STUDIES OF WILD TYPE AND MUTANT MUSCLE NICOTINIC RECEPTORS

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Abstract

Single channel patch clamping and maximum likelihood fitting to kinetic schemes have been used to characterise the adult human muscle nicotinic receptor. Both the wild type receptor and a disease-causing receptor mutation were studied.

The mutation underlies a case of the muscle degenerative disease termed slow channel congenital myasthenic syndrome. The mutation at positions εL78P is a ‘gain of function’ mutations that result in extended burst durations compared with wild type receptors. The mutations affects both agonist dissociation and channel gating, despite the fact that the mutation is not within the classical acetylcholine binding site or within the pore region.

The choice of kinetic scheme to describe the receptor has also been investigated. A scheme incorporating two different binding sites indicates that the dissociation rates of acetylcholine are different at the two binding sites of the human receptor. The effect of using an EC50 constraint as opposed to fixing a rate constant was examined, as was the effects of including either an agonist-blocked state or a desensitised state. In schemes examining agonist association and dissociation to and from open channels, it is shown that the rate of dissociation of acetylcholine is negligible compared with the other kinetic parameters involved in channel activation. In all of the schemes tested, consistent values were obtained for the rates of diliganded channel opening, diliganded channel closing, and the total dissociation rate of agonist from the diliganded receptor. In addition the effect of conotoxin-GI on the nicotinic receptor was examined at the single channel level. It is shown that this toxin increases the proportion of moniliganded openings and has asymmetric effects on acetylcholine dissociation from the two binding sites.
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Thanks must also go out to the other members of the group, past and present. Special thanks must go to the now departed Chris Hatton; he’s not dead he just works in a different laboratory now. It was Chris who taught me the practical aspects of patching and he was a very good role model as a dedicated experimentalist. He was also a good drinking companion. Stephanie Schorge has sat at the other end of the bench from me for much of my time here, she has been a great friend to have in the laboratory and guided me through the fun of being a graduate student. Thanks must go to Philippe Behe, who has answered many of my random (and sometimes idiotic) questions with impeccable grace. I extend my thanks to the other members of the group, Sergio Elenes, Remigijus Lape, and Ioana Vais, for their help over the years. They are a thoroughly nice bunch of people. Thanks should also go out to the members of Lucia Sivilotti’s group with whom I have discussed work and life with throughout my PhD.

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1. INTRODUCTION

1.1 Ion Channels

The flow of ions between electrically separate compartments is essential for life. With the evolution of cellular-based life forms, organisms could become much more complex by regulating their intracellular constituents in a controlled way. The boundary of the cell, constructed of a lipid bilayer provided an essential barrier to keep metabolites, ions, proteins, nucleic acids and organelles within the cell. With this ability to maintain the local concentrations of molecules came an electrical isolation. The lipid membrane is highly impermeable to charged molecules and is an insulator and thus mechanisms which allowed charged molecules to be exchanged between the environments separated by insulating lipid membrane were required. In the case of small, common atomic ions, proteins in the form of channels through the membrane have been utilised. These ion channels are present in virtually all organisms, from bacteria to humans. The flux of small ions between two compartments that are electrically isolated results necessarily in a current flow, and an associated voltage change. It is this voltage change that has been utilised in higher organisms, most notably animals, as a signalling mechanism in the nervous system.

1.1.1 Ion Channel Classification

Ion channels can be classified by two criteria, the mechanism that activates the channel to allow the passage of ions, and secondly the types of ions that can pass through the channel. Channels can be activated (gated) by ligand binding, voltage changes, mechanical stress or temperature. The ligand-gated channels can be further subdivided based on the types of ligand they bind and their gross structure, into the super families of nicotinic-like channels, glutamate, and purinergic receptors. The nicotinic-like channels, also termed the cys-loop receptors (Karlin & Akabas, 1995) are all characterised by their pentameric structure, with each subunit contributing a large extracellular domain, a smaller intracellular domain, and four transmembrane domains termed M1 to M4, to the overall complex, shown in figure 1.1. They all contain two disulphide-bonded cysteine residues thirteen residues apart in primary sequence, within the extracellular domain. This super family can be divided into different types of channels base on their primary endogenous ligand. There are acetylcholine receptors, glycine receptors, γ-aminobutyric acid (GABA) receptors, 5-hydroxytryptamine 3 (5-
Figure 1.1. (A) All members of the nicotinic super family of ligand-gated receptors form pentameric complexes with a central ion conducting pore. Shown here are the subunits of the adult muscle nAChR the muscle nAChR as seen facing the muscle endplate. There are two ACh binding sites (shown in purple) lying at the interfaces of the αδ and αε subunits. (B) Each subunit of the nicotinic super family of ion channels consists of a large N-terminal domain, and four transmembrane segments termed M1 to M4.
HT₃ receptors, glutamate receptors and histidine receptors. In addition there is a further group represented by a single member termed the zinc-activated channel (ZAC) which has no known ligand but which has currents that are potentiated by zinc (Davies et al., 2003). The different types of receptor also differ in their conduction properties, depending on the class of organism, some are cation-permeable whilst other are anion permeable, table 1.1.

<table>
<thead>
<tr>
<th>Receptor agonist</th>
<th>Ion selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Cation</td>
</tr>
<tr>
<td>5-HT (invertebrate only)</td>
<td>Anion</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Cation</td>
</tr>
<tr>
<td>Acetylcholine (invertebrates only)</td>
<td>Anion</td>
</tr>
<tr>
<td>GABA</td>
<td>Anion</td>
</tr>
<tr>
<td>GABA (invertebrates only)</td>
<td>Cation</td>
</tr>
<tr>
<td>Glutamate (invertebrate only)</td>
<td>Anion</td>
</tr>
<tr>
<td>Glycine (vertebrate only)</td>
<td>Anion</td>
</tr>
<tr>
<td>Histamine (invertebrate only)</td>
<td>Anion</td>
</tr>
<tr>
<td>ZAC (human and dog only)</td>
<td>Cation</td>
</tr>
</tbody>
</table>

Table 1.1. The members of the nicotinic super family of ligand-gated ion channels and their ion selectivity.

1.2 Nicotinic Acetylcholine Receptors (nAChRs)

The nAChR has been an important molecule in the advancement of both pharmacology and neuroscience. Its activities were the first to be attributed to a receptor, or 'receptive substance' as it was termed at the time (Langley, 1878; Langley, 1905). Using nicotine and curare it was demonstrated that the abolition of muscle activity via nerve stimulation could occur, but importantly the abolition of contraction when the muscle was directly stimulated did not occur ((Conti, 2002) which references Bernard 1872). The endogenous agonist on the nAChR, acetylcholine (ACh) was the first neurotransmitter to be discovered (Dale et al., 1936). nAChRs were the first ligand-gated ion channel to be investigated using electrophysiology (Fatt & Katz, 1951), and the first protein in synaptic transmission to have its role determined (Fatt & Katz, 1952). It was also the first ion channel or receptor to be chemically isolated (Changeux et al., 1970). It was the first ion channel to be cloned (Noda et al., 1982; Noda et al., 1983; Claudio et al., 1983), and the first ion channel to have its structure determined at high resolution (Toyoshima & Unwin, 1988; Unwin, 1993; Unwin, 1995), although it was not the first ion channel to have its three dimensional crystal structure solved, that honour went to the KcSA potassium channel (Doyle et al., 1998).
1.2.1 Function of Mammalian Muscle nAChRs
The muscle synapse consists of the presynaptic terminal of a motor neurone, the synaptic cleft, and the postsynaptic muscle endplate. The endplate is invaginated to form junctional folds which lie directly opposite the active sites of vesicle fusion in the presynaptic bulb. Muscle nAChRs are concentrated on the junctional folds of the muscle endplate at a density of approximately 10000/\mu m^2 (Salpeter & Loring, 1985). Extrasynaptically the density falls to approximately 100/\mu m^2 (Salpeter et al., 1988).
During synaptic transmission ACh is released from the motor neurone presynapse into the synaptic cleft from where it can diffuse and bind to nAChRs. The activation of nAChRs by ACh triggers the opening of the receptor’s cation-permeable pore. The synaptic current produced by nAChRs is rapidly activating, and decays exponentially. The exponential time course of decay is determined by the single channel kinetic properties of the receptor, in which the lifetimes of the different states are exponentially distributed. As the sum of a random number of exponentially distributed components is itself exponentially distributed, this leads to the exponential decay of the synaptic current. The ion flux during activation of nAChRs depolarises the membrane of the endplate, which triggers a muscle action potential. This propagating action potential travels along the muscle T tubules activating voltage-gated Ca^{2+} channels that are physically coupled to ryanodine receptors embedded within the membrane of sarcoplasmic reticulum. Activation of the RyRs leads to Ca^{2+} efflux from the sarcoplasmic reticulum into the cytosol. This large increase in cytosolic Ca^{2+} activates the contractile apparatus of the muscle. The reduction of the activity of nAChRs at the endplate following ACh release into the synaptic cleft arises primarily due to the rapid degradation of ACh by hydrolysis into acetate and choline by acetylcholinesterases (AChEs). The A_{12} form of AChE containing twelve catalytic subunits and anchored to the basal lamina via a collagen tail is the predominant form of AChE at mammalian neuromuscular junctions (Sketelj & Brzin, 1985). The net effect of this enzyme’s presence is to remove ACh rapidly (compared with the activity of the nAChRs), thus the nAChRs are exposed to only a brief pulse of agonist.

1.2.2 Basic Structure of nAChRs
The electric fish, Torpedo was originally the organism of choice to study nAChRs due to the large amounts of concentrated nAChR in electric organs formed from modified muscle tissue. Like all other members of the nicotinic super family of ion channels, the Torpedo nAChR is a pentameric transmembrane complex. The main consideration
when discussing *Torpedo* receptors and human receptors is that the *Torpedo* receptors always contain γ subunits, as opposed to ε subunits in adult mammals. In humans nAChRs are coded by sixteen different genes. These genes code for nine α subunits, four β subunits, and single δ, ε, and γ subunits. The proteins encoded by these genes can combine in many different combinations to produce pentameric nAChRs with a variety of different properties in different tissues and cells. Under normal conditions in skeletal muscle the nAChRs are composed of two α1 subunits, a β1, a δ, and an ε subunit arranged around a central cation conducting pore as shown in figure 1.1A (Karlin & Akbas, 1995; Hucho *et al.*, 1996; Corringer *et al.*, 2000). All of the muscle nAChR subunits are glycosylated (Vandlen *et al.*, 1979). Glycosylation highlights one of the more interesting differences across species found in nAChRs. In both cobras and their famous predators, the mongooses there is a glycosylation consensus sequence several residues N-terminal to a region of the ACh binding site. Both the cobra and mongoose are immune to the potent nAChR competitive antagonist, α-bungarotoxin (α-BuTx) from the venom of the banded krait (a snake closely related to cobras). Furthermore this glycosylation signal can be inserted into the α-BuTx-susceptible mouse nAChRs and there is a 140-fold reduction in the affinity of α-BuTx for the receptors (Kreienkamp *et al.*, 1994).

The subunit composition of muscle nAChRs is not constant. During foetal development, a γ subunit is expressed instead of the ε subunit (Sakmann, 1978; Fischbach & Schuetze, 1980; Witzemann *et al.*, 1989). Shortly after birth the γ subunit expression declines to be replaced by the expression of the ε subunit. The molecular activity driving this switch in transcription of the γ subunit to the ε subunit is unknown, however it is vital for normal muscular transmission, shown by atrophy and premature death in mice in which the ε subunit has been deleted (Witzemann *et al.*, 1996). In addition the γ subunit may also be expressed during adulthood in regions where the muscle endplate becomes denervated (usually due to injury). The main differences between nAChRs containing the ε subunit compared to the γ subunit are in conductance and burst length (Gu *et al.*, 1990; Mishina *et al.*, 1986; Witzemann *et al.*, 1987; Hamill & Sakmann, 1981), around 40 pS for γ-containing channels and around 60 pS for ε-containing channels. Burst duration is approximately ten-fold longer in γ-containing receptors, at 11 ms, compared with around 1 ms for ε-containing receptors.
As well as the expression of fetal and muscle nAChR subunits, there is evidence of α4 and β2 expression in muscle. These subunits, usually regarded as neuronal nAChRs have been found to be expressed in rat skeletal muscle (Sala et al., 1996), however their physiological role is unknown (Kimura, 1998).

1.2.3 Regulation of nAChRs

At functioning muscle endplates nAChRs are maintained at high densities by several regulatory mechanisms. Rapsyn acts to cluster nAChR at positions in the post synaptic membrane (Froehner et al, 1990) and it also links nAChRs to the cytoskeleton via dystroglycan (Cartaud et al, 1998). Rapsyn binds to the long M3-M4 intracellular loop of nAChRs (Maimone & Merlie, 1993; Maimone & Enigk, 1999). Maintenance of nAChR expression at the endplate is achieved through two counteracting mechanisms. The acetylcholine receptor-inducing activity protein (ARIA protein), a member of the neuregulin family of proteins has a role in maintaining nAChR expression at the muscle endplate, (Sandrock, et al., 1997). It believed to be secreted from motor neurones where it is found concentrated in the presynaptic nerve terminals (Sandrock et al, 1995) and to act on transmembrane receptor tyrosine kinases to stimulate expression of nAChR subunits in the region of ARIA secretion, i.e. in the region of the motor neuron synapse. Down regulation of nAChRs occurs in an activity-dependent manner. The increase in cytosolic Ca\(^{2+}\) due to muscle stimulation activates not only the contractile apparatus but also protein kinase C (PKC), (Huang et al, 1992). Targets of this kinase include various myogenic factors that are inactivated by PKC-mediated phosphorylation. These myogenic factors bind to regions termed E boxes along the genes encoding the nAChR subunits enhancing the expression of nAChR subunits (Piette et al., 1990), and thus the synaptic activity-dependent inactivation of these myogenic factors acts to reduce nAChR expression (Huang et al, 1994).

nAChRs contain an endoplasmic reticulum (ER) retention signal to aid surface expression of correctly folded complexes. The amino acid residue sequence PL(Y/F)(F/Y)XXN at the extracellular end of M1 is conserved across nAChR subunits. In correctly folded receptors this signal is buried within the complex, and thus receptors are not retained within the ER (Wang et al., 2002).
1.3 Detailed Structure of nAChRs

1.3.1 The Transmembrane Region

The M2 segments from each of the five subunits comprise the actual ion conducting pathway through the membrane. Within the M2 segments there are three rings (around the subunits) of conserved anionic amino acid residues termed the extracellular, intermediate, and cytoplasmic rings, (Imoto et al., 1986; Imoto et al., 1988; White & Cohen, 1992), shown in primary structure in figure 1.2 and in tertiary structure in figures 1.3A and 1.3B. The intermediate ring is believed to be a major determinant of the ion selectivity filter of the channel (Konno et al., 1991). In addition there is a conserved ring of hydroxylated amino acids (serine or threonine) which has been suggested to be part of the selectivity filter (Villarroel et al., 1992a; Villarroel & Sakmann, 1992b), shown in figure 1.3C.

The muscle nAChR is permeable to cations, mainly to Na\(^+\) and K\(^+\). There is some Ca\(^{2+}\) flux through muscle nAChRs, although the ratio of permeability to Na\(^+\) is only around 0.2 (Adams 1980). Recently the three dimensional structure of the transmembrane segments of the Torpedo nAChR in the shut state was solved to 4 Å (Miyazawa et al., 2003), shown in figures 1.3, 1.4C, and 1.4D. This structure confirmed the results of many experiments that the M2 segments line the ion conduction pathway. In addition it showed that the narrowest region of the pore, approximately halfway along the M2 helices is the gate of the channel and is formed primarily by residues at the levels of αL251 and αV255. The diameter within this region of the pore is 6 to 7 Å which is too narrow to allow passage of a hydrated Na\(^+\) or K\(^+\) ion. Due to the presence of hydrophobic side chains at the narrowest region of the pore, ions cannot pass through without a hydration shell, unlike ions passing through the KcSA K\(^+\) channel (Doyle et al., 1998) which utilise backbone carbonyl groups to act as surrogate hydration shells for cations. It is these properties which prevent the passage of ions whilst the channel is in the shut state. The nAChR transmembrane structure also showed that the inner ring of M2 helices do not make rigid contacts with an outer ring consisting of the M1, M3, and M4 segments. When the pore opens, the M2 segments rotate clockwise (as seen from the synaptic cleft) whilst the outer ring of helices remain relatively static (Miyazawa et al., 2003). The structure also demonstrated that the transmembrane helices extend some 10 Å above the surface of the lipid bilayer.
Figure 1.2. The amino acid sequence of the pore-lining M2 segments of the most commonly studied nAChR subunits. Position 251 (Torpedo α subunit numbering), shown in bold is at the narrowest point of the channel. Three conserved rings of charged amino acids termed the cytoplasmic, the intermediate, and the extracellular rings have been identified (Imoto 1988). In addition a conserved ring of hydroxyl residues has been implicated in channel conductance (Villarroel, 1992b). Alignments were constructed by Paul J. Groot-Kormelink.
Figure 1.3. (A) The pore-lining M2 segments from each subunit viewed 'side on' from the membrane. (B) Three M2 segments of the Torpedo nAChR viewed 'side on, into the pore'. The three rings of charged residues identified by (Imoto et al, 1988) are shown in green. (C) The M2 segments of the Torpedo nAChR viewed as if facing the endplate. The ring of threonine and serine residues identified as being involved in the selectivity filter (Villaroel et al, 1992b) are shown as space filled spheres. All of the structures represented here are from the cryoelectron microscopy structure of the nAChR transmembrane domain (protein databank accession code 1OED, Miyazawa et al, 2003). They have been manipulated and viewed using the RasTop programme.
Figure 1.4. The structure of AChbp, viewed from the synaptic cleft (A) and side on through the membrane (B) (protein databank accession number 119B) (Brejc et al, 2001). The structure of the membrane spanning segments of the Torpedo nAChR (protein databank accession number 1OED) (Miyazawa et al, 2003), viewed from the synaptic cleft (C), and ‘side on’ (D). The subunits of AChbp have been coloured the same as their homologous subunits in the nAChR.
There has been some evidence that M1 may also line part of the pore (Akabas & Karlin, 1995). However these were based on the accessibility of M1 residues to hydrophilic reagents and it was assumed the only water accessible residues in the M segments were exposed to the channel lumen. It can now be seen from the atomic structure that it is possible that there is a narrow aqueous layer separating the inner and out rings of helices at the N-terminal end of M1, (Miyazawa et al, 2003).

The M4 segment faces the lipid bilayer and not the ion conducting pore. Many point mutations within M4 have been shown to alter single channel properties (Li et al, 1990; Bouzat et al., 1994; Lasalde et al., 1996; Bouzat et al 1998), although effects have been difficult to ascribe specifically to either agonist binding or gating. It has been assumed in several cases that mutations of the lipid facing residues in M4 will affect gating at the nearby M2 segment rather than ACh binding in the distal N-terminal region, although this may well not be the case.

In addition the extracellular loop between M2 and M3 in nAChRs has been shown to play a role in channel gating (Campos-Caro et al., 1996; Grosman et al, 2000a), highlighted by cases of congenital myasthenia (Croxen et al., 1997). This loop has also been implicated in channel gating in other members of the nicotinic superfamily of ion channels. In glycine receptors (Lynch et al., 1995; Rajendra et al, 1995; Lewis et al, 1998) mutations in this region can cause startle disease, and mutations in this loop in GABA<sub>C</sub> receptors can affect gating, (Kusama et al., 1994).

Little is known regarding the structure of the M3-M4 intracellular loop. No atomic resolution structures of this or homologous regions have been obtained. It has been predicted to contain three alpha helices (Le Novere et al., 1999), and has several sites for phosphorylation (Swope et al., 1992). From the crystal structure of the transmembrane domain and the low resolution structure of the entire complex it may well form a 'basket' structure with several openings perpendicular to the plane of the membrane. This can be hypothesised as the cytosolic ring of positive charges that have a role in conduction (Imoto et al., 1988) are positioned away from the narrow pore in the crystal structure of the transmembrane domain omitting the M3-M4 loop (Miyazawa et al., 2003), shown in figure 1.3B.
1.3.2 The Extracellular N-Terminal Region

Each complete muscle nAChR has two binding sites for ACh, (Karlin, 1967; Colquhoun & Sakmann, 1985; Jackson, 1988; Sine et al, 1990) located in the N-terminal domain at the interfaces between the α and δ, and the α and ε subunits (or α and γ subunits in the mammalian fetal form and the Torpedo receptor), (Pedersen & Cohen, 1990). These ACh binding pockets were initially localised by affinity labelling (Kao et al., 1984). A reagent was used that specifically labels cysteine residues and it identified one of the characteristic adjacent cysteine residues found in all nAChR α subunits. Subsequent studies utilising affinity labeling and site-directed mutagenesis led to the identification of six regions (termed loops) making up the ACh binding sites, shown in table 1.2.

<table>
<thead>
<tr>
<th>Loop</th>
<th>Residue</th>
<th>Labeling method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>αY93</td>
<td>DDF, ACh mustard</td>
<td>(Galzi et al, 1990; Galzi, 1991; Middleton &amp; Cohen, 1991)</td>
</tr>
<tr>
<td>B</td>
<td>αW149</td>
<td>DDF, SDM</td>
<td>(Galzi et al, 1991; Dennis et al., 1988)</td>
</tr>
<tr>
<td>C</td>
<td>αY190</td>
<td>DDF, nicotine, lophotoxin</td>
<td>(Middleton &amp; Cohen, 1991; Abramson et al., 1989; Dennis et al., 1988; Galzi, 1991)</td>
</tr>
<tr>
<td>C</td>
<td>αC192</td>
<td>MBTA, DDF, nicotine</td>
<td>(Kao et al., 1984; Middleton &amp; Cohen, 1991; Dennis et al., 1988; Galzi, 1991)</td>
</tr>
<tr>
<td>C</td>
<td>αC193</td>
<td>MBTA, DDF</td>
<td>(Kao et al., 1984; Dennis et al., 1988; Galzi et al, 1991)</td>
</tr>
<tr>
<td>C</td>
<td>αY198</td>
<td>nicotine</td>
<td>(Middleton &amp; Cohen, 1991)</td>
</tr>
<tr>
<td>D</td>
<td>γW55</td>
<td>(+)-TC, nicotine, DMT</td>
<td>(Chiara et al., 1998; Bren &amp; Sine, 1997; Chiara &amp; Cohen, 1997; Xie &amp; Cohen, 2001)</td>
</tr>
<tr>
<td>D</td>
<td>δW57</td>
<td>(+)-TC, nicotine</td>
<td>(Chiara &amp; Cohen, 1997; Xie &amp; Cohen, 2001)</td>
</tr>
<tr>
<td>E</td>
<td>δL111</td>
<td>Bz2 choline</td>
<td>(Wang et al., 2000)</td>
</tr>
<tr>
<td>E</td>
<td>δR113</td>
<td>(+)-TC</td>
<td>(Chiara et al, 1999)</td>
</tr>
<tr>
<td>E</td>
<td>δL121</td>
<td>Cysteine-modifying reagents, DMT</td>
<td>(Sine, 1997)</td>
</tr>
<tr>
<td>E</td>
<td>γL109</td>
<td>Bz2 choline</td>
<td>(Wang et al., 2000)</td>
</tr>
<tr>
<td>E</td>
<td>γY111</td>
<td>(+)-TC, SDM, and cysteine-modifying reagents</td>
<td>(Chiara et al, 1999; Sine, 1993; Sine, 1997; Wang et al., 2000)</td>
</tr>
<tr>
<td>E</td>
<td>γL119</td>
<td>Cysteine-modifying reagents, DMT</td>
<td>(Sine, 1997)</td>
</tr>
<tr>
<td>E</td>
<td>εL119</td>
<td>Cysteine-modifying reagents, DMT</td>
<td>(Sine, 1997)</td>
</tr>
<tr>
<td>F</td>
<td>δD180</td>
<td>SDM</td>
<td>(Martin et al, 1996; Czajkowski et al., 1993)</td>
</tr>
<tr>
<td>F</td>
<td>γD174</td>
<td>SDM</td>
<td>(Martin et al, 1996)</td>
</tr>
</tbody>
</table>

Table 1.2. Identification of six loops of residues that contribute to the ACh binding sites of the nAChR by affinity labelling and site-directed mutagenesis. Abbreviations used in the table, Bz2 choline (4-benzoylbenzoylcholine), DDF (p-(N,N-dimethyl)aminobenzenediazonium fluoroborate), DMT (dimethyl d-tubocurarine), (+)-TC (+(+)^{3}H)tubocurarine), MBTA (4-(N-maleimido)benzyltri^{3}H)methylammonium), SDM (site-directed mutagenesis).

