relation to the gating mechanism. The profound effects of mutating this leucine to a hydrophilic amino acid on the agonist sensitivity of the receptor and its desensitisation properties were first reported for the recombinant homomeric α7 neuronal nicotinic receptor by Revah *et al.* (1991). In the muscle type receptor, progressive replacement of leucines by serines (Labarca *et al.*, 1995) or by threonines (Filatov & White, 1995) increases, by roughly uniform increments, the sensitivity of the channel (i.e. decreases the EC_{50} for ACh). A similar effect is seen in neuronal nicotinic receptors that contain α3, β4, and β3 subunits (Boorman *et al.*, 2000). However, other experiments (e.g. Wilson & Karlin, 1998) have been interpreted to indicate that the gate is located closer to the cytoplasmic membrane surface.

The snail ACh-binding protein

Before going on to discuss the agonist binding site, we shall discuss the snail acetylcholine-binding protein (AChBP). Glial cells in the snail, *Lymnea stagnalis*, produce and secrete this protein, which is a homopentamer having structural homology with the large N-terminal portion of the extracellular domain of ion channels in the nicotinic superfamily. The protomer of AChBP is composed of 210 amino acids and has 20-23% sequence identity with the muscle-type ACh receptor subunits. It contains most of the residues that were previously suspected to be involved in ACh binding to the receptor. Its crystal structure was solved recently to 2.7Å resolution (Brejc *et al.*, 2001), revealing the protomer to be organized around two sets of β-strands, forming 'Greek key' motifs, folded into a curled β-sandwich.

The β-sandwich can be divided into inner- and outer-sheet parts, shown in blue and red in Figure 4., which are covalently linked together through a disulphide bond. The 'cys-loop' disulphide bond (C128 - C142 in *Torpedo* and human α1 subunits) plays an important structural role in stabilizing the three-dimensional fold (Brejc et al., 2001), and is absolutely conserved among all members of the ion channel superfamily.

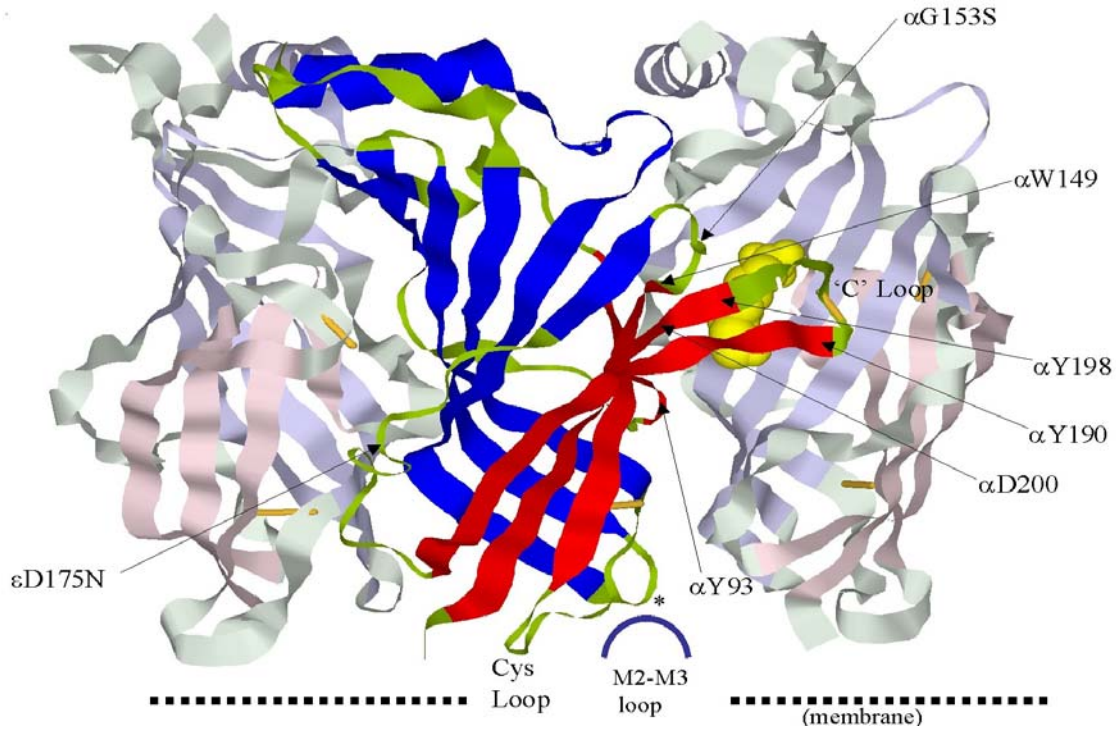

Figure 4

Three subunits of the *Lymnaea* AChBP viewed perpendicular to the five-fold axis of symmetry, and from the outside of the pentamer. The inner and outer sheets of the beta sandwich are coloured blue and red respectively whilst the putative ligand HEPES is coloured purple. The approximate positions of the alpha carbons of residues discussed in the text are marked with arrows on the foremost subunit. The approximate position of the cell membrane, and of the M2-M3 loop are shown diagrammatically. The inner beta sheet (blue) is thought to rotate after agonist binding, and to interact with the M2-M3 loop (as indicated by the asterisk).

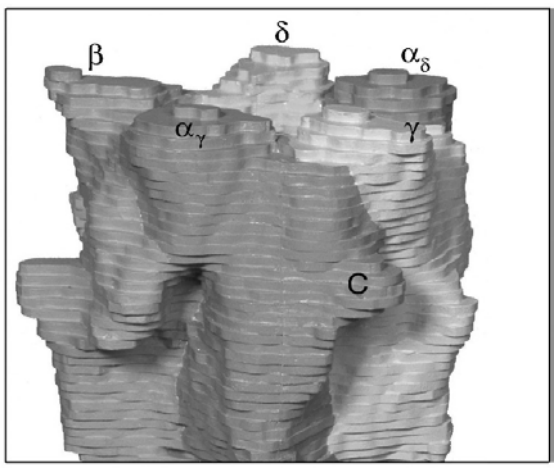

Figure 5.

Wooden model of the extracellular part of the ACh receptor, based on the 4.6Å map of the shut channel (Miyazawa *et al.*, 1999). The membrane surface is at the bottom of the figure. C denotes the C-loop region of the receptor (see also ACh binding protein, Figs. 4 and 6), which makes part of the ACh-binding site. The width of each wooden slab corresponds to 2Å (from Unwin et al., 2002).

AChBP has been crystallised only with HEPES, rather than ACh, bound, and "owing to low occupancy and limited resolution, the precise orientation of the HEPES molecule cannot be definitely resolved" (Brejc *et al.*, 2001). In overall appearance, it is very similar to the extracellular domain of the receptor (Figure 5). The C-loop is particularly prominent both in AChBP (Figure 4) and in the receptor, where it is shown as the projection labelled C in Figure 5. The C-loop contains several conserved residues that are thought to be part of the acetylcholine binding site: two adjacent cysteines that are characteristic of α subunits, homologues of αC192, αC193, two tyrosines (αY190, αY198) and an aspartic acid (αD200). It is orientated more tightly against the neighbouring subunit in AChBP than it is in the (unliganded) receptor. These residues on the C-loop, and other nearby aromatic residues (see below) that form part of the binding site in AChBP, are homologues of residues that have been postulated to form the binding site of nicotinic receptors, on the basis of mutational studies and/or photo-affinity labelling studies. One exception is H145 (see Fig 6A), which aligns with αY151, which had not been thought to be important. Also some residues postulated to be part of the binding site by other methods do not appear to be in the AChBP binding site (e.g. αY86).

ACh binding region of the receptor

The two ACh binding sites are located in the extracellular domain, about 30Å from the membrane surface, or 45Å from the gate. Although the actual ligand-binding site has not yet been identified definitively within the three-dimensional structure of the receptor, ACh is expected to bind through cation-π interactions, where the positive charge of its quaternary ammonium moiety interacts with electron-rich aromatic side-chains (Zhong *et al.*, 1998). The recently solved structure of AChBP (Brejc *et al.*, 2001), discussed above, shows that the 'signature' aromatic residues lie in a pocket next to the interface with the anticlockwise-positioned protomer, as seen from the 'synaptic cleft'. The pocket identified in AChBP would lie behind the protruding densities, labelled C in Fig. 5, near the αγ- and αδ- interfaces. The densities at C can also be identified with the C-loop structure in AChBP (see Figure 4), although they do not curve around so tightly towards the neighbouring subunits, making a more open cleft in the (unliganded) receptor.

The key aromatic residues at the binding site are most likely: αY93, αW149, αY190 and αY198. These residues are located in three separate loops of the polypeptide chain (Corringer *et al.*, 2000), designated A (Y93), B (W149) and C (Y190 and Y198). All were identified as being near the agonist binding site by labelling with a small photo-activatable ligand that covalently reacts with the receptor upon UV-irradiation and acts as a competitive antagonist (Dennis *et al.*, 1988), and the first three are highly conserved in aligned positions of all muscle and neuronal α subunits. Experiments in which a series of unnatural tryptophan derivatives were substituted in place of the natural residues, have suggested that the side-chain of αW149 is in van der Waals contact with the quaternary ammonium group of ACh in the bound state of the receptor (Zhong *et al.*, 1998). Chemical labelling has also shown that the pair of adjacent cysteines (αC192 and αC193) is likely to be close to the binding site (Kao & Karlin, 1986).

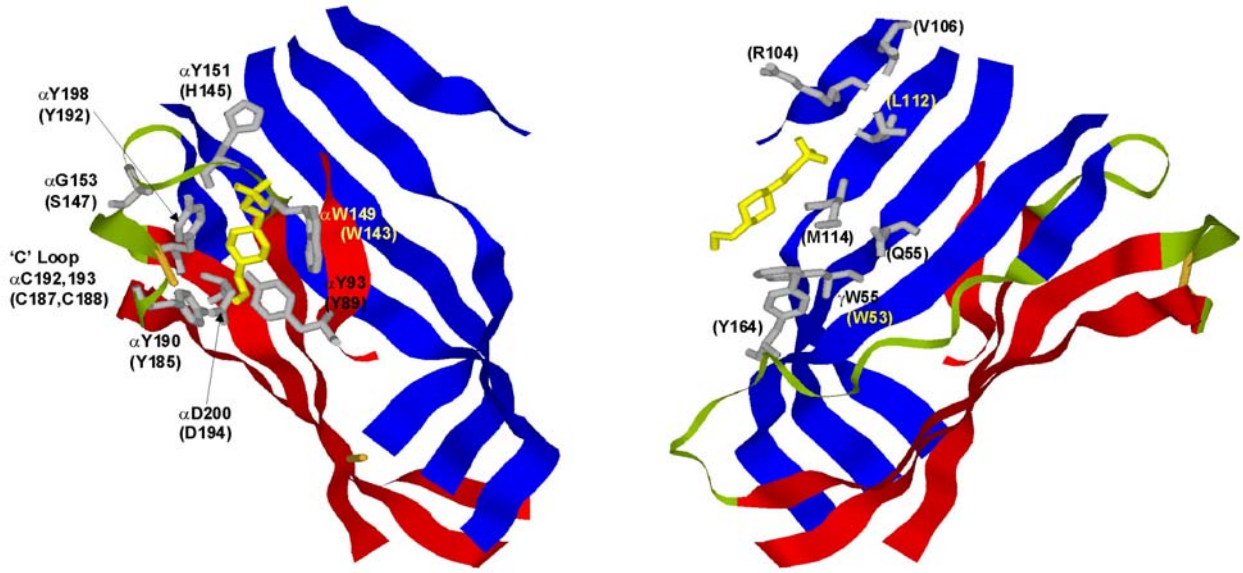

Figure 6

The binding site of AChBP. The ligand (HEPES) is in yellow. Blue and red regions denote the inner- and outer-shee beta sandwich (Unwin et al., 2002). The views in A and B are with the 5-fold axis vertical, and the 'membrane' at the

A. The structure shown is analogous to the binding site of the the *Torpedo* or human α1 subunit, to which the numbering of identified residues refers. The parenthesised numbers refer to AChBP.
Surface of the neighbouring protomer, that faces the binding site of AChBP. In the receptor this would be part of the γ, ε or δ subunit. The parenthesised numbers refer to the snail protein. W53 aligns with W55 in mouse γ, ε and δ nicotinic receptor subunits, but most of the residues have no obvious analogue in the γ, ε or d subunits of the nicotinic receptor. For example, Q55 aligns with mouse γ E57, εG57 and δD57; L112 aligns with mouse γ Y117 or δT119, and Y164 is A or G in the nicotinic subunits.

Figure 6A shows the binding site region for the AChBP with the ligand (HEPES), to show the position of the residues mentioned above. The 'plus side' of the interface (Fig. 6A) is analogous with the α subunit of the nicotinic receptor.

The most important residues in neighbouring subunits that influence ACh binding are W57 of the δ subunit and the homologous W55 of the γ subunit (Chiara & Cohen, 1997; Xie & Cohen, 2001). The 'minus side' of the AChBP interface, shown in Figure 6B, would be the γ/ε or the δ subunit in the receptor, but in the AChBP it is another identical subunit. The residues shown to be in contact with the ligand, labelled in Fig 6B, mostly have no obvious analogue in the ACh receptor. The one important exception is W53 which corresponds to the tryptophan residues mentioned above. Consequently the snail protein model is rather less helpful about the non-α side of the receptor interface.

The mechanism of activation

The structural transition from the shut to the open-channel form of the receptor has been analysed at 9Å resolution by comparing the three-dimensional map of the shut form, as described above, with that of the open form, obtained by spraying ACh onto the tubes and then freezing them rapidly within 5ms of spray impact (Unwin, 1995). The rapid freezing combined with minimal delay was needed to trap the activation reaction and minimise the number of receptors that would become desensitised.

A detailed comparison of the two structures indicated that the binding of ACh initiates two interconnected events in the extracellular domain. One is a local disturbance, involving all five subunits, in the region of the binding sites, and the other an extended conformational change, involving predominantly the two α subunits, which communicates to the transmembrane portion. These experiments give a picture of the receptor in either of the two states, i.e. the shut- and open-channel forms, and thus provide no direct information relating to the possibility that the binding to one site might affect the binding to the other *before* the channel opens (see Hatton et al 2003). However, there is a tight association of αγ with the neighbouring γ subunit (Unwin *et al.,* 2002) which is next to αδ; thus some coupling is quite possible.

In the membrane, the exposure to ACh did not bring about any obvious alteration to the outer structure facing the lipids, whereas the M2 helices switched quite dramatically to a new configuration in which the bends, instead of pointing towards the axis of the pore, had rotated (clockwise) over to the side, as shown in Figure 7.

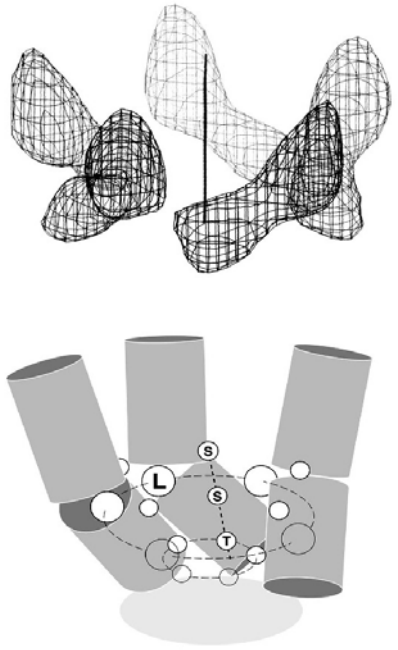

Figure 7.
Transient configuration of M2 helices around the open pore. (a) A barrel of α-helical segments, having a pronounced twist, forms in the cytoplasmic leaflet of the bilayer, constricting the pore maximally at the cytoplasmic membrane surface. The bend in the rods is at the same level as for the closed pore, but instead of pointing inwards has rotated over to the side. (b) Schematic representation of the most distant three rods. A tentative alignment of the amino acid sequence with the densities suggests that a line of polar residues (serines and threonine; see Fig. 2) should be facing the open pore (from Unwin, 1995).

This rearrangement had the effect of opening up the pore in the middle of the membrane, and making it narrowest at the cytoplasmic membrane surface, where the α-helices now came close enough to associate by side-to-side interactions around the ring. Thus there appear to be two alternative configurations of M2 helices around the pore: one (the shut configuration) stabilized by side-to-side interactions near the middle of the membrane, and the other (the open configuration) stabilized by side-to-side interactions close to the cytoplasmic membrane surface. These limited sets of interactions, combined with the rigid α-helical folds, might be important in ensuring the precise permeation and fast gating kinetics characterising acetylcholine-gated channels.

A tentative alignment of the M2 sequence with the densities in the cytoplasmic leaflet suggests that a line of small polar (serine or threonine) residues would lie almost parallel to the axis of the pore when the channel opens (Fig.7), an orientation that should stabilize the passing ions by providing an environment of high polarisability. The threonine residue at the point of maximum constriction (*Torpedo* αT244), when substituted by other residues of different volume, has a pronounced effect on ion flow, as if it were at the narrowest part of the open pore (Villarroel & Sakmann, 1992). The diameter of this most constricted portion of the channel, based on permeability measurements made with small uncharged molecules of different size, is about 10Å (Dwyer *et al.*, 1980; Cohen *et al.*, 1992). This value is similar that indicated by the structural results.

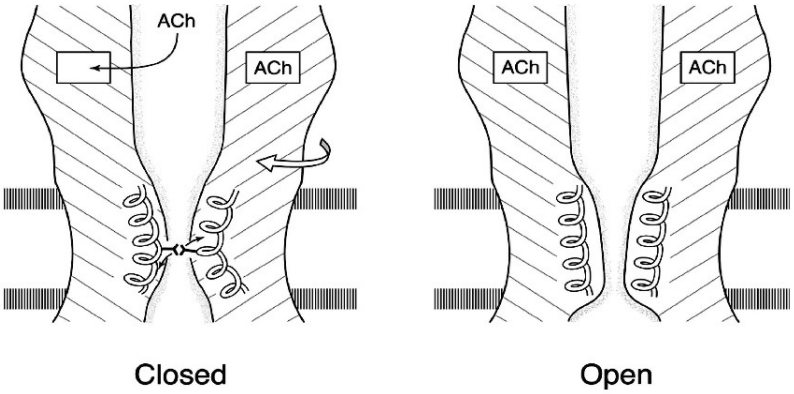

Figure 8
Simplified model of the channel opening mechanism suggested by time-resolved electron microscopic experiments. Binding of ACh to both α subunits initiates a concerted disturbance at the level of the binding pockets, which leads to small (clockwise) rotations of the α subunits at the level of the membrane. The rotations destabilize the association of bent α-helices forming the gate, and favour the alternative mode of association (Fig. 7), in which the pore is wider at the middle of the membrane and most constricted at the cytoplasmic membrane surface (adapted from Unwin, 1998).

A simple mechanistic picture of the structural transition, derived from these studies, would be as illustrated in Figure 8.. Firstly ACh triggers a localized disturbance in the region of the binding sites. Secondly, the effect of this disturbance is communicated by axial rotations, involving mainly the α subunits, to the M2 helices in the membrane. Third, the M2 helices transmit the rotations to the gate-forming side-chains (see Fig. 4), drawing them away from the central axis; the mode of association near the middle of the membrane is thereby disfavoured, and the helices switch to the alternative side-to-side mode of association, creating an open pore.

A more precise description of the extended conformational change, linking to the transmembrane portion, has recently been derived by a comparing the 4.6Å structure of the extracellular domain with the crystal structure of the AChBP. It is found that, to a good approximation, there are two alternative extended conformations of the receptor subunits - one characteristic of either α subunit before activation, and the other characteristic of the three non-α subunits – and that the binding of ACh converts the structures of the two α subunits to the non-α form (Unwin et al., 2002). Evidently, the α subunits are distorted initially by their interactions with neighbouring subunits, and the free energy of binding overcomes these distortions, making the whole assembly more symmetrical, analogous to the ligand-bound AChBP.

This transition to the activated conformation of the receptor involves relative movements of the inner and outer parts of the β-sandwich, which compose the core of the α subunit (see Figures 4-6), around the cys-loop disulphide bond, as shown in Figure 9.

Figure 9 near here

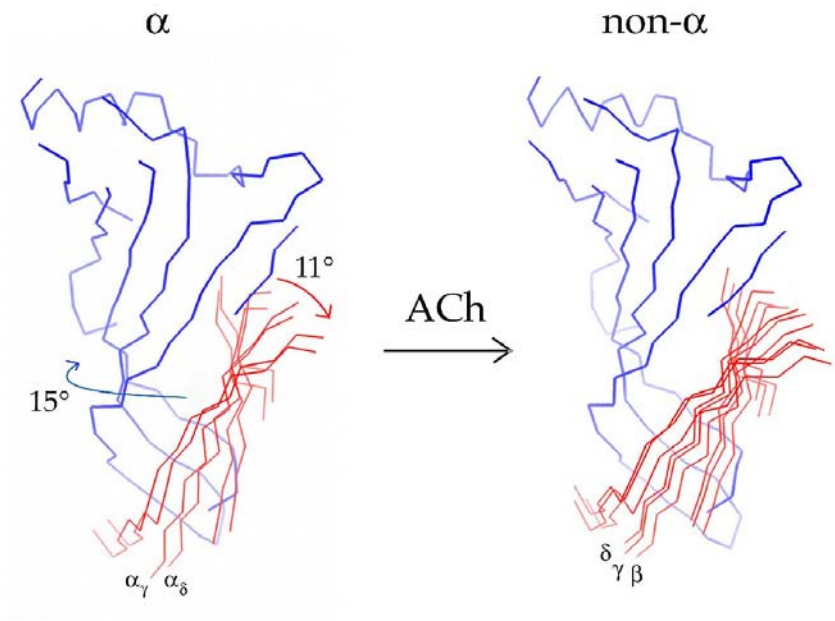

Figure 9
Arrangement of the inner (blue) and outer (red) β-sheet parts of the (a) α and (b) non-α subunits (see Figs, 4 and 6), after fitting to the densities in the 4.6Å map of the receptor. The arrangement of the sheets in the α subunits switches to that of the non-α sheets when ACh binds. The views are in the same direction, towards the central axis from outside the pentamer. Arrows and angles in (a) denote the sense and magnitudes of the rotations relating the α to the non- α sheets. The traces are aligned so that the inner sheets are superimposed. (adapted from Unwin et al., 2002).

Most strikingly, there are 15-16° clockwise rotations of the polypeptide chains on the inner surface of the vestibule next to the membrane-spanning pore. The M2 segments, and also the M2-M3 loops lie directly under these rotating elements. The importance of the M2-M3 loop for gating was first suggested by the group of Schofield as a plausible interpretation of the mechanism by which startle mutations in this area impair the agonist sensitivity of another member of the nicotinic superfamily, the glycine receptor (Rajendra *et al.*, 1995). Thus, Lynch *et al.* (1997), on the basis of a scanning alanine mutagenesis study and macroscopic dose-response curves, suggested that *both* M1-M2 and M2-M3 loops are involved in gating. The single channel work of Lewis *et al.* (1998), based on a preliminary plausible model of

the glycine receptor activation mechanism, confirmed that a mutation in M2-M3 (αK276E) predominantly changes gating. In the nicotinic receptor too, there is evidence that the M2-M3 region is important in coupling the binding-reaction to gating (Grosman *et al.*, 2000). Thus it seems that coupling does not occur directly through M1, which does not appear to move significantly, but via an interaction of the M2-M3 loop with a part of the extracellular chain associated with the inner sheet, probably the loop between the β1 and β2 strands (see asterisk in Fig. 4 and Brejc *et al.*, 2001) and/or the cys loop.

How does binding of ACh bring about the extended conformational change in the α subunits, converting them into a non-α form? A likely possibility, consistent with the three-dimensional maps and also with the results of biochemical experiments using binding-site reagents (Karlin, 1993), is that the C-loop is drawn inwards by the bound ACh, bringing it closer to its location in the (non-α) protomer of AChBP. The joined outer sheet could in this way be reorientated and stabilized in the configuration it would have in the absence of subunit interactions, hence favouring a switch towards the relaxed, non-α form of the subunit. Whatever the precise details, the movements that result in the open shut transition must be fast, because it is known that the whole transition from shut to open takes less than 3 μs (how much less is not known) to complete once it has started (Maconochie *et al.*, 1995), and the channel often shuts briefly (average 12 μs) and re-opens (see below).

Function and structure in muscle receptors

The high resolving power of single ion channel measurements means that the function of ion channels is probably understood better than that of most enzymes. On the other hand, the lack of detailed crystal structures has hampered efforts to relate these functional measurements to the protein structure. That situation is improving rapidly.

The nature of the problem: separation of binding and gating

Methods for recording the currents through single ion channels were developed by Neher & Sakmann (1976) and Hamill *et al.* (1981). The theoretical basis for their interpretation was developed initially by Colquhoun & Hawkes (1977; 1982). Far more is known about the muscle-type nicotinic acetylcholine receptor than about any other. The first attempts to investigate the mechanism of action of acetylcholine (ACh) itself were made by Colquhoun & Sakmann (1981; 1985) By that time it was already well known that there were two binding sites for ACh. Single channel measurements made it clear that the channel *can* open with only one ACh molecule bound (though much less efficiently than with two bound). This is something that is essentially impossible to detect unambiguously from whole cell measurements. Perhaps more importantly, these measurements allowed a distinction to be made between the initial binding of the agonist, and the subsequent conformational change. This distinction is absolutely crucial for understanding the action of agonists in terms of classical ideas of affinity and efficacy, and it is the crucial logical basis for the use of mutation studies to identify the position of the ligand-binding site. This "binding-gating" problem has been reviewed by Colquhoun (1998). The problem has been solved most fully for the muscle-type nicotinic acetylcholine receptor, and there are some reasonably good estimates for GABA and glycine receptors. For other ion channels, and for all G protein-coupled receptors, the problem is still unsolved.

The binding-gating problem in its simplest form can be described in terms of the del Castillo & Katz (1957) mechanism. This describes the binding of a single agonist molecule (A) to a receptor (R), with an equilibrium constant $K = k_{-1}/k_{+1}$. The binding may be followed by a conformation change from the shut to the open state, with an equilibrium constant $E = \beta/\alpha$, thus

$$\mathrm{R} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{AR} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \mathrm{AR}^* \quad . \qquad (1)$$

The fraction of channels that are open at equilibrium is related hyperbolically to the concentration of agonist, with maximum $E/(1 + E)$. The fraction of receptors that have an agonist bound to them at equilibrium is also related hyperbolically to the concentration of agonist (with maximum 1). The concentration that is needed to produce 50% of the maximum effect, for both response *and* binding, is $K_{eff} = K/(1 + E)$. Thus the effects of binding (K) and gating (or efficacy, E) cannot be separated by either functional or binding experiments at equilibrium.

Binding experiments do not measure agonist binding (in any sense that is useful for learning about the binding site, or for elucidating structure-function relationships). In the context of a Monod-Wyman-Changeux type mechanism (Monod *et al.,* 1965), it is true that an agonist that is very selective for the open state will give, in a macroscopic binding experiment, an equilibrium constant for binding that approaches the true (microscopic) affinity for the *open* state. However, to get from the shut to the open state requires a change in conformation that is potentially affected by mutations in any part of the molecule that moves. And this change in conformation is what determines the microscopic affinity for the open state. Thus, if we want to know about the binding site itself (as opposed to other regions that change shape on opening) then we need to know the microscopic affinity for the *shut* state, and this cannot be obtained from a ligand-binding experiment with an agonist. For a more detailed discussion of this question see Colquhoun (1998).

Methods for measurement of function

Solving the binding-gating problem is equivalent to finding a reaction mechanism that describes the actual reaction mechanism of the receptor (to a sufficiently good approximation), and then estimating values for the rate constants in that mechanism. If anything has been learned in the last 40 year it is that it is futile to imagine that firm conclusions can be drawn about channel function without a physically-realistic mechanism. Null methods that circumvent the need for detailed knowledge of mechanism work well for antagonists (the Schild method, Arunlakshana & Schild, 1959), but they do *not* work for agonists (Colquhoun, 1987; Colquhoun, 1998). In many ways the qualitative step of identifying the mechanism is harder that the quantitative problem of estimating rate constants, though the latter can be hard enough. Mechanisms like that in (1) can be ruled out straight away for the nicotinic receptor because it is known that two agonist molecules must be bound to open the channel efficiently. Schemes A and B in Figure 10 are the two that have been most commonly used.

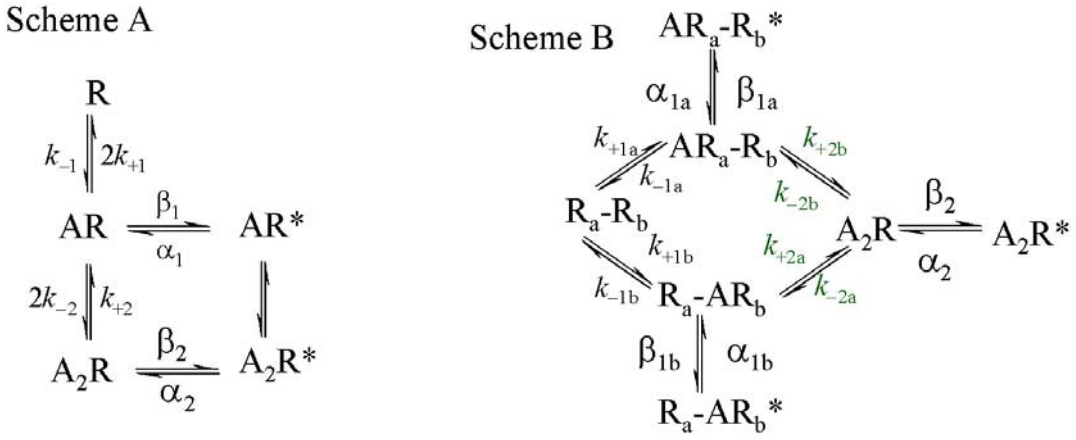

Figure 10

Two reaction schemes that have been widely used to represent the activation of the nicotinic receptor. R represents the inactive (shut) receptor, R* the active (open) receptor, and A the agonist. The rate constants for the individual reaction steps are denoted *k* (for association and dissociation), or α (for shutting) and β (for opening).

A. In this case the two binding sites for ACh are supposed to be the same, though the possibility is allowed that there may be cooperativity in the process of binding to the shut receptor, so binding of the second ACh molecule may not have the same rate constants as binding of the first.
B. The two binding sites for ACh are assumed to be different from the start, so two distinguishable mono-liganded states exist. In its most general form, this mechanism also allows for cooperativity of the binding reaction, in the sense that the rates for binding to site *a* may depend on whether or not site *b* is occupied (and *vice versa*).

In Figure 10A, the two binding sites for ACh are supposed to be the same, though the possibility is allowed that there may be cooperativity in the process of binding to the shut receptor, so binding of the second ACh molecule may not have the same rate constants as binding of the first. In Figure 10B (which in most cases is the more realistic) the two binding sites for ACh are assumed to be different from the start, so two distinguishable mono-liganded states exist. In its most general form, this mechanism also allows for cooperativity of the binding reaction, in the sense that the rates for binding to site *a* may depend on whether or not site *b* is occupied (and *vice versa*). In almost all work it has been assumed that such cooperativity is absent, so the following constraints are applied.

$$k_{-2a} = k_{-1a}\ , \quad k_{-2b} = k_{-1b}\ , \quad k_{+2b} = k_{+1b}\ . \tag{2}$$

These, together with the microscopic reversibility constraint, assure also that

$$k_{+1a} = k_{+2a} \tag{3}$$

In addition to the states that are shown in these schemes, we need to add, at higher agonist concentrations, states that represent open channels that have become blocked by ACh molecules. Block by ACh is fast, the blockages lasting only for 15-20 μs or

so on average (similar to the spontaneous short shuttings of the receptor). The affinity for block of the open channel is very dependent on membrane potential but is usually around 1 mM (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985). All other agonists and antagonists that have been tested can also produce block, some with much higher affinity than ACh (e.g. (+)-tubocurarine, Colquhoun *et al.*, 1979); suxamethonium, Marshall *et al.*, 1990). The channel block by (+)-tubocurarine has a particularly high affinity, which seems strange since it is too big to fit in the pore. Presumably it must bind further out (though still within the electric field), where the channel/vestibule is wider, and in such a way that ions cannot pass it.

Desensitisation

The schemes in Fig. 10 do not contain desensitised states, i.e. states in which the agonist is still bound to the channel, but the channel is closed in a conformation distinct from that of the (unliganded) shut-channel form. This omission can be justified by the facts that (a) we can measure the things that we need without having to include desensitised states, (b) desensitisation is probably of no physiological importance for nicotinic receptors (Magleby & Pallotta, 1981), and, in that sense, is of peripheral interest, and (c) desensitisation is a complex and ill-understood phenomenon, so it is hard to describe an adequate reaction mechanism for it. It has been known for some time that desensitisation is a complex phenomenon that develops on many different time scales, from milliseconds to minutes (e.g. Katz & Thesleff, 1957; Cachelin & Colquhoun, 1989; Butler *et al.*, 1993; Franke *et al.*, 1993)). Recently the extent of this complexity has been shown elegantly by single channel methods (Elenes & Auerbach, 2002). There is clearly not just one desensitised state, but many, though nothing is known yet about the structural differences between these states. It is simply not feasible to fit the many rate constants, and fortunately it is not necessary to do so in order to learn about channel activation.

Fitting rate constants

In earlier work, inferences about rate constants were made from single channel recordings by *ad hoc* methods, and only rough corrections were possible for the fact that brief events (too fast for the bandwidth of the recording) are not detectable in single channel recordings. Inferences were made from distributions of quantities such as the distribution of apparent open and shut times, the distribution of the apparent number of openings per burst, and the distribution of burst length. Use of these univariate distributions (i.e. obtaining the time constants by fitting each distribution separately) does not make the best use of the information in the record because the lengths of openings and shuttings are correlated in just about every sort of ion channel in which the question has been examined, and use of bivariate distributions is necessary to extract all the information (Fredkin *et al.*, 1985). A method that extracts all of the information in the record was first proposed by Horn & Lange (1983), but it could not be used in practice because of the missed event problem.