Many of the results regarding the location and nature of the ACh binding sites have been verified following the crystal structure determinations of the ACh binding protein.
(AChbp)(Brejc et al., 2001), shown in figures 1.4A, 1.4B, 1.5, 1.6, and 1.7. The homopentameric AChbp was identified in the snail *Lymnaea stagnalis*. The protein is secreted from glial cells into the synaptic cleft where it is believed to act as an ACh buffer, and therefore its release may regulate synaptic transmission at these synapses (Smit et al., 2001). It shows remarkable similarity to the nicotinic super family of ligand-gated ion channels; its amino acid sequence has 24% homology with the α7 nAChR subunit, it is the same approximate length as the N-terminal domain of nAChR subunits, it forms a pentameric structure with a central cavity, and the dimensions of this pentamer very closely correspond with those found for the Torpedo nAChR obtained by cryoelectron microscopy. Many of the residues initially identified in nAChRs as being within the ACh binding sites are found in the AChbp located around pockets at the subunit interfaces. The predictions of these studies of nAChRs regarding which residues are within the ACh binding site have proved in many cases to be remarkably accurate when it is considered that very few of them made any attempt to separate binding effects from gating effects. However some residues previously attributed to be within the binding site have been shown through the AChbp structure to be outside of the ACh binding site, for example δD59 in the mouse nAChR (O'Leary et al., 1994). Alignments of the protein sequence of AChbp and the human, mouse, and Torpedo muscle subunits (those that have been investigated the most) are shown in figure 1.8. Residues that have been shown to be involved in binding are highlighted. When the AChbp was first crystallised (at 3.8 Å), a molecule of the buffer hepes was found to be occupying each of the ACh binding sites. It is believed that this occurred as although hepes is not particularly analogous to ACh, both do contain a quaternary ammonium ion, and hepes was included in the crystallisation buffer at the high concentration of 100 mM. Pharmacological studies on AChbp show that it can bind a range of known nAChR agonists and antagonists, (EC50 or IC50 values are given in brackets), such as ACh (4.2 µM), nicotine (98 nM), choline (190 µM), α-bungarotoxin (2.6 nM) and atropine (5.3 µM), (Smit et al., 2001). Therefore the crystal structure of AChbp is likely to be an excellent approximation to the structure of nAChRs. Searches of the human genome have not yielded any homologues to AChbp in humans. Since the publication of this immensely important crystal structure, many groups have used it as a base to model a range of ligand-gated ion channels (Sine et al., 2002a; Le Novere et al., 2002; Cromer et al., 2002; Reeves et al., 2003).
Figure 1.5. Three subunits of the AChbp viewed ‘side on’. Each subunit consists of two twisted β-sheets (shown coloured red and blue). The homologous positions of some of the major residues shown to be in the binding site of the nAChR α subunit are shown, hepes is coloured yellow. The star indicates the region of nAChRs believed to interact with the M2-M3 loop.
Figure 1.6. The 'plus' face of the ACh binding site of the AChbp viewed facing from the 'minus' face. The residues are numbered according to the Torpedo α subunit, the bracketed residues are the AChbp numbering. The two twisted β-sheets are shown in red and blue, loops between β-sheets in green, and hepes in yellow.
Figure 1.7. The ‘minus’ face of the ACh binding site of the AChbp viewed ‘side on’ from outside of the receptor. The residues are numbered according to the *Torpedo* γ subunit, the bracketed residues are the AChbp numbering. The two twisted β-sheets are shown in red and blue, loops between β-sheets in green, and hepes in yellow.
Figure 1.8. Alignment of the ACh binding loops from AChbp and the most studied nAChR receptor subunits (Torpedo $\alpha$ subunit numbering). Residues of the AChbp that line the ACh binding pocket directly are shown in purple. Alignments were constructed by Paul J. Groot-Kormelink. Residues in blue have been shown to be labelled with agonists or antagonists.
Structures of an actual nAChR have also been obtained. Using cryoelectron microscopy the structures of the entire Torpedo nAChR have been obtained in the closed conformation (at 4.6 Å) (Miyazawa et al., 1999), in the desensitised conformation (at 9 Å) (Unwin, 1993), and recently for the membrane spanning region of the receptor (at 4 Å) (Miyazawa et al., 2003). These structures show good similarity to the crystal structure of AChbp. However there are some differences, for example tunnels connecting the ACh binding site to the internal face of the vestibule leading to the ion conduction pathway were detected from the Torpedo structures. These were absent in the crystal structure of the AChbp. There is also an additional loop of seven to fourteen amino acids found in β, δ, ε, and γ nAChR subunits that is absent from the AChbp. This loop has been shown to contain residues that directly interact with the competitive antagonist waglerin-1, and may be structurally involved in the ACh binding site (Molles et al., 2002).

1.4 Pharmacology of nAChRs

The nAChR has become a common target for the venom of many predatory animals throughout evolution. Toxins from snakes, snails, wasps and coral can all act as antagonists at nAChRs. They have proved to be invaluable tools in the analysis of nAChR structure and function. Probably the most useful antagonist of nAChRs has been α-bungarotoxin (α-BuTx), isolated from the venom of the banded krait (Bungarus multicinctus). The incredibly high affinity of α-BuTx for muscle nAChR (Changeux et al., 1970; Miledi et al., 1971), with a $K_D$ of less than 1 nM allowed this toxin to be used to physically isolate nAChRs from Torpedo electric organ. This in turn led to the eventual sequencing and cloning of the members of nAChR family.

There has been much debate about whether the two ACh binding sites of nAChRs are functionally equivalent. This has been complicated by apparent species differences. At the simplest level the binding sites must be different, as one is formed at the αδ interface whilst the other is formed at the αε interface, however there seems to be some debate as to in whether these differences are significant enough to give the binding sites different detectable kinetic properties. In both the adult mouse and frog both sites are usually assumed to be identical (Salamone et al., 1999; Auerbach et al., 1996; Wang et al., 1997). However in adult human (Hatton et al., 2003), fetal mouse (Jackson,
1988; Zhang et al, 1995), and Torpedo (Sine et al, 1990) receptors the sites have been shown to have different properties.

In terms of competitive antagonists the sites are certainly different with several natural toxins having differing affinities for the two sites. Possibly the earliest known nAChR toxin, tubocurarine isolated from the plant Chondodendron tomentosum has been used to highlight differences between the binding sites. Tubocurarine was originally identified as a powerful toxin by South American tribes that used various forms of curare to coat the tips of blow darts. The tubocurarine derivative dimethyltubocurarine (DMT) has been used to identify differences in the two ACh binding sites, this toxin has much higher affinity for the $\alpha\gamma$ site than for the $\alpha\delta$ site in mouse muscle nAChRs (Sine, 1993; Bren & Sine, 1997; Chiara & Cohen, 1997). Interestingly the 'classical' competitive antagonist tubocurarine has been shown to act also as a channel blocker (Colquhoun et al., 1979), the channel blocking action is voltage-dependent, whilst the competitive antagonist action is voltage-independent.

The nAChR agonist epibatidine has also been shown to have different affinities for the two different sites on mouse nAChRs (Prince & Sine, 1998). There is a 15-fold difference between the $\alpha\delta$ site and the $\alpha\varepsilon$ site, and 75-fold difference between the $\alpha\delta$ and $\alpha\gamma$ sites. The $\alpha$-conotoxin MI is a very site-selective antagonist at the fetal mouse receptor, with $K_D$ values of approximately 2 nM and 15 $\mu$M for the $\alpha\delta$ and $\alpha\gamma$ site respectively (Kreienkamp et al., 1994; Groebe et al., 1995; Sugiyama et al., 1998). $\alpha$-conotoxin GI and $\alpha$-conotoxin SIA both show greater affinity at the $\alpha\gamma$ binding site compared to the $\alpha\delta$ binding site (Hann et al., 1997). $\alpha$-conotoxin EI has a much higher affinity for the Torpedo $\alpha\delta$ site than for the $\alpha\gamma$ site (Martinez et al, 1995). The conotoxins are produced by an interesting group of organisms called cone snails. The five hundred or so species of cone snails together are estimated to produce several tens of thousands of different toxic peptides, (Olivera, 1997) termed conotoxins which are potent antagonists of many different ion channels and receptors. These toxins are used by the snails to immobilise prey such as fish, marine worms, and molluscs. A potential problem of being a predatory marine snail is that fish prey can move much more rapidly than the snails. This apparent problem is overcome by rapidly inducing excitotoxic shock and by blocking of neuromuscular transmission in the prey animal. The excitotoxic shock is induced by utilising toxins that block inactivation of voltage-gated Na$^+$ channels (Shon et al, 1995) and by blocking certain K$^+$ channels, thereby inhibiting
K⁺ efflux from neurones (Terlau et al., 1996). The neuromuscular block is achieved by utilising antagonists of presynaptic voltage-gated Ca²⁺ channels (Olivera et al., 1994), postsynaptic muscle nAChRs (McManus et al., 1981), and skeletal muscle voltage-gated Na⁺ channels (Spence et al., 1977). The overall effect of injecting these toxins into a passing fish is almost instantaneous paralysis. All of the conotoxins are characterised by being short peptides with several intramolecular disulphide bonds creating a tightly packed molecule, they are divided into several subgroups depending on the sequence arrangement of their disulphide forming cysteine residues. There are several conotoxins that have high affinity (nanomolar) for muscle nAChRs but low (micromolar) affinity for many of the neuronal nAChRs (Luetje et al., 1990; Johnson et al., 1995). Also as outlined above several of the conotoxins have very different affinities for the two different ACh binding sites of muscle nAChRs. As well as blocking the ACh binding sites, there is a conotoxin that can block nAChRs as a non-competitive antagonist. This conotoxin, ψ-conotoxin PIIIE is a channel blocking antagonist (Shon et al., 1997).

There are also conotoxins that show specificity for neuronal nAChRs over muscle nAChRs, such as α-conotoxin MII (Cartier et al., 1996) and α-conotoxin GIC (McIntosh et al., 2002) which are both specific for α3β2 nAChRs, and α-conotoxin IMI which is specific for α7 nAChRs.

Another example of toxins that can discriminate between different ACh binding sites are the waglerins. Originally isolated from the venom of Wagler’s pit viper (*Tropidolaemus wagleri*) they are of interest as prior to their discovery, no toxins from the viper family of snakes had been believed to contain toxins with high affinity to nAChRs. Waglerin-1 (Wtx-1), (Taylor et al., 1998) highlights how the specificity of an antagonist can be highly dependent on the species from which the nAChR has been derived. Wtx-1 has a affinities of 10 nM and 20 μM for the mouse αε and αδ ACh binding sites respectively, a 2000-fold difference. However, these are reduced to an 80-fold difference, (1 μM at the αε site and 80 μM at the αδ site), in the rat nAChRs, (Molles et al., 2002). Of relevance to extrapolating results from rodents to human receptors, their is a 70-fold difference in the affinities of Wtx-1 for the human receptor binding sites, but the highest affinity is for the αδ site.

(Ackermann & Taylor, 1997) have suggested there are differences in the two binding sites based on mutated NmmI toxin. NmmI is an α-neurotoxin from the Mozambique
spitting cobra (*Naja mossambica mossambica*). The α-neurotoxins are polypeptides with masses around 7 kD, they contain 4 to 5 disulphide bonds and have 3 loops which contain the binding determinants. Using fetal mouse receptors, recombinant NmmI mutants, and α-BTX competition assays they were able to construct NmmI mutants with very different affinities for the two binding sites. They used α-CTX-MI to block the α/γ site, as α-CTX-MI has over a 10000-fold difference in affinity for the two sites (Ohno *et al.*, 1995) to determine the affinity of the toxin for each site, shown in table 1.3.

<table>
<thead>
<tr>
<th>NmmI form</th>
<th>Change in αδ Kd compared to wild type</th>
<th>Change in αγ Kd compared to wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E10A</td>
<td>2-fold decrease</td>
<td>2-fold decrease</td>
</tr>
<tr>
<td>K27E</td>
<td>13-fold increase</td>
<td>390-fold increase</td>
</tr>
<tr>
<td>R33E</td>
<td>680-fold increase</td>
<td>16000-fold increase</td>
</tr>
<tr>
<td>K47A</td>
<td>11-fold increase</td>
<td>11-fold increase</td>
</tr>
</tbody>
</table>

Table 1.3. Point mutations in the NmmI toxin have highlighted the differences in the affinity of the two mouse fetal nAChR ACh binding sites for antagonist (Ackermann & Taylor, 1997).

Polyamines have also been shown to bind to nAChRs. (Bixel *et al.*, 2001) used photoaffinity labelling with MR44 to determine the binding site of a polyamine on the *Torpedo* nAChR. MR44 is a photoactivatable philanthotoxin-433 (PhTx-433) analogue that binds to nAChRs with high affinity, whilst PhTx-433 is a neuroactive polyamine-containing toxin from venomous digger wasps (*Philanthus triangulum*). They found that two molecules of ^125^I-MR-44 bind per receptor complex, the bound sequence was close to the agonist binding site but the head group of MR-44 was localised to the vestibule.

Local anaesthetics can also have effects on nAChRs (Adams, 1976; Dilger *et al.*, 1993). The binding site of the general anaesthetic azioctanol was determined using photoaffinity labelling. The primary site of incorporation was αE262, positioned at the synaptic cleft end of M2 (Pratt *et al.*, 2000).

The binding site for non-competitive inhibitors (NCI's) e.g. chlorpromazine, ethidium bromide, triphenylmethylphosphonium (TPMP⁺) is within the ion channel pore.
Photoaffinity labelling using TPMP⁺ and 3H-chloropromazine have shown that they preferentially bind to M2 residues (Hucho et al, 1986).

### 1.5 Kinetics of Binding and Gating

It was predicted in the 1970s (Colquhoun & Hawkes, 1977) that with any plausible rate constants, ion channel openings should occur not as single openings, but in bursts of openings, i.e. multiple openings occur in sequence, separated by brief shutoffs of a duration orders of magnitude lower than shutoffs seen between activations. This has turned out to be the case for nAChR (Colquhoun & Hawkes, 1981; Sakmann et al, 1981) and for all other sorts of ion channels that have been studied. The bursting characteristics of a channel allow events to be assigned to a single channel molecule and estimates of the microscopic rate constants to be obtained.

#### 1.5.1 Estimating Rate Constants of Reaction Schemes

In the summaries of kinetic studies on nAChRs presented below, the nomenclature of labelling rate constants is the same as in scheme 1 for consistency (see appendix I). There have been two main approaches in estimating individual transition rate constants of nAChRs. The simpler method makes use of assumptions about which observed states, i.e. open or shut, correspond approximately to which actual physical states in a kinetic scheme, such as diliganded open or diliganded shut states. Physical rate constants can then be calculated from the parameters obtained by fitting open or shut time duration histograms with mixtures of exponential components. Methods of this sort have been used by (Colquhoun & Sakmann, 1985; Wang et al., 1997; Akk et al., 1999).

A procedure employed by Auerbach and colleagues is to obtain estimates of β₂ the channel opening rate constant, from plots of effective open time against agonist concentration. The effective open time is defined as the inverse of the slowest time constant of the intraburst closed time duration histogram. As agonist concentration is increased the apparent open time should approach β₂ asymptotically as the channel will be spending all of the time in the diliganded states. Plots of ACh concentration and effective opening rate are fitted with equation 1.1,
\[ \beta' = \frac{\beta_2}{1 + \left( \frac{EC_{50}}{[ACh]} \right)^n} \]  

(1.1)

where \( n \) is the Hill coefficient and \( EC_{50} \) refers to the ACh concentration at which \( \beta_2 \) is half-maximal. This is a purely empirical equation, it does not describe the kinetic schemes used in the analysis. An additional problem is that this method of estimating \( \beta_2 \) is that the fitted equation does not take into account any ACh blocked states or desensitised states, both of which are apparent at the high ACh concentrations needed for the curve to approach the asymptote. Also, in some instances the curve is seen not to plateau, even at very high agonist concentrations (Akk et al, 1999; Salamone et al., 1999).

A preferable method to those described above is the direct estimation of rate constants from the idealised single channel record (as described in the methods section). This alters a set of rate constants until the likelihood of the rate constants describing the single channel record has been maximised. The method has the advantage that there are fewer assumptions about which visible states represent which kinetic states and it also takes into account the sequential nature of transitions, information which is lost when examining open and closed time duration histograms.

1.5.2 Kinetics of Wild Type Muscle nAChRs

Little work has been conducted on the kinetics of human nAChRs. This stems historically from the lack of availability of the human cDNA clones. Originally, most work was on tissue from Torpedo or frog. Later as molecular cloning became common, the Torpedo and mouse nAChRs could be expressed in cell lines. It is presumably due to the availability initially of nAChRs from other species that the kinetics of the human receptor has been less studied. This situation has begun to be reversed with the discovery that the genetic basis of some forms of congenital myasthenia are due to mutations in human nAChRs.

Studies of the frog muscle endplate (Colquhoun & Sakmann, 1985) showed from the fitting of exponentials to open and shut state histograms that at least three shut states and two open states are needed to describe nAChRs. Simply from the fact that two molecules of ACh are required for efficient activation of nAChRs it can be deduced that
there are at least three shut states; unliganded, monoliganded, and diliganded. Scheme A includes these states (Karlin, 1967; Colquhoun & Sakman, 1985). It incorporates two equivalent binding sites, interactions between the two sites whilst the channel is still shut, and monoliganded as well as diliganded openings. Estimates of the rate constants were obtained by examining the burst characteristics, in which it was assumed that,

$$\tau_f = \frac{1}{(\beta_2 + 2k_{-2})} \text{ and } n_b = \frac{\beta_2}{2k_{-2}}$$

(1.2)

where $\tau_f$ is the mean length of brief shuttings assumed to be sojourns in A$_2$R within bursts and $n_b$ is the mean number of shuttings within bursts. There was an approximate correction for missed events. These calculations yielded values of 30600 s$^{-1}$ for $\beta_2$, 714 s$^{-1}$ for $\alpha_2$, and 8150 s$^{-1}$ for $k_{-1}$ for frog muscle nAChRs at 11 °C.

Further studies on the kinetics of nAChRs found at the frog endplate investigated the values of the equilibrium constants, (Colquhoun & Ogden, 1988). In this study cluster length at relatively high ACh concentrations (5 µM to 1 mM) were examined to obtain $P_o$ curves. The cluster length decreased with increasing ACh concentration and $P_o$ peaked at 0.87, at a concentration of approximately 100 µM ACh. Estimates were obtained for diliganded efficacy and binding equilibrium constants of ACh binding and ACh block. Several kinetic schemes were examined, that incorporated identical (scheme B) or different binding sites (scheme C), or also two independent subunits. Estimates of equilibrium constants were determined by fitting $P_o$ curves with
expressions relating the occupancy of the diliganded open state at equilibrium with ACh concentration and the equilibrium constants. A wide range of estimates for $E_2$ were obtained; 32 for the linear scheme with identical binding sites, 1100 for the linear schemes with different binding sites, and 500 for the non-linear scheme with different binding sites. However the values of 1100 and 500 were specified as being indeterminate. In all three schemes the equilibrium constant for block of the pore by ACh ($K_{Block}$) was estimated as approximately 1.3 mM with membrane potentials in the range of $-95$ mV to $-130$ mV. Values of 24 $\mu$M to 77 $\mu$M were obtained for the equilibrium binding constant of one site, whilst in schemes where the binding sites were allowed to have different affinities, the low affinity site had equilibrium dissociation constants in the range of 3.5 mM

![Scheme B](image)

![Scheme C](image)

A scheme with two identical ligand binding steps and diliganded openings has been used several times in other studies. (Akk et al, 1999) used adult mouse nAChRs and maximum likelihood fitting to this kinetic scheme (scheme D). $\beta_2$ was fixed at 60000 s$^{-1}$, and $\alpha_2$ was estimated at 1321 s$^{-1}$. The binding and unbinding rates of ACh were estimated at 111 $\mu$M$^{-1}$ s$^{-1}$ and 18020 s$^{-1}$ respectively.
A scheme that describes two identical binding sites, a single species of monoliganded opening, a single species of diliganded opening and a diliganded blocked state (scheme E) was used to estimate the rate constants describing the adult mouse nAChR (Wang et al., 1997).

\[
\begin{align*}
R & \xrightarrow{k_{+1}} AR & \xrightarrow{k_{+2}} A_2 R & \xrightarrow{\beta_2} A_2 R^* & \xrightarrow{k_{+Block}} A_2 R_{Block} \\
& \xleftarrow{k_{-1}} & \xleftarrow{k_{-2}} & \xleftarrow{\alpha_2} & \xleftarrow{k_{-Block}} \\
& \xleftarrow{\alpha_1} & \xleftarrow{\beta_1} & & \\
& & & & \text{Scheme E}
\end{align*}
\]

The diliganded channel opening rate (\(\beta_2\)) was obtained by measuring the mean duration of brief shuttings (\(1/(\beta_2 + 2k_{-2})\)) and the number of brief shuttings per burst (\(\beta_2 / 2k_{-2}\)) and solving for \(\beta_2\) (as in (Colquhoun & Sakmann, 1985) and (Milone et al., 1997)) using the values from fitting histograms of burst properties with sums of exponential components obtained in the presence of 1 \(\mu\)M ACh. A value of 48900 s\(^{-1}\) was obtained. This rate was fixed and used in maximum likelihood fitting to scheme E to obtain the other rate constants.

In addition, fitting to scheme F was carried out. This scheme incorporates two binding sites but with the possibility of the two binding sites being different before any ligand has bound (Colquhoun & Sakmann, 1985; Milone et al., 1997), but leading to identical monoliganded openings.