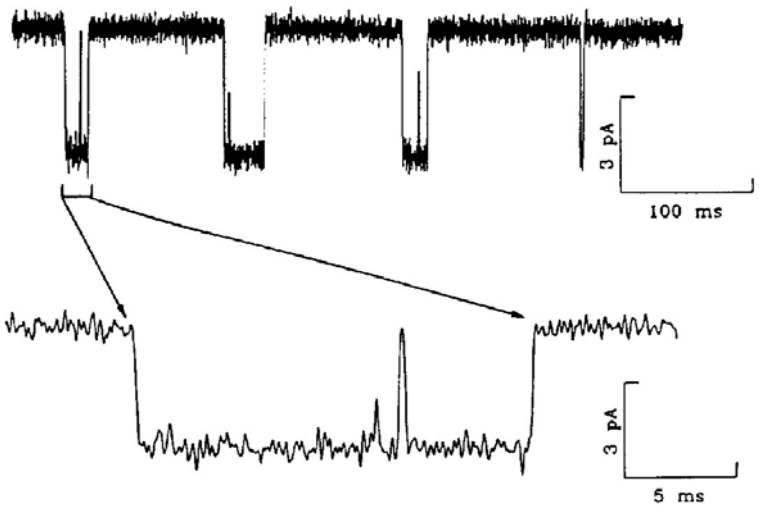

Figure 11
Four individual activations of the muscle nicotinic receptor by acetylcholine. The first activation is enlarged to show that it is not a single opening, but (at least) three openings in quick succession. In the enlarged part the durations of the openings are 10.7, 1.0 and 5.7 ms.. These are separated by shuttings of 0.061, and 0.289 ms (filtering of the record is such that the 61 μs shut time is too short to reach the baseline)/ Recording from frog muscle (*cutaneus pectoris*) endplate (ACh 100 nM, −100 mV, Colquhoun & Sakmann, unpublished).

Recordings from muscle-type nicotinic receptors contain many brief closures (see Figs. 11 and 13) with a mean lifetime of around 15 μs at 20° C, and since the shortest event that can be detected reliably is around 20 -30 μs, the majority of these are missed (since the durations are exponentially distributed it is possible the estimate the mean even when observations as short as the mean are missed). Methods have improved since then. Now an exact method for allowing for missed events is available, so it is possible to analyse an entire observed recording by maximum likelihood methods that extract all of the information and which incorporate missed event correction. There are some other methods under development, in particular methods based on the theory of Hidden Markov processes, but none are in routine use, and none apart from maximum likelihood methods have had any systematic investigation of the properties of the estimators. A brief description of the maximum likelihood approach will be given next.

Maximum likelihood estimation of rate constants from single channel records

'Likelihood' means the probability (density) of the observations given a hypothesis concerning the reaction mechanism and the values of the rate constants in it. Two programs are available for doing such calculations, MIL from Buffalo (the lab of Auerbach & Sachs) (http://www.qub.buffalo.edu/index.html), and HJCFIT (http://www.ucl.ac.uk/Pharmacology/dc.html) from UCL. Both programs work on similar principles, both can fit several data sets simultaneously, and MIL may be faster, but the UCL version has a number of advantages over MIL in other respects. For example (a) it uses exact missed event correction rather than an approximation, (b) it uses exact 'start and end of burst' vectors that improve accuracy when low concentration records have to be fitted in bursts (because of lack of knowledge of the number of channels in the patch), (c) the final fit can be tested not only by plotting open and shut time distributions but also conditional distributions and dependency plots which show how well the fit predicts correlations in the observations, and (d) it is the only method for which the quality of the estimates has been tested by repeated simulations.

The method of maximum likelihood allows the rate constants in a specified reaction mechanism to be estimated *directly* from an idealised single channel record. There is no need to plot open and shut time distributions etc beforehand. The principles of the methods currently in use have been described by Hawkes *et al.*, (1990), Hawkes *et al.* (1992), Colquhoun *et al.* (1996), Qin *et al.* (1996); (see also Colquhoun & Hawkes, 1995). The HJCFIT approach has been tested by Colquhoun et al (2003), and used by Hatton et al. 2003 and by Beato *et al.* (2002). The MIL program has been used in many publications from the Auerbach and Sine labs.

Many mutants of nicotinic receptors, both naturally occurring and artificial, have been investigated. The need for methods such as those just described is emphasisied by the fact that only a minority of these mutants have been investigated by methods that make a serious attempt to distinguish binding and gating effects (many of the recent studies come from the labs of Auerbach & Sine). There is little point in trying to relate the results to receptor structure if this has not been done, so we shall next discuss some of the better characterised mutants, as well as the wild type receptor. In particular, several mutations that were found to reduce the potency of ACh were initially guessed to have affected ACh binding, and therefore the mutated residues were presumed to be in the ACh binding site. However re-examination has shown that some of these mutations actually affect conformation change rather than binding, and conversely some residues that were not thought to be part of the binding site seem to have a big effect on binding. Some reassessment of structure-activity relationships therefore seems desirable.

The wild type nicotinic acetylcholine receptor (muscle type)

The scheme shown in Fig 10B seems to be adequate to describe the behaviour of the wild type human receptor, though the results of Hatton et al. (2003) suggest that the entire concentration range can be fitted well only if either (a) the rate constants for binding to site *a* depend on whether site *b* is occupied, or (b) an extra shut state with a lifetime of around 1 ms is added to the right of the open state (Salamone *et al.,* 1999).

Diliganded receptors

From the point of view of the structure-function relationships of proteins, the difference between the two ACh binding sites is of great interest. However from the physiological point of view it is not very important. Singly-liganded openings are brief, and except at very low concentrations, rare. They contribute next to nothing to the endplate current that is responsible for neuromuscular transmission. From the physiological point of view, the rates that matter most are the opening and shutting rate constants for the doubly-liganded channel (α_2 and β_2) and the total rate at which agonist dissociates from the doubly-liganded receptor ($k_{-2a} + k_{-2b}$; see Fig 10). After exposure to the transient high concentration of ACh released from a nerve ending, most receptor molecules will be in the doubly-liganded states, and these three values determine the length of each individual opening, the number of re-openings and the lengths of the short shut periods that separate each opening. In other words, they are sufficient to determine the characteristics of the predominant doubly-liganded bursts of openings (channel 'activations') that are responsible for neuromuscular transmission. These three rates are, fortunately, the easiest to determine (see Colquhoun et al., 2003).

For the wild type receptor of most species, the channel opening rate constant β_2 is 50000 to 60000 s^{-1} at 20° C (e.g. Salamone *et al.*, 1999; Hatton et al 2003). This is not much different from the value originally found in frog muscle (30000 s^{-1} at 11° C; Colquhoun & Sakmann, 1985). In the lab of Auerbach & Sachs, this value is usually estimated by extrapolation to infinite concentration of the reciprocal of the durations of shut times within bursts. This method has the drawback that the extrapolation has to be done with the wrong equation (the Hill equation), and that it is often hard to get close to saturation. It is not, in any case, necessary, because β_2 is easy to estimate directly, simultaneously with the other parameters, by the maximum likelihood method (Hatton et al 2003; Colquhoun et al. 2003). In practice, however, there is not much disagreement about the value of β_2 . Most values are in the range 40000 – 60000 s^{-1}. Fast concentration jump methods give similar values to those found by single channel analysis. e.g 15000 s^{-1}. (Liu & Dilger, 1991), 30000 s^{-1} (Franke *et al.*, 1993), or 30000 - 100000 s^{-1}. (Maconochie & Steinbach, 1998). These measurements provide valuable confirmation that the proposed interpretation of the single channel observations is essentially right. The channel shutting rate, α_2, is about 1500 to 2000 s^{-1} at 20° C, so individual openings last about 0.5 – 0.7 ms on average. The total dissociation rate from doubly-liganded receptors is 14000 to 15000 s^{-1}. These numbers imply that an average doubly-liganded channel activation consists of about 4.8 openings (each of 0.6 ms), separated by 3.8 brief shuttings (each of 14.4 μs), so the mean length of the activation is about 2.9 ms. These numbers seem to be similar in human, rat and frog, but some species variation is possible. It is hard to compare values for mean open times in the literature because most are not corrected for missed brief shuttings. For this reason, it is safer to compare values for burst lengths, but they are often not given.

Monoliganded receptors

It is clear that the two ACh binding sites differ, and this has been found to be the case for most subtypes of the receptor. There has, however been little unanimity about the extent to which they differ (see also Edmonds *et al.*, 1995). There seems to be a particularly large difference for the *Torpedo* receptor (Sine *et al.*, 1990). Binding of a single ACh molecule is sufficient to produce brief openings of the channel (though with very low efficacy, Colquhoun & Sakmann, 1981), and in the adult human receptor he most obvious sign that the sites differ lies in the fact that two classes of singly liganded openings are detectable (see Figure 16), one much briefer than the other (Hatton et al., 2003). The shorter one is barely resolvable, but has been demonstrated more clearly in ultra-low noise recordings from mouse embryonic receptors (in myoballs) by Parzefall *et al.* (1998). Colquhoun & Sakmann (1985) found little evidence for a difference in frog muscle receptors. Using more recent methods, it has been suggested that there is little difference between the two sites in the adult form of the mouse receptor (Salamone *et al.*, 1999). These authors found a significantly better fit if the sites were not assumed to be equivalent, but had similar equilibrium constants for binding. However this study used only high agonist concentrations and ignored singly-liganded openings, so it is unlikely to be very sensitive to differences between the sites. The extent of the difference between the two sites for ACh may be species-dependent, or perhaps the inconsistent reports

merely reflect the difficulty of the electrophysiological experiments. The fact is that it is hard to distinguish all of the separate rate constants for the two sites by electrophysiological methods (the shortest open times are on the brink of resolvability), and even with the best forms of analysis now available it is not possible to resolve the 13 free parameters in the scheme in Fig. 10B, without imposing the (possibly untrue) constraint of independence of the sites (see eqs. 1 and 2) (Colquhoun *et al.* 2003).

Binding experiments have given very convincing evidence for the two binding sites being different in their ability to bind *antagonists*. Although studies with antagonists do not tell us directly about how ACh will behave, they do have the enormous advantage that there is no binding-gating problem with antagonists and their microscopic binding affinities can be measured much more directly than for agonists.
For example, the small peptide α-conotoxin MI binds with much higher affinity to the α/δ site than to the α/γ site of mouse muscle receptors (Sine *et al.*, 1995a); see also the discussion on conotoxins below. This interaction seems to involve particularly Y198 on the α subunit and S36, Y113 and I178 on the δ subunit (Bren & Sine, 2000). Site selectivity is opposite for tubocurarine and its derivative metocurine (dimethyltubocurarine), which have higher affinity for the α/γ (or α/ε) site than for the α/δ site of mouse muscle or *Torpedo* receptors (Sine, 1993; Hann *et al.*, 1994; Bren & Sine, 1997). Note that site selectivity is species dependent for α-conotoxin MI, which targets α/γ (rather than α/δ) in *Torpedo* receptors (Groebe *et al.*, 1995).

Some well-characterised mutations

In the enzyme acetylcholinesterase, there is evidence that the charged quaternary ammonium group of ACh interacts with an aromatic residue, tryptophan (Silman *et al.*, 1994). The evidence concerning this interaction is much less certain in the nicotinic receptor, but the putative binding site region contains a number of aromatic amino acids that have been investigated. Several of them are labelled by photo-affinity reagents (Devillers-Thiery *et al.*, 1993).

αW149. An ingenious study by Zhong *et al.*, (1998) used unnatural amino acids to conclude that αW149 was "the primary cation-π binding site" in the nicotinic receptor. This sort of study cannot, however, distinguish between effects on binding and gating, because it relies on macroscopic EC_{50} data only. A single channel study by Akk (2001) found an 80-fold increase in EC_{50} for the αW149F mutation, but only a 12-fold weakening in binding affinity. The remainder of the potency reduction resulted from impairment of gating, the opening rate constant, β_2, of the doubly-occupied channel being reduced 93-fold.
In the AChBP, the analogous residue, W143, forms part of the wall of the HEPES binding site (Fig. 6A).

αY93. Properties of the αY93F mutation have been described by Auerbach *et al.* (1996), Akk & Steinbach (2000) and Akk (2001). This mutation increases the EC_{50} for ACh by 39–fold, with a 4-fold increase in the dissociation equilibrium constant estimated on the assumption that the two binding sites are equivalent (a 13-fold reduction in the association rate constant, plus a 3-fold reduction in the dissociation rate constant). Gating was quite strongly affected: there was a 50-fold decrease in

channel opening rate constant (β_2), and 2-fold increase in the closing rate constant (α_2), and therefore about 100-fold reduction in the gating equilibrium constant, $E = \beta_2/\alpha_2$. Even allowing for the fact that the EC_{50} depends roughly on $\sqrt{E}$ (see Colquhoun, 1998), the major effect is on gating rather than binding.
In the AChBP, the analogous residue, Y89, forms part of the bottom half of the HEPES binding site (Fig. 6A).

αY190. This tyrosine is on the C-loop and is close to the pair of adjacent cysteine residues (C192 and C193) that characterises α subunits. The mutation Y190F (like Y93F and W149F) also decreases macroscopic ACh binding (Tomaselli *et al.*, 1991), and increases the EC_{50} by 184-fold (in embryonic mouse muscle receptor, Chen *et al.*, 1995). These effects were originally attributed to changes in the binding. Chen *et al.* (1995) found that the equilibrium dissociation constant for binding to the *shut* receptor was indeed increased about 70-fold (they fitted a mechanism with two sequential bindings so this factor refers to the product of the two binding equilibrium constants, which is what matters for doubly-occupied receptors). However they also found large effects on gating: a 400-fold decrease in channel opening rate (β_2) and a 2-fold increase in shutting rate (α_2), so there was an 800-fold reduction in the gating equilibrium constant, $E = \beta_2/\alpha_2$. Qualitatively similar mixed effects were seen when Y190 was replaced by W, S or T (Chen *et al.*, 1995).
In the AChBP, the analogous residue, Y185, forms part of the bottom half of the HEPES binding site (Fig. 6A).

αY198. Although αY198 (which is also in the C-loop) has been proposed to interact directly with ACh in its binding site (O'Leary & White, 1992; Sine *et al.*, 1994), single channel analysis of αY198F shows hardly any effect on the dissociation equilibrium constant (and only 2-fold slowing of the rates), with a larger, but still modest effect on gating (Akk *et al.*, 1999).
In the AChBP, the analogous residue, Y192, forms part of the wall of the HEPES binding site (Fig. 6A)

αD200. O'Leary & White (1992) suggested that gating changes account for the modest increase in EC_{50} observed in αD200N. This conclusion was based on the complete loss of the efficacy of partial agonists in mutant receptors. This interpretation was confirmed by the single-channel study of Akk *et al.* (1996) who observed a profound decrease in β_2 (100- and 400-fold for the adult and embryonic mouse muscle receptor, respectively). This was accompanied by a small (3-fold) increase in the closing rate α_2, which further decreased E_2 to a value between 0.1 and 0.2 for both embryonic and adult receptors. The binding of ACh was investigated using the scheme in Fig 10A, with omission of the singly-liganded open state (a reasonable approximation given that most experiments were at high enough concentration to make them rare). This scheme describes cooperativity of binding rather than non-equivalence of sites, but it should give reasonable estimates of the total dissociation rate from the doubly-liganded state. The results suggested a slight *increase* in affinity for ACh for adult mouse receptor and little change in embryonic mouse receptor. The effects of the mutation are virtually entirely as result of impaired gating.

In the AChBP there is also aspartate at the equivalent position (D194) (see Fig. 6A). It is at the end of the 'C loop' and almost 10Å from the nearest part of the HEPES

ligand. This residue forms a hydrogen bond with K139, and this may be important for keeping C-loop in an appropriate position for ACh binding. The C-loop seems to be mobile in the unbound structure but comes inwards upon binding, moving the outer sheet with it to initiate the extended conformational change. One might expect residues on the C-loop (Y190, Y198, D200), residues near the inner/outer-sheet interface (especially G153), and residues at the α/γ or the α/δ subunit interfaces (Y93, W149), to be important for coupling the ACh-binding reaction to gating, because they are in locations where relative movements occur. Also the pair of Cys residues, which can switch conformations about their disulphide bond, might play a role in the conformational change by stabilizing alternative configurations of the C-loop.

αG153S. This is a naturally occurring 'gain-of-function' mutation in humans, in which it causes a slow channel congenital myasthenic syndrome (SCCMS; Engel *et al.*, 1982). It is also interesting because it is one of the mutations the effects of which are almost entirely on binding. It leads to prolonged decay of miniature end-plate currents (MEPCs) as a result of the channel activations (bursts) being about 15-fold longer than in wild type, on average (Sine *et al.*, 1995b; Croxen *et al.*, 1997). Single channel analysis indicates that the main reason for the prolonged bursts of openings, in receptors that contain αG153S, is slowed dissociation of ACh from the doubly-liganded shut state, so the channel re-opens more often (Sine *et al.*, 1995b; Salamone *et al.*, 1999). The fits in the latter paper suggest a 30-fold decrease in equilibrium dissociation constant for ACh binding, attributable mainly to a reduced dissociation rate from the doubly-liganded shut state, with relatively little effect on gating (the opening rate β_2 is hardly changed by the mutation, but openings become somewhat shorter, resulting in about 3-fold increase in E_2).

Despite this rather selective effect on binding, the residue (S147) in AChBP that is analogous with αG153, does not seem to form part of the binding site (see Figs. 4 and 6), but is separated from it by at least 10Å. Nevertheless, this residue seems to be in a critical location in terms of linking binding to gating. It is on the loop connecting the inner sheet to the outer sheet part of the β-sandwich, and the relative movements are greatest in this region when ACh binds.