The rate constants obtained were exactly the same as when obtained fitting to scheme E. The monoliganded gating rate constants were estimated at 216 s\(^{-1}\) for opening (\(\beta_1\)) and 3320 s\(^{-1}\) for closing (\(\alpha_1\)). This predicts a monoliganded opening will have a duration of 300 \(\mu\)s. The rate constants for ACh binding (\(k_+\)) and unbinding were (\(k_-\)) estimated at 129 \(\mu\)M\(^{-1}\) s\(^{-1}\) and 21900 s\(^{-1}\) respectively. The rate constants for ACh binding and unbinding to the pore were estimated at 9.67 \(\mu\)M\(^{-1}\) s\(^{-1}\) and 74100 s\(^{-1}\), predicting an equilibrium dissociation constant of 7.7 mM for ACh block of the pore at a membrane potential of \(-70\) mV.
Further estimates of the kinetics of mouse nAChRs were obtained by (Salamone et al., 1999). $\beta_2$ was determined by measuring effective opening rate at a range of ACh concentrations as described previously. $\alpha_2$ was determined by using maximum likelihood fitting of kinetic parameters to scheme G. This scheme assumes that the agonist binding sites are initially different but are also independent, i.e. the binding of agonist to the $a$ site is the same whether or not there is agonist bound at the $b$ site. It does not include any monoliganded openings. However it has been shown from simulations that the monoliganded opening and closing rates are difficult to estimate reliably (Colquhoun et al, 2003).
Fits were done assuming the binding sites were equivalent, that is \( k_{+a} = k_{+b}, k_{-a} = k_{-b} \), and assuming they were non-equivalent, that is \( k_{+a} \neq k_{+b}, k_{-a} \neq k_{-b} \). They showed that the opening and closing rates of the channel, and the rates of entry and exit from desensitisation are not affected as to whether the binding sites are assumed to be equivalent or not.

In addition the equilibrium dissociation constants of the two binding sites, when assumed to be non-equivalent were very similar, however both the rates of ACh association and dissociation were different at the different sites.

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>Non-equivalent sites</th>
<th>Equivalent sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{+a} (\mu M^{-1} s^{-1}) )</td>
<td>97</td>
<td>158</td>
</tr>
<tr>
<td>( k_{-a} (s^{-1}) )</td>
<td>18026</td>
<td>25267</td>
</tr>
<tr>
<td>( k_{+b} (\mu M^{-1} s^{-1}) )</td>
<td>291</td>
<td>-</td>
</tr>
<tr>
<td>( k_{-b} (s^{-1}) )</td>
<td>43010</td>
<td>-</td>
</tr>
<tr>
<td>( K_a (\mu M) )</td>
<td>186</td>
<td>160</td>
</tr>
<tr>
<td>( K_b (\mu M) )</td>
<td>148</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.4. Estimates of the rate constants of the mouse nAChR (Salamone et al., 1999).

Studies of the rate constants describing desensitisation of the nAChR are intrinsically difficult, due to the non-conducting state of desensitisation and the existence of multiple desensitised states (Elenes & Auerbach, 2002) in a cyclic reaction scheme that allows recovery from desensitisation without reopening of the channel (Katz & Thesleff, 1957).

(Grosman & Auerbach, 2001), using adult mouse nAChRs estimated the rates of dissociation from the open channels and the rate of entry into desensitisation using both a linear (scheme H) and a cyclic reaction scheme, (scheme I).

\[
\begin{align*}
R & \xrightarrow{k_{+1}} AR & A_2 & \xrightarrow{\beta_2} A_2 R^* \\
& \xrightarrow{k_{-1}} & A_2 R & \xrightarrow{\alpha_2} & A_2 R^* \\
\end{align*}
\]

Scheme H
Using the linear model, the slowest time constant of the burst length distribution measured at low ACh concentrations (termed $\tau_{\text{burst}}^{\text{linear}}$) can be approximated by equation 1.3, this model ignores the intraburst shut time durations, dissociation of agonist from the open state, and desensitisation.

$$\tau_{\text{burst}}^{\text{linear}} \equiv \left(1 + \frac{\beta_2}{2k_a}\right) \frac{1}{\alpha_2} \quad (1.3)$$

When the efficacy of diliganded openings ($E_2$) is high, as in many gain of function mutations of nAChRs then there is a higher chance of desensitisation or agonist dissociation from the open state terminating a burst. In these cases an approximation for the time constant of the burst length was derived, (equation 1.4).

$$\tau_{\text{burst}}^{\text{cyclic}} \equiv \left(\beta_D + 2k_{(o)} + \frac{\alpha_2}{1 + \frac{\beta_2}{2k_-}}\right)^{-1} = \tau_{\text{burst}}^{\text{linear}} \frac{\sigma \tau_{\text{burst}}^{\text{linear}}}{\sigma + \tau_{\text{burst}}^{\text{linear}}} + 1 \quad (1.4)$$

where $\sigma = 2k_{(o)} + \beta_D$

Wild type receptors and a series of mutants with the 12' position of M2 within the δ subunit mutated, were fitted as a function of $\alpha_2$ and $\beta_2$ with equation 1.4. This led to estimates of 56870 s$^{-1}$ for $2k_{-}$ and 39 s$^{-1}$ for $\sigma$. For this fitting the values of $\alpha_2$ and $\beta_2$
for wild type were taken as 2000 s$^{-1}$ and 50000 s$^{-1}$ respectively, these values were from (Salamone et al., 1999), obtained as described previously.

The $\alpha_2$ and $\beta_2$ values of the mutated receptors were taken from estimates obtained previously (Grosman & Auerbach, 2000b; Grosman & Auerbach, 2000c) in the presence of choline (CCh), and adjusted to represent the receptors activated by acetylcholine by assuming,

$$\frac{\alpha_2^{\text{(mutant receptor + CCh)}}}{\alpha_2^{\text{(wild type receptor + CCh)}}} = \frac{\alpha_2^{\text{(mutant receptor + ACh)}}}{\alpha_2^{\text{(wild type receptor + ACh)}}}$$

(1.5)

$$\frac{\beta_2^{\text{(mutant receptor + CCh)}}}{\beta_2^{\text{(wild type receptor + CCh)}}} = \frac{\beta_2^{\text{(mutant receptor + ACh)}}}{\beta_2^{\text{(wild type receptor + ACh)}}}$$

(1.6)

The rate of entry into desensitisation ($\beta_D$) was calculated as the reciprocal of the time constant of the longest open time per cluster distribution for wild type and several $\delta 12'$ mutants. The mean value obtained for these receptors was 14.8 s$^{-1}$ for $\beta_D$. According to equation 1.4, this gives a value of 24 s$^{-1}$ for $2k_{\text{off}}$.

The kinetics of human nAChRs have been examined, (Ohno et al., 1997) looked at wild type receptors and several receptors with mutations responsible for congenital myasthenic syndrome (CMS). Maximum likelihood fitting of the kinetic parameters to a linear scheme incorporating cooperativity of binding and blocked diliganded state (scheme J) was performed. $\alpha_2$ was estimated at 1100 s$^{-1}$ and $\beta_2$ was estimated at 42900 s$^{-1}$. The blocking equilibrium constant ($K_{\text{Block}}$) was estimated as 3.2 mM at $-70$ mV and 22 $^\circ$C, this was comprised of the values of 155000 s$^{-1}$ for $k_{\text{-Block}}$ and 48 $\mu$M$^{-1}$ s$^{-1}$ for $k_{\text{+Block}}$. These rates predict that the blocked shut state has a lifetime of 6.5 $\mu$s, which is in reasonable agreement with the value of 20 $\mu$s obtained in the frog nAChRs at 11 $^\circ$C (Colquhoun & Sakmann, 1985). Since a linear scheme with two different sets of association and dissociation rates was used (i.e. $k_{+1} \neq k_{+2}$ and $k_{-1} \neq k_{-2}$) this does not allow the sites to have different binding properties, but does allow the occupation of one site to affect the binding properties of the other site.
1.5.3 Kinetics of Mutated Muscle nAChRs

Many single amino acid residue positions of the nAChR have been studied using site-directed mutagenesis in detail. (Akk, 2001) looked at the kinetics of mutant adult mouse nAChRs using scheme H.

\[
\begin{align*}
R \xrightarrow{k_{-1}} & AR & A_2R & \xrightarrow{\beta_2} & A_2 R^* & \xrightarrow{k_{-block}} & A_2 R_{block}
\end{align*}
\]

\pmb{\beta_2} was obtained by calculating the maximum effective opening rate at high concentrations of ACh as described previously, the other rate constants were obtained by fitting single channel data to scheme H. Compared with wild type the binding site mutation aY93F mutation showed a 50-fold decrease in \( \beta_2 \), a 2-fold increase in \( \alpha_2 \), a 3-fold decrease in the ACh dissociation rate, and a 13-fold increase in the ACh association rate. These are slightly smaller effects than measured previously for this mutation (120-fold decrease in \( \beta_2 \), 9-fold decrease in ACh dissociation, (Akk et al, 1999). The aW149F mutation showed a 90-fold decrease in \( \beta_2 \), no change in \( \alpha_2 \), a 2-fold increase in the ACh dissociation rate and an 8-fold decrease in the ACh association rate. Therefore both mutations, shown previously by labelling studies to be within the binding sites have predominantly gating effects.

The ammonium moiety of ACh may have cation-\( \pi \) interactions with one of the many aromatic residues within the binding site, e.g. aW149 (Zhong et al, 1998) although these are based on assuming that aW149 is primarily involved in binding rather than gating. Using the same kinetic scheme and similar fitting methods the aY198F mutation (another mutation that resides within the ACh binding site) was found to mainly affect ACh dissociation, (Akk et al, 1999). Compared to wild type receptors, in this mutation \( \beta_2 \) was found to be decreased 3-fold and the ACh dissociation rate was decreased 9-fold.
αY190 in the ACh binding site has been shown to have dual roles in ACh binding and gating. The mutation αY190F causes a 400-fold decrease in β2, a 2-fold increase in α2, and a 70-fold increase in the ACh equilibrium dissociation constant (when fitting to a sequential kinetic scheme) (Chen et al, 1995).

αD200, another residue within the ACh binding site has been shown to affect mainly gating of the channel. The mutation αD200N causes over a hundred fold decrease in β2 in both the adult and fetal mouse nAChR (Akk 1996).

There evidence from site-directed mutagenesis experiments and antagonist competition assays that that the charged moiety of ACh interacts with the negatively charged residues δD180 and γD174 on the minus face of the binding site, (Czajkowski et al., 1993; Czajkowski & Karlin, 1995). The homologous mutation in the mouse ε subunit, εD175N has also been studied (Akk et al, 1999). Using a kinetic scheme that assumed equivalent binding sites, maximum likelihood fitting was used to demonstrate that the mutation decreased the efficacy, E2, 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.

1.6 nAChR Gating

The loop connecting M2 and M3 has been implicated in channel gating in several ligand-gated ion channels, including channels containing α7 (Campos-Caro et al., 1996), and in glycine channels (Lynch et al., 1995; Rajendra et al 1995; Lewis et al, 1998). It should be remembered that the terms ‘gating’ and ‘gate’ can be ambiguous. For example there is little doubt that around the midpoints of the M2 segments there is a gate that acts to prevent ion conduction in the closed states. However the term gating can refer not only to the movements of this narrow region, but also the transduction of the conformational change around the ACh binding sites to the channel pore. These gating events represent wells in an energy landscape of gating, and the movements involved may not necessarily involve a neat sequential movement of different parts of the protein but could just as easily be spread over the whole receptor.
Several studies have used linear free energy relationships (LFERS) to probe the gating of nAChRs. This method makes inferences from observations of whether differences in an equilibrium constant in a series of mutants results in changes in the forward rate constant or the backward rate constant, or both. The studies give information regarding the energetic state of an amino acid residue whilst in the transition state between two stable states (typically the diliganded closed and diliganded open states of nAChRs).

A series of mutations at the 12' position of M2 in the adult mouse muscle nAChR δ subunit were constructed and the ΔG of channel opening calculated (Grosman & Auerbach, 2000c). Brønsted plots were constructed to determine the extent of the gating reaction during the transition state at the positions mutated. These plots are based on the fact that the change in the free energy of the transition state for a particular reaction step (such as channel opening) given a perturbation in the system (in this case mutation of a single amino acid) is composed of two elements. These elements are the change in free energy of the two systems in the open state given the perturbation, and the change in the free energy of the closed state given the perturbation. The elements are scaled relative to each other by the introduction of the Φ term.

\[
(G^\text{tp} - G^\text{t}) = \varphi(G^\text{open} - G^\text{open}) + (1 - \varphi)(G^\text{closed} - G^\text{closed})
\]  

(1.7)

This can be rearranged and expressed in rate constants (Leffler, 1953)(Grosman et al., 2000d) as,

\[
\log \beta_2 = \varphi \log \frac{\beta_2}{\alpha_2} + C, \text{ where } C \text{ is a constant.}
\]

(1.8)

Therefore the slope of a plot of log β₂ / α₂ and log β will have a slope of Φ.

It was found from Brønsted plots that at this position the channel is approximately 30% (a Φ value of 0.3) 'open like' during the transition state. However there are several assumptions made during LFER that it is important to be aware of. The existence of fractional Φ values is open to interpretations. A fractional Φ value could indicate that during gating the transition state is proportionally 'like' either the closed or open state. It could also indicate that that there are two different transition states with the Φ value representing the proportion of events in which one of the transition states is accessed. It
is also assumed that the mutations do not affect the reaction pathway taken, and that the mutations do not significantly alter the structure of the open or closed states, i.e. the start and end points of the reaction pathway are the same in all mutated receptors. These are essentially the same assumptions that are made when comparing a wild type and a mutant channel using maximum likelihood fitting of single channel data to kinetic schemes.

This work was extended by looking at a series of δM2 mutants and examining their LFERs (Cymes et al, 2002). Mutations were engineered at eleven different positions in the M2 segment. Three of these positions (10’, 11’ and 15’) were not amenable to Φ analysis. Brønsted plots could not be constructed for the 11’ and 15’ positions. At the 11’ position, no changes in efficacy could be detected and thus a Brønsted plot could not be constructed, at the 15’ position the kinetic behaviour was found to be heterogeneous and therefore “a single representative kinetic behaviour” could not be attributed to the range of mutations in this position. At the 10’ position a nonlinear Brønsted plot was obtained and when fitted with a parallel two state transition model, the parameters were very ill defined and a phi value of 2.1 (which makes no physical sense) was obtained. The general trend found was that residues towards the intracellular side resemble the closed state (Φ ≈ 0, range of -0.18 ± 0.23 to 0.03 ± 0.05 for 2’ to 10’) whilst residues towards the extracellular end of M2 are more open like (Φ ≈ 0.3, range of 0.24 ± 0.09 to 0.38 ± 0.01 for 12’ to 19’). Importantly the limits of the ability to estimate accurate Φ values obtained when using Brønsted plots with only two values was examined. This was done by calculating all of the possible two-point Brønsted plot Φ values of twelve mutations made at the 12’ position. The conclusions were that at this position at least a ten-fold difference in equilibrium constants was needed to obtain accurate Φ values from a two point Brønsted plot.

1.6.1 Transduction of ACh Binding into Opening of the Pore
The cryoelectron images of the Torpedo nAChR in the open and closed states have been compared to the AChbp crystal structure (Unwin et al, 2002). The study showed that the AChbp subunits had similar conformations to the non-α subunits of the nAChR, but the inner β-sheets were rotated approximately 15° clockwise compared to the α subunits in the absence of ACh. The outer β-sheets were tilted approximately 11° clockwise compared to the α subunits. No significant differences in the conformations of the two
α subunits could be detected. Binding of ACh is believed to convert the α subunits to "non α subunit" conformations making the whole complex more symmetrical.

By positioning models based on the structures AChbp and the transmembrane region of the Torpedo nAChR clues are being revealed as to how ligand binding is communicated to the pore to induce and structural change which allows the passage of ions (Miyazawa et al., 2003). It appears that the residue V44 within the loop linking the β1 and β2 strands of the AChbp can dock into a pocket formed from αS269 and αP272 of the nAChR M2 segment. Presumably the rotation of the inner β-sheet leads to rotation of the M2 segments and a widening of the pore around the gate region. A sequence of events for opening the pore has been suggested (Miyazawa et al., 2003),

1. ACh binding causes a change in conformation of the α subunits which allows their inner β-sheets to rotate clockwise.
2. The rotations of the inner β-sheets are transmitted to the M2 segments which weakens the hydrophobic interactions between the residues forming the gate.
3. The M2 segments form new stable interactions with the outer ring of transmembrane helices.

1.7 Voltage Sensitivity of nAChRs

Although nAChRs are ligand-gated ion channels, the membrane potential can also influence channel activity (Magleby & Stevens, 1972). Studies on frog endplates showed that the length of short gaps within bursts elicited by ACh do not vary with voltage and thus it seems unlikely that either β2 or ACh dissociation are voltage-dependent (Colquhoun & Sakmann, 1985). If one of these rates were voltage-dependent, then the other rate would have to also be voltage-dependent, but with opposite polarity and to the same extent. Work on the actions of tubocurarine demonstrate that its competitive antagonism action is not voltage sensitive (Colquhoun et al., 1979), and in as much as it is assumed that the binding sites of competitive antagonists overlap the binding sites of agonists this suggests that the agonist binding site is not influenced by the membrane potential.
The apparent channel lifetime of frog nAChRs is seen to decrease over the range of -120 mV to +100 mV, this indicates that $\alpha_2$ (the channel shutting rate) increases with increasing depolarisation (Colquhoun & Ogden, 1988). This phenomenon has also been seen in mouse nAChRs. Increasing depolarisation from -30 mV to -100 mV increased the burst length of adult mouse nAChRs from 0.7 ms to 2.0 ms (Auerbach et al., 1996).

To determine the effect of voltage on specific rate constants maximum likelihood fitting to scheme H (a simple three state sequential scheme) was carried out. To determine $\alpha$, the mutant $\alpha$D200N was used (described by (Auerbach et al., 1996)). This mutant has a low value of $\beta_2/k_{-2}$ (less than 0.05), therefore the mean burst length ($\tau$) was assumed to be approximately equal to the inverse of $\alpha_2$. Using $\alpha$D200N (in embryonic receptor) showed decreased burst lengths and increased shut times compared to wild type. However the voltage sensitivity was little changed, 56 mV being required for an e-fold change in $\tau$ (compared to 63 mV for wild type $\gamma$-containing receptors). To determine the voltage sensitivity of $\beta_2$, the $\alpha$Y93F mutant was used to determine the plateau of the effective opening rate. This was found not to vary over the range of -25 mV to -100 mV, and thus $\beta_2$ was determined to be voltage-independent. Using equation 1.9, estimates of $z\delta$ were obtained, where $z$ is the amount of charge required to terminate an opening and $\delta$ is the fraction of the electric field that this charge must move through. In wild type adult, wild type fetal, $\alpha$D200N, and $\alpha$Y93F $z\delta$ was calculated to be approximately 0.4.

$$\tau = \tau_0 \exp\left(\frac{z\delta V}{kT}\right)$$

(1.9)

A $z\delta$ value of 0.4 could represent two charges moving 20% through the membrane (as the authors suggest), or it could be 5 charges moving through 8% of the membrane. The $z\delta$ values of several receptors with mutations in the pore-lining M2 segment were also estimated to be approximately 0.4. This value seemed to be independent of mutation position or whether $\epsilon$ or $\gamma$ subunits are used. The direction of the charge transfer detected is either positive outward or negative inward. The voltage-dependence of channel closing cannot be assigned to a specific part of the receptor.
1.8 Desensitisation of nAChRs

Desensitisation can be defined as the lack of activation following prolonged application of agonist. In terms of the wild type muscle nAChR it is not believed to be physiologically relevant due to the short lifetime of ACh within the synaptic cleft. However it needs to be understood as it occurs under some experimental conditions (often as an unwanted phenomenon) and it seems more physiologically relevant in ion channels other than the muscle nAChR. For example it may be more important at CNS synapses where ACh plays less of a role in fast synaptic transmission. Choline can act as an agonist on α7 receptors and thus the rapid desensitisation seen with α7 receptors may serve to limit the effects of residual choline in the extracellular space. Finally desensitisation may be of pathological importance in some naturally occurring nAChR mutations (Ohno et al., 1996). At the single channel level desensitisation manifests itself as long shut times between bursts at high concentrations of agonist (Sakmann et al., 1980), causing the openings to clusters into bursts. It is because of the desensitisation that the clusters of bursts give rise to long stretches of activations that originate from a single individual channel.

Desensitisation is usually considered to proceed from the diliganded open state of the receptor (Dilger et al., 1993). Desensitisation is also cyclic process (Katz & Thesleff, 1957; Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1989; Franke et al., 1993), that is the receptor can recover back to the unliganded resting state without passing back through the diliganded open state. The simplest scheme that accounts for this was proposed by (Katz & Thesleff, 1957) and this demands that the desensitised form of the receptor has a higher affinity for agonist than the resting form. It is also known that there are several different desensitised states of the nAChR can exist over a range of timescales, (Magleby & Pallotta, 1981; Feltz & Trautmann, 1982; Cachelin & Colquhoun, 1989; Elenes & Auerbach, 2002). This range of time scales suggests that there are many different desensitised states.

To describe the fast and slow components of desensitisation commonly seen from whole cell experiments, it has been suggested that there are two fundamentally different desensitised states. (Magleby & Pallotta, 1981) have suggested that the application of ACh can convert some receptors into a ‘desensitisable state’ which upon application of further ACh can be converted to ‘desensitised receptors’. An extension of this idea that
allows for a nonlinear arrangement of entering two different desensitised states, and also
counts for the binding of two ACh molecules has been proposed (scheme K). It is
shown below, redrawn from (Feltz & Trautmann, 1982), the equilibrium arrows have
been drawn as single lines for clarity.

![Scheme K](image)

The entry rates into desensitisation of the adult mouse nAChR from both the shut
diliganded receptor ($\beta_{D(s)}$) and the open diliganded receptor ($\beta_D$) have been estimated
(Auerbach & Akk, 1998). In this study it was concluded that desensitisation proceeds
mainly from the diliganded open state by considering the properties of the time constant
of the duration of clusters multiplied by the probability of being open within a cluster
($\tau_P o$), outlined below,

1. $\tau_P o$ showed no dependence on ACh concentration.
2. Plots of $(\tau_P o)^{-1}$ against the occupancies of the unliganded, monoliganded, and
diliganded states showed a negative correlation, no correlation, and a positive
   correlation respectively. The occupancies were calculated from a scheme that
   consists of two identical sequential ACh bindings and a diliganded openings and
   using estimates of equilibrium dissociation of 100 $\mu$M or 160 $\mu$M (depending on
   the composition of the extracellular solution) and a value of 50 for $E_2$, these
   estimates were obtained from previous studies, (Auerbach et al., 1996; Wang et
   al., 1997). The positive correlation seen with the diliganded states indicates that
desensitisation mainly occurs from diliganded states. This was concluded since
if transitions between states visited in a cluster are rapid compared to entry
transition rates into desensitised states then the distribution of mean cluster
duration will tend to a single exponential with the following relationship,
\[ \tau^{-1} = \sum P_i k_{iD(i)} \], where \( P \) is the occupancy of state \( i \), and \( k_{iD(i)} \) is the entry rate into desensitisation from state \( i \) and thus \((\tau c P_o)^{-1}\) should increase linearly with occupancy.

3. A plot of \((\tau c P_o)^{-1} \equiv \beta_d + \frac{\beta_{D(i)}}{E_2}\) showed no correlations between \((\tau c P_o)^{-1}\) and \( E_2 \).

The \( E_2 \) values for this plot were obtained from a range of wild type and mutant receptors with different agonists and at different membrane potentials by either dose-response curves or single channel kinetic analysis. The equation of this plot was derived from the state occupancies of the diliganded states calculated in (2) and \( \tau^{-1} = \sum P_i k_{iD(i)} \). The rate of entry into desensitisation from the diliganded open state was 3.18 s\(^{-1}\) (\( \beta_d \)), compared to -0.08 s\(^{-1}\) for the rate of entry into desensitisation from the diliganded shut state (\( \beta_{D(i)} \)), this value was not significantly different from 0 s\(^{-1}\).