αN217K. This is another naturally occurring SCCMS mutant in man; and like αG153S, it is a 'gain-of-function' mutation. MEPCs recorded with intracellular microelectrodes decayed biexponentially, the slower component being approximately 7-fold longer than control (Engel *et al.*, 1996). Wang *et al.* (1997) used single channel analysis (with the MIL program), to fit the rate constants in mechanisms A and B (Fig. 10). The potency of ACh is increased 20-fold (EC_{50} is reduced 20-fold) in adult human receptors that contain αN217K . This appears to result almost entirely from an increase in the microscopic binding affinity, and in particular from a slowing of dissociation from the doubly-liganded receptor. The gating effects were even smaller than for αG153S. The main channel opening rate, β_2, was slowed by approximately 40% in the mutant, whilst α_2 was slowed by approximately 50%, so there was hardly any change in the main efficacy term, E_2.

This result is somewhat surprising since αN217 is nowhere near the area that is normally considered to be the binding site. Indeed it is not even in the extracellular region at all, but is buried several amino acids down in the predicted first

transmembrane domain (M1), as indicated in Figure 12. It has no analogue in the AChBP (which is only 210 amino acids long).

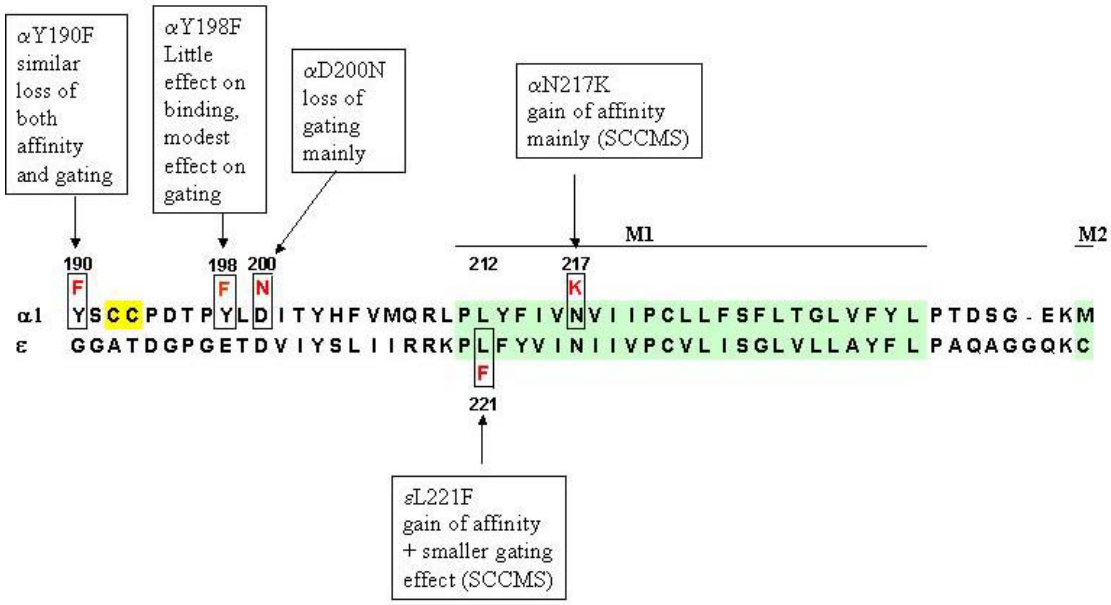

Figure 12
Aligned sequences of the human α1 and ε subunits, to show the position of some of the mutations that are discussed in the text. The (approximate) position of the M1 region is shown, and the beginning of M2

However there is still some uncertainty about which residues in M1 are actually within the lipid membrane and it could be that the boundary of M1 is actually closer to α217 than the conventional position shown in Fig. 12). It is possible (but not proven) that this mutation in M1 points to, and interacts with the M2 region which moves during gating (whereas M1 is not thought to move).

Mutations in the epsilon subunit

Much evidence has suggested that the ACh binding sites are close to subunit interfaces, and the AChBP structure seems to confirm this view. Most of the residues of the α subunit that were thought to be closely involved in binding do indeed appear in or near the binding site for HEPES shown in Fig 6A, which is on one side of the subunit-subunit interface. The other side of the interface (see Fig. 6B) is thought to be formed from the γ or ε subunit for one site, and from the δ subunit for the other site.

In the AChBP, all the subunits are the same, and for the most part their interface residues do not align in any very convincing way with the γ, ε or δ subunits. The one exception is W53 (see Fig 2A) which aligns with W55 in the mouse γ, ε and δ subunits. The γ W55 residue contributes to the binding of the *Naja naja* α-toxin (Osaka *et al.*, 2000), but no binding-gating studies have been done on mutations at this position. An additional problem on this side of the interface is that Brejc *et al.* (2001) state that "the loop F region has an unusual conformation, but as it is relatively weakly resolved, its precise analysis is difficult".

εD175N . Position 175 in the mouse ε subunit was thought to be of interest because of studies on the homologous position in the δ subunit, δD180 (Czajkowski *et al.*, 1993; Czajkowski & Karlin, 1991). They mutated all of the aspartate and glutamate residues to asparagine or glutamine respectively, in a region known to be proximal to the binding site by cross linking studies. The biggest effect seen was an eighty-fold increase in the EC_{50} for ACh produced by the δD180N mutation.

Akk *et al.*, (1999) constructed the homologous mouse mutation εD175N to test the effects on the rate constants of binding and gating. Using high concentrations of ACh and P_{open} curves, the EC_{50} of εD175N was found to be increased ten-fold relative to the wild type receptor. Using a simple kinetic scheme that assumed equivalent binding sites, maximum likelihood fitting was used to demonstrate that the mutation decreased the efficacy, E_2, nearly eighty fold. There were binding effects too, *both* the association and dissociation rates being reduced more than ten-fold, so the equilibrium dissociation constant for binding was essentially unchanged. The authors suggested that the mutation affects the mobility of ACh around the binding site, but *not* the affinity of the binding site for ACh.

The AChBP contains no obvious analogue of εD175, and so casts no light on its role in binding.

εL221F. This is another gain-of-function SCCMS mutation that has been found in two unrelated families, and which causes myasthenic symptoms (Oosterhuis *et al.*, 1987). Ultrastructural studies revealed degenerated junctional folds and diffusely thickened endplate basal lamina, as in other forms of SCCMS.
The εL221 residue is located near the N terminal end of M1 and is therefore presumably very close to, if not actually within, the cell membrane (though, as discussed above, there is some uncertainty about where M1 starts). Like other SCCMS mutants it causes prolongation of the single channel activations (bursts) produced by ACh and consequent slowing of the decay of MEPCs (Croxen *et al.,* 2002). The time constant for decay of MEPCs increased from 1.7 ms to approximately 15 ms.
Typical activations of wild type and mutant receptor are shown in Figure 13.

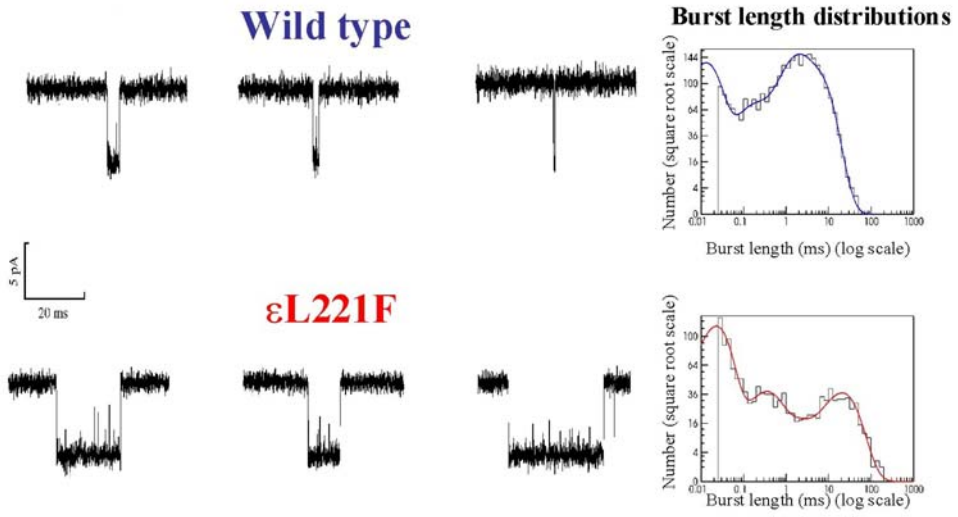

Figure 13
Typical activations of wild type and εL221F human muscle nicotinic receptors by ACh (10-30 nM). It is obvious that the bursts of openings (activations) are longer on average for the mutant receptor. This is shown by the typical distributions of the durations of bursts shown on the right.

. What is *not* obvious to the naked eye is whether the activations are longer because the individual

openings are longer, or because the channel re-opens more often (in fact the latter is the predominant effect).
Hatton et al, (2003) used maximum likelihood estimation of rate constants from single channel records, using HJCFIT. Various mechanisms were tested, including those shown in Fig 10, and variants of these that included either channel block, or the extra shut state that Auerbach and his colleagues have found to be necessary to fit some records. This postulates an extra shut state, connected directly to the doubly-liganded open state that describes isomerisation into a short-lived (about 1 ms) shut state that could (though this is merely semantic) be described as a very short lived desensitised state. A wide range of ACh concentrations was tested (30 nM to 30 μM for wild-type; 1 nM to 30 μM for εL221F).

When low concentrations were fitted separately, good fits could be obtained with the mechanism in scheme B (Fig 10), with the assumption that the binding to site *a* was the same whether site *b* was occupied or not. This assumption of independent binding to the two different sites was effected by using the constraints in eqs. 2 and 3. The same was true when high concentration records were fitted alone, though somewhat different values were obtained for some rates. As might be expected as a result of this, it did not prove possible to fit both high and low concentrations simultaneously with this scheme. To achieve this it was necessary to either (a) relax the constraints in eqs 2, 3, or (b) add the extra short-lived shut state (see above) to scheme B. It is not, at present, possible to tell which of these schemes is closer to the truth. The first option requires that the rates of association and dissociation for binding to site *a* depended on whether site *b* was occupied or not (and *vice versa*), and this implies that two binding sites, which are quite a long way apart, should be able to interact *before* the occurrence of the major conformation change that accompanies the opening of the channel. This is, physically, a somewhat unattractive idea, though by no means impossible. The second option also has an unattractive feature in that it involves postulating a rather arbitrary shut state without any good physical or structural reason.

Fortunately it is not necessary to decide between these options in order to obtain estimates of the main doubly-liganded parameters, α_2, β_2 and total dissociation rate from the doubly-liganded shut channel.

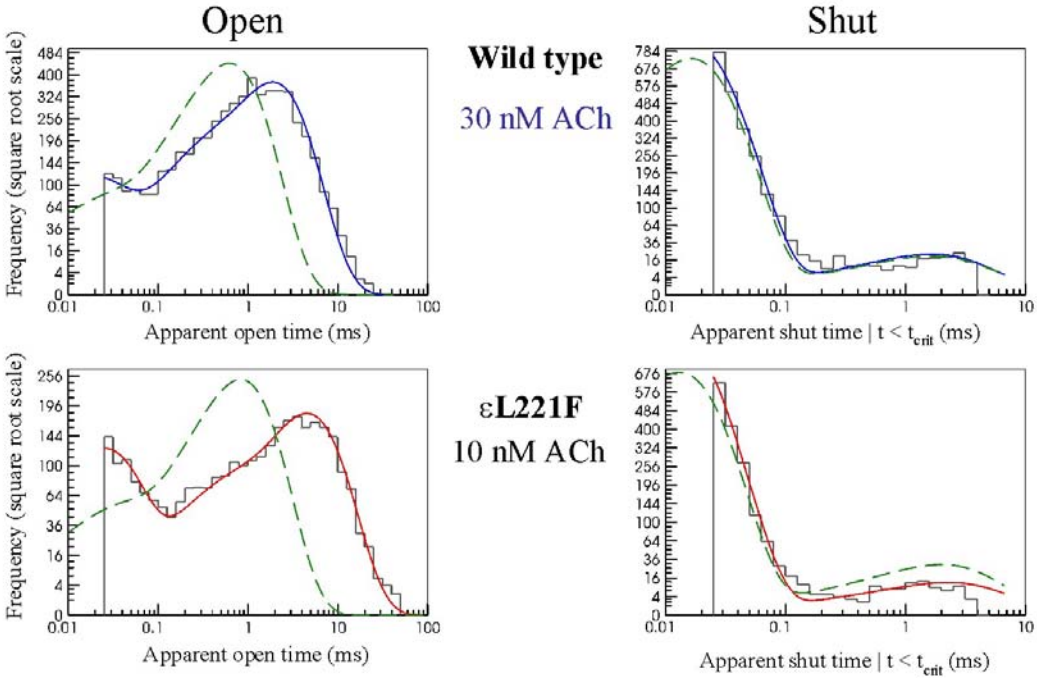

Figure 14Distributions of (logarithms of) the apparent open time (left) and apparent shut time (right) for wild type human receptors (top) and for mutant εL221F receptors (bottom). The histogram shows the experimental observations. The continuous lines were not fitted directly to the data in the histograms, but were calculated from the rate constants for the mechanism that was fitted (Fig 10, scheme B with the two sites constrained to be independent). The distributions were calculate with appropriate allowance for missed events (HJC distributions; Hawkes *et al.*, 1990; Hawkes *et al.*, 1992). The fact that they superimpose well on the histograms shows that the mechanism was a good description of the observations. The dashed lines show the distributions calculated from the fitted rate constants in the conventional way (Colquhoun & Hawkes, 1982), without allowance for missed events, so they are our estimate of the true distributions of open and shut times. (Hatton *et al.*, 2000).

Figures 14 and 15 show the results of using HJCFIT to estimate the rate constants in Fig. 10B from low concentration single channel recordings (see legends for details). Figure 16 shows a carton representation of the mechanism, with examples of activations caused by occupancy of either site alone, or of both sites. The numbers shown in Figure 16 are estimates of the rate constants for typical fits.

The effect of the εL221F mutation was, surprisingly (given its location) mainly on binding. The total dissociation rate was decreased from 15000 s^{-1} for wild type to 4000 s^{-1} for the mutant receptor. A small gating effect was seen too. The doubly liganded opening rate, β_2, was increased from 50000 s^{-1} to 73000 s^{-1}, whilst α_2 decreased from 1900 to 1200. This combination leads to a 2-fold increase in E_2, the efficacy of doubly liganded gating.

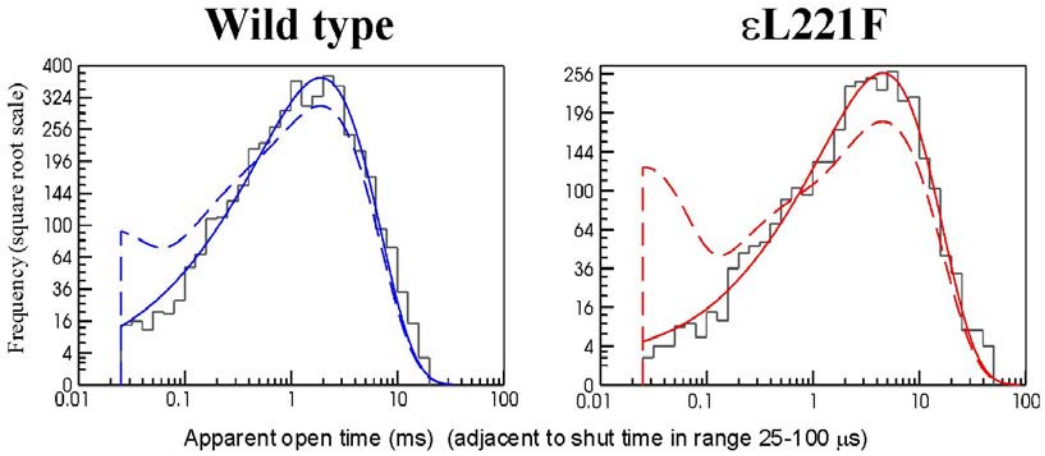

Figure 15
Conditional open time distributions
The histograms show the observed open times only for openings that were adjacent to short shut times (25-100 μs in duration). The continuous lines were not fitted to these histograms, but are the HJC conditional distributions of apparent open times calculated from the fitted rate constants (Colquhoun *et al.*, 1996). The fact that they superimpose well on the histograms shows that the fit predicts correctly the negative correlation between adjacent apparent open and shut times. The dashed lines show the calculated HJC distributions of *all* open times (as shown as solid lines in Fig, 14, left).