From the previous three sets of calculations, it was concluded that \((\tau c P_o)^{-1}\) is a direct measure of the rate of entry into desensitisation from diliganded open channels. It was also concluded that rate of recovery from desensitisation is \(\approx 0.01\) s\(^{-1}\). This was calculated by dividing the mean frequency of clusters of the main population of clusters (0.078 s\(^{-1}\)) by the number of channels in the patch. As the experiments were performed in the cell-attached configuration the number of channels was not known. It was assumed to be \(\approx 10\). Due to the assumptions made to calculate the rate of recovery from desensitisation, the value of 0.01 s\(^{-1}\) cannot be considered robust.

The rate of entry into desensitisation of adult mouse nAChRs has been estimated using single channel kinetics and a cyclic reaction scheme to be 14.8 s\(^{-1}\), (Grosman & Auerbach, 2001). A description of how this rate was determined is described earlier in "kinetics of binding and gating".

Evidence for desensitisation proceeding mainly from the diliganded open state in fetal mouse nAChR has been presented by (Franke et al., 1993). In these studies the inverse of the time constant of entry into desensitisation (1/\( \tau_D \)) was linearly correlated with \( P_{open} \). Simulations demonstrated that desensitisation proceeding from the diliganded
open state also produced a linear relationship between $\tau_d$ and $P_{open}$. This linear relationship was not present in simulations where desensitisation proceeded from the unliganded resting state or where desensitisation was due to binding of a third ACh molecule to the diliganded open receptor. However, a linear scheme was considered unsuitable as no re-openings of the ion channel were seen following removal of desensitising concentrations of ACh and so it was concluded a cyclic scheme is required to describe desensitisation.

(Elenes & Auerbach, 2002) showed that the shut time distributions at high ACh concentrations of adult mouse nAChRs required at least five components, this may indicate at least four diliganded desensitised states if it is assumed that at this concentration all of the receptors are mainly diliganded. The fifth and longest shut state will be a function of the number of channels in the patch. All possible non-cyclical models comprising of five diliganded desensitised and a diliganded open state were tested using maximum likelihood fitting to one patch. Although the models could not be distinguished a common feature of all the fits were equilibrium constants of desensitisation that ranged from $<0.1$ to $>10$. In addition, the two extreme cases from this population of models, that of a star and a linear scheme were examined in more detail.

The results from fitting the linear model suggest that the first four desensitised states have lifetimes in the range of 0.7 ms to 630 ms and the open time has a lifetime of 14 ms (this refers to 'burst lifetime' as only one diliganded open state, and no non-desensitised shut states were included). The fifth desensitised state was assumed to be a
function of the number of channels in the patch. Fitting to the star model predicted a similar range of desensitised state lifetimes, and similar values for each individual lifetime.

There is evidence indicating that the structure of the nAChR differs in the resting shut state and the desensitised shut state. Cryoelectron microscopy shows that the δ subunit of the *Torpedo* nAChR is tilted after being exposed to CCh for a few minutes (Toyoshima & Unwin, 1988). However this structure may only represent the slow state(s) of desensitisation. Also this structure is at low resolution (18 Å) and so fine details of the positions of secondary structure cannot be determined.

Use of the photoaffinity antagonist 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine ([125I]-TID) has also highlighted differences in the structure of the nAChR in the resting and desensitised states (White & Cohen, 1992). In the resting state, two residues facing the pore in M2 were labeled in the β and δ subunits of *Torpedo* nAChRs. In the presence of 50 µM carbamylcholine, in which the receptors should be desensitised the labeling was extended into the N-terminal of the M2 segments to include a further two pore facing residues in each of the β and δ subunits.

Differences between the structure of the resting and desensitised mouse fetal receptors have also been investigated using the substituted cysteine accessibility method (SCAM). It was found that in the desensitised state, the residues acting to occlude the ion conduction pathway are more extensive then in the resting shut state, ranging from αG240 to αL251 (Wilson & Karlin, 2001). In the shut resting conformation of the channel residues αG240 through to αT244 (mouse muscle numbering) were found to form a barrier that inhibits the passage of ions through the channel (Wilson & Karlin, 1998). Differences around the ACh binding site have also been detected between resting and desensitised states shown by increased photolabeling of binding site residues upon desensitisation of the receptor (Galzi *et al*, 1991).

**1.9 Neuronal nAChRs**

Although the muscle nAChR is the most extensively studied ligand-gated ion channel, it is by no means the most abundant. Of immense interest are the neuronal nAChRs. These receptors, closely related evolutionarily and functionally to muscle nAChRs are
found predominantly in the nervous system. However, single channel analysis of neuronal nAChRs is not easy (Sivilotti, 2000). Neuronal nAChRs tend to run down when looking at the single channel properties (Buisson et al., 1996). Due to this run down it is difficult to obtain enough transitions to construct reproducible burst length histograms. In addition multiple conductance states are often seen, although the levels cannot always be assigned unambiguously. There is the further complication of the stoichiometry of the channels being measured, injection of say α4 and β2 cRNA into oocytes could in theory lead to multiple channel species, the problem increases when three different subunits are injected. Therefore it is not surprising that many inferences about neuronal nAChRs are made based on evidence from muscle type nAChRs, and for this reason an overview of their roles is presented here.

The neuronal nAChRs are comprised of nine α (although humans lack the α8 subunit that is found in chicks) and three β subunits and are expressed within both the central and peripheral nervous systems. The most abundant and widespread of the neuronal subunits are α4 and β2, being found throughout the central nervous system. Other subunits can have more localised expression, for example α10 has only been found in the cochlea (Elgoyhen et al., 2001), whilst α6 is only found within catecholaminergic nuclei of the brain. α3, α5, α7, β2, and β4 are all expressed in peripheral ganglia to varying extents (Conroy & Berg, 1995; Poth et al., 1997; Vernallis et al., 1993). Neuronal nAChRs are also found outside of the nervous system, for example α7 subunits have been reported in bronchial epithelial cells (Zia et al., 1997), lymphocytes, vascular endothelial cells, thymus cells, and in skeletal muscle during the switch from γ subunit to ε subunit expression (Fischer et al., 1999).

The complete range of stoichiometries of the neuronal nAChRs is far from known. However, certain combinations of subunits have been found to be localised together in regions of the nervous system, and importantly have been shown to produce functional channels in heterologous expression systems. For example, α4 and β2 subunits coassemble to form receptors that have been found to mediate tonic inhibition by GABA in the CA1 region of the hippocampus (Alkondon et al., 1999). It has also been discovered that some neuronal nAChR subunits can form triplet receptors, such as the α3, β4, and β3 subunits (Groot-Kormelink et al., 1998). The simplest stoichiometry found is with the homomeric nAChRs, such as those formed by α7 and α9. The
homomeric neuronal nAChRs have properties which readily distinguish them from the heteromeric neuronal nAChRs. They have a high affinity for α-BuTx (Keyser et al., 1993), although around 10-fold less than muscle nAChRs it is still much greater than for heteromeric neuronal nAChRs. Also homomeric neuronal nAChR have a higher Ca$^{2+}$ permeability ratio than any other nAChRs.

1.9.1 Ca$^{2+}$ Permeability of Neuronal nAChRs
All nAChRs are permeable to Ca$^{2+}$, however the Ca$^{2+}$ permeability tends to be greater in neuronal nAChRs compared to muscle nAChRs. This could mean that Ca$^{2+}$ entering through some neuronal nAChRs act primarily as a second messenger rather than simply as a charge carrier (Mulle et al., 1992; Vernino et al., 1992; Vernino et al., 1994). The pCa$^{2+}$/pNa$^{+}$ ratio for muscle nAChRs is around 0.12, compared to 1.1 for α3β4 receptors, (Costa et al., 1994), whilst α7 receptors have a pCa$^{2+}$/pNa$^{+}$ ratio as high as 20, (Seguela et al., 1992). This Ca$^{2+}$ influx can have a physiological role, as shown by the activation of a Ca$^{2+}$-dependent chloride conductance in the medial habenula (Mulle et al., 1992).

1.9.2 Inward Rectification of Neuronal nAChRs
Most neuronal nAChRs in contrast to muscle nAChRs display inward rectification when in whole cell mode, that is they pass very little current at positive potentials, (Mathie et al., 1990). This rectification is absent in outside-out patches which suggests that intracellular factors are responsible for the inward rectification. The inward rectification seen with α7 receptors is mainly due to Mg$^{2+}$ as it can be reduced in a dose-dependent manner with intracellular application of the Mg$^{2+}$ chelating agent CDTA (Bonfante-Cabarcas et al., 1996; Forster & Bertrand, 1995). The rectification seen at other neuronal nAChRs is thought to be due to block by the intracellular polyamine spermine (Haghighi & Cooper, 1998).

1.9.3 Activation of Neuronal nAChRs
As well as being activated by ACh neuronal nAChRs can be activated by choline, one of the products of ACh hydrolysis and present at high concentrations in serum. Choline is a full agonist at α7 receptors, but is an ineffective agonist at many other nAChR receptors (Papke et al., 1996).
Many substances have been found to be able to modulate nAChR elicited currents. Ca\(^{2+}\) in the millimolar range has been found to be a positive allosteric effector at some neuronal nAChRs (Vemino et al., 1992), and has also been shown to affect the open probability of neuronal nAChRs, (Amador & Dani, 1995). The binding site for modulating Ca\(^{2+}\) has been found to reside at the interface between two \(\alpha 7\) subunits (Galzi et al., 1996). Hormones such as progesterone and testosterone have also been shown to modulate neuronal nAChRs, although in a negative fashion (Bertrand et al., 1991; Valera et al., 1992).

1.9.4 Neuronal nAChRs in Synaptic Transmission
Unlike the postsynaptic muscle nAChRs which are involved in ‘classical’ synaptic transmission, neuronal nAChRs have much more complex roles, acting post, pre, and extra-synaptically. Postsynaptic roles for neuronal nAChRs have been shown both in the hippocampus (Frazier et al., 1998) and in retinal ganglion cells (Feller et al., 1996). Electron microscopy has shown \(\alpha 3\) subunits concentrated on the postsynaptic membranes (Loring et al., 1988), and \(\alpha 7\) subunits have been detected on pseudodendrites protruding from the ciliary soma that are not associated with any postsynaptic density (Jacob & Berg, 1983; Loring et al, 1985). However it appears that both \(\alpha 3\) and \(\alpha 7\) subunits are utilised in synaptic transmission as synaptic currents recorded from embryonic ciliary neurons show a biphasic current. The large rapidly inactivating component of this current can be blocked by \(\alpha\)-BuTX, (\(\alpha\)-BuTX is a poor antagonist of \(\alpha 3\) receptors but an effective antagonist of \(\alpha 7\) receptors), (Ullian et al., 1997). \(\alpha 7\) receptors have also been shown to act presynaptically in autonomic ganglia. Application of nicotine to synapses formed between visceral motor neurones and sympathetic ganglion neurones results in an increase in the rate of spontaneous excitatory postsynaptic currents (epsces) without any increase in the current amplitudes (indicating a presynaptic effect). This presynaptic effect is due to \(\alpha 7\) receptors as it could be blocked with nanomolar concentrations of \(\alpha\)-BuTX, (McGehee et al., 1995). \(\alpha 7\) receptors may also be activated perisynaptically. Although there is efficient hydrolysis of ACh in the synaptic cleft by ACh esterase, \(\alpha 7\) receptors may be activated by choline within the extrasynaptic space. In addition arachidonic acid is produced in an activity-dependent manner from postsynaptic neurones, and this has been shown to modulate \(\alpha 7\) function (Vijayaraghavan et al., 1995).
Presynaptic neuronal nAChRs in the brain seem to be more common than the postsynaptic nAChRs. They have been shown to be present at presynapses releasing either glutamate (McGehee et al., 1995), GABA (Lena et al., 1993), ACh (McGehee et al., 1995), 5-HT, or noradrenaline (Li et al., 1998) and in many brain areas. Not only are neuronal nAChRs present presynaptically but they have also been shown to modulate synaptic transmission presynaptically. At hippocampal synapses nicotine application increased the frequency of epscs without affecting their amplitude suggesting a presynaptic effect. This was an effect that could be blocked by α-BuTX, indicating that it was caused by α7 receptors (Gray et al., 1996).

1.9.5 Neuronal nAChRs in Development

Neuronal nAChRs are believed to play a role in regulating the development of the foetus. They are expressed at high levels from early on in development, and both pre and postnatal nicotine treatment can disrupt morphologically and functional development of the nervous system. Throughout adult life nAChR expression is stable once they first begin to be expressed after differentiation of neuronal precursor cells. However there is often a reduction in the expression of nAChRs during pathological aging, although it is not known whether this is a cause of the pathology or a symptom.

1.9.6 Neuronal nAChRs and Disease

Several disease conditions have been either positively identified to be due to neuronal nAChRs or have strong links with nAChR physiology. The rare condition, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is caused by mutations in the neuronal nAChR subunits. The S248F substitution mutation in α4 shows reduced Ca^{2+} permeability, faster desensitisation, and slower recovery in α4β2 receptors compared to wild type α4β2 receptors (Steinlein et al, 1995). The insertion of a leucine residue towards the extracellular side of the M2 segment of α4 subunits can also cause ADNFLE (Steinlein et al., 1997). This insertion results in decreased Ca^{2+} permeability and increased ACh potency at α4β2 receptors. The mutation V287L within the M2 segment of β2 subunits has also been linked to ADNFLE, this mutation exerts a dominant negative effect on α4β2 receptors, it reduces desensitisation of the receptors (Fusco et al., 2000). The antiepileptic drug carbamazepine has been used as treatment in ADNFLE cases, and it acts as a non-competitive antagonist at α4β2 receptors, (Picard et al, 1999).
Neuronal nAChRs have been implicated in both Alzheimer’s disease and Parkinson’s disease, accompanying both of these conditions is the loss of high affinity nicotine binding sites (Whitehouse et al., 1988), which is thought to be due to a loss of α4β2 receptors. In both Alzheimer’s disease and Parkinson’s disease various cholinergic pathways are degraded, such as those in the basal forebrain, and the nucleus basalis of Meynert which provides the main source of cholinergic innervation into the cortex (Mesulam & Geula, 1988). To date there is little data to suggest why these cholinergic pathways are degraded in these conditions, and whether this loss is a cause or an effect of the diseases.

1.10 Muscle nAChRs and Disease

As outlined above nAChRs are involved in a great many disease states. It is not surprising that most of these disease states that involve the neuronal nAChRs attract a great deal of interest in both the pharmaceutical industry and the popular press. It is also not surprising that most information about nAChRs involved in disease is from the much easier to study and functionally better defined muscle nAChRs.

1.10.1 Myasthenia gravis (MG)
The symptoms of MG include generalised muscle weakness, especially of the ocular muscles. Many patients have autoantibodies directed against the muscle nAChR. Muscle weakness arises due to these antibodies binding the nAChRs and activating the complement cascade that leads to destruction of the neuromuscular junction (Lang & Vincent, 2003). The disorder can also be caused by autoantibodies to the muscle specific tyrosine kinase (MuSK) that it localised at the neuromuscular junction (Hoch et al., 2001).

1.10.2 Congenital Myasthenic Syndromes (CMS)
CMS are broadly categorised as disease that produce muscle weakness with an underlying genetic basis, that is the patients lack the autoantibody to nAChRs seen in patients with the more common condition myasthenia gravis. Spontaneous mutations can rise in many proteins that lead to muscle activity being detrimentally affected. Of these, only those due to alterations in the muscle nAChRs shall be considered in depth, although it is worth noting that CMS can also arise due to mutations in endplate acetylcholinesterase (AChE) deficiency (Ohno et al., 2000), choline acetyltransferase
deficiency (Ohno et al., 2001), and rapsyn deficiency (Ohno et al., 2002). In addition some case of CMS have an unknown molecular mechanism. Studies of CMS can involve clinical observation, in vitro whole cell electrophysiology, single channel electrophysiology, cytochemical and ultra structural studies, linkage analysis and mutation analysis.

Mutations causing CMS are found in all of the nAChR subunits and in many different regions of each subunit. They can be classified as either slow channel (SCCMS) or fast channel syndromes. SCCMS are due to over activity of the nAChRs, characterised by a slower decay of the endplate current compared to wild type endplates, whilst the fast channel syndromes are characterised by a faster endplate current decay. There are also mutations that lead to low expression of particular nAChR subunits. Most low expression mutations are due to insertions or deletions, although some point mutations due occur. Mutations can also occur in the promoter regions of nAChR genes.

Deficiency of nAChRs at the muscle endplate leads to an increased number of endplate regions, and rapsyn expression is usually also low. Quantal release is usually higher than normal and in mutations that lead to low expression of epsilon subunits, the gamma subunit is present (Engel et al., 1996; Ohno et al., 1997). Most CMS disorders that result in a loss of nAChRs at the muscle endplate are due to mutations in the ε subunit (Engel et al., 1996; Ohno et al., 1997; Ohno et al., 1998; Chiara et al., 1998; Milone et al., 1998). This may be because upregulation of the γ subunit can help to alleviate the effects of the ε mutations, and thus patients with ε subunit mutations are more likely to survive than those with mutations in other subunits.

The slow channel syndromes all result from gain of function mutations. The symptoms can appear early in life and peak within a decade, alternatively symptoms can be absent up until middle age and then progress in severity. Most patients show varying degrees of weakness in the ocular, shoulder, limb, cervical, wrist and finger muscles. Patients fatigue easily and can have difficulty walking and breathing. Prolonged activation of the nAChRs results in prolonged endplate currents and potentials, which in turn leads to one or more repetitive compound muscle action potentials (CMAPs). Repetitive stimulation of muscle endplates often results in decremental decreases in the CMAPs. There is usually degradation of the muscle end plates. Excessive accumulation of calcium has also been detected at some endplates (Magleby & Zengel, 1982) and has been detected in the endplates of a SCCMS transgenic mouse (Gomez et al., 1997).
this calcium overload that is believed to be responsible for the degeneration of junctional folds and nuclear apoptosis (Leonard & Salpeter, 1979). In vitro microelectrode studies show prolonged excitatory postsynaptic potentials (EPPs) and delayed decay of excitatory postsynaptic currents (EPSCs). The mutant receptors have longer open periods than the wild type receptors, and some mutations can cause the receptors to open even in the absence of agonist, (Ohno et al., 1995), resulting in a near continuous cation influx into the endplate region. The pathological effects are due to the compromised safety margin of neurotransmission which can lead to degenerating junctional folds and depolarisation block during physiological stimulation due to the summation of excitatory postsynaptic potentials (EPPs). Quinidine, a nAChR open channel blocker, has been used clinically to treat patients with slow channel CMS (Fukudome et al, 1998;Harper & Engel, 1998).

Many of the mutated receptors underlying SCCMS may have their detrimental effects exacerbated by activation via choline. Choline is a component of serum and one of the products of ACh hydrolysis has been shown to activate homologous mouse nAChRs containing the SCCMS mutation εT264P and those containing αS269I (Zhou et al, 1999). However, it is unclear of the physiological role of choline in activating SCCMS, as the concentration of choline in the synaptic cleft is unknown.

Fast channel syndromes appear to be rarer than the slow channel syndromes. Mutations responsible for fast channel CMS have been located in the α, δ, and ε subunits (αV285I (Wang et al., 1999), δE59K (Brownlow et al., 2001), εP121L (Ohno et al., 1996)). The symptoms tend to be similar to the slow channel syndromes, for example the αV285I fast channel mutation leads to weakness of ocular, facial, neck, and limb muscles (Wang et al., 1999) and patients fatigue easily (Ohno et al., 1997; Wang et al., 1999).

However there is little of the destruction of junctional folds seen in slow channel patients, which adds evidence to the fact that in slow channel patients it is the over activity of the receptor that leads to endplate destruction. As with many of the slow channel mutations, analysis of αV285I fast channel mutant has yielded information about the wild type receptor. Using maximum likelihood fitting to a kinetic scheme it was found that the fast channel mutant αV285I affects mainly the gating of the channel. This is surprising as this residue is within the M3 segment of nAChRs. In addition mutations constructed in equivalent positions in the β, δ, and ε subunits also show effects on gating, although the effects of mutating δ are much less pronounced.
indicating that gating does not occur through equivalent contributions from all of the subunits.

In comparison to slow channel patients, many fast channel patients usually respond well to treatment with cholinesterase inhibitors which act to increase the lifetime of ACh within the synaptic cleft and thus reversing the attenuation of the ACh signal caused by the fast channel mutation.

Several point mutations that cause CMS have been studied in detail. The effects of some of these are described below.

αG153S, (Salamone et al., 1999). Single channel records of nAChRs with this mutation were fitted to a kinetic scheme like scheme 1 but lacking monoliganded openings. β2 was unaffected but α2 was reduced 3-fold. The major effects were to reduce dissociation rate by 23 and 5 fold at the two sites. According to the AChbp crystal structure the homologue of αG153 (S147) is not within the ACh binding site, shown in figure 1.6. However it is on a loop two residues away from H145, the backbone of which does line the binding site. It can be speculated that as every n and n+2 amino acid residues in a sequence tend to be orientated in the same hemisphere along protein chains, the introduction of the side of serine (instead of the single hydrogen of glycine) could lead to distortion of the peptide backbone at this position.

αV156M (Croxen et al., 1997). This mutation leads to limb, facial, and neck weakness. One patient examined also had difficulty with chewing and swallowing and wasting of forearm and hand muscles. Electromyography did not give repetitive compound muscle action potentials in response to a single stimulus unlike many forms of SCCMS. Analysis of mepps from muscle biopsies showed the decay time constant was slowed three-fold.

αN217K (Engel et al., 1996; Wang et al., 1997). This residue is in the M1 segment towards the extracellular N-terminal domain. The effect of the mutation on the channel kinetics were determined by using maximum likelihood fitting to a linear scheme accounting for identical binding sites (the mutation was engineered into mouse nAChRs). The main effect was an approximate 10-fold decrease in the dissociation rate of ACh. There was a slight increase in the gating efficacy, caused by a slight decrease
in $\beta_2$ and a two-fold reduction in $\alpha_2$. The results highlight the fact that a mutation which is not in the 'recognised' ACh binding site can have effects on the binding site.

$\alpha V249F$ (Milone et al., 1997). The patient with this mutation is severely disabled with endplate myopathy, prolonged decay of endplate currents, and the nAChRs have prolonged openings. The mutation is within the transmembrane segment M2 but is not at the level of the gate, nor is it believed to face the channel lumen. This mutation has a multitude of effects; it has an increased rate of diliganded channel opening, a decreased rate of diliganded channel closing, and a decreased rate of agonist dissociation. All of these changes in microscopic rates act to increase the activity of receptors containing the $\alpha V249F$ substitution.

$\alpha T254I$ (Croxen et al., 1997). This mutation causes generalised weakness, wasting of finger muscles, atrophy of cervical muscles, and respiratory problems due to diaphragmatic weakness. As with several other CMS mutations there was a deterioration in the patients condition during pregnancy. Electromyography showed that single nerve stimuli elicited multiple responses. This mutation is located within M2, the pore-lining segment of the nAChR. Single channel analysis showed that there was a 7-fold increase in the slowest time constant of the burst length distribution.