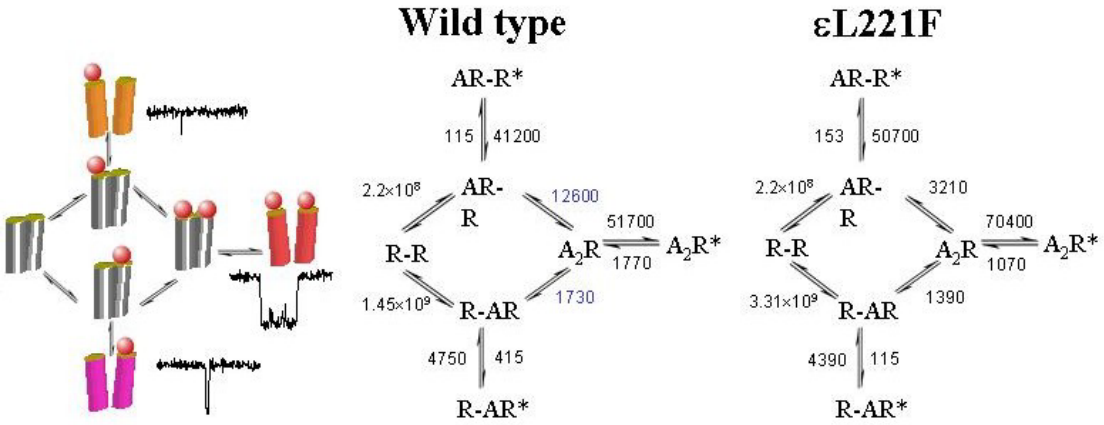

Figure 16
Left: cartoon representation of the reaction scheme in Fig 10B, with examples of the sort of channel activations that are produced by the wild type receptor with either one, or both, of the binding sites occupied by ACh.
Middle and right: the reaction scheme in Fig. 10B, with the results of a particular HJCFIT fitting marked on the arrows (the binding sites were assumed to be independent).

Macroscopic currents

Once an appropriate mechanism has been chosen, and values for its rate constants have been estimated, then programs exist to calculate the time course of macroscopic currents under any specified conditions (e.g. SCALCS from http://www.ucl.ac.uk/Pharmacology/dc.html) . Thus it can be seen whether the fitted mechanism is capable of predicting the time course of synaptic currents. The resolution of single channel experiments is so much greater than for measurements of macroscopic currents that it is not possible to go the other way. Figure 17 shows the calculated response to a 0.2 ms pulse of 1 mM ACh. for both wild type and the εL221F mutant. Despite the complexity of the mechanism (the curve has 6 exponential components), the decay phase is very close to a single exponential curve in both cases. This calculation predicts that the mutation will cause a seven-fold slowing of the decay of the synaptic current, much as observed for miniature end-plate currents measured on a biopsied muscle fibre from a patient with the εL221F mutation (Oosterhuis *et al.*, 1987).
The relationship between single channel currents and macroscopic currents has been considered both experimentally and theoretically by Wyllie *et al.* (1998), who give the general relationship that relates the two sorts of measurement.

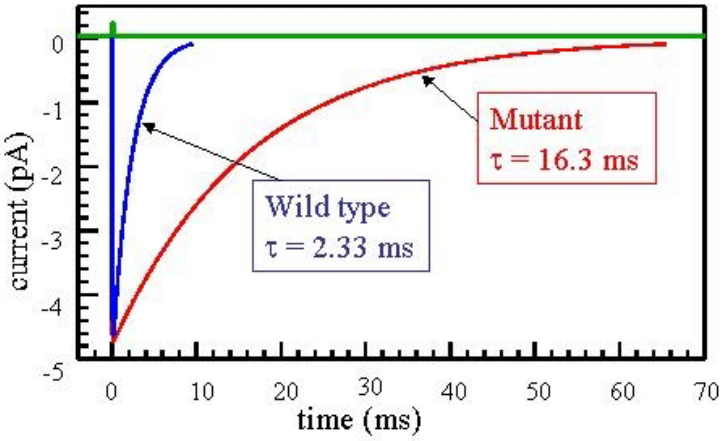

Figure 17
The predicted macroscopic current. The rate constants that have been fitted to results from equilibrium recordings (see Figs. 14-16) were used to calculate the macroscopic response to a 0.2 ms pulse of ACh (1 mM), as in Colquhoun & Hawkes (1977). This calculation predicts that the mutation will cause a seven-fold slowing of the decay of the synaptic current, much as observed (Oosterhuis *et al.*, 1987).

Summary of effects of mutations

Before considering the effect of mutations, it is first necessary to ask how accurately it is possible to distinguish binding from gating. The results of single channel analysis of the sort described are consistent with those of macroscopic jumps, and simulations (with HJCFIT) show that this method can clearly give good estimates, at least of the main doubly-liganded rate constants. For the method to give misleading results the reaction mechanisms on which it is based (like those in Fig. 12) would have to be in some way seriously wrong
Obviously it is to be expected that mutations in residues that form part of the physical binding site will affect agonist binding, and most of those discussed above do so. They mostly affect gating too, but this is not unreasonable. Since the act of binding has to be transmitted to other parts of the molecule to trigger the large conformation

change that occurs on opening, it is perhaps not at all surprising that residues such as Y190, which are almost certainly form part of the binding site itself, also influence the gating process. It is harder to explain why αY198F shows hardly any effect on binding, though according to the analogy with AChBP it is part of the binding site. On the other hand, the lack of effect of αD200N on binding, yet strong effect on gating, is consistent with its position some distance from the binding site, as predicted from the AChBP structure, but at the base of the C-loop where it could play a role in initiating the extended conformational change.

It is salutary, though, to notice that one of the purest binding effects is produced by the αG153S mutation, and it is unlikely that this residue is part of the binding site. Of course it is usually easy to produce *post hoc* rationalisations of such results. A glycine to serine mutation would be expected to reduce the rotational freedom around the peptide bond in this position. This increase in rigidity as well as the introduction of a larger more polar side chain may disrupt the structure of the binding site without actually being in the binding site.

Even more extreme examples are provided by the αN217K and εL221F mutations. Both seem to have greater effects on binding than on gating, yet they are about 30 Å from the binding site, and close to, or buried in, the cell membrane. No sense can be made of this at present, beyond making the obvious statement that it appears that the binding site can be influenced at a distance.

The optimistic way to look at the outcome of the work that has gone into mutational studies of the binding site, is that a reasonably accurate picture of the site appears to have emerged, even when the arguments have been somewhat irrational (like looking only at the EC_{50} of agonists). However this approach has, as expected, lead to some wrong conclusions too. The less optimistic conclusion that has to be drawn is that even when things are analysed by the best available methods, it is not possible to infer that a residue is part of the binding site, as exemplified by the cases of the αG153S, αN217K and εL221F mutations. Perhaps now that we are just beginning to understand the physical movements of chains and residues that accompany the process by which binding is translated into a conformation change, it may become possible to explain, and even predict some of the effects that are seen. For the moment, though, it is still necessary to engage in quite a lot of *post hoc* rationalisation. As always, comparisons with enzymes are interesting. Functional assessment of enzymes has to be done at a much cruder level than can be done with channels. However enzymes have the advantage that complete structures of many mutants have been determined. Despite this, the ability to rationalise the effect of mutations is still limited (Shortle, 1992).

Antagonists of the muscle type nicotinic receptor

There are two main types of antagonist action, competitive block, and ion channel block. Every antagonist that has been tested (and indeed every agonist too) can produce block of the ion channel, apparently by plugging the pore. Despite this fact, the competitive action is much more important for their clinical effects than the channel block action (unlike some of the antagonists of autonomic neuronal nicotinic receptors). This is still true even when the antagonist has a higher affinity for the

open channel pore than it has for the receptor binding site. For example, (+)-tubocurarine (TC) has an equilibrium constant of 0.34 μM for competition at the frog receptor (not voltage dependent), but an equilibrium constant of 0.12 μM for the open ion channel at –70 mV (or 0.02 μM at –120 mV, Colquhoun *et al.*, 1979). Like most channel blockers it can block the channel only when it is open (or at least faster when it is open –the selectivity for the open channel is far from complete for many channel blockers). However at the concentrations of TC that are used, the *rate* at which block develops is quite slow, and under physiological conditions the channels are open (and so susceptible to block) for a very short time only. This means that during normal neuromuscular transmission the equilibrium level of channel block cannot be attained, and the competitive action is far more important (Magleby *et al.*, 1981).

It is relatively easy to obtain equilibrium constants for the binding of antagonists, either by equilibrium binding methods (there is no binding-gating problem for antagonists), or from measurements of responses, by the Schild method. Nevertheless it is still unfortunately common for nothing but IC_{50} values to be given, and since these must inevitably depend on the nature of the tissue, and on concentration of agonist, this is not very helpful. A "Cheng-Prusoff" type correction cannot be applied to responses for an ion channel, which show cooperativity. An additional complication is that in order to understand the action of antagonists under physiological conditions, under which the application of agonist is very brief, we need the association and dissociation rate constants, not just their ratio, the equilibrium constant.

Rates of action of channel blockers

There is a lot of information available about the rate of action of channel blocking antagonist because this is relatively easy to determine by single channel methods, and can often be obtained also by macroscopic methods like voltage jump relaxations (e.g. Colquhoun *et al.*, 1979), or noise analysis (Colquhoun & Sheridan, 1981).

Which method is best will depend on the nature of the channel blocker. The average duration of a blockage may be 10 μs (for carbachol) up to 3 s (for TC). If the mean length of a blockage is in the range of 10s of microseconds up to several milliseconds, then blockages produce obvious interruptions in the single channel record that can be measured as a component of the shut time distribution. If, on the other hand, the blockages are very long then it becomes hard to distinguish which shut times correspond to blockages, and macroscopic relaxation methods are better.

Rates of action of competitive blockers

Knowledge of the competitive mechanism of action is far older than knowledge of channel block. Competitive block was already formulated quantitatively in 1937 (Gaddum, 1937). In contrast with channel blocking agents, which were discovered only in the 1970s (Blackman, 1970; Armstrong & Hille, 1972; Adams, 1976). It was soon discovered how to make robust estimates of the equilibrium constants for competitive antagonists from measurements of responses (Schild, 1947; Arunlakshana & Schild, 1959), and soon after by direct measurement of ligand binding (Paton &

Rang, 1965). Despite this there have been very few measurements of the *rate constants* for the association and dissociation of competitive antagonists.

Attempts to measure the rate constants for competitive block have a long history. Probably the first was by Hill (1909), in the famous paper in which he gave the first derivation of the Langmuir binding equation. He got it wrong, as most papers since have done. The usual reason for getting it wrong is that the observed rates are limited by diffusion rather that by receptor association and dissociation. The fact that the molecules are bound tightly as they diffuse makes the diffusion far slower than simple calculations might predict (when TC is present in a synaptic cleft at a concentration equal to its equilibrium constant, so half the sites are occupied, there is only one free molecule for every 200 or so that are bound). Worse still, under these conditions, diffusion plus binding may slow association and dissociation rates to roughly similar extents (Colquhoun & Ritchie, 1973), so the test that is often applied, that the ratio of the rate constants should be similar to the equilibrium constant determined independently, may give quite misleading agreement. Single channel methods are almost totally useless for solving this problem, because a competitive blocker produces long shut times in the record even when they dissociate and associate rapidly (long periods are spent shuttling, possibly rapidly, between various shut states; unpublished observations). When, as is usual, we do not know how many channels are present in the patch, these long shut periods cannot be distinguished from those caused by, for example, desensitisation. Channel activations are not changed by the competitive antagonist, they are just rarer. The problem, for nicotinic antagonists, seems to have been solved at last by Wenningmann & Dilger (2001) and Demazumder & Dilger (2001). They used concentration jump methods (exchange time 100-200 μs on bare pipette), on the embryonic ($\alpha_2\beta\gamma\delta$) mouse receptor in the BC3H1 cell line. They worked at −50 mV to minimise the effects of channel block. They measured occupancy of the receptors by TC and pancuronium by applying a pulse of ACh at various times after addition or removal of the antagonist.

They do not try to describe quantitatively the response to the test pulse, but rather assume that it is sufficiently fast that at its peak there will have been essentially no change in occupancy by antagonist. Insofar as this is true, the peak response to the test pulse (as fraction of control) should be a reasonable measure of the fraction of channels not occupied by antagonist (and in this case the IC_{50} should be close to the equilibrium constant for antagonist binding, K_B). They also assume that block of *one* site (the high affinity one mostly, if they differ) is sufficient to block the response.

For (+)-tubocurarine they find the association rate constant $k_{+B} = 1.2 \times 10^8$ $M^{-1}s^{-1}$, and the dissociation rate constant to be $k_{-B} = 5.9$ s^{-1}, so $\tau_{off} = 170$ ms. The ratio of these gives $K_B = 50$ nM, which was not greatly different from the estimate of K_B obtained from the IC_{50} (41 nM). Despite the size of the TC molecule, the association rate constant is quite fast, similar to that for ACh. At a clinical concentration that blocks, say, 90% of receptors (0.44 μM) there will be 59 associations per second (per free receptor), each occupancy lasting, on average 170 ms, and the time constant for the association reaction will be about 17 ms.

For pancuronium, Wenningmann & Dilger (2001) found an association rate constant 2.7×10^8 $M^{-1}s^{-1}$, and the dissociation rate constant to be $k_{-B} = 2.1$ s^{-1}, so $\tau_{off} = 476$ ms. The ratio of these gives $K_B = 7.8$ nM, similar to the estimate of K_B obtained from

the IC_{50} (5.5 nM). Again the association rate constant is quite fast, similar to that for ACh. At a concentration that blocks 90% of receptors (0.09 μM) there will be 21 associations per second (per free receptor), each occupancy lasting, on average 480 ms, and the time constant for the association reaction will be about 48 ms.

NEURONAL NICOTINIC RECEPTORS: GENES

The first striking difference between muscle and neuronal nicotinic receptors lies in the sheer number of genes that code for neuronal subunits, comprising nine α (α2 to α10) and three β subunits (β2 to β4 see Table 1). The important questions then become first of all what combination of subunits *can* form functional receptors when expressed together and, even more importantly, which of these combinations are present in neuronal cells and matter to the physiological and pharmacological role of neuronal nicotinic receptors.

A first approach to determine the rules that govern assembly of the neuronal subunits is to find which combinations form functional receptors in heterologous expression systems.

- Some α subunits can form homomeric receptors, i.e. give functional responses when expressed alone. Among mammalian subunits these are α7 and α9.
- Other α subunits (α2, α3, α4 and α6) can only form a functional receptor if expressed with a β subunit (β2 or β4); these heteromeric α/β receptors have a stoichiometry of 2 α to 3 β (Anand *et al.*, 1991; Cooper *et al.*, 1991; Boorman *et al.*, 2000) and by analogy with muscle receptors are likely to have a topology of αβαββ
- α10 can participate to the formation of a receptor only if expressed with α9 (Elgoyhen *et al.*, 2001; Sgard *et al.*, 2002)stoichiometry as yet unknown)
- α5 and β3 can form a receptor only if expressed together with a pair of "classical" α and β subunits (i.e. α2-α4 plus β2 or β4); these receptors are formed by 2 copies each of the "classical" α and β subunits plus one copy of α5 or β3 (thus α5 or β3 take the place of a "classical" β subunit; (Ramirez-Latorre *et al.*, 1996; Boorman *et al.*, 2000; Groot-Kormelink *et al.*, 2001).

These rules of assembly seem to hold broadly for both *Xenopus* oocytes and mammalian cell lines (for a review of the differences see Sivilotti *et al.*, 2000).

The information from the heterologous expression work must be viewed in the context of the actual pattern of expression of different subunits in central and peripheral neurones. In situ hybridization data show that α4 and β2 are the most widespread and abundant of the "heteromeric type" subunits in the CNS, whereas α3 and β4 are the most important subunits in autonomic ganglia and chromaffin cells. This distinction is not absolute (for a recent review see Sargent, 2000). Other subunits may have a more discrete localization, appearing in specific CNS areas: this is the case for α6 (which is concentrated in catecholaminergic nuclei of the brain, Le Novère et al., 1996) and β3 (present in substantia nigra, striatum, cerebellum and retina; (Sargent, 2000; Forsayeth & Kobrin, 1997)). Transcripts for the α7 subunit are present both in the CNS and in the peripheral nervous system, whereas the expression of α9 and α10 is confined to the cochlea.

The restrictions in the subunit distribution go some way to offset the most important limitation of the data from heterologous expression experiments, namely that they only tells us what the *minimal* subunit requirement is for a functional nicotinic receptor. Nevertheless, it is not uncommon for a native cell to express many of the known subunits. Does this mean that the receptors on the surface of such a neurone are a mosaic of "minimal" combination receptors or that more complex

combinations are possible and/or favoured in those circumstances? For instance, what will be the subunit composition of nicotinic receptor on a cell that expresses the α3, β2 and β4 subunits? Will the majority of receptors on the cell surface comprise all the subunits or will such a complex α3β2β4 heteromer be rare relative to α3β2 and α3β4 receptors?

There is indeed evidence that subunit combinations even more complex than the ones outlined above do form in native neurones (the list that follows is by no means exhaustive). For instance, immunoprecipitation showed that a fraction of the major type of nicotinic receptor in ciliary ganglion neurones (α3β4α5) contains also β2 subunits (Conroy & Berg, 1995). Evidence that nicotinic receptors containing four different subunits (α4, β2, β3 and β4) can form comes also from work on CNS-type subunits by Forsayeth & Kobrin (1997). Finally, in autonomic neurones, another major type of nicotinic receptor is based on α7 subunits and comprises more than one pharmacological class of receptors: one class resembles the recombinant homomeric α7 receptor, whereas the other(s) may result from coassembly with other subunits (Yu & Role, 1998; Cuevas & Berg, 1998; Cuevas *et al.*, 2000)).

This problem is recognised by the current nomenclature convention for neuronal nicotinic receptors, which allows referring to a receptor type as – for example- α7*, where the asterisk means that α7 may not be the only subunit present.

General properties of Neuronal receptors

It is difficult to relate the numerous receptor subtypes which are possible on the basis of cloning and heterologous expression data to the functional properties of actual native receptors. Biophysical properties and the pharmacological tools available so far allow us to make only broad distinctions between classes of native receptor subtypes. They cannot distinguish all those native receptor subtypes than those that are in principle possible. One reason for this limitation is that the doubt remains that heterologous expression of three or more subunits may lead to the expression of heterogeneous receptor mosaics rather than the a complex combination in a form pure enough to be characterized.