$\alpha S269I$ (Oosterhuis et al., 1987; Croxen et al., 1997). This mutation caused arm weakness during pregnancy, mild hand muscle wasting, repetitive responses to single muscle stimuli, and prolonged decay of epps. The mutation is within the M2-M3 loop. There is a 7-fold increase in the slowest time constant of the burst length duration distribution compared to wild type nAChRs.

$\delta P250Q$ (Shen et al., 2002). This mutation is at the C-terminal end of transmembrane segment M1. It causes a fast channel CMS, and reduces nAChR surface expression. This mutation is one of the more severe; symptoms include delayed motor development, high arched palate, weakness of the neck, limb, and masticatory muscles, severe ophthalmoplegia, and facial diplegia. In addition the patient also required ventilator support for the first four months of life. Siblings of the patients in this study died in infancy from muscle weakness and respiratory complications, however they were not tested for the mutation. Muscle biopsies from the patient showed that the structure of the endplates was relatively normal but decreased amplitudes of mepps and mepcs were
recorded. \( \alpha \text{-BuTX} \) binding studies showed decreased amounts of nAChR at the endplates. The estimates of rate constants of \( \delta P250Q \) and also \( \epsilon P245Q \) were obtained by using maximum likelihood fitting to scheme E. \( \epsilon P245Q \) was constructed as previous studies have shown that \( \epsilon P245L \) (in the homologous position to \( \delta P250Q \)) causes a slow channel CMS (Ohno et al., 1997).

They found that \( \delta P250Q \) had \( \beta_2 \) reduced from \( 51000 \text{ s}^{-1} \) in the wild type to \( 30000 \text{ s}^{-1} \), whilst \( \epsilon P245Q \) had no effect on \( \beta_2 \) although ACh dissociation was slightly slowed. Due to these studies it was concluded that the fast and slow channel effects of \( \delta P250Q \) and \( \epsilon P245L \) show that the contributions of the penultimate prolines in M1 to gating are not equivalent.

\( \epsilon L221F \) (Oosterhuis et al., 1987; Hatton et al., 2003). This mutation leads to mild ptosis and weakness of the shoulders and fingers which increased in severity during pregnancy. The mutation causes destruction of the muscle endplates and the decay of mepcs is slowed 9-fold compared to wild type nAChRs (Croxen et al., 2002). The main kinetic effect of the mutation is on dissociation of agonist rather than gating. The total dissociation rate was reduced from \( 15000 \text{ s}^{-1} \) to \( 4000 \text{ s}^{-1} \).

1.11 Aims of the Thesis

The thesis aims to investigate the single channel properties of wild type human muscle nAChRs and the \( \epsilon L78P \) mutant nAChR which is responsible for a slow channel congenital myasthenic syndrome. Single channel properties will be examined by describing distributions of open period durations, shut time durations, and burst length durations with exponential components. The microscopic rate constants dictating the activity of the human muscle nAChR will be investigated using maximum likelihood
fitting of the single channel data to various different kinetic schemes. The properties of the human nAChR are important due to species differences that are present between human, mouse, frog, and *Torpedo* receptors. In addition the use of using maximum likelihood fitting to determine the effects of the competitive antagonist α-conotoxin-GI on the wild type receptor will be investigated.
2 Methods

2.1 Cell Culture and Transfections

Human embryonic kidney cells (HEK-293) obtained from either the American Type Culture Collection (ATCC) or the European Collection of Cell Cultures (ECCAC) were cultured in a medium consisting of Dulbecco's modified eagle medium containing 10% bovine calf serum (v/v) and 1% penicillin streptomycin solution (at 10000 units/ml penicillin and 10 mg/ml streptomycin). The cells were maintained at 37 °C in 5% CO₂.

Cells were split when they reached 70% to 90% confluence in the flasks. To split the cells medium was removed and the cells were briefly rinsed in Dulbecco's phosphate-buffered saline (PBS). The cells were then incubated in trypsin for 1 minute at 37 °C to detach them from the flask, resuspended in medium and centrifuged at 1000 r.p.m. for 5 minutes. The resulting cell pellet was then resuspended in fresh medium and a few drops of cell suspension were added to fresh flasks or 40 mm petri dishes containing a coverslip coated with poly-L-lysine (0.01 % w/v).

Cells were either plated onto coverslips 3 to 4 hours before transfection or simultaneously with the transfection mix. The calcium phosphate method was used to transfect the cells. For each petri dish, 3 µg of total DNA was used (at approximately 0.5 µg / ml). This consisted of pcDNA3.1 plasmids (Invitrogen) containing inserts encoding the nAChR subunits α1, β1, δ, ε, enhanced green fluorescent protein (eGFP), and the non-coding plasmid pcDNA3.1 in the ratio of 2:1:1:1:65:30. This DNA mix was added to 60 µl of 340 mM CaCl₂. This mixture was in turn added drop wise to 66 µl of 2 × Hepes-buffered saline (280 mM NaCl, 50 mM (N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]) (Hepes), 2.8 mM Na₂HPO₄, pH 7.2 with NaOH). The solution was left for 1 minute and then added to the petri dish. Cells were then incubated overnight at 37 °C in 5% CO₂ and the medium replaced next morning. Cells were patched between 16 and 56 hours following the transfection procedure.
2.2 Electrophysiology

2.2.1 Cell Attached Single Channel Recording

Cells emitting green fluorescence were patched in the cell-attached configuration in an extracellular solution containing, 142 mM KCl, 10 mM hepes, 5.4 mM NaCl, 1.7 mM MgCl₂, 1.8 mM CaCl₂, pH adjusted to 7.4 with KOH at 19 °C. Borosilicate pipettes (GC150F-7.5, Clark Capillaries) were pulled to give a resistance of 8 MΩ to 12 MΩ and their tips coated with Sylgard (Dow Corning). Immediately before patching the pipettes were fire-polished and back-filled with extracellular solution containing ACh chloride. ACh was made up as 500 mM solutions, and stored at 4 °C for a maximum of two weeks before being discarded. Cells were patched at approximately -100 mV. Currents were amplified using an Axopatch 200A (Axon Instruments) coupled to a CV 201AU headstage (Axon Instruments). Data were filtered initially at 10 kHz and recorded on digital audio tape (DAT) using a DTR-1204 digital tape recorder (Biologic Science Instruments). Before digitisation data were further filtered at 5 kHz to 8 kHz using an 8th order Bessel filter (built in house), giving a total filtering of 4.8 kHz to 6.2 kHz. Data were digitised at 50 kHz to 80 kHz using a CED 1401 interface (Cambridge Electronic Design) and the CONSAM program (I. Vais and D. Colquhoun).

2.2.2 Whole Cell Recording

Cells emitting green fluorescence were patched in the whole cell configuration in an extracellular solution containing, 142 mM KCl, 10 mM hepes, 5.4 mM NaCl, 1.7 mM MgCl₂, 1.8 mM CaCl₂, pH adjusted to 7.4 with KOH. Borosilicate pipettes (GC150TF-7.5, Clark Capillaries) were pulled to a resistance of 2 MΩ to 3 MΩ. Immediately before patching the pipettes were fire-polished and back-filled with an intracellular solution containing 110 mM KCl, 10 mM hepes, 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 2.5 mM NaCl, pH adjusted to 7.3 with KOH. Applications of agonist and / or antagonists were made via a custom built U-tube delivery system with a tube diameter of approximately 250 µm positioned as near to the cell as possible. Before each application of drug the U-tube was flushed with the new solution. Currents were amplified using an Axopatch 200A (Axon Instruments) coupled to a CV 201AU headstage (Axon Instruments). Data were filtered initially at 1 kHz and recorded on digital audio tape (DAT) using a DTR-1204 digital tape recorder (Biologic Science Instruments). Data from individual whole cell experiments was used to construct dose response curves fitted with the Hill equation,
each point on the dose response curve was the mean of three observations. The individual curves were then normalised and it is the mean normalised values are shown in the results section.

2.3 Single Channel Analysis

Following an initial inspection of the digitised single channel trace, the amplitudes and the duration of the open and closed states were measured using the SCAN program to give an idealised record. The transitions were fitted with convolved step-response functions using a least squares criterion. The EKDIST program was used to impose retrospectively a resolution of 20 µs to 40 µs, and to construct stability plots and histograms of channel properties. Amplitude histograms were fitted with two Gaussian components.

The duration of an 'open period' was defined as the length of time for which the channel remained open (regardless of conductance), the period ending when a shut time longer than the imposed resolution occurs. Bursts were defined as a sequence of openings and shuttings that end once a shut time longer than t crit occurs. The t crit values were chosen so as to ensure that an equal proportion of intervals from both the low and high components were misclassified, (Colquhoun & Sakmann, 1985). In some cases this was not possible due to the bisection algorithm employed not converging. In these cases t crit was chosen so that an equal number of intervals from the low and high components were misclassified, (Magleby & Pallotta, 1983;Clapham & Neher, 1984). The number of intervals fitted for each patch ranged from 2000 to 50000. For a typical sets of fits the mean number of transitions fitted per patch was 17500 ± 2600.

The open period, shut time, and burst length histograms were fitted with mixtures of n exponential probability density functions (pdfs) of the general form,

\[ f(t) = a_1 \tau_1^{-1} \exp\left(\frac{-t}{\tau_1}\right) + a_2 \tau_2^{-1} \exp\left(\frac{-t}{\tau_2}\right) + \ldots + a_n \tau_n^{-1} \exp\left(\frac{-t}{\tau_n}\right) , \]  

using the Simplex algorithm to maximise the likelihood of the parameters. The maximum likelihood \( L \) was calculated using the expression

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\[ \ln L(\tau) = \ln f(t_1) + \ln f(t_2) + \ldots \ln f(t_N), \]  

(2.2)

where \( t_N (n = 1, \ldots, N) \) are the observed interval lengths (dwell times).

Numerical results in the text are given as means ± standard deviations of the mean (s.d.m.) (also known as standard error) except for rate constants which are displayed as the mean ± coefficient of variance of the mean (c.v.m.) for readability. Error bars on graphs are the s.d.m. The mean values of parameters which are a ratio of two rate constants (such equilibrium constants) were obtained by calculating the mean of the ratios from each experiment.

To assess differences between the means, randomisation tests or Student’s \( t \) tests were performed. The randomisation tests were carried out using the RANTEST program with 50000 random samples of the data.

**2.3.1 Theory of Fitting Single Channel Data to Kinetic Schemes**

One of the key aims in studying the single channel kinetics of an ion channel is to derive expressions that relate the rate constants of a reaction scheme to observable subsets of protein states. Matrix notation is ideal for these purposes. Once the standard results have been obtained it is theoretically simple to calculate how well a set of rates constants describes single channel data, and subsequently the rates can be varied until the likelihood of the observations is maximised. The likelihood of the observations is defined as the probability (density) of making the observations, given a particular mechanism and set of rate constants. The results can be expressed in wholly scalar form as a mixture of exponentials, analogous to equation 2.1 and this allows a visual comparison between the mathematical description of the rates and the data they are being fitted to.

Unfortunately there is a limit to the resolution of measurements that can be obtained, therefore events shorter than this resolution will not be detected, referred to as missed events. Taking these missed events into account considerably increases the complexity of the relationships between the rate constants and the observed data. The procedures for describing data at zero resolution will be described, these methods are much simpler and are useful for laying down the fundamental ideas and the notation used in the
analysis. The complications that arise when considering missed events will be described further on.

In the case of muscle nicotinic receptors, single channels generally only show two observable states, a conducting open state, and a non-conducting shut state. It is known that both the observable open and shut states comprise several different stable states, albeit with indistinguishable conductances. Therefore the very simplest mechanism, one with only two states will be used to obtain the basic results, this will then be expanded to more complex schemes, such as scheme 1 shown below which forms the basis of the kinetic analyses performed in this study.

The two states to be considered to derive the basic results will be $A_2R$ and $A_2R^*$ (states 1 and 4 in the above scheme). The usual assumption is that the transitions between states can be described as a Markov process with discrete states in continuous time (Colquhoun & Hawkes, 1977). The change in state of a channel is a random process that occurs with a frequency that depends on the rate constants (in this case $\alpha_2$ or $\beta_2$).
With these assumptions the probabilities of a change in state during a time interval
denoted $\Delta t$ are,

\[
\text{Prob \ [channel in state 1 at } t \text{ and is in state 4 at } t + \Delta t] = \alpha_2\Delta t + o(\Delta t)
\]  \hspace{1cm} (2.3)

\[
\text{Prob \ [channel in state 4 at } t \text{ and is in state 1 at } t + \Delta t] = \beta_2\Delta t + o(\Delta t)
\]  \hspace{1cm} (2.4)

In the usual notation for stochastic processes, $o(\Delta t)$ is a remainder term that takes into
account multiple transitions between states 1 and 4 occurring during $\Delta t$. As $\Delta t$ tends
to zero (as will be required in the derivation later on) this remainder terms becomes
negligible relative to $\Delta t$. The above equations can therefore be rewritten as,

\[
\alpha_2\Delta t = \lim_{\Delta t \to 0} \left[ \text{Prob\ (channel is in state 4 between } t \text{ and } t + \Delta t
\right.
\left. \text{ I channel is in state 1 at } t \right]
\]  \hspace{1cm} (2.5)

\[
\beta_2\Delta t = \lim_{\Delta t \to 0} \left[ \text{Prob\ (channel is in state 1 between } t \text{ and } t + \Delta t
\right.
\left. \text{ I channel is in state 4 at } t \right]
\]  \hspace{1cm} (2.6)

Since the system (in the case of these two states) must be in either state 1 or 4 then,

\[
1 - \alpha_2\Delta t = \lim_{\Delta t \to 0} \left[ \text{Prob\ (channel is in state 1 between } t \text{ and } t + \Delta t
\right.
\left. \text{ I channel is in state 1 at } t \right]
\]  \hspace{1cm} (2.7)

\[
1 - \beta_2\Delta t = \lim_{\Delta t \to 0} \left[ \text{Prob\ (channel is in state 4 between } t \text{ and } t + \Delta t
\right.
\left. \text{ I channel is in state 4 at } t \right]
\]  \hspace{1cm} (2.8)

Defining $P_{ij}(\Delta t)$ as the probability of a transition between states $i$ and $j$ during a period of
$\Delta t$ gives the following expressions for the two states being considered here,

\[
P_{11}(\Delta t) = 1 - \alpha_2\Delta t
\]  \hspace{1cm} (2.9)

\[
P_{14}(\Delta t) = \alpha_2\Delta t
\]  \hspace{1cm} (2.10)

\[
P_{41}(\Delta t) = \beta_2\Delta t
\]  \hspace{1cm} (2.11)

\[
P_{44}(\Delta t) = 1 - \beta_2\Delta t
\]  \hspace{1cm} (2.12)
In a more general way, $P_{ij}(t)$ can be defined,

$$P_{ij}(t) = \text{Prob}[\text{in state } j \text{ at } t \text{ l in state } i \text{ at } 0] \quad (2.13)$$

$P_{ij}(t)$ cannot be calculated explicitly, however the rate of change of $P_{ij}(t)$ can be calculated by using the Chapman-Kolmogorov equation which is derived below. Starting from the situation in which at 0 and $t + \Delta t$ the channel is in state 1,

$$P_{i1}(t + \Delta t) = \text{Prob}[\text{in state } 1 \text{ at } t + \Delta t \text{ l in state } 1 \text{ at } 0] \quad (2.14)$$

This can be broken down into two parts,

$$P_{i1}(t + \Delta t) = \text{Prob}[\text{in state } 1 \text{ at } t + \Delta t \text{ l in state } 1 \text{ at } t] \times \text{Prob}[\text{in state } 1 \text{ at } t \text{ l in state } 1 \text{ at } 0] + \text{Prob}[\text{in state } 1 \text{ at } t + \Delta t \text{ l in state } 4 \text{ at } t] \times \text{Prob}[\text{in state } 4 \text{ at } t \text{ l in state } 1 \text{ at } 0] \quad (2.15)$$

The above equation describes the two mutually exclusive situations of the channel being in state 1 at 0, $t$, and $t + \Delta t$, or the channel being in state 1 at 0 and $t + \Delta t$ but in state 4 at $t$. The above equation can be rewritten as,

$$P_{i1}(t + \Delta t) = [P_{i1}(\Delta t)] \times [P_{i1}(t)] + [P_{i4}(\Delta t)] \times [P_{i4}(t)] \quad (2.16)$$

Substituting the rate constants $\alpha_2$ and $\beta_2$ into the above terms and rearranging gives equations 2.17 through to 2.20.

$$P_{i1}(t + \Delta t) = (1 - \alpha_2 \Delta t)P_{i1}(t) + \beta_2 \Delta t P_{i4}(t) \quad (2.17)$$

$$P_{i1}(t + \Delta t) = P_{i1} - \alpha_2 \Delta t P_{i1}(t) + \beta_2 \Delta t P_{i4}(t) \quad (2.18)$$

$$P_{i1}(t + \Delta t) = P_{i1}(t) - \Delta t[\alpha_2 P_{i1}(t) + \beta_2 P_{i4}(t)] \quad (2.19)$$

$$\frac{P_{i1}(t + \Delta t) - P_{i1}(t)}{\Delta t} = -\alpha_2 P_{i1}(t) + \beta_2 P_{i4}(t) \quad (2.20)$$
By making $\Delta t$ tend to 0, the above equation becomes a differential equation,

$$\frac{dP_{11}(t)}{dt} = -\alpha_2 P_{11}(t) + \beta_2 P_{14}(t)$$ \hspace{1cm} (2.21)

Analogous derivations can be found for all of the other transition probabilities. This leads to twenty eight differential equations describing scheme 1. Due to the rapidly growing number of equations, matrices are utilised. This allows results to be given in a general form that is valid for any mechanism, with any number of states. This not only reduces the amount of writing but also of making many of the results to be derived later appear (almost) intuitive.

In matrix notation, equation 2.21 can be written as follows, and it can be shown that this result is valid not only for the two state case for which it has been derived, but for any mechanism. It is known as the Chapman-Kolmogorov equation.

$$\frac{dP(t)}{dt} = P(t)Q$$ \hspace{1cm} (2.22)

This is an entirely general equation that will apply to any scheme used, as long as $P$ is a matrix whose elements are the transition probabilities between the states, and $Q$ is a matrix whose elements are the rate constants for transitions between all of the different states in a scheme. The states in the scheme are numbered, and by convention 'activated' (in the case of ion channels, open) states are assigned the lowest numbers. In general, the off-diagonal elements of $Q$ are the rate constants that describe the frequency of transitions from state $i$ to state $j$ (it is then convenient to define the diagonal elements to make the rows sum to zero). The application of the Chapman-Kolmogorov equation to single channel analysis, and the subsequent development of the standard notation are described by (Colquhoun & Hawkes, 1977; Colquhoun & Hawkes, 1981; Colquhoun & Hawkes, 1982). The solution to the Chapman-Kolmogorov equation is, as long as $Q$ is constant,

$$P(t) = \exp(Qt)$$ \hspace{1cm} (2.23)
2.3.2 Evaluating exp(Qt)

Calculating exp(Qt) is essential in deriving the likelihood expression and the distributions of the open and shut times. As exp(Qt) cannot be calculated accurately from its usual series expansion, it is usually calculated by using the spectral expansion theorem,

$$\exp(Qt) = \sum_{n=1}^{k} \exp(\lambda_m t) A_m$$  

(2.24)

In the above expression $\lambda_m$ are the $k$ eigenvalues of the $Q$ matrix. The eigenvalues are the roots of the characteristic polynomial that can be found from a matrix. The eigenvalues $Q$ are real and negative in case of single channel analysis, and have the dimensions of frequency, $s^{-1}$. Usually the eigenvalues of the $-Q$ matrix are used so that the rate constants are positive, and their reciprocals are time constants. From each eigenvalue, column ($c_m$) and row ($r_m$) eigenvectors are obtained such that

$$\lambda_m c_m = Q c_m \quad \text{and} \quad \lambda_m r_m = r_m Q$$  

(2.25, 2.26)

From the column and row eigenvectors, the spectral expansion matrices ($A_m$) can be calculated,

$$A_m = c_m r_m$$  

(2.27)

Once the spectral expansion matrices have been calculated these along with the eigenvalues of the $Q$ matrix can be used to evaluate any function of $Q$, in particular exp(Qt).

Equations such as 2.23 are the basis of the general analysis of macroscopic current and noise, but for single channels, it must be allowed for the fact that that several states are indistinguishable (have the same conductance), so we are dealing with an aggregated Markov process. Scheme 1 (used predominantly throughout this text) will now be used first to illustrate how modifications need to be introduced to cope with more complex schemes. Scheme 1 contains three open states and four shut states, however there are only two distinguishable states, those corresponding to open and those
corresponding to closed. This problem is incorporated into the matrix methods by partitioning the initial $Q$ matrix pertaining to this mechanism into identifiable subsets of states,

$$Q = \begin{bmatrix}
-\alpha_2 & 0 & 0 & \alpha_2 & 0 & 0 & 0 \\
0 & -\alpha_{1a} & 0 & 0 & \alpha_{1a} & 0 & 0 \\
0 & 0 & -\alpha_{1b} & 0 & 0 & \alpha_{1b} & 0 \\
\beta_2 & 0 & 0 & -(\beta_{2a} + k_{2a} + k_{2b}) & k_{-2a} & k_{-2b} & 0 \\
0 & \beta_{1a} & 0 & k_{+2a} & -(\beta_{1a} + k_{+2a} + k_{1a}) & 0 & k_{-1a} \\
0 & 0 & \beta_{1b} & k_{+2b} & 0 & -(\beta_{1b} + k_{+2b} + k_{-1b}) & k_{-1b} \\
0 & 0 & 0 & 0 & k_{+1a} & k_{+1b} & -(k_{+1a} + k_{+1b})
\end{bmatrix}$$

The subsets of states labelled in the $Q$ matrix pertain to transitions between open states ($A$), short lived shut states ($B$), long lived shut states ($C$), all shut states ($F$), and burst states ($E$) where

$$F = B \cup C, \quad E = A \cup B$$

The rate constants in the top left region of the $Q$ matrix correspond to transitions between open states (none in this case) $Q_{AA}$, those in the top right region corresponds to transitions from open to closed states $Q_{AF}$, those in the bottom left region corresponds to transitions from closed to open states $Q_{FA}$, and those in the bottom right corresponds to transitions between closed states $Q_{FF}$. We can now defines a variant of the $P$ matrix, denoted $^AP(t)$ that allows for transitions within subsets of states, thus

$$^AP_j(t) = P[\text{system in } A \text{ throughout } (0,t) \text{ and in state } j \text{ at time } t]$$

\[ \text{in state } i \text{ at time } 0], \, i,j \in A \quad (2.30) \]

Similarly,

$$^AP_j(t + \Delta t) = \sum_{k \in A} \{ P[\text{system in } A \text{ throughout } (0,t) \text{ and state } k \text{ at } t]$$

\[ \text{in state } i \text{ at time } 0] \times P(\text{state } j \text{ at } t + \Delta t \mid \text{state } k \text{ at } t)\}, \, i,j \in A \quad (2.31) \]
The second factor varies on depending on whether \( i = j \) or not. When \( i = j \) then it represents the probability that the system remains within state \( k \) during \( \Delta t \) which is formally stated as

\[
1 - P(\text{leaving } k) = 1 + q_{kk} \Delta t + o(\Delta t) \tag{2.32}
\]

If \( i \neq j \) in the second factor becomes,

\[
P(\text{leaves state } i \text{ for state } j) = q_{ij} \Delta t + o(\Delta t) \tag{2.33}
\]

In matrix format equation this can be stated as,

\[
P_{AA}(t + \Delta t) = P_{AA}(t)[I + Q_{AA} \Delta t + o(\Delta t)] \tag{2.34}
\]

Rearranging the above equation gives equation 2.35 which is analogous to the previously derived Chapman-Kolmogorov equation that referred to the whole \( Q \) matrix, (equation 2.22),

\[
\frac{dP_{AA}(t)}{dt} = P_{AA}(t)Q_{AA} \tag{2.35}
\]

and unsurprisingly, when solved, assuming \( Q_{AA} \) is constant, an analogous result to equation 2.23 is obtained,

\[
P_{AA}(t) = \exp(Q_{AA} t) \tag{2.36}
\]

The previous derivations referred to the system remaining in subset \( A \), albeit with the possibility of transitions between subsets of states within \( A \). It is also necessary to define the probabilities of this occurring and then proceeding to leave \( A \) for \( B \). This is formalised below,

\[
g_{j}(t) = \lim [P(\text{remaining in } A \text{ from time 0 to time } t \text{ and leaving } A \text{ for state } j \text{ between } t \text{ and } t + \Delta t \text{ in state } i \text{ at time 0}) / \Delta t], \ i \in A, \ j \in B \tag{2.37}
\]
The elements $g_{ij}(t)$ are the elements of a matrix $G_{AB}(t)$ which can be found using,

$$G_{AB}(t) = P_{AA}(t)Q_{AB}$$  \hspace{1cm} (2.38)

Thus from previous results,

$$G_{AB}(t) = \exp(Q_{AA}t)Q_{AB}$$  \hspace{1cm} (2.39)

The same derivations can be used to find other $G$ matrices. For example the $G$ matrix describing transitions from any open state to any shut state,

$$G_{AF}(t) = \exp(Q_{AA}t)Q_{AF}$$  \hspace{1cm} (2.40)

2.3.3 Initial Vectors

To perform the likelihood calculation and also to plot the HJC distributions as scalar expressions it is necessary to calculate initial vectors. These vectors (denoted $\varphi_0$) have elements that are the probabilities of an opening starting in each of the open states. The probability of the system starting in any of the open states depends on the relative occupancy of the closed states, and the transition rates from all of the closed states to the open states and can be calculated as follows.

$$\varphi_0 = \frac{p_{F}(\infty)Q_{FA}}{p_{F}(\infty)Q_{FA}u_A}$$  \hspace{1cm} (2.41)

The numerator consists of the vector $p_F(\infty)$ the elements of which are the occupancies of the closed states at equilibrium, and $Q_{FA}$, which is a sub matrix of $Q$ that contains all of the transition rates from the shut states to the open states. The denominator which includes the unit vector $u_A$ is the sum of the numerator terms and thus normalises the expression so that the probabilities add to one.

The occupancies of each state, at time $t$, denoted $p_i(t)$, can be written as a vector, $p(t)$. The occupancies in the steady state (which, for mechanisms like scheme 1, implies equilibrium) can be found from
\[
\frac{dp(\infty)}{dt} = p(\infty)Q = 0
\] (2.42)

Several standard methods for solving this equation exist, (Colquhoun & Hawkes, 1995).

2.3.4 Calculation of the Likelihood

The likelihood of a sequence of channel opening and closings is calculated using sequence of \( G \) matrices that have elements that are the probabilities of transitions occurring from one subset of states (e.g. open states) to another subset of states (e.g. closed) within a time interval, allowing for any number of transitions between states within the same subset (Colquhoun et al., 1996). The first term is an initial vector \( \phi_0 \), the elements of which are the probabilities of a series of openings starting in each of the open states.

\[
L = \phi_0 G_{AP}(t_{\alpha})G_{FA}(t_{\beta})G_{AP}(t_{\gamma})...u_f
\] (2.43)

Equation 2.43 describes the likelihood calculation for ideal resolution. However to describe data at non ideal resolution missed events must be taken into account. The calculations of the \( G(t) \) matrices at non ideal resolution (termed \( ^*G \), for extended interval due to missing brief transitions which act to extend the apparent intervals) are considerably more complex. The exact solution of \( ^*G \) is a piecewise solution (Hawkes et al., 1990). There is one solution for intervals between \( t_{res} \) and \( 2t_{res} \), a different solution for intervals between \( 2t_{res} \) and \( 3t_{res} \) and so on. The different solutions involve multiplying eigenvalues of \( Q \) by increasingly complicated polynomials. This eventually leads to instability in the equations at high multiples of \( t_{res} \). However there is an asymptotic approximation which can be used for longer intervals (Hawkes et al., 1992). This approximation is very accurate for intervals longer than \( 2t_{res} \) or \( 3t_{res} \), and it has the great virtue that, unlike the exact solution, it is a mixture of exponentials and the number of exponential components is the same as it would be for the ideal \( (t_{res} = 0) \) distribution, though the time constants and areas are different because of the missed events. In the likelihood calculations in this text the exact solution is used for intervals below \( 3t_{res} \), whilst the approximation is used for longer intervals.
2.3.4 Plotting the HJC Distributions

General results of deriving the functions describing the open time, shut time, and burst length distributions in terms of rate constant have been derived (Colquhoun & Hawkes, 1981; Colquhoun & Hawkes, 1982).

Open time, \( f_o = \varphi_o \exp(Q_{AA}t)(-Q_{AA})u_A \)  \hspace{1cm} (2.44)

Shut time, \( f_s = \varphi_f \exp(Q_{FF}t)(-Q_{FF})u_F \)  \hspace{1cm} (2.45)

Burst length, \( f_b = \varphi_b [\exp(Q_{EE}t)]_{AA} (Q_{AB}G_{BC} + Q_{AC})u_C \)  \hspace{1cm} (2.46)

In the above expression \( \varphi \) is analogous to \( \varphi \) (derived previously), with all shut states (F) replacing all open states (A). The initial vector for the burst length distribution (\( \varphi_b \)) is found as follows,

\[
\varphi_b = \frac{p_c(\infty)(Q_{CB}G_{BA} + Q_{CA})}{p_c(\infty)(Q_{CB}G_{BA} + Q_{CA})u_A}
\]  \hspace{1cm} (2.47)

This equation contains the occupancy of the long lived shut states (\( p_c \)) as bursts are preceded by occupancy in a long lived shut state. The remaining terms describe the transitions to the open states (A) either via the short lived shut states (B) or directly from the long lived shut states. The above functions can be converted into scalar form to allow superimposition of the functions onto the respective single channel record histograms (Colquhoun & Hawkes, 1981; Colquhoun & Hawkes, 1982).

When short events are missed, analogues of these distributions must be used that allow for this omission. These are described as HJC distributions, and the HJC distributions of open and shut times, calculated from fitted rate constants, are what are superimposed on the observed distributions.
3.1 Introduction

When analysing single channel data the first step is to produce an idealised version of the data. This record contains the amplitudes and durations of all apparent open times and the durations of all apparent shut times. From this record histograms can be constructed which give information on the different populations of events. It is a standard technique to fit these open and shut time duration histograms with sums of exponentials. Ideally the number of exponential components needed to fit a distribution indicates the minimum number of relevant states in the kinetic scheme of the receptor. This is because the lifetime of an individual state is exponentially distributed, however only the minimum number of states can be determined from the fitting of exponential components. Although not providing direct information about the states, the fitting of exponentials to histograms is useful to gain quantitative information about the properties of the channel. As well as open and shut time duration histograms, burst length duration histograms were also constructed as these give more physiological information than the open or shut time duration histograms alone. This is because physiological activations of nAChRs in the muscle endplate consist of a distribution of a series of openings (bursts) separated by very short shuttings; it is the charge carried within the total burst that is responsible for the subsequent voltage change and generation of a muscle action potential. The time constants of decay of the nAChR-mediated current will be the same as the time constants describing the duration of bursts (Wyllie et al., 1998). In addition, the estimates of total burst length are much less prone to error due to the limits of resolution than the estimates of open period and shut time durations. Brief missed transitions within a burst will not significantly alter the measured length of the burst. However, brief transitions missed from open periods or shut times will act to concatenate events which have durations much longer than the applied resolution.

3.2 Results

Figure 3.1 shows single channel traces of human wild type muscle nAChRs recorded in the cell-attached configuration at a range of ACh concentrations. The openings clearly occur in bursts of openings. Before the construction of histograms the stability of the
Figure 3.1. Sample single channel traces of wild type nAChRs at 50 nM, 100 nM, 1 μM, 10 μM, 50 μM ACh. At high ACh concentrations channel activations contain more open periods, but are also clustered more than at low ACh concentrations. In both columns channel amplitudes are approximately 6 pA (openings are downward deflections). The left column traces are 1 s long, the right column traces are 100 ms long. The current amplitude scales are identical in all the traces.
patches was examined by plotting amplitude of openings, open time duration, or $P_{\text{open}}$ against the number of transitions. Typical stable patches are shown in figure 3.2.

The single channel experiments in this work were performed in the cell-attached configuration. Whilst recording currents in this way the resting cell membrane potential of the cell is unknown, however by measuring the amplitudes of single channel openings at different holding potentials it is possible to calculate the conductance of the channel by plotting a current / voltage (I/V) curve. Ohm's law states that,

$$V = IR$$

where $V$ is the voltage, $I$ is the current passed, and $R$ is the resistance. Conductance ($g$) is the inverse of resistance, substituting this into Ohm's law and rearranging gives,

$$gV = I$$

Therefore a plot of voltage against current for an ohmic channel will yield a straight line with a slope of $g$. As individual cells can be expected to have different although similar resting potential, I/V curves were plotted for each cell, their individual slope conductance's calculated by fitting a straight line using the least squares method. The slope conductance of the muscle nAChRs in the presence of extracellular solution (see Methods) was calculated to be $69.9 \pm 1.2 \text{ pS}$, $n = 8$. An example of the changes in amplitude of channel currents with voltage is shown in figure 3.3.

3.2.1 Open Period Durations

Open period durations are defined as the length of time that the channel remains open, until a shut state occurs that is longer than the imposed resolution occurs regardless of the current amplitude of the open state(s). This is justified as muscle nAChRs consistently show one predominant conductance level. The open period durations of wild type human muscle
Figure 3.2. The stability of the single channel amplitudes (A), mean open times (B), and $P_{\text{open}}$ (C) over the lifetime of the patches were inspected to ensure that they did not vary.
Figure 3.3. Examples of fitted amplitude histograms from a single patch at a range of membrane potentials (left column). Above, the slope of the IV plot gives the channel conductance in this patch as 69.7 pS. The overall means channel conductance was 69.9 ± 1.2 pS, n = 8.
nAChRs at varying ACh concentrations were analysed individually at 30 μs resolution. In some cases it was possible to fit the open period duration histograms with just one or two exponential components (especially for patches at the higher concentrations of ACh), however for most patches three components were required. Figure 3.4 shows example open period duration histograms fitted with three exponential components. The overall mean open period duration is approximately 1.2 ms and is insensitive to ACh concentration as shown in figure 3.5A, and is logically therefore a function of the ACh dissociation rates and the parameters describing movements of the receptor, i.e. the gating rate constants, (excluding such effects as phosphorylation which are not believed to occur on the timescales being studied here). The values of the time constants and their areas are given in table 3.1.

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>Mean open period (ms)</th>
<th>( \tau_1 ) (ms)</th>
<th>( a_1 ) (%)</th>
<th>( \tau_2 ) (ms)</th>
<th>( a_2 ) (%)</th>
<th>( \tau_3 ) (ms)</th>
<th>( a_3 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>6</td>
<td>1.26 ± 0.20</td>
<td>0.042 ± 0.098</td>
<td>19.7 ± 5.1</td>
<td>0.715 ± 0.187</td>
<td>33.3 ± 8.8</td>
<td>3.81 ± 1.17</td>
<td>47.1 ± 11.4</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>1.17 ± 0.18</td>
<td>0.033 ± 0.016</td>
<td>28.5 ± 7.7</td>
<td>0.739 ± 0.219</td>
<td>36.5 ± 11.4</td>
<td>3.03 ± 0.79</td>
<td>34.9 ± 9.0</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>1.11 ± 0.25</td>
<td>0.053 ± 0.022</td>
<td>28.1 ± 13.0</td>
<td>0.819 ± 0.313</td>
<td>45.0 ± 16.9</td>
<td>2.95 ± 0.91</td>
<td>27 ± 12.8</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>1.21 ± 0.04</td>
<td>0.054 ± 0.023</td>
<td>8.6 ± 1.8</td>
<td>0.603 ± 0.094</td>
<td>28.9 ± 5.2</td>
<td>1.63 ± 0.094</td>
<td>62.5 ± 5.1</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>1.11 ± 0.12</td>
<td>0.045 ± 0.035</td>
<td>17.1 ± 7.1</td>
<td>0.630 ± 0.151</td>
<td>28.1 ± 8.8</td>
<td>1.708 ± 0.202</td>
<td>54.9 ± 7.6</td>
</tr>
</tbody>
</table>

Table 3.1. The mean open period of wild type nAChRs and the individual values of the three time constants and their areas used to fit open period histograms, (examples shown in figure 3.4). Errors shown are the standard deviations of the mean, also known as the standard error. All patches were at 30 μs resolution.

The individual time constants are well separated, on average there is a 15-fold difference between \( \tau_1 \) and \( \tau_2 \), and a 4-fold difference between \( \tau_2 \) and \( \tau_3 \). Figures 3.5B and 3.5C show the effects of ACh concentration on the values of the time constants and their relative areas. The first two time constants change little with increasing ACh concentration, whilst the longest time constant decreases slightly at the higher ACh concentrations. The area of the slowest time constant increases with ACh concentration, presumably as this time constant is describing the diliganded openings, which will be more frequent than monoliganded openings at high concentrations.
Figure 3.4. Examples of wild type nAChR open time histograms at a range of ACh concentrations. Although some patches (especially at the higher concentrations) could be fitted with a single exponential component, here they have all been fitted with three exponential components which is the minimum number that can adequately describe the data at all concentrations.
Figure 3.5. (A) There is little change in the overall mean open period duration at different ACh concentrations. (B) Changes in the values of the open time constants over a range of ACh concentrations. The first two time constants are not affected by ACh concentration, whilst the third decreases at high ACh concentrations. (C) Changes in the relative areas of the three open period duration constants. The increase in area of the third time constant compensates for the decrease in its \( \tau \) value so there is little change in the overall mean open time.
However, this increase in the relative area of the longest time constant compensates for the decrease in the value of the time constant so that the overall mean open period duration changes little over the range of concentrations tested. There was a slight decrease in the area of the shortest time component at the higher ACh concentrations, this is expected as there will be less monoliganded openings at high ACh concentrations. This effect can be seen clearly in section 5.2.1 in which bursts with a single opening are analysed.

3.2.2 Shut Time Durations
The shut time durations of individual patches containing wild type nAChRs over a range of ACh concentrations were analysed. The shut time duration distributions at low concentrations (50 nM and 100nM) showed two principal components, separated by a much smaller component. In all case at least three exponential components were needed to adequately fit the histograms. Figure 3.6 shows the shut time duration distribution of a patch in the presence of 50 nM ACh. It is clear by eye that the two component fit is not an accurate description of the data, whilst the three component fit is much better, even though the increase in log likelihood between these two fits is less than 1%. At higher concentrations of ACh, a further two shut time components were needed to describe the data. These extra components indicate the presence of long-lived desensitised state(s), as the long shut time durations persist at high concentrations of ACh, even when the absolute frequency of activations should be increased. In all patches at all concentrations the longest shut time constant was extremely variable, e.g. a range of 31 ms to 1.6 s was observed for the 100 nM patches. This component is dependent on the number of receptors present in the patch, and so cannot be used to estimate rate constants (see Methods). The more receptors there are in a patch, then the greater the absolute frequency of activations will be. This frequency is extremely variable at identical ACh concentrations and thus the number of receptors in a patch is extremely variable. As at least 5 components were need to fit the high concentration patches, the low concentration patches were also fitted with 5 components to allow comparisons between patches at different concentrations. Table 3.2 shows the areas and time constants for five component fits to the shut time duration distributions, figure 3.7 shows the five component fits to example shut time duration histograms at each of the ACh concentrations tested.
Figure 3.6. The shut time histogram of a patch in the presence of 50 nM ACh. In both panels the histogram is identical, the top panel has been fitted with two exponential components whilst the lower panel has been fitted with three exponential components, visibly producing a much better fit.
Figure 3.7. Examples of shut time histograms of wild type nAChRs at a range of ACh concentrations. Patches at low concentrations of ACh were fitted well with three exponential components, whilst five exponential components were needed at concentrations of 1 μM and above.
Figure 3.8. (A), the values of the shut time constants at a range of ACh concentrations. Although the shut time constants are variable they do not seem dependent on ACh concentration. (B), the mean values of the first four time constants for all of the patches. Although figure 3.8A shows that some are quite variable, it can be seen that they are still all well separated from each other. (C), changes in the relative areas of the five shut time constants at a range of ACh concentrations. Apart from the area of the fastest time constant, the areas are quite variable across the range of ACh concentrations.
Figure 3.8 plots the values of the time constants and their areas, (the last time component had been omitted from figure 3.8B as it has little significance regarding individual channel functioning). The shut time constants are well separated and have little dependence on concentration. The mean values of the fastest four time constants over all concentrations are 16 μs, 190 μs, 2.9 ms, and 47 ms.

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>$\tau_1$ (ms)</th>
<th>$a_1$ (%)</th>
<th>$\tau_2$ (ms)</th>
<th>$a_2$ (%)</th>
<th>$\tau_3$ (ms)</th>
<th>$a_3$ (%)</th>
<th>$\tau_4$ (ms)</th>
<th>$a_4$ (%)</th>
<th>$\tau_5$ (ms)</th>
<th>$a_5$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>6</td>
<td>0.015 ± 0.003</td>
<td>46.5 ± 11.6</td>
<td>0.199 ± 0.096</td>
<td>12.7 ± 10.4</td>
<td>0.839 ± 0.346</td>
<td>1.1 ± 0.3</td>
<td>32.2 ± 9.97</td>
<td>12.4 ± 5.4</td>
<td>139 ± 47.5</td>
<td>27.3 ± 12.6</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>0.016 ± 0.001</td>
<td>60.1 ± 4.9</td>
<td>0.079 ± 0.025</td>
<td>6.6 ± 2.8</td>
<td>1.47 ± 0.936</td>
<td>1.2 ± 0.5</td>
<td>60.0 ± 50.8</td>
<td>2.3 ± 9.97</td>
<td>398 ± 252</td>
<td>29.0 ± 3.3</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.016 ± 0.001</td>
<td>71.2 ± 3.6</td>
<td>0.246 ± 0.113</td>
<td>3.8 ± 1.3</td>
<td>8.51 ± 2.10</td>
<td>18.1 ± 3.2</td>
<td>102 ± 643</td>
<td>5.1 ± 0.5</td>
<td>1030 ± 643</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.018 ± 0.001</td>
<td>63.0 ± 3.4</td>
<td>0.265 ± 0.063</td>
<td>4.5 ± 1.1</td>
<td>2.89 ± 0.412</td>
<td>27.5 ± 4.0</td>
<td>22.1 ± 620</td>
<td>3.8 ± 0.5</td>
<td>1470 ± 919</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>0.014 ± 0.002</td>
<td>74.0 ± 3.6</td>
<td>0.164 ± 0.077</td>
<td>9.3 ± 4.1</td>
<td>1.28 ± 0.204</td>
<td>14.4 ± 3.1</td>
<td>18.1 ± 4.31</td>
<td>1.7 ± 1.3</td>
<td>327 ± 85.4</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3.2 Time constants and their relative areas used to fit the shut time duration histograms of nAChRs at a range of ACh concentrations. All are at 30 μs resolution.

3.2.3 Definition of a Burst

Ion channels observed at the single channel level typically exhibit bursting behaviour. That is during a single burst there are brief flickers from the open state to the closed state. The term burst can be used in two different but overlapping contexts. When bursts are defined in terms of quantitatively describing the activity of a receptor (as in fitting to sums of exponential components) then a burst is the activity that would be expected after physiological activation due to a brief pulse of ACh, this can be referred to as an ‘activation’. This is different from the definition used in section 4 when fitting directly to a kinetic scheme, in that case the term burst refers to a section of the single channel record that is believed to have originated from one single channel molecule. Even in the case of quantitatively describing channel activity the definition of a burst is not always clear cut. In the perfect example a single channel record will contain a series of openings with brief closures to the closed state, separated by shuttings many orders of magnitude greater than the brief shuttings. However, in terms of quantitatively describing the receptor, without reference to a particular reaction mechanism it appears that there are several different definitions of bursts possible for nAChRs. This arises as
the shut time duration distributions need at least three components to fit at low ACh concentrations, and at least five components at high ACh concentrations. Therefore when describing 'burst' properties it is essential that information regarding how the bursts were defined is included. This information takes the form of a $t_{\text{crit}}$ value, the calculation of which is described in the methods section. The fundamental property of a $t_{\text{crit}}$ is that it separates the shut time duration histogram into two parts; to the left of the $t_{\text{crit}}$ value are those shut times deemed to be within a burst, to the right of the $t_{\text{crit}}$ value are those shut times deemed to be outside of the burst. As there are varying numbers of shut time components depending on the concentration of ACh used, each shut time duration histogram will have several $t_{\text{crit}}$ values. For example, a $t_{\text{crit}}$ value separating the second and third components, a $t_{\text{crit}}$ value separating the third and fourth components, and a $t_{\text{crit}}$ value separating the fourth and fifth components. Examination of the shut time duration distributions at all ACh concentrations tested shows that there are always at least three components. The values of the time constants of the first two do not seem to depend on concentration. However at higher concentrations, the third component appears shifted to the left, whilst two much longer components appear. Therefore in the analysis of bursts carried out here, several different types of bursts have been defined. First, the shut time duration histograms were fitted with the minimum number of components to describe the data satisfactorily. This differs from section 3.2.2 where all of the histograms were fitted with five components to allow direct comparisons of the shut time constants across a wide range of ACh concentrations. The properties of different kinds of bursts were determined, bursts were designated as being short, intermediate, or long. Short bursts were defined as those containing only the first two shut time components, thus the $t_{\text{crit}}$ value is chosen so as to lie between the second and third shut time constants. Intermediate bursts were defined by a $t_{\text{crit}}$ value lying between the third and fourth shut time constants, and long bursts were defined by a $t_{\text{crit}}$ value lying between the fourth and fifth shut time constants. Intermediate and long bursts were typically only observed at the higher ACh concentrations. Short bursts were observed at all concentrations.