The problem is further compounded by the difficulty of carrying out kinetic studies on neuronal nicotinic receptors (mostly because of rundown): this means for instance that we do not know anything about agonist binding affinities or efficacy and how these change with subunit composition (or indeed with inherited mutations).

The first broad functional distinction is between homomeric-type and heteromeric-type receptors (the edges are blurred when we take into account the existence of α7* receptors, but the distinction is clear for recombinant receptors). The most distinctive properties for homomeric receptors in this context are their sensitivity to the antagonist effects of α-bungarotoxin and methyllycaconitine (the latter selective at concentrations up to approximately 1 nM), and their faster desensitisation and higher calcium permeability than heteromeric α/β receptors.

More detailed distinctions among the different types of recombinant heteromeric α/β combinations can be done on the basis of differences in agonist sensitivity. For instance cytisine is both potent and efficacious as an agonist on receptors that contain the β4 subunit but is poorly efficacious on β2-containing receptors (Luetje & Patrick, 1991, see discussion of agonists below). It is hard to predict what the sensitivity to cytisine would be for a complex heteromeric receptor,

i.e. a receptor containing both β2 and β4 subunits. Help in this respect may come from the increasing availability of competitive antagonists selective for the different ligand-binding interfaces, such as conotoxins (see below). At present the most useful ones are α-conotoxin MII (specific for α3β2 interfaces) and AuIBA (specific for α3β4 interfaces). It is reasonable to assume that a complex heteromeric receptor would be blocked even if only one interface were occupied by the antagonist, but there is no proof of that at present.

It is hard to identify a distinctive and consistent contribution of subunits such as α5 or β3 to nicotinic receptor properties. This may be because these subunits may not participate to the formation of the receptor binding site as either classical α- or β-type subunits. In the case of α5, the most consistent change appears to be a speeding of desensitisation. Increases in calcium permeability and single-channel conductance have also been reported, but the changes in agonist potency observed (in absolute or relative terms) depend on the specific combination expressed and are therefore not very useful as a diagnostic tool for identifying subunit combinations in native receptors (for a review see Sivilotti *et al.*, 2000). Similarly, recombinant β3-containing receptors have higher single-channel conductance, but differ little in other receptor properties (Beato, Boorman & Groot-Kormelink, personal communication). It is worth noting that single channel conductance, calcium permeability and speed of apparent desensitisation are not distinctive enough to be useful in receptor classification in most cases (see below).

Finally, co-expressing α10 with α9 in oocytes is reported to increase receptor expression, speed of desensitisation and sensitivity to external calcium (Elgoyhen *et al.*, 2001) and to change the receptor sensitivity to α-bungarotoxin and (+)-tubocurarine (Sgard *et al.*, 2002).

Biophysical properties

Calcium

Calcium has multiple effects on nicotinic receptors. Not only it is to some extent permeant, but it affects the receptor single channel conductance and modulates the agonist response of neuronal type receptors.

All nicotinic receptors are somewhat calcium permeable: the most permeable are neuronal homomeric receptors (α7, α9) and the least permeable embryonic muscle receptors. It must be noted that the measurement of relative calcium permeability by the simplest technique (reversal potential shift induced by changes in extracellular calcium concentrations) is error-prone for neuronal nicotinic receptors because their extreme inward rectification makes it difficult to measure reversal potentials accurately. A further technical difficulty (for recombinant receptors) arises from the presence in *Xenopus* oocytes of a calcium-dependent chloride conductance that has to be suppressed or minimised by either intracellular calcium chelation or chloride depletion. Some of these problems can be overcome expressing the receptors in mammalian cell lines and using ratiometric measurements of intracellular calcium and coupled with whole-cell recording, in order to obtain a measure of what proportion of the nicotinic current is carried by calcium (a measure that has also the advantage of being physiologically more relevant).

Bearing in mind these cautions, the permeability to calcium is around 1/10 to 1/5 of that to sodium or caesium ions for mammalian muscle receptors of the embryonic type (native or recombinant), and is much higher (0.5-0.9) for the adult form of the receptor (Vernino *et al.*, 1992; Costa *et al.*, 1994; Villarroel & Sakmann,

1996). This is in agreement with estimates that calcium ions carry approximately 2% and 4% of the total nicotinic current through embryonic and adult muscle receptors, respectively (at physiological calcium concentrations and holding potentials; Decker & Dani, 1990; Vernino *et al.*, 1994; Ragozzino *et al.*, 1998)).

The calcium permeability of ganglion-type nicotinic receptors is reported to be similar to that of adult muscle in experiments on superior cervical ganglion, intracardiac ganglia and chromaffin cells, with values for calcium permeability (relative to sodium or caesium) between 0.5 and 1 and fractional current measurement between 2.5 and 4.7% (Fieber & Adams, 1991; Zhou & Neher, 1993; Vernino *et al.*, 1992; Vernino *et al.*, 1994; Nutter & Adams, 1995; Rogers & Dani, 1995). This is in broad agreement with recombinant work on heteromeric receptors or native receptors likely to be heteromeric (Mulle *et al.*, 1992a; Costa *et al.*, 1994; Ragozzino *et al.*, 1998). Some reversal potential method studies suggest much higher calcium permeability (vs. adult muscle receptors) for superior cervical ganglion neurones (Trouslard *et al.*, 1993) or heteromeric recombinant receptors (Kuryatov *et al.*, 1997), particularly if the α5 subunit is expressed (Gerzanich *et al.*, 1998).

Nicotinic receptors formed by α7 or α9 are by far the most calcium permeant. Thus for recombinant or native α7-like receptors reported values for relative calcium permeability range from 6 to 20 (Bertrand *et al.*, 1993; Sands *et al.*, 1993; Castro & Albuquerque, 1995; Fucile *et al.*, 2000) with fractional current carried by calcium as high as 20% (Delbono *et al.*, 1997); for work on the M2 determinants of this high calcium permeability see Bertrand *et al.* (1993). This would mean that α7 receptors are almost as calcium permeable as the NMDA-type of glutamate receptor. Equally high calcium permeability was reported for α9-type receptors (Katz *et al.*, 1999; Jagger *et al.*, 2000) expressed alone or with α10 (Sgard *et al.*, 2002).
Finally, what matters most from the physiological point of view is that direct calcium entry through nicotinic receptors can be sufficient to act as a postsynaptic signal, for instance activating calcium-dependent SK potassium channels in outer hair cells of the cochlea (Oliver *et al.*, 2000) or contributing to apamin sensitive hyperpolarisation in rat otic ganglion (Callister *et al.*, 1997).

Note that increasing the concentration of extracellular calcium reduces the single channel conductance of both muscle and neuronal nicotinic receptors (Bregestovski *et al.*, 1979; Lewis, 1979; Mathie *et al.*, 1987; Neuhaus & Cachelin, 1990; Mulle *et al.*, 1992a; Vernino *et al.*, 1992).

Finally, changes in extracellular calcium (in the low millimolar range) can modulate nicotinic responses. Increases in calcium concentration strongly enhance macroscopic responses of either native or recombinant heteromeric nicotinic receptors to low ACh concentrations, decreasing the EC_{50} to ACh and increasing the Hill slope of the curve (Mulle *et al.*, 1992b; Vernino *et al.*, 1992; Zhou & Neher, 1993; Buisson *et al.*, 2000). This effect is not seen with muscle embryonic channels (Vernino *et al.*, 1992). In native α7* receptors the modulation has been reported to be biphasic- with potentiation at sub-millimolar calcium concentrations and depression at higher concentrations (Bonfante-Cabarcas *et al.*, 1996). The sequence determinants for this effect have been investigated for chick recombinant α7/5HT3 chimaeric receptors by Galzi *et al.* (1996) who have identified residues α7 161-172 as particularly important: in the AChBP these residues are on the minus face, at the end of loop 9, which is near the extracellular end of the pore. Le Novère *et al.* (2002) proposed, on the basis of

their modelling of the α7 subunit on the AchBP, that the binding site for calcium is formed at the subunit interface by residues belonging to different subunits. These residues include some identified by Galzi *et al.* (1996), such as E44 and E172, but also D43 and D41. Of these, E44 and D43 would be on the (+) side and E172 and D41 on the (-) side.

Single-channel conductance

The main structure determinants for the single channel conductance of neuronal nicotinic receptors are likely to be (as for muscle receptors) the residues in positions 4′, 1′ and 20′ of M2 (this numbering system for M2 residues is defined in Figure 3; for review see Buisson *et al.*, 2000). The total charge on each of these rings of charges has an important effect on conductance. It is worth noting that theM2 sequence is well conserved across neuronal nicotinic subunits. In particular, the residue in 4′ is negatively charged in all except the α5 and α9 subunits (which have a neutral residue in this position) and 1′ is always negatively charged. A difference is seen in the 20′ residue, which is negatively charged for all subunits except β2 and β4. This may be the reason for the conductance increase observed in channels containing α5 or β3 (Ramirez-Latorre *et al.*, 1996; Sivilotti *et al.*, 1997; Beato, Boorman & Groot-Kormelink, personal communication): if these subunits replace a classical β subunit they will produce a –2 change in the charge on the external ring.
While single channel conductance is a useful diagnostic criterion for the classification of other ionotropic receptors (for instance native NMDA receptors), this does not apply to neuronal nicotinic channels. Conductance levels are not very distinctive, because even recombinant receptors that should in principle be homogeneous have multiple conductance levels, and these levels overlap considerably for different combinations. In addition, the same combination expressed in different heterologous system can give rise to different conductances (Lewis *et al.*, 1997) and conductances are exquisitely sensitive to divalent ions concentrations, making it difficult to compare data that haven't been obtained in identical recording solutions. Characterization of channel conductances is also hindered by the phenomenon of "rundown" in the excised patch configuration, i.e. the disappearance of channel activity, which appears to be mostly agonist independent and may be triggered by patch excision. These factors make it very difficult to use single channel conductance (and worse still kinetics) as a criterion for the identification of specific subunit combinations in native receptors.

Inward rectification

One striking property of neuronal nicotinic channels is the extreme inward rectification of the macroscopic current-voltage relation. In contrast muscle type receptors show only modest inward rectification that can be accounted for almost entirely by the fact that the main channel shutting rate becomes slower as the membrane is hyperpolarized (about e-fold per 60-100 mV; Colquhoun & Sakmann, 1985). In neuronal receptors rectification is so extreme that there is hardly any whole-cell current at all at potentials between –10 and +60 mV: in functional terms, neuronal nicotinic channels could legitimately be described as 'discordance detectors' because they pass little current at depolarised potentials. Inward rectification has been reported for a variety of native and recombinant neuronal nicotinic receptors (with the notable exception of α9* receptors from the cochlea, Jagger *et al.*, 2000). A clue for

the understanding of its cause came from the absence of rectification in excised patches and the linear current-voltage relationship of the single channel conductance (when the artificial intracellular medium does not contain magnesium ions). This suggested that rectification is caused either by channel block by intracellular components (that are not present in artificial intracellular solutions) and/or by voltage-dependence of the channel kinetics (Mathie *et al.*, 1990). We now know that both are important: the major role is played by channel block by micromolar concentration of the intracellular polyamine, spermine (Haghighi & Cooper, 1998), with a minor contribution by intracellular magnesium ions (Mathie *et al.*, 1990; Ifune & Steinbach, 1990; Ifune & Steinbach, 1992) and by the voltage dependence of the channel P_{open}. While this work was carried out on native neuronal nicotinic receptors of autonomic ganglia (and on recombinant α4β2 and α3β4 channels), it is likely that similar mechanisms underlie the rectification of other neuronal nicotinic receptors (Alkondon *et al.*, 1994).

The M2 determinants for inward rectification have been investigated in recombinant chick α7 receptors by Forster & Bertrand (1995)).

NATIVE NEURONAL NICOTINIC RECEPTORS: PHYSIOLOGICAL ROLE

Neuronal nicotinic receptors are found on a variety of classes of neurones, both in the peripheral and in the central nervous system, and on non-neuronal cells (for a review of the latter see Wessler *et al.*, 1998).

Peripheral nervous system

In the peripheral nervous system, these receptors mediate fast synaptic transmission at autonomic ganglia and at efferent cholinergic synapses onto cochlear outer hair cells.

AUTONOMIC GANGLIA

α3 receptors*

The pattern of subunit expression, immunoprecipitation and antisense data all agree in recognising a major role to α3β4* type receptors in autonomic ganglion neurones, including chromaffin cells (Listerud *et al.*, 1991; Rust *et al.*, 1994; Conroy & Berg, 1995; Campos-Caro *et al.*, 1997; but see Skok *et al.*, 1999 and Klimaschewski *et al.*, 1994). In chick ciliary ganglia these receptors contain also the α5 subunit: additionally a significant fraction of them contains both the α5 and the β2 subunit (reviewed in Berg *et al.*, 2000).

This class of receptor has traditionally been thought to be the major or indeed the only type of receptor involved in synaptic transmission in ganglia because of the resistance of synaptic transmission to α-bungarotoxin (Brown & Fumagalli, 1977) and the subsynaptic location of these receptors (reviewed in Temburni *et al.*, 2000). These data were confirmed by the profound autonomic defect observed in mice in which the α3 had been knocked out (Xu *et al.*, 1999a). Similar problems were observed in mice lacking *both* the β2 and the β4 subunits, whereas mice lacking only one of these β subunits had a relatively normal autonomic phenotype (Xu *et al.*, 1999b). It is worth noting that the range of subunit expressed in ganglia (and the variability of the range from one neurone to the other, Poth *et al.*, 1997) offers scope for considerable heterogeneity within this class of receptors (see for instance Britt & Brenner, 1997).

α7 receptors*

It is common for autonomic ganglion neurones to express also α7-type receptors. In embryonic chick ciliary ganglion, these receptors have a location distinct from α3* receptors, as they are specifically targetted to spine mats on the soma of the postsynaptic neurone (for a review see Berg *et al.*, 2000). It is not clear to what extent postsynaptic densities are localised on these spines, but there is good evidence that blocking α7 receptors damages the reliability with which most neurones (two-thirds of the neurones in E13-14 chick ciliary ganglion) follow frequencies of presynaptic stimulation equal to or greater than 1 Hz (Chang & Berg, 1999). The situation for adult *mammalian* ganglion neurones is less clear, but these cells do express both classical α7 receptors (i.e. homomeric) and α7* receptors, which differ in their slower desensitisation and quicker recovery from α-bungarotoxin block (see for intracardiac and superior cervical ganglia (Cuevas & Berg, 1998; Cuevas *et al.*, 2000; Cuevas *et al.*, 2000). Transcripts for the α7 subunit are also present in chromaffin cells (García-Guzmán *et al.*, 1995), but functional studies have so far failed to demonstrate functional α-bungarotoxin-sensitive receptors on these cells (Nooney *et al.*, 1992a; Afar *et al.*, 1994; Nooney & Feltz, 1995; Tachikawa *et al.*, 2001) although binding sites for α-bungarotoxin can be revealed by autoradiography (Criado *et al.*, 1997). While α7 null mice are viable and do not display gross phenotypic defects (Orr-Urtreger *et al.*, 1997), they do have a subtle autonomic deficit, manifest as an impairment of baroreflex responses. This impairment is limited to responses, such as tachycardia, mediated by the sympathetic nervous system (Franceschini *et al.*, 2000).

Additionally, functional nicotinic receptors have been found on the preganglionic terminals of embryonic chick ganglia (Coggan *et al.*, 1997) and on the axon terminals of postganglionic superior cervical ganglion neurones in culture, where it was found that receptors on terminals differ in agonist sensitivity from the somatic ones (Kristufek *et al.*, 1999).

Cochlea

Outer hair cells receive cholinergic input from the olivary complex. Synaptic transmission here is mediated by α9 (or possibly α9α10) nicotinic receptors: their high calcium permeability and their coupling to a calcium-dependent potassium conductance mean that the cholinergic inward current is swamped by the outward potassium current (see Ashmore, 1994; Fuchs & Murrow, 1992). Both the native receptors on outer hair cells and recombinant α9 and α9α10 nicotinic receptors are unusual, in that they are insensitive to nicotine. These receptors are blocked by the glycine receptor antagonist strychnine (likely to be competitive, reported IC_{50} around 20 nM) and by the GABA receptor antagonist bicuculline, albeit less potently (IC_{50} around 1 μM; (Housley & Ashmore, 1991; Elgoyhen *et al.*, 1994; Elgoyhen *et al.*, 2001).

Central Nervous System

While nicotinic receptors are much less abundant in the CNS than acetylcholine muscarinic receptors, they are nevertheless widespread and found (for instance) in cortical areas, including the hippocampus, in the thalamus, basal ganglia, cerebellum and retina. Particularly high levels of high affinity receptor binding are found in the habenula and interpeduncular nucleus (reviewed by Sargent, 2000).

Despite the widespread presence of neuronal nicotinic receptors, for a long time their only known physiological role in the CNS was in mediating the excitation of Renshaw

cells by motoneurone axon collaterals (Eccles *et al.*, 1954). The situation has now changed, particularly with respect to the demonstration of a synaptic role especially for α7-like receptors.

Receptor types

In many areas of the CNS, the most represented type of heteromeric nicotinic receptor is likely to be α4β2 or α4β2*: these receptors correspond to high affinity binding sites for [^{3}H]nicotine. This is likely to be the synaptic receptor on Renshaw cells, which are immunopositive for the α4 and β2, but not the α7 subunit, in agreement with the insensitivity of their synaptic responses to methyllycaconitine (Dourado & Sargent, 2002).

Judging from the distribution of the different subunits, other receptor types may be important in discrete locations of the CNS. A case in point is that of the α6 subunit, which is represented in basal ganglia and catecholaminergic neurones (Göldner *et al.*, 1997; Klink *et al.*, 2001; Le Novère *et al.*, 1996; Léna *et al.*, 1999). Combinations of the α3β4 type are also thought to be important in discrete CNS areas i.e. in the habenula and interpeduncular nucleus. Homomeric receptors of the α7 type are also present in the CNS and correspond to the binding sites for [^{125}I] α–bungarotoxin.

Identifying nicotinic subunit combinations in the central nervous system is an area of intense research which makes use of all the tools available, such as biophysical and pharmacological characterization of functional receptor responses, *in situ* hybridisation and single-cell RT-PCR, antisense and transgenic techniques.

In particular, knockout transgenic mice lacking the α3, α4, α7, α9, β2 and β4 subunits have been bred (Xu *et al.*, 1999a; Marubio *et al.*, 1999; Orr-Urtreger *et al.*, 1997; Vetter *et al.*, 1999; Picciotto *et al.*, 1995; Xu *et al.*, 1999b, respectively). In addition, mice bearing the L9'T mutation in the α4 or the α7 subunits have been obtained (Labarca *et al.*, 2001; Orr-Urtreger *et al.*, 2000): in both strains homozygous mice die soon after birth. A discussion of the implications of these data for our understanding of the diversity and the physiological and pharmacological roles of nicotinic receptors can be found in Cordero-Erausquin *et al.* (2000) and Zoli *et al.* (1998).

We shall focus our review of receptor types to a specific area, the mammalian hippocampus and to electrophysiological evidence.

The first descriptions of nicotinic responses in hippocampus refer to agonist responses recorded in long-term primary cultures (Zorumski *et al.*, 1992; Alkondon & Albuquerque, 1993). Three main types of agonist responses were described, broadly corresponding to α7, α4β2 and α3β4-like responses (reviewed in Albuquerque *et al.*, 2000).

Of these the pure α7-type (type IA) is by far the most common. Type I current was described by other groups in acute slices of rat hippocampus (CA1 or dentate gyrus) in response to pressure applied ACh (Jones & Yakel, 1997; Frazier *et al.*, 1998a; McQuiston & Madison, 1999). It is a fast-desensitising response, distinctive in its marked sensitivity to the antagonists α-bungarotoxin (10 nM), MLA (1 nM) and α-conotoxin ImI and its resistance to mecamylamine and dihydro-β-erythroidine (both 1 μM). Another property that links these receptors to recombinant α7 homomers is the

sensitivity to the agonist effect of choline (1 mM; for the choline selectivity for α7 see Papke *et al.* (1996).

In acute hippocampal slices, this current is abolished by 100 nM α-bungarotoxin. It is rarely if at all present on principal neurones, but common in interneurones (50% of all interneurones have a pure type IA; (Jones & Yakel, 1997; Frazier *et al.*, 1998a; McQuiston & Madison, 1999) and may be especially important in interneurones that control input onto the pyramidal cell dendrites. Note that fast α7-like responses have been described in pyramidal cells in culture (Albuquerque *et al.*, 2000) or in mouse CA1 pyramidal neurones (acute slices, Ji *et al*., 2001). Other properties of this current include sensitivity to other classical α7 antagonists such as MLA (2 nM; McQuiston & Madison, 1999); 10 nM (Jones & Yakel, 1997; Frazier *et al.*, 1998a) and α-conotoxin ImI (200-500 nM) and resistance to mecamylamine (0.5-1 μM), and dihydro-β-erythroidine (100-150 nM). The α7 involvement is confirmed by the disappearance of type I currents in α7 knockout mice (Orr-Urtreger *et al.*, 1997).

In hippocampal cultures, other less common types of nicotinic responses were the slower α-bungarotoxin resistant types II and III. Of these, II is the most common (10% of neurones in primary culture), and may correspond to α4β2*. It is very sensitive to dihydro-β-erythroidine (10 nM) and is decreased by high concentrations of methyllycaconitine (100 nM). It is also blocked by mecamylamine (1 μM). The most rare and slow responses were termed type III (2% of hippocampal neurones in culture): these may correspond to α3β4* receptors, are sensitive to 1 μM mecamylamine or 20 μM tubocurarine and resistant to 100 nM methyllycaconitine. This classification is likely to hold outside the hippocampus as well, as shown by results in normal and β2-knockout mice (see for instance Zoli *et al.*, 1998), who distinguish a fourth type of nicotinic response, similar to type III, but with faster desensitisation at high nicotine doses and different properties in equilibrium binding assays with agonists).

In acute slices, a mixed response, that comprises fast α7-like and slow components has been described in interneurones of the *stratum oriens* (36% of all interneurones). The slow response is sensitive to mecamylamine (1 μM) and to a certain extent to dihydro-β-erythroidine (100 nM), but resistant to the α3β2 antagonist α-conotoxin MII (200 nM). In its moderate sensitivity to dihydro-β-erythroidine this response may resemble more α3β4-like responses (type III) than α4β2, type II ones (McQuiston & Madison, 1999), but it has been suggested that sensitivity to all antagonists is lower in slices than in cultured dissociated neurones (Alkondon *et al.*, 1999): the apparent difference could be simply due to access problems, as no *Kd* data are available (for a review of the difficulty in comparing IC_{50} values see Sivilotti *et al.*, 2000). Note that it is still controversial whether *pyramidal* cells do have nicotinic responses: there are reports of both fast α7-type responses (Albuquerque *et al.*, 2000; Ji *et al.*, 2001) and slow responses (Alkondon *et al.*, 1999).
Recent work shows that of these receptors, the α7 type seems to be the most important for synaptic transmission in the hippocampus. α7 immunoreactivity is present at nearly all the synapses in CA1 *stratum radiatum* (including GABAergic and glutamatergic ones, Fabian-Fine *et al.*, 2001) and α7-like receptors mediate fast synaptic transmission onto CA1 interneurones (Alkondon *et al.*, 1998 ;Frazier *et al.*, 1998b). The identification was based on the sensitivity of synaptic currents (both

evoked and spontaneous) to the antagonists methyllycaconitine (50-150 nM) and α-bungarotoxin (100 nM) and to desensitising concentrations of the selective α7 agonist choline. Such cholinergic currents were relatively rare, being found only in 17/125 stratum radiatum interneurones, but represented 10% of the total evoked synaptic current in these cells. It is possible that the rarity of these currents is due to the difficulty in recruiting cholinergic afferents, given that in most interneurones α7 responses to ACh application could be detected (see also Buhler & Dunwiddie, 2001). Nicotinic synaptic currents of the α7 type were also detected in pyramidal cells (Hefft *et al.*, 1999) in acute or organotypic hippocampal slices. Here the contribution of nicotinic currents to the total postsynaptic current was very modest (less than 3% of total).

While it is natural that one should look for nicotinic fast synaptic transmission in the central nervous system, in analogy to the peripheral role of these receptors, central nicotinic receptors are often present at a presynaptic level (reviewed by McGehee & Role, 1996; Wonnacott, 1997; Kaiser & Wonnacott, 2000). Thus in many brain areas pharmacological activation of these channels produces an increase in spontaneous release of a variety of transmitters, including catecholamines, GABA, 5HT, glutamate and ACh itself. This is due either to direct calcium entry through nicotinic channels located on presynaptic terminals or by the firing of sodium-dependent action potentials (which eventually reach terminals) by depolarisation produced by pre-terminal nicotinic receptors. These two mechanisms can be distinguished on the basis of the tetrodotoxin sensitivity of the latter (see for instance Léna *et al.*, 1993).

In most preparations direct electrophysiological recording from presynaptic structures is not possible and hence characterisation of presynaptic (or preterminal) nicotinic receptors has to rely on neurochemical measurements of transmitter release, postsynaptic recording of the effects of such release or presynaptic intracellular calcium measurement. There is evidence that both α7 and non-α7 receptor types can play a presynaptic role. For instance, a predominant α7 involvement was reported by McGehee *et al.* (1995) for glutamate release in chick habenula/interpeduncular nucleus co-cultures and by Gray *et al.* (1996) for mossy fiber terminals in rat hippocampus slices. Nevertheless, the type of nicotinic receptor involved depends both on the brain region and on the nature of the terminal, i.e. on the transmitter released. A particularly striking example is that of the rat dorsal raphe nucleus, where the nicotinic receptors involved in noradrenaline release are sensitive to 100 nM methyllycaconitine (i.e. α7-like), whereas those involved in 5HT release are not (Li *et al.*, 1998). Indeed, depending on the pattern of nicotine application, *both* α7 and non-α7 receptors may enhance glutamate release in rat hippocampal micro-island cultures (Radcliffe & Dani, 1998). Nicotinic enhancement of GABA release in hippocampus is likely to be mediated by both α7 and non-α7 receptors (Alkondon *et al.*, 1999; Maggi *et al.*, 2001).
Catecholaminergic terminals bear non-α7 nicotinic receptors, which are thought to be of either the β2* or the β4* type in the case of dopaminergic and noradrenergic terminals, respectively. A thorough review of the pharmacology of these receptors can be found in Kaiser & Wonnacott (2000).

Because nicotinic receptors can both modulate the release of a variety of transmitters and directly depolarise postsynaptic neurones, the functional consequences of their

pharmacological activation can be both subtle and widespread. It has been reported that activation of presynaptic nicotinic receptors results in increases in the amplitude of submaximal glutamatergic synaptic currents (McGehee & Role, 1995; Bordey *et al.*, 1996; Radcliffe & Dani, 1998; Mansvelder & McGehee, 2000) or in the increase in the non-NMDA component and decrease in the NMDA one (Fisher & Dani, 2000). Important modulatory effects on synaptic plasticity have also been described (Mansvelder & McGehee, 2000; Ji *et al.*, 2001).

The widespread presence of nicotinic receptors in the central nervous system coupled with the relative rarity of a classical synaptic role means that it is still difficult to describe a clear physiological role for these receptors. This is particularly true for receptors in presynaptic locations, for which the level and temporal pattern of exposure to the neurotransmitter are unknown. Approaches that are casting light on this problem include mouse knockout models and transmitter depletion by blockers of vesicular transport processes such as vesamicol: the combination of these techniques has recently shown that normal evoked dopamine release in striatal slices is strongly dependent on endogenous cholinergic mechanisms that involve the activation of β2-containing nicotinic receptors (Zhou *et al.*, 2001).

Central neuronal nicotinic receptors are the target for the pharmacological actions of nicotine in tobacco. Plasma levels of nicotine in smokers go through short-lasting peaks superimposed onto a sustained lower concentration that rises steadily through the day. This is likely to result in a complex pattern of activation and desensitisation and this is accompanied by long-term regulatory effects on the number of nicotinic receptors (reviewed in Dani *et al.*, 2001; Hyman *et al.*, 2001). Knockout models suggest that it is the β2-containing receptor type that has a primary role in sustaining nicotine self-administration in mice (Picciotto *et al.*, 1998).

Autosomal dominant nocturnal frontal lobe epilepsy: a central nicotinic defect.
Clues on the physiological role of neuronal nicotinic receptors come from the identification of a form of human epilepsy that can be caused by mutations in either the α4 or the β2 nicotinic subunits. This rare syndrome, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), was the first idiopathic epilepsy to be identified as a monogenic disorder (Steinlein *et al.*, 1995), and consists of seizures which occur during light sleep. While these can be quite distinctive, it is not uncommon for the seizures to be misdiagnosed as nightmares or other sleep-related disturbances (for a review see Sutor & Zolles, 2001). As other mendelian forms of epilepsy, this syndrome is very heterogeneous: thus it is unclear whether it is due exclusively to defects in central neuronal nicotinic receptors. In at least one family this syndrome is linked to 15q24, a chromosomal locus that does not contain either α4 or β2 genes, but rather a cluster of *peripheral* neuronal nicotinic subunits (i.e. α3, β4 and α5; Phillips *et al.*, 1998), which have a restricted expression pattern in the CNS. Furthermore, the autosomal dominant inheritance pattern is obscured by incomplete penetrance (estimated at 75%) and the actual symptoms are extremely variable from one patient to the other *within* the same family (Sutor & Zolles, 2001).

Five mutations have been identified so far, all either in the pore-lining domain, M2, or in the short linker that connects it with M3. The characterisation of the functional consequences of the mutations (by heterologous expression in *Xenopus* oocytes) has been carried out using mostly macroscopic techniques, because of the difficulty in

obtaining excised patch recordings for neuronal receptors. Again intrinsic technical difficulties mean that (with few exceptions), the electrophysiological data are from 'all mutant' receptors (i.e. *not* the sort of receptor that patients – who are heterozygous- would have). Furthermore, the data come from expression of $\alpha4\beta2$ receptors and may not be entirely predictive of the behaviour of native receptors which may contain also other nicotinic subunits. Finally, it must also be said that there is considerable divergence in the reported effects of the same mutation between one lab and the other and between the effects of different mutations which in man produce similar phenotypes.

Three mutations have been reported for the $\alpha4$ subunit, all in the M2 region. Two are mis-sense mutations (at 6′ S248F, Steinlein *et al.*, 1995) or at 10′ (S252L; this mutation hasn't been characterised electrophysiologically, Hirose *et al.*, 1999), whereas one is the insertion of a Leu after 17′ (Steinlein *et al.*, 1997). The two ADNFLE mutations known for the $\beta2$ subunit are at the same residue (22′) of the M2-M3 linker, which is a region likely to be important in receptor gating. These are V287L (De Fusco *et al.*, 2000) and V287M (Phillips *et al.*, 2001).

Studies that used recombinant expression of mutant $\alpha4\beta2$ receptors described a variety of effects for these mutations, encompassing increases in ACh EC_{50} for S248F and V287M (Kuryatov *et al.*, 1997; Bertrand *et al.*, 1998; Phillips *et al.*, 2001) and decreases for the 776ins3 Leu insertion (Bertrand *et al.*, 1998). A reduction in the maximum current elicited by ACh was reported for S248F, but not for the 776ins3 Leu insertion mutant (Bertrand *et al.*, 1998; Figl *et al.*, 1998). A potential 'gain of function' effect was seen for the $\alpha4$ mutations and consisted of a 'wind-up' or increase in the response to low agonist concentrations upon repeated application (Kuryatov *et al.*, 1997; Figl *et al.*, 1998).

It is difficult to interpret the varied biophysical effects of the epilepsy mutations on recombinant receptors *in vitro* with the pathogenesis of the actual disease. Is this disease due to loss or gain of nicotinic receptor function? Which is the most important of these changes? The uncertainty is inevitable given that at present we cannot tell whether the major physiological role of central nicotinic receptors is postsynaptic or presynaptic. If the postsynaptic role is the most important, we must try to argue on the effect of mutations on the receptor response to brief saturating transients of ACh. On the other hand, presynaptic receptors may be activated by concentrations of transmitter which are lower and in conditions closer to equilibrium. Furthermore, it is entirely possible that the same central nicotinic receptors play both roles to different extents in different CNS areas.
Finally, it must also be borne in mind the case of muscle receptors - both gain of function and loss of function mutations can produce congenital myasthenia.

Agonists for neuronal nicotinic receptors
The different combinations of recombinant neuronal nicotinic receptors differ in their sensitivity to agonists. The list of nicotinic agonists is long, and comprises both natural compounds such as choline, nicotine, cytisine, lobeline, epibatidine, anabaseine and synthetic compounds such as tetramethylammonium (TMA), 1,1-dimethyl-4-phenylpiperazinium (DMPP) and carbachol, to name but a few.

We will consider the different combinations in groups, that is homomeric α7 receptors on one hand and heteromers on the other (i.e. central type α4β2 receptors and ganglionic type α3β4 receptors), highlighting the compounds that are most useful for receptor classification purposes, irrespective of species differences using functional assays. It is worth noting that classical pharmacology shows that using agonists for receptor classification is fraught with problems. This is because the functional EC_{50} of an agonist on the same receptor will depend on several experimental variables, and especially on the rate of application (discussed in Sivilotti *et al.*, 2000). The usefulness of the technique is greater if agonist potency ranks are determined, especially at their low-concentration limit (in order to reduce the confounding effects of agonist self-block and desensitisation, see Covernton *et al.*, 1994). Particular caution is needed also because our knowledge of the relative potency of agonists comes from recombinant expression of pure homomeric or 'pair' heteromeric receptors. We do not know how agonist potency would be changed in a complex heteromeric receptor, i.e. one which contained two different interface binding sites.
In the case of neuronal nicotinic receptors, the choice to use agonists is dictated by the paucity of suitably selective competitive antagonists, a situation that may changing with the increased availability of an increased range of conotoxins (see below).