3.2.4 Burst Length durations

Of the twenty six patches used in burst analysis, short bursts could not be defined for one 10 μM patch and one 50 μM patch due to the $t_{\text{crit}}$ algorithms not converging. Of the remaining twenty four patches, twenty could be fit well with three components, examples are shown in figure 3.9. There was no apparent concentration dependence of
Figure 3.9. Short burst lengths (defined in the text) of nAChRs at a range of ACh concentrations. At higher concentrations there appears to be a slight rightward shift, produced by an increase in the relative area of the slowest component.
Figure 3.10. (A), mean length of short bursts (defined in the text) of nAChRs across a range of ACh concentrations. (B), the mean values of the time constants describing short bursts across a range of ACh concentrations. They are independent of concentration. (C), the areas of the time constants in (B). The relative area of the shortest time constant decreases with increasing concentration, whilst the relative area of the longest component increases with increasing ACh concentration. (D), mean values of the three time constants across all ACh concentrations showing them to be well separated.
the short bursts, shown in figure 3.10A, although the burst lengths ranged from 0.8 ms to 2.4 ms. Overall, across all concentrations the mean short burst length was 1.83 ± 0.19 ms. Figures 3.10B and 3.10D show that the time constants did not vary with concentration and are well separated, the values of the time constants and their associated areas are given in table 3.3. However the area of the fastest component decreased at higher ACh concentrations whilst the area of the slowest component increased with increasing ACh concentration, figure 3.10C. The fastest component, with a mean across all ACh concentrations of 40 ± 7 μs primarily consists of ‘bursts’ containing a single monoliganded opening, and therefore it is expected that the frequency of these should be reduced at higher ACh concentrations. The middle component is expected to be comprised mainly of bursts that correspond to a single diliganded openings, the mean length being 0.79 ± 0.12 ms. The slowest component, with a mean of 3.30 ± 0.38 ms corresponds to multiple diliganded openings occurring in quick succession.

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>Mean burst length (ms)</th>
<th>τ₁ (ms)</th>
<th>a₁ (%)</th>
<th>τ₂ (ms)</th>
<th>a₂ (%)</th>
<th>τ₃ (ms)</th>
<th>a₃ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>6</td>
<td>1.64 ± 0.30</td>
<td>0.056</td>
<td>23.3</td>
<td>0.847</td>
<td>32.3</td>
<td>3.748</td>
<td>40.5</td>
</tr>
<tr>
<td>0.1</td>
<td>4</td>
<td>1.41 ± 0.30</td>
<td>0.032</td>
<td>29.7</td>
<td>0.615</td>
<td>26.2</td>
<td>2.860</td>
<td>44.1</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0.80 ± 0.57</td>
<td>0.036</td>
<td>15.7</td>
<td>0.966</td>
<td>44.2</td>
<td>3.043</td>
<td>40.0</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>2.38 ± 0.70</td>
<td>0.033</td>
<td>10.8</td>
<td>0.525</td>
<td>20.3</td>
<td>3.280</td>
<td>68.9</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>2.15 ± 0.34</td>
<td>0.030</td>
<td>11.6</td>
<td>1.070</td>
<td>33.0</td>
<td>3.243</td>
<td>55.5</td>
</tr>
</tbody>
</table>

Table 3.3 Time constants and their relative areas fitted to short bursts. These bursts had a mean τ_{cal} value of 0.53 ± 0.14 ms.

To clarify this the effect of concentration on the areas of the different time constants of only those short bursts that contained a single open period were examined. This procedure takes into account the fact that diliganded activations can occur in bursts of openings whilst monoliganded openings invariably occur as single openings and thus all ‘bursts’ of monoliganded openings will consist of a single opening. Figure 3.11 shows the ratio of the areas of the slowest component (area 3) and either the fastest component (area 1) or the intermediate component (area 2) at different ACh concentrations. Both
Figure 3.11. Analysis of the short bursts (defined by $t_{crib}$) was repeated examining the bursts that consisted of a single open period. The subsequent histograms were fitted with three exponential components and the ratio of the areas of the slow component (area 1) and the two fast components (areas 1 and 2) were compared at different ACh concentrations. In both cases the proportion of long bursts increased relative to that of the faster bursts at higher ACh concentrations.
graphs show slight positive slopes that are significantly different from zero, \( R = 0.93, \) and \( P<0.05 \) for both graphs). This indicates that there are proportionally more bursts consisting of a long open period duration component than either of the two types of short bursts at high ACh concentrations.

Bursts defined by the inclusion of the three fastest components from the shut time duration histograms ('intermediate bursts') were seen at ACh concentrations of 1 µM, 10 µM, and 50 µM, but not in the nanomolar range, example burst length histograms are shown in figure 3.12. The mean length of these bursts when fitted with four exponential components were \( 11.5 \pm 4.3 \text{ ms} \) \( n = 4 \), \( 15.0 \pm 2.3 \text{ ms} \) \( n = 5 \), and \( 7.0 \pm 2.4 \text{ ms} \) \( n = 5 \) for 1 µM, 10 µM, and 50 µM patches respectively, shown in figure 3.13A, and table 3.4. The overall mean length of intermediate bursts across these concentrations of ACh is \( 11.2 \pm 1.8 \text{ ms} \), six-fold greater than the mean length of the short bursts. The four time constants and the areas of the components of the intermediate bursts were comparable across the three concentrations tested as shown in figures 3.13B and 3.13C. The overall means values of the four time constants are 20 µs, 308 µs, 2.4 ms and 25.4 ms. The mean areas of each of the time constants shown in figure 3.13E show that the 20 µs and 25.4 ms bursts account for nearly 75 % of all intermediate bursts observed.

<table>
<thead>
<tr>
<th>[ACh] (µM)</th>
<th>n</th>
<th>( \tau_1 ) (ms)</th>
<th>( a_1 ) (%)</th>
<th>( \tau_2 ) (ms)</th>
<th>( a_2 ) (%)</th>
<th>( \tau_3 ) (ms)</th>
<th>( a_3 ) (%)</th>
<th>( \tau_4 ) (ms)</th>
<th>( a_4 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4</td>
<td>0.015 ± 0.004</td>
<td>34.7 ± 9.7</td>
<td>0.173 ± 0.065</td>
<td>10.3 ± 3.7</td>
<td>2.418 ± 0.339</td>
<td>11.9 ± 3.9</td>
<td>26.4 ± 8.7</td>
<td>43.1 ± 7.0</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.033 ± 0.006</td>
<td>20.5 ± 4.2</td>
<td>0.468 ± 0.071</td>
<td>16.6 ± 5.4</td>
<td>7.338 ± 2.448</td>
<td>26.1 ± 13.4</td>
<td>48.7 ± 18.3</td>
<td>36.8 ± 12.7</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>0.024 ± 0.007</td>
<td>28.9 ± 11.6</td>
<td>0.415 ± 0.152</td>
<td>16.4 ± 7.5</td>
<td>2.308 ± 0.321</td>
<td>12.0 ± 4.4</td>
<td>24.7 ± 10.0</td>
<td>42.8 ± 13.7</td>
</tr>
<tr>
<td>Mean of all concentrations</td>
<td>14</td>
<td>0.020 ± 0.003</td>
<td>31.5 ± 5.9</td>
<td>0.308 ± 0.076</td>
<td>13.7 ± 3.5</td>
<td>2.357 ± 0.176</td>
<td>11.9 ± 2.3</td>
<td>25.4 ± 5.1</td>
<td>42.9 ± 6.2</td>
</tr>
</tbody>
</table>

Table 3.4 Time constants and their relative areas fitted to intermediate length bursts. These bursts had a mean \( t_{crit} \) value of 7.7 ± 2.3 ms.

Bursts defined by the inclusion of the four fastest components from the shut time duration histograms ('long bursts') were seen only in patches that were in the presence of 50 µM ACh. Because these long bursts occur infrequently in the patches there are
Figure 3.12. Intermediate burst length histograms of nAChRs at a range of ACh concentrations. The histograms have been fitted with four exponential components and show a decreased proportion of short bursts at the higher concentrations.
Figure 3.13. (A), mean length of intermediate bursts of nAChRs (defined in the text) across a range of ACh concentrations. (B), the mean values of the time constants describing short bursts across a range of ACh concentrations. They are independent of concentration. (C), the areas of the time constants in (B), they change little with ACh concentration. (D), the mean value of the time constants across all ACh concentrations shows that they are well separated in time. (E), the mean areas of the four time constants across all ACh concentrations. This graph show that almost 75 % of the intermediate bursts consist of either very short or very long 'intermediate' bursts.
too few events to fit histograms with exponential components. However, this is advantageous as a long burst consists of a long stretch of channel that can be sure to have originated from an individual channel molecule. Taking just the actual values of the bursts, the mean length of long bursts is $70 \pm 42$ ms, $n = 5$. The high variability results in intrinsic variability between patches and also although the long burst length distributions have too few events to fit accurately, they clearly contain several components as shown in figure 3.14.

There was some slight concentration-dependence of the short bursts, whilst the intermediate and long bursts were concentration-dependent, as these populations of bursts could not be detected in patches with ACh in the nanomolar range. This implies that more states are being accessed from the diliganded states of the receptor, i.e. the occupancies of the state(s) undetectable at low concentrations are increased.
Figure 3.14. Histograms showing the distributions of long bursts from patched in the presence of 50 μM ACh. At this concentration there are long desensitised periods between bursts and therefore the histograms haven’t been fitted with exponential components due to the low number of events and subsequent variability. However it is obvious by eye that there are several components in the distribution of long bursts.
4. nAChR Binding and Gating Rate Constants

4.1 Introduction

It is desirable to describe a biological process, such as the activation of an ion channel in terms of kinetic parameters which relate well to actual physical processes that are occurring. In this context, microscopic rate constants describing the binding and unbinding of ACh, and transitions from closed to open channels are ideal. To determine these constants it is first necessary to introduce a state scheme that adequately describes nAChRs. The scheme should not only describe data obtained by electrophysiological evidence but should also incorporate as much as possible what is known about the receptor from biochemical, pharmacological, physiological, and structural data.

In as much as a kinetic scheme is a model of a protein’s behaviour, it must be remembered that a useful kinetic model will not just contain states that can physically exist, but ideally only contain states that are physically relevant. In this thinking the states in a kinetic scheme represent only those states that form a subgroup of all states, being defined as such as those with ‘considerably greater stability’ than all other physically possible states. The states thus represent low energy wells in the free energy landscape of possible protein conformations. Scheme 1, which is the scheme that has been used predominantly in these studies could be expanded to include many more states and pathways. Which extra states and pathways are necessary to describe accurately the behaviour of the receptor is a valid question.

4.1.1 A Scheme with Two Different Binding Sites

The scheme (scheme 1) used most commonly throughout this work was introduced in 1985 (Colquhoun & Sakmann, 1985), and has been used in several studies of the nicotinic receptors since, (Milone et al., 1997; Hatton et al, 2000; Croxen et al., 2002; Hatton et al., 2003;). It incorporates the fact that two ACh molecules can bind to the receptor at two different sites. In addition it includes the fact that although virtually all of the synaptic current at the muscle endplate is due to the opening of diliganded channels, it is possible for openings to occur when one ACh molecule is bound at either of the sites. The two different sites referred to in the scheme as \( a \) and \( b \) correspond to the \( \alpha-\delta \) and the \( \alpha-\varepsilon \) ACh binding sites, although no assumptions are made as to which of the ACh binding sites the \( a \) and the \( b \) sites refer. There are several points that need to
be mentioned regarding what the scheme doesn't include, but which have been included in other nAChR schemes.

First, thermodynamics dictates that nAChRs must exist an unliganded open state. However, it is entirely consistent with the dictates of thermodynamics that this state is rarely visited, and single channel experiments in the absence of agonist failed to yield any channel activations. Therefore it is sensible to omit an unliganded open state from the scheme.

Secondly, there are no desensitised states in this scheme. It is know that the nAChR desensitises (there is a reduction in response in the continued presence of agonist). At the microscopic level desensitisation is known to involve many nonconducting states, maybe as many as five or six, (Elenes & Auerbach, 2002), it is also known that nAChRs can recover from desensitisation without passing through a conducting state (Katz & Thesleff, 1957). To date desensitisation has not been adequately accounted for in a kinetic scheme for the nAChR. Fortunately desensitisation only becomes apparent in the single channel record at high concentrations of agonist, for example at about 10 μM
for ACh. Therefore, at least for patches at lower concentrations of ACh it can be
ignored. For patches at higher concentrations it has been found that it is still not
necessary to include desensitised states to describe the receptor, shown in section 8, and
also by (Colquhoun et al, 2003; Hatton et al, 2003).

The third omission to this scheme is that there is no agonist blocked state of the channel.
At high enough concentrations (around 300 μM) of ACh, the occurrence of ion channel
block becomes apparent from the single channel records. The positively charged ACh
molecules occlude the ion conducting pore of the channel when the interior of the cell is
at negative potentials relative to the external environment. The agonist-blocked state
was omitted as no decrease in the apparent amplitude of the openings was observed in
the experiments presented in this section. A decrease in the apparent amplitude of the
openings would be expected due to the very fast dissociation of ACh from the pore, and
the high rate of ACh association at high ACh concentrations which would lead to many
very short blocked events. This decrease in apparent amplitude of openings is
characteristic of block due to the very fast nature of block in the case of nAChRs and
ACH. Section 7 details fits to the data using a scheme that includes an agonist blocked
pore state.

4.1.2 Connections Between States
In scheme 1 each rate constant describes the frequency of transitions between two stable
states, either the binding or unbinding of a ligand, or the opening or closing of the ion
channel. It is possible to add four more rate constants to scheme 1, without altering the
number of states. In theory it is possible for ligand to bind when the channel in one of
the monoliganded open states. As is shown in section 9 these extra rates are not needed
to describe the receptor and are very slow.

4.1.3 Fixed Rate constants and Constrained Rate constants
It is not known how many channels are in a cell attached patch. It is not easy to
determine the number of nAChRs in an outside-out patch either. Because of this
limitation the absolute rate of channel activations cannot be known. This is why only
‘within burst’ shut times can be used. The longest shut times seen are almost entirely
dependent on the number of channels in a particular patch. Therefore it follows that at
least one of the initial (binding of a first molecule of ligand) association rate constants
cannot be determined. Therefore $k_{+1a}$ was fixed at a physically realistic value simply to
aid the fitting process. It has been shown (Colquhoun et al., 2003; Hatton et al., 2003) that using a fixed forward rate as opposed to using a macroscopic EC50 value to determine the absolute rate of channel activations has little affect on the estimation of the diliganded parameters. However fixing the EC50 can affect the estimates of some of the binding and moniliganded gating rate constants. In particular inaccurate estimates of the true EC50 by a little as two-fold can increase the number of microscopic parameters that are estimated incorrectly (Colquhoun et al., 2003).

It was assumed that agonist binding at the two sites was independent, that is the two sites did not exhibit any cooperativity, defined as,

\[
\begin{align*}
  k_{+\alpha} &= k_{+2\alpha} \\
  k_{-\alpha} &= k_{-2\alpha} \\
  k_{+\beta} &= k_{+2\beta} \\
  k_{-\beta} &= k_{-2\beta}
\end{align*}
\]

In addition the association and dissociation rates were constrained to obey microscopic reversibility, that is,

\[
k_{+\alpha} \times k_{+2\beta} \times k_{-2\alpha} \times k_{-\beta} = k_{+\beta} \times k_{+2\alpha} \times k_{-2\beta} \times k_{-\alpha}
\]

4.2 Results

The rate constants obtained from fitting the idealised single channel traces to scheme 1 are shown in table 4.1. The data are the means of fits to individual cell attached patches (as opposed to fitting several patches together simultaneously) at a range of ACh concentrations (50 nM to 50 µM). Some simultaneous fitting of patches at different concentrations was attempted. However in several cases the HJC distributions did not accurately describe the experimental data, therefore it was decided to present all of the results as the results from individual fits. The data presented here includes data previously reported, (Hatton et al., 2003).

Some of the rate constants are estimated very well whilst others show a great deal of variability. This is shown in the range of c.v.m.s of the parameters; the diliganded
parameters have a range of 4% to 5%, the binding and unbinding rate constants have a range of 15% to 22%, and the monoligated gating parameters have a range 25% to 62%. Overlays of the HJC distributions onto the open and shut times histograms are shown in figure 4.1 and can be seen to describe the data well. It should be noted that the HJC distributions in figure 4.1 are not fitted to the actual histograms, but are the overlaid scalar functions describing scheme 1 with the rate constants that maximised the likelihood of the observations. Their overlay onto the histograms is simply for visual comparison. In addition to the HJC distributions, figure 4.1 also shows the expected distributions given ideal resolution (dotted lines). These distributions differ considerably from the open time histograms. This is because of the difficulty in detecting a short shuttling within an activation, which when missed would act to overestimate the length of an open time. The difference between the ideal resolution distribution and the shut times is less pronounced than for the open times. This is mainly because the shortest openings are longer than the short shuttings. This also occurs as it is easier to detect a short opening than a short shuttling, presumably because the shut level of the baseline of a single channel record tends to have a lower r.m.s. noise value than that of an open channel. The overlay of the ideal resolution histograms highlights the importance of considering events missed due to the limited resolution of the experiments.

<table>
<thead>
<tr>
<th>n</th>
<th>$\alpha_1$ (s$^{-1}$)</th>
<th>$\beta_1$ (s$^{-1}$)</th>
<th>$E_2$ (s$^{-1}$)</th>
<th>$\alpha_{1a}$ (s$^{-1}$)</th>
<th>$\beta_{1a}$ (s$^{-1}$)</th>
<th>$E_{1a}$ (s$^{-1}$)</th>
<th>$\alpha_{1b}$ (s$^{-1}$)</th>
<th>$\beta_{1b}$ (s$^{-1}$)</th>
<th>$E_{1b}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>1950 ± 5%</td>
<td>50500 ± 4%</td>
<td>26.8 ± 5%</td>
<td>76400 ± 25%</td>
<td>3420 ± 51%</td>
<td>0.318 ± 57%</td>
<td>27800 ± 62%</td>
<td>642 ± 62%</td>
<td>0.179 ± 69%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>n</th>
<th>$k_{2a}$ * (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{-2a}$ (s$^{-1}$)</th>
<th>$K_a$ (µM)</th>
<th>$k_{2b}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{-2b}$ (s$^{-1}$)</th>
<th>$K_b$ (µM)</th>
<th>Total Dissociation (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>100 ± 15%</td>
<td>5230 ± 15%</td>
<td>52.3</td>
<td>3270 ± 22%</td>
<td>11100 ± 10%</td>
<td>76.3 ± 27%</td>
<td>16300 ± 4%</td>
</tr>
</tbody>
</table>

Tables 4.1. Values obtained for the kinetic parameters in scheme 1 by using maximum likelihood fitting to patches in the presence of a 50 nM, 100 nM, 1 µM, 10 µM, and 100 µM ACh. The values for $k_{2a}$, $k_{-2a}$, $k_{2b}$, and $k_{-2b}$ are not shown as these were constrained to be equal to $k_{2a}$, $k_{-2a}$, $k_{2b}$, and $k_{-2b}$ respectively. Total dissociation refers to the sum of $k_{-2a}$ and $k_{-2b}$. * = fixed parameter and therefore no c.v.m. is given.
Figure 4.1. Example HJC distributions overlaid onto open time (left column) and shut time (right column) histograms of wild type nAChRs at a range of concentrations. The blue lines are the HJC distributions calculated from maximum likelihood fitting to the idealised single channel traces. The dotted red line shows the form the HJC distribution would take given infinite resolution. The HJC distributions describe the open and shut time histograms very well.
The estimated rate constants given in table 4.1 predict an EC50 value of 3.8 μM for the human nAChR. This compares reasonably well to macroscopic measurements in which whole cell dose response curves predict an EC50 of 12.8 μM, shown in figure 4.2. Although it should be remembered that included in this EC50 calculation is the $k_{+2a}$ rate that was fixed during the fitting process.

4.2.1 Diliganded Parameters

It has been shown from simulations, (Colquhoun et al., 2003) that $\alpha_2$, $\beta_2$, and the dissociation rates from the diliganded shut state should be well estimated. The values obtained for these rates ($\alpha_2 = 1950 \text{ s}^{-1}$, $\beta_2 = 50500 \text{ s}^{-1}$, total dissociation = 16300 $\text{ s}^{-1}$) agree with those obtained in other studies given the different experimental conditions (Colquhoun & Sakmann, 1985; Wang et al., 1997; Akk 1999; Salamone et al., 1999). The values obtained for the diliganded parameters predict mean bursts properties of 4 openings (calculated as $1 + (\beta_2 / (k_{-2a} + k_{-2b})))$ of 0.5 ms duration, separated by brief 15 μs sojourns to the diliganded shut state. Together it is the diliganded gating rates and the total dissociation rate (the diliganded parameters) that almost entirely determine the physiological functioning of the nAChR. Using the values from table 4.1, the predicted synaptic current in response to a brief pulse of ACh was calculated, shown in figure 4.3. The single channel rate constants predict a macroscopic decay constant of around 2.1 ms. This compares well to that of approximately 2.8 ms obtained as the time constant of decay of human endplates at the same membrane potential, (Cull-Candy et al., 1979).

4.2.2 Binding Parameters

The individual ACh dissociation rates from the diliganded receptor differ 2.5-fold from each other, (5230 $\text{ s}^{-1}$ for the $a$ site, 11100 $\text{ s}^{-1}$ for the $b$ site). The association rate constant of the $a$ site was fixed in the fitting process due to the lack of information regarding the number of channels in the patches. It therefore follows that $K_a$ has little physical meaning as it is calculated from $k_{+2a}$. However, the association rate constant of the $b$ site is well estimated, and from the binding parameters it can be concluded that the $b$ site has an affinity of around 76 μM for ACh.

4.2.3 Monoliganded Parameters

The monoliganded gating parameters are much less well estimated than the other rate constants in the scheme. This is due to the limited resolution of the experiments and the short durations of the two types of monoliganded opening. In particular the value
Figure 4.2. nAChR whole cell dose response curve obtained using ACh as the agonist. The curve was constructed from the normalised values of six cells, with each point being the mean of three measurements in each cell at each ACh concentration. The points are fitted with the Hill equation, which gives an EC50 value of 12.8 μM and a Hill slope of 1.2. This EC50 value compares reasonably well with that of 3.8 μM, calculated from the mean values of the microscopic rate constants in table 4.1.
Figure 4.3. The simulated macroscopic synaptic current in response to the activation of a nAChR by a 0.1 ms pulse of 1mM ACh (green line) using the microscopic rate constants from table 4.1. The rates predict a macroscopic time constant of decay of 2.1 ms.
obtained for $\beta_{1b}$ (and therefore also $E_{1b}$) is unreliable. This is because $\beta_{1b}$ is sensitive to errors in fixing $k_{+2b}$ at the wrong value, (Colquhoun et al, 2003).

4.2.4 Effects of ACh Concentration on Rate Constant Estimates

Table 4.2 shows a break down of the parameters by ACh concentration, figures 4.4, 4.5, and 4.6 show these results graphically. Rate constants by their very definition should be independent of agonist concentration, and it is encouraging to see that there is no concentration dependence for most of the parameters. However there does seem to be some concentration dependence of the $\alpha_2$, $\beta_2$ and the $k_{+2b}$ rates. These anomalies suggest that the mechanism being used here, although accurately describing the data still has some caveats. In particular, at high ACh concentrations both $\alpha_2$ and $\beta_2$ (rates that should not be affected by the agonist concentration) increase slightly. A log-linear regression line fit to the $\alpha_2$ values gives a R value of 0.92 and a slope of $289 \ln (x)$ which is significantly different from zero, ($P<0.05$). A log-linear fit to the $\beta_2$ data points did not give a slope that was significantly different from zero. The diliganded efficacy, $E_2$ (simply $\beta_2$ divided by $\alpha_2$) shows a slight concentration dependence, the log-linear fit gave a slope of $-0.88 \ln (x)$, ($P = 0.05$). As $\alpha_2$ and $\beta_2$ are positively correlated it may be the case that in reality only one of these two rates is concentration-dependent, and the other simply appears to have the same relationship due to the correlation between the parameters. These results suggest that there are concentration dependent pathways leading away from the diliganded open state, and due to the mechanism used, this could only lead to a state not included in scheme 1. This state may correspond to a diliganded open channel with agonist occluding the pore. This possibility is examined in section 7. Alternatively it could involve concentration dependent steps to desensitised states, although this seems unlikely as, other than the two (already occupied) ACh binding pockets, and the mouth of the pore, there are no further recognised sites on nAChRs for ACh to bind. There is clear concentration dependence of $k_{+2b}$ that is harder to rationalise although the relationship is apparent. The plots showing the monoliganded gating parameters against ACh concentration do not show any concentration dependence, however they do highlight the high amount of variability in the estimation of these parameters.
Tables 4.2. As table 4.1 but here the values of the rate constants have been separated on the basis of the ACh concentration they were measured at. As in table 4.1 the values for $k_{+a}$, $k_{-a}$, $k_{+b}$, and $k_{-b}$ are not shown as these were constrained to be equal to $k_{+2a}$, $k_{-2a}$, $k_{+2b}$, and $k_{-2b}$ respectively. Total dissociation refers to the sum of $k_{+a}$ and $k_{-a}$. * = fixed parameter.