Broadly speaking, choline is the most useful agonist for α7 receptors (Mandelzys *et al.*, 1995; Papke *et al.*, 1996; Alkondon *et al.*, 1997), as it is a full agonist on these receptors (EC_{50} 1.6 mM vs. 0.13 mM for ACh; Alkondon *et al.*, 1997) but is ineffective or a very poor partial agonist on α3β4 and α4β2-type receptors.
On the other hand, cytisine is both efficacious and potent as an agonist on heteromers containing the β4 subunits (i.e. ganglion-type receptors), but is only a partial agonist on β2 heteromers (Luetje & Patrick, 1991; Papke & Heinemann, 1994; Covernton *et al.*, 1994). On the latter receptor type, the maximum current to cytisine is no more than 25% of that produced by ACh (the precise value depends on the α subunit, the species from which the clones are derived and the nature of the functional assay: the range of values reported is 1%-25%).
Most other agonists do not show such a great level of selectivity as the ones discussed above. It is nevertheless worth mentioning the non-selective agonists 1,1-dimethyl-4-phenylpiperazinium (DMPP) for its widespread use, and epibatidine (from the skin of an Ecuadorean tree frog, *Epidobates tricolor*) for its extreme potency, orders of magnitude greater than other agonists, especially for the heteromeric receptors.

Antagonists
There is a great paucity of data for the affinity of competitive antagonists of neuronal nicotinic receptors. Functional studies usually report antagonist IC_{50}*s*: an IC_{50} value may be (marginally) quicker to obtain than dissociation constants in a Schild-type design, but an IC_{50} value depends on the agonist concentration used. Given that comparison of IC_{50}*s* across different preparations is difficult, differences in receptor types can only be argued if relatively large IC_{50} differences are observed and if the agonist concentration involved is similar. This consideration is important especially if synaptic responses as studied and compared to agonist applications, given that synaptic currents are likely to be produced by very short (submillisecond) rises in ACh concentration to very high levels. Furthermore, IC_{50} experiments do not tell us anything about the actual mechanism of action of the antagonist (i.e. competitive vs. open channel block, see the discussion of antagonists of muscle receptors). This

means that much potentially valuable information on the binding site is lacking. This is true not only for functional studies, but also for binding studies. In most cases, binding assays for neuronal nicotinic antagonists use displacement of a labelled *agonist* by the antagonist: this is because there are no selective antagonists of sufficiently high affinity that can be used in such work (S.Wonnacott personal communication). The resulting *Ki* is the equivalent of an IC_{50} *i.e.* not a true dissociation constant. The exception is α7-type receptors, for which labelled α-bungarotoxin and methyllycaconitine can be used (Davies *et al.*, 1999).
Because of the limitations in the data available in the literature, we shall focus on the antagonists that are most useful for receptor classification. Traditionally, the main group is that of competitive antagonists that selectively block α7 and other homomeric receptors, namely α-bungarotoxin, methyllycaconitine (at low concentrations) and α-conotoxin ImI. For a recent review of toxin antagonists of neuronal nicotinic receptors see (McIntosh, 2000)

α-bungarotoxin is one of the components of the poison of the banded krait, *Bungarus multicinctus* (74 amino acids, MW 8000). The affinity of this toxin for the homomeric α7 receptor appears to be high in binding assays (1-2 nM, Davies *et al.*, 1999), but considerably lower than that for muscle-type receptors. In practice, α-bungarotoxin is used at concentrations between 10 and 100 nM in order to block α7-type receptors. This block is nearly irreversible for 'pure' α7 receptors. Indeed, quick reversal of the block by removal of the antagonist has been taken to indicate the presence of a different receptor, i.e. α7* which may contain subunits other than α7 (Cuevas *et al.*, 2000). Other homomeric-type receptors, such as α9 and α9/α10 are also sensitive to nanomolar concentrations of α-bungarotoxin (Elgoyhen *et al.*, 1994; Elgoyhen *et al.*, 2001; Johnson *et al.*, 1995; Sgard *et al.*, 2002).
Another toxin from the banded krait is κ-bungarotoxin (66 amino acids): this is a competitive blocker of neuronal receptors, particularly potent (nearly irreversible) on α3β2 receptors. The main difficulty in using κ-bungarotoxin lies in its limited availability, mostly because of the difficulty in eliminating contaminant α-bungarotoxin in the purification from crude venom (κ-bungarotoxin is not commercially available to our knowledge). Recombinant expression in yeast may improve availability of the pure toxin.

It is interesting to note that an endogenous molecule related to snake neurotoxins, *lynx1,* is present in the rodent CNS, where it is surface-anchored and expressed by neurones positive for nicotinic α4β2 and α7 receptors. In recombinant systems, the effects of co-expressing this molecule with α4β2 receptors are complex: increases in EC_{50} and in the relative frequency of the biggest conductance and speeding of desensitisation during sustained agonist application have been reported (Miwa *et al.*, 1999; Ibañez-Tallon *et al.*, 2002).

Methyllycaconitine is an alkaloid derived from *Delphinium brownii* (Alkondon *et al.*, 1992): it is a competitive antagonist selective for α7 and α7* receptors, effective at low nanomolar concentrations (2-5 nM, Davies *et al.*, 1999; Yum *et al.*, 1996). Work on the macroscopic kinetics of the onset and offset of antagonist action on recombinant α7/5HT3 chimaeras suggests methyllycaconitine affinity may be an order of magnitude higher than that for pure α7 receptors (Palma *et al.*, 1996). Additionally, this study was consistent with the idea that homomeric receptors have

indeed got five binding sites for the alkaloid (i.e. up to one per subunit), if it was assumed that binding of one antagonist molecule is enough to block the response. Heteromeric receptors are also blocked by methyllycaconitine, but at much higher concentrations (tens of nM); recombinant α4β2 receptors recover from methyllycaconitine block with a time course consistent with the presence of two antagonist binding sites (Palma *et al.*, 1996).

It is worth mentioning that the glycine receptor antagonist strychnine is also a good competitive antagonist at homomeric receptors, both of the α7 and α9/α10 type: strychnine is effective on these receptors at sub-micromolar concentrations, such as are commonly used in order to suppress glycine receptor activity in native preparations (Séguéla *et al.*, 1993; Peng *et al.*, 1994; Elgoyhen *et al.*, 1994).

When it comes to heteromeric receptors, many antagonists have little useful selectivity. The exceptions are dihydro-β-erythroidine and the rapidly growing family of the α-conotoxins (see below). An important point is that many of the antagonists available have channel blocking properties (see for instance mecamylamine and hexamethonium).

Dihydro-β-erythroidine

Dihydro-β-erythroidine is an alkaloid obtained from the seeds of several species of the genus *Erythrina*. Its mechanism of action on neuronal nicotinic receptors is likely to be competitive (Bertrand *et al.*, 1992), but no Kd values have been reported in the literature to our knowledge. Even with the limitations of the IC_{50} approach, it is clear that the compound has a marked selectivity for some types of heteromeric receptors. Thus it is a poor antagonist of both α3β4 (IC_{50} range 14-23 μM, Chavez-Noriega *et al.*, 1997; Harvey & Luetje, 1996) and α7 receptors (IC_{50} range 2-20 μM, Bertrand *et al.*, 1992; Chavez-Noriega *et al.*, 1997; Virginio *et al.*, 2002). On the other hand, dihydro-β-erythroidine is effective at submicromolar concentrations on recombinant α4β2 and α4β4 receptors (IC_{50} values below 0.4 μM, Harvey *et al.*, 1996; Buisson *et al.*, 1996; Chavez-Noriega *et al.*, 1997; Chavez-Noriega *et al.*, 2000) and is perhaps slightly less potent on α3β2 receptors (IC_{50} 0.4-1.6 μM; Harvey & Luetje, 1996; Chavez-Noriega *et al.*, 1997); all these data were obtained at equilibrium, against agonist concentrations between EC_{20} and EC_{50}, depending on the study). For a mutagenesis study of the α subunit residues that determine the difference in sensitivity between α3β2 and α3β4 combinations see Harvey & Luetje, (1996).

Conotoxins

Recent work on this family of compounds has given rise to some of the most useful nicotinic antagonists, because of the exquisite selectivity of some of these peptides for individual binding interfaces and because of their likely competitive mechanism of action.

The *Conus* genus of marine snails provides an enormous variety of small peptide toxins (estimated at 200-500 per species). These are active on disparate voltage- and ligand-gated ion channels (for reviews see McIntosh *et al.*, 1999; McIntosh, 2000; McIntosh & Jones, 2001). The venom is used by the snail to hunt its prey, which, depending on the snail species, can be worm, mollusc or fish. The active principles are small peptides which are maintained in a specific configuration by one or more disulfide bonds. The peptides with more than one disulfide bond are called

conotoxins, and they are further subdivided into superfamilies on the basis of the pattern of disulfide bonds in the molecule. Conotoxins that are competitive antagonists of nicotinic receptors belong to the A superfamily (see McIntosh *et al.*, 1999). Interestingly, the exquisite specificity of these compounds means that the data reported for receptors of a particular species cannot be extrapolated even to a related mammalian species, given that small differences in the binding domain sequences can markedly change the sensitivity to a conotoxin (McIntosh, 2000).

Conotoxins that act on muscle receptors

A first grouping is that of toxins that act only on muscle-type nicotinic receptors: these are derived from the fish-hunting species *C. geographus, ermineus, magus* and *striatus*. Chemically most of these toxins belong to the α group and the 3/5 subfamily (see McIntosh *et al.*, 1999).

Of this group, the toxins which are available commercially (as of June 2002) are α-conotoxins GI and MI (which are the most-extensively characterised), SI and SIA.

Both α-conotoxin GI, α-conotoxin MI and α-conotoxin SIA have a strong selectivity for the α/δ interface of mouse embryonic muscle receptor The highest affinity is observed for GI and MI, and the range of equilibrium constants reported is 1-5 nM for the α/δ site vs. 8-58 μM for the α/γ site (Sine *et al.*, 1995a; Groebe *et al.*, 1995; Hann *et al.*, 1997). Note that the selectivity is reversed for *Torpedo* (Hann *et al.*, 1994). No effect is reported for either α-conotoxin GI and MI at 5 μM on a variety of rat homomeric and heteromeric neuronal nicotinic receptors expressed in oocytes (Johnson *et al.*, 1995).

A peptide from another fish-hunting snail, *C. ermineus*, α–conotoxin EI (belonging to the 4/7 subfamily of the αA group), has a similar preference for mouse muscle α/δ interfaces, but is much less selective than α–conotoxin GI or MI. Interestingly, this conotoxin targets α/δ also in *Torpedo* receptors (Martinez *et al.*, 1995), contrary to the behaviour of α-conotoxins GI, MI and SIA.

Conotoxins that act on neuronal receptors

(note that IC_{50} values in this section were obtained in oocytes against very brief applications of near-maximal concentrations of ACh; as discussed for tubocurarine in the section on muscle receptors, this method would give acceptable estimates of the true equilibrium constant of the antagonist if its receptor occupancy cannot equilibrate with the agonist during the agonist application)

A worm-eating species, *C.imperialis*, produces α-conotoxin ImI, which is an effective antagonist of homomeric type neuronal nicotinic receptors, such as rat α7 or α9 (reported IC_{50}s 0.22 and 1.8 μM, respectively). This commercially available 12 amino acid amidated peptide is a very weak blocker of mouse muscle-receptors (IC_{50} 51 μM) and is ineffective at 5 μM on heteromeric rat neuronal receptors (Johnson *et al.*, 1995).

Another commercially available conotoxin which is selective for neuronal nicotinic receptors is α-conotoxin MII, a 16 amino acid α4/7 peptide from *C.magus*. This toxin is selective for rat α3β2 (IC_{50} 0.5 nM) *vs.* other heteromeric and homomeric combinations of rat neuronal subunits. Only at much higher concentration (200 nM), this toxin had a small effect on rat recombinant α4β2, α3β4, α4β4 or muscle

receptors (20-30% reduction in the maximal ACh response). Rat homomeric α7 receptors are also resistant to α-conotoxin MII (IC_{50} 200 nM; Cartier *et al.*, 1996).

Finally, α-conotoxin AuIB (15 amino acids, from *C.aulicus*, at present not commercially available) is selective for the rat α3β4 interface, but is not very potent (IC_{50} 0.5-0.75 μM). Rat α7 receptors are approximately 10-fold less sensitive than α3β4, while other heteromeric neuronal combinations and muscle receptors are at least 100-fold less sensitive (Luo *et al.*, 1998).

Trimetaphan
This sulfonium ganglion blocker still has a limited use in clinical practice (as i.v. infusion) to induce controlled hypotension in surgery.
Trimetaphan produces a voltage-independent block in a variety of autonomic ganglion preparations and is likely to have a competitive mechanism of action (Ascher *et al.*, 1979; Gurney & Rang, 1984; Nooney *et al.*, 1992b). The approximate Kd value reported is 1.44 μM (Ascher *et al.*, 1979). Our own work with human recombinant α3β4 receptors indicates a *Kd* in the region of 70 nM (Boorman, Groot-Kormelink & Sivilotti, in preparation). Little is known of the selectivity of trimetaphan on different receptor combinations: the only data available (Cachelin & Rust, 1995) suggest that α3β4 receptors are more sensitive than α3β2. Trimetaphan is known to be a poor antagonist of nicotinic receptors on outer hair cells in the cochlea (Erostegui *et al.*, 1994), now known to be of the α9/α10 type.

Methonium compounds
The polymethylene bistrimethyl ammonium series has been investigated sine the 19th century (see Colquhoun, 1997). This series of compounds consists of two quaternary ammonium groups joined by a polymethylene chain of variable length. They work on both muscle type and neuronal type nicotinic receptors, some as agonists and others as antagonists. There actions were characterised by (Paton & Zaimis, 1949) and (Paton & Zaimis, 1951). In fact these papers were perhaps the first to give a clear demonstration of how different the muscle and neuronal types of receptor really are.
On the muscle receptor, hexamethonium was a weak antagonist, but the most potent member of the series was decamethonium was, which, unusually, worked as an agonist. The mechanism of block by depolarisation was elucidated by Burns & Paton (1951). Their observations can now be explained in more detail, as result of inexcitability of the muscle fibre membrane, close to the neuromuscular synapse, brought about by inactivation of perijunctional sodium channels, caused by prolonged depolarisation of the end plate region. On the neuronal receptor (in peripheral ganglia) hexamethonium was the most potent of the series, but it worked in a quite different way, as an antagonist. Subsequent work has shown that all of these compounds, including those which are agonists, can block the open ion channel to a greater or lesser extent. In fact, as first envisaged by Blackman, (1970); all the compounds that are antagonists work primarily by channel block; none of them are good competitive antagonists (though it is still not unknown for textbooks to describe them as 'competitive', simply because they are not agonists). Decamethonium also blocks the neuronal receptor channel but it is a weaker antagonist than hexamethonium because it dissociates more rapidly (Ascher *et al.*, 1979)). The slower dissociation (and higher potency) of hexamethonium on ganglion receptors was shown by (Gurney & Rang, 1984) to be a result of the fact that hexamethonium

(but not decamethonium) was small enough to be trapped in the channel. It was as if, the blocker having entered the channel while it was open, the channel could then shut again, trapping the hexamethonium inside, and slowing its dissociation. In fact the hexamethonium can barely escape at all unless the channel is opened again by an agonist: recovery from block required both agonist application and membrane depolarisation.

Mecamylamine

This secondary amine compound merits a mention because it is the most used antagonist in behavioural studies, thanks to its ability to cross the blood-brain barrier (for a review see Young *et al.*, 2001). Mecamylamine was originally developed as ganglion blocker and antihypertensive, but, like trimetaphan, its clinical use is now very limited, although it has been suggested as a possible therapeutic agent in Tourette's syndrome.

Heterologous expression data show that mecamylamine is not selective for the different receptor types (see for instance Chavez-Noriega *et al.*, 1997; Chavez-Noriega *et al.*, 2000), and is effective at low micromolar concentrations. At concentrations greater than 1 μM mecamylamine is an open channel blocker on recombinant α4β2 receptors (Bertrand *et al.*, 1990), and on nicotinic receptors of intracardiac ganglia (Fieber & Adams, 1991) and chromaffin cells (Nooney *et al.*, 1992b; Giniatullin *et al.*, 2000). Indeed, there is now evidence that, like hexamethonium, mecamylamine gives rise to a persistent block because it is trapped in the channel. Thus, recovery is speeded up by combining agonist application with membrane depolarisation: modelling of use dependence and time course of recovery suggests that channels which have trapped blocker open more slowly (Giniatullin *et al.*, 2000). Nevertheless, when mecamylamine is applied at very much lower concentrations to rat parasympathetic ganglion neurones, it is more effective against *low* agonist concentrations (Ascher *et al.*, 1979; Fieber & Adams, 1991). This observation is inconsistent with channel block and suggests a competitive mechanism at these low concentrations, with a *Kd* of the order of 25-50 nM.

Chlorisondamine

This compound acts as open channel blocker on neuronal nicotinic receptors (see for instance for autonomic ganglia, Amador & Dani, 1995). It is often used for *in vivo* studies because of its very long lasting effects (Clarke *et al.*, 1994), which are probably due to trapped channel block (as shown at the frog neuromuscular junction, Neely & Lingle, 1986).

(+)-tubocurarine

Neuronal nicotinic receptors are blocked by micromolar concentrations of (+)-tubocurarine, which is an effective, slow dissociating open channel blocker on ganglionic receptors (Ascher *et al.*, 1979). Nevertheless, there is no evidence that the block involves 'trapping' of tubocurarine (Giniatullin *et al.*, 2000). This compound is likely to have additional effects, other than simple open channel block, as effects compatible with a partial agonist action have been described for both ganglionic and recombinant α3β4 receptors (Nooney *et al.*, 1992b; Cachelin & Rust, 1994).

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