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>$\alpha_2$ (s⁻¹)</th>
<th>$\beta_2$ (s⁻¹)</th>
<th>$E_2$</th>
<th>$\alpha_{1b}$ (s⁻¹)</th>
<th>$\beta_{1b}$ (s⁻¹)</th>
<th>$E_{1b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>7</td>
<td>1590 ± 11%</td>
<td>40800 ± 13%</td>
<td>26.6</td>
<td>4000 ± 16%</td>
<td>12.0 ± 56%</td>
<td>3.24 × 10⁻³</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>1620 ± 9%</td>
<td>47900 ± 9%</td>
<td>30.3</td>
<td>4350 ± 20%</td>
<td>13.4 ± 27%</td>
<td>7.97 × 10⁻³</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>2080 ± 4%</td>
<td>57200 ± 7%</td>
<td>27.7</td>
<td>27500 ± 38%</td>
<td>2880 ± 99%</td>
<td>0.893</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>2140 ± 6%</td>
<td>54500 ± 5%</td>
<td>25.5</td>
<td>140000 ± 87%</td>
<td>730 ± 87%</td>
<td>0.152</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>2660 ± 11%</td>
<td>57800 ± 3%</td>
<td>22.4</td>
<td>54400 ± 78%</td>
<td>1080 ± 71%</td>
<td>0.119</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>$\alpha_{1a}$ (s⁻¹)</th>
<th>$\beta_{1a}$ (s⁻¹)</th>
<th>$E_{1a}$</th>
<th>$\alpha_{1b}$ (s⁻¹)</th>
<th>$\beta_{1b}$ (s⁻¹)</th>
<th>$E_{1b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>7</td>
<td>67200 ± 53%</td>
<td>7020 ± 99%</td>
<td>2.75 × 10⁻²</td>
<td>4400 ± 16%</td>
<td>12.0 ± 56%</td>
<td>3.24 × 10⁻³</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>74000 ± 26%</td>
<td>464 ± 35%</td>
<td>6.91 × 10⁻³</td>
<td>4350 ± 20%</td>
<td>13.4 ± 27%</td>
<td>7.97 × 10⁻³</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>13700 ± 77%</td>
<td>6220 ± 41%</td>
<td>2.14</td>
<td>27500 ± 38%</td>
<td>2880 ± 99%</td>
<td>0.893</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>143000 ± 43%</td>
<td>2540 ± 87%</td>
<td>1.84 × 10⁻²</td>
<td>10600 ± 65%</td>
<td>572 ± 41%</td>
<td>0.152</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>42000 ± 76%</td>
<td>1080 ± 61%</td>
<td>7.14 × 10⁻²</td>
<td>140000 ± 87%</td>
<td>730 ± 75%</td>
<td>0.119</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[ACh] (μM)</th>
<th>n</th>
<th>$k_{+2a}$ (μM⁻¹ s⁻¹)</th>
<th>$k_{-2a}$ (s⁻¹)</th>
<th>$K_a$ (μM)</th>
<th>$k_{+2b}$ (μM⁻¹ s⁻¹)</th>
<th>$k_{-2b}$ (s⁻¹)</th>
<th>$K_b$ (μM)</th>
<th>Total Dissociation (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>7</td>
<td>100 ± 22%</td>
<td>2680 ± 22%</td>
<td>26.8</td>
<td>6100 ± 25%</td>
<td>15700 ± 18%</td>
<td>17.2</td>
<td>18400 ± 13%</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>100 ± 19%</td>
<td>3330 ± 19%</td>
<td>33.3</td>
<td>5680 ± 28%</td>
<td>12700 ± 11%</td>
<td>59.9</td>
<td>16100 ± 6%</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>100 ± 37%</td>
<td>9520 ± 37%</td>
<td>95.0</td>
<td>2820 ± 36%</td>
<td>5520 ± 21%</td>
<td>3.08</td>
<td>15000 ± 16%</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>100 ± 27%</td>
<td>5440 ± 27%</td>
<td>54.4</td>
<td>1260 ± 30%</td>
<td>10100 ± 16%</td>
<td>127</td>
<td>15500 ± 4%</td>
</tr>
<tr>
<td>50</td>
<td>4</td>
<td>100 ± 25%</td>
<td>8350 ± 25%</td>
<td>83.4</td>
<td>2550 ± 21%</td>
<td>7390 ± 21%</td>
<td>287</td>
<td>15700 ± 4%</td>
</tr>
</tbody>
</table>
Figure 4.4. Effects of ACh concentration of the diliganded gating parameters. There is some significant concentration dependence of $\alpha_2$ but not of $\beta_2$ ($P<0.05$ and $P>0.05$ respectively). The diliganded efficacy shows some possible concentration dependence, $P = 0.05$. 
Figure 4.5. Effects of ACh concentration of the monoliganded gating parameters. The greatest concentration dependence appears to be that of $\alpha_{1b}$, although these data points only give an $R^2$ value of 0.56. These graphs highlight the large amount of variability in the estimates of the monoliganded parameters.
Figure 4.6. The effects of ACh concentration of the binding parameters. Most parameters show no concentration dependence. However, $k_{+25}$ shows a striking relationship, it dramatically decreases at higher concentrations in a non-linear fashion.
5. **Effects of the congenital myasthenic syndrome mutation εL78P on the kinetics of human muscle nAChRs**

5.1 **Introduction**

A single homozygous nucleotide point mutation of thymine to cytosine at position 233 of the ε subunit DNA results in the substitution of a proline residue for a leucine residue at position 78 in the protein, (Croxen *et al.*, 2002). This mutation underlies a rare form of slow channel congenital myasthenia, and this is the first recorded case of a slow channel syndrome that is recessively inherited. The mutation was originally identified in a 29 year old female patient who failed to breathe following a general anaesthetic. Six to seven years before she had noticed muscle weakness. Subsequent examinations identified bilateral ptosis, limitation of eye movements, weakness of facial and hand muscles, and weakness at the neck, shoulders and hips. Electromyography showed an increased decrement of response at 3 Hz stimulation. Antibodies to nAChRs were not detected. One of her siblings reported a similar failure to breathe after administration of a general anaesthetic.

5.2 **Results**

*5.2.1 Single Channel Properties of εL78P nAChRs*

Cell attached patches were used to study the single channel properties of human nAChRs containing the εL78P mutation, single channel traces are shown in figure 5.1. The εL78P activations were visibly longer than the wild type activations. The open period duration histograms of εL78P receptors with 100 nM ACh could be fitted adequately with three, and in one case, four exponential components. The values of the individual time constants and their areas are shown in table 5.1, the histograms and the fits of exponential components are shown in figure 5.2. The εL78P receptors contained a greater proportion of short openings compared with wild type receptors, however an approximate doubling of the longest time constant means that the overall mean open time duration is little different between εL78P receptors and wild type receptors, at 1.17 ms and 1.15 ms respectively.
Figure 5.1. Single channel traces of wild type (top) and εL78P (bottom) nAChRs in the presence of 100 nM ACh. Openings are downward deflections. The εL78P nAChRs have much longer activations than the wild type nAChRs.
Figure 5.2. Single channel properties of wild type (left column) and εL78P nAChRs. The proportion of both very short and very long open time durations are increased in the εL78P receptors. In both cases the histograms have been fitted with three exponential components. Shut time duration histograms fitted with five exponential components. At this concentration (100 nM ACh) the εL78P receptors have a smaller proportion of intermediate length shut time durations.
<table>
<thead>
<tr>
<th>nAChR</th>
<th>n</th>
<th>Mean open period (ms)</th>
<th>$\tau_1$ (ms)</th>
<th>$a_1$ (%)</th>
<th>$\tau_2$ (ms)</th>
<th>$a_2$ (%)</th>
<th>$\tau_3$ (ms)</th>
<th>$a_3$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6</td>
<td>1.17 ± 0.18</td>
<td>0.033 ± 0.016</td>
<td>28.5 ± 7.7</td>
<td>0.739 ± 0.219</td>
<td>36.5 ± 11.4</td>
<td>3.03 ± 0.79</td>
<td>34.9 ± 9.0</td>
</tr>
<tr>
<td>eL78P</td>
<td>4</td>
<td>1.15 ± 0.85</td>
<td>0.009 ± 0.003</td>
<td>76.0 ± 15.6</td>
<td>0.208 ± 0.007</td>
<td>7.6 ± 4.4</td>
<td>7.40 ± 0.59</td>
<td>16.4 ± 13.0</td>
</tr>
</tbody>
</table>

Table 5.1. The mean open period of wild type nAChRs (from section 3) and eL78P receptors, and their individual values of the three time constants and areas used to fit open period histograms, (examples shown in figure 5.2). Errors shown are the standard deviations of the mean (standard error).

The shut time duration histograms of all of the eL78P patches could be fitted with three to five exponential components, shown in figure 5.2. The values of the five component fits are shown in Table 5.2 and compared with wild type receptors, also at 100 nM and fitted with five exponential components. The eL78P receptors had a greater proportion of short shut times compared to wild type. The proportion of intermediate shut times was greater in the wild type receptors than in the eL78P receptors.

<table>
<thead>
<tr>
<th>nAChR</th>
<th>n</th>
<th>$\tau_1$ (ms)</th>
<th>$a_1$ (%)</th>
<th>$\tau_2$ (ms)</th>
<th>$a_2$ (%)</th>
<th>$\tau_3$ (ms)</th>
<th>$a_3$ (%)</th>
<th>$\tau_4$ (ms)</th>
<th>$a_4$ (%)</th>
<th>$\tau_5$ (ms)</th>
<th>$a_5$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6</td>
<td>0.016 ± 0.001</td>
<td>60.1 ± 4.9</td>
<td>0.079 ± 0.025</td>
<td>6.6 ± 2.8</td>
<td>1.47 ± 0.936</td>
<td>1.2 ± 0.5</td>
<td>60.0 ± 50.8</td>
<td>2.3 ± 0.9</td>
<td>398 ± 252</td>
<td>29.0 ± 3.3</td>
</tr>
<tr>
<td>eL78P</td>
<td>5</td>
<td>0.011 ± 0.001</td>
<td>88.2 ± 2.2</td>
<td>0.217 ± 0.114</td>
<td>2.4 ± 0.5</td>
<td>0.83 ± 0.15</td>
<td>0.8 ± 0.2</td>
<td>34.6 ± 10.1</td>
<td>0.7 ± 0.3</td>
<td>315 ± 55</td>
<td>7.9 ± 2.2</td>
</tr>
</tbody>
</table>

Table 5.2. Shut time duration histograms of wild type and eL78P receptors in the presence of 100 nM ACh were both fitted with five components. The eL78P receptors had a greater proportion of short shut times compared to wild type. The longer shut time were more variable between the two types of receptor, the proportion of intermediate shut times ($\tau_2$, $\tau_3$, and $\tau_4$) was reduced in the eL78P, making up only 3.9% of the distribution, compared to 10.1% in the wild type receptors. Errors shown are the standard deviations of the mean (standard error).

Burst length duration histograms were constructed for receptors containing the eL78P mutation. The bursts were defined by fitting the shut time duration histograms with four or five components and then specifying a $t_{crit}$ value so that all but the longest shut time was included in the burst lengths. The burst length duration histograms obtained could be fitted adequately with four, or in most cases five exponential components, the
parameters are compared with wild type burst length duration parameters (from table 3.3) in table 5.3. Figure 5.3 shows that at 100 nM ACh the distributions of the burst length durations of wild type and εL78P receptors are very different. The mean burst length of wild type receptors at this ACh concentration was 1.41 ± 0.3 ms, 4-fold shorter than the mean burst length duration of εL78P receptors which was 5.75 ± 0.02 ms. The majority of the bursts from εL78P receptors consist of very short burst length durations (τ_{fastest} = 8 μs, area = 82 %). These are expected to be isolated single-liganded openings; being so short they will have little influence on the endplate current during physiological activation of nAChRs. Virtually all of the synaptic current is due to multiple diliganded open periods occurring in quick succession (a ‘typical’ burst). Therefore the burst length durations have been reexamined, and histograms of the distributions constructed excluding bursts that consist only of a single open period. Wild type receptors distributions were consistently fitted with a single exponential component, but εL78P receptors required two clear exponential components, shown in table 5.4 and figure 5.3.

<table>
<thead>
<tr>
<th>nAChR</th>
<th>n</th>
<th>τ₁ (ms) ± τ₁ (%a₁)</th>
<th>τ₂ (ms) ± τ₂ (%a₂)</th>
<th>τ₃ (ms) ± τ₃ (%a₃)</th>
<th>τ₄ (ms) ± τ₄ (%a₄)</th>
<th>τ₅ (ms) ± τ₅ (%a₅)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>4</td>
<td>0.032 ± 0.013</td>
<td>29.7 ± 12.7</td>
<td>0.615 ± 0.260</td>
<td>26.2 ± 7.0</td>
<td>2.860 ± 0.457</td>
</tr>
<tr>
<td>εL78P</td>
<td>5</td>
<td>0.008 ± 0.002</td>
<td>82.3 ± 8.5</td>
<td>0.424 ± 0.319</td>
<td>4.8 ± 2.4</td>
<td>1.09 ± 0.42</td>
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Table 5.3. Burst length duration parameters of wild type and εL78P nAChRs at 100 nM ACh. The εL78P receptors required five exponential components to describe their burst properties adequately whilst the wild type receptors could be described with three exponential components. Errors shown are the standard deviations of the mean (standard error).
Burst length durations

Burst length durations of bursts containing one or more open periods

Figure 5.3. Single channel burst properties of wild type (left column) and eL78P nAChRs. The eL78P receptors have a very different distribution of bursts compared with wild type receptors; they also have an increased proportion of very short and very long bursts. Wild type receptors required three exponential components to describe their burst length distributions, eL78P required five exponential components. Examining the distributions of burst lengths of bursts containing more than one open period revealed that eL78P receptors are described with two exponential components whilst wild type receptors can be described with a single exponential component. These distributions omit single-liganded ‘bursts’ as well as double-liganded ‘bursts’ that consist of a single open period.
Table 5.4. Burst length durations parameters of bursts containing more than one open period of wild type and eL78P nAChRs at 100 nM ACh. The histograms of the eL78P receptors are described by two exponential components whilst the wild type receptors could be described a single exponential component. The overall mean length of these bursts was increased 8-fold. Errors shown are the standard deviations of the mean (standard error).

5.2.2 Determining Rate Constants of eL78P Receptors

To determine the rate constants determining channel activity of the eL78P receptors maximum likelihood fitting using scheme 1 was carried out, with the same constraints and fixed rates as described in section 4 for analysing the wild type channels. Briefly, one of the forward association rates was fixed at a plausible value, the two agonist binding sites were assumed to be independent, and the rates were forced to obey microscopic reversibility. Fig 5.4 shows the HJC distributions overlaid onto open and shut time histograms and compares them with histograms from wild type channels. As stated in section 4, some concentration dependence of the rate constants was detected when using this model to describe the wild type receptor. To allow direct comparisons between the wild type and the eL78P containing receptor, all the eL78P patches were obtained in the presence of 100 nM ACh, and table 5.4 compares these values with the mean values obtained from wild type receptors in the presence of 100 nM ACh.

Table 5.5 shows that many of the kinetic parameters in scheme 1 are affected by the eL78P mutation. Considering the most robustly estimated parameters, (those describing the diliganded properties of the receptor) it can be seen that the eL78P mutation decreases the rate of closing of the diliganded receptor ($\alpha_2$) two-fold. There is no significant effect on the opening rate of the diliganded receptor ($\beta_2$). The reduction in $\alpha_2$ is reflected also as in increase in $E_2$, the efficacy of the diliganded channel. In addition there is a four-fold slowing of the total rate of ACh dissociation from the diliganded closed receptor. Together it is the decreases in both $\alpha_2$ and the total dissociation rate that account for the very long bursts seen in the eL78P receptors, i.e. the channel is more likely to stay open, and agonist is less likely to dissociate and so the
Figure 5.4. Example HJC distributions overlaid onto open time (left column) and shut time (right column) histograms of wild type nAChRs and εL78P nAChRs with 100 nM ACh. The blue lines are the HJC distributions calculated from maximum likelihood fitting to the idealised single channel traces. The dotted red line shows the form the HJC distribution would take given infinite resolution. The HJC distributions describe the open and shut time histograms well.

Figure 5.5. The distribution of bursts lengths with more than one open period of εL78P nAChRs calculated from the estimated rate constants from table 5.5. The peaks are in approximately the same positions as found for fitting two exponential components directly to the histogram of length of bursts with more than one opening (figure 5.3), however their areas are different.
channel is more likely to reopen. Considering the individual binding sites, there is an eight-fold slowing of ACh dissociation at the $a$ site and a 4-fold slowing at the $b$ site.

There is a four-fold slowing of the rate of ACh association at the $b$ site. No information is available as to the effects of the $eL78P$ mutation on ACh association at the $a$ site as this value was fixed during the fitting process and following from this the values obtained for $K_a$ the equilibrium dissociation constant for the $a$ site are not useful.

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<td>4.01</td>
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<td>-</td>
<td>0.001</td>
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Table 5.5. Values obtained for the kinetic parameters in scheme 1 by using maximum likelihood fitting to patches in the presence of 100 nM ACh. The P values given were calculated by performing randomisation tests (see methods). The major physiological effects of the mutation were to decrease $\alpha_3$ and decrease the total rate of ACh dissociation from the binding sites. $* = $ fixed parameter and therefore no c.v.m. is given.
The eL78P mutation has no significant effects on the closing rates of the monoliganded open states. However it does affect the monoliganded opening rate, $\beta_{1a}$, and this leads to an increase in the efficacy of the single ligand openings elicited from the $a$ site. Although changes are seen in $\beta_{1b}$ these are not thought to be relevant as it has been shown by simulations that under the fitting conditions used here this parameter is cannot be estimated accurately when using a fixed ACh association rate. This is because errors in the rate at which one of the ACh association rates is fixed at can only be compensated for by altering $\beta_{1b}$ without altering the relative frequencies of the two monoliganded open states, (Colquhoun et al, 2003). However it should be remembered that the estimates for the values of the monoliganded parameters are variable even in the wild type receptors.

5.2.3 Calculated Conditional Burst Length Distributions of Wild Type and eL78P nAChRs

Using the estimated rate constants for the eL78P nAChR from table 5.5 the distribution of burst lengths with more than one opening was calculated given ideal resolution, it is shown in figure 5.5. It consists of nine exponential terms, six represent the normal burst length distribution calculated in the normal way from microscopic rate constants and three terms represent the distribution of the open periods durations of the first opening in a burst, which is subtracted from the normal burst length distribution to give the distribution of bursts lengths with more than one open period. The distribution shows two clear peaks at 0.3 ms and 49 ms, similar values to the time constants determined by fitting exponential components to the actual histograms constructed from the idealised single channel record (table 5.4, figure 5.3). However the areas of the components are different, when fitting to the histograms the two components had approximately equal areas, however the calculated distribution have unequal areas. To investigate this further conditional burst length distributions were constructed. These distributions describe the burst length distributions, conditional on starting in each of the three open states. They have been calculated for the wild type receptor and eL78P receptor, shown in figure 5.6, the parameters are shown in table 5.6.

There is a single peak for both wild type and eL78P nAChRs, with means of 2.2 ms and 49.4 ms respectively in the burst length distributions conditional on starting in the diliganded open state. For bursts that begin in either of the monoliganded open states of the situation is a little more complicated. Bursts that start in the monoliganded $b$ open
Figure 5.6. Burst length distributions of wild type (left column) and εL78P (right column) nAChRs conditional on starting in either the diliganded open state (top), monoliganded open state \( a \) (middle), or the monoliganded open state \( b \) (bottom). The distributions were calculated from the rate constants estimated from maximum likelihood fitting to scheme 1.
state of the wild type nAChR have two components that have area greater than 1 %, with time constants of 30 μs and 90 μs. For the wild type nAChR for bursts starting in the monoliganded \( a \) state there are three clear components (with appreciable area). The longest has a time constant of 2.2 ms and corresponds to bursts that are initiated in the brief monoliganded \( b \) state (with an estimated lifetime of 36 μs) and then proceed via shut states to a sequence of transitions between the diliganded shut and the diliganded open states.

The conditional burst length distributions of \( \varepsilon \)L78P nAChRs dependent on starting in the monoliganded open states show several peaks. For those bursts originating in the monoliganded \( b \) state there are two approximately equal components with significant area, with time constants of 30 μs and 0.3 ms. For those bursts originating in the monoliganded \( a \) state there are three components with significant area. Interestingly the predominant component leads to the very long bursts that involve multiple transitions between the diliganded open and shut states. This demonstrates that most of the disease-causing bursts of the \( \varepsilon \)L78P nAChR are actually initiated from one of the monoliganded states.

<table>
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<th></th>
<th>Conditional on starting in the diliganded open state</th>
<th>Conditional on starting in the ( a ) monoliganded state</th>
<th>Conditional on starting in the ( b ) monoliganded open state</th>
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<td></td>
<td>( \tau ) (ms)</td>
<td>Area (%)</td>
<td>( \tau ) (ms)</td>
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<tr>
<td>Wild type</td>
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<td>0.01</td>
</tr>
<tr>
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<td></td>
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<td>2.23</td>
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<tr>
<td>( \varepsilon )L78P</td>
<td>49.44</td>
<td>102</td>
<td>( 6 \times 10^{-3} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49.44</td>
</tr>
</tbody>
</table>

Table 5.6. The conditional burst length distributions were calculated from the microscopic rate constants for wild type nAChRs and \( \varepsilon \)L78P nAChRs. Each distribution is the sum of six exponential components, many of these components have effectively zero area so only those components with areas greater than 1 % are shown here.

The initial vector for bursts for the \( \varepsilon \)L78P nAChR shown below gives the probabilities of a burst starting in each of the open states, from left to right the probabilities correspond to the diliganded open, monoliganded \( a \) open, and monoliganded \( b \) open states.
\[ \phi_b = (0.56 \times 10^{-2} \quad 0.35 \times 10^{-1} \quad 0.96) \]  

Virtually all bursts of the eL78P nAChR start in the monoliganded \( b \) state, these will mainly correspond to bursts with a single open state, and the not the long pathogenic bursts seen in the single channel records. However 6-fold more bursts are initiated from the monoliganded \( a \) open state than from the diliganded open state. According to the burst length distributions conditional on open state, approximately 60% of the bursts initiated from the monoliganded \( a \) open state will proceed to very long lived bursts. Although most bursts start in the monoliganded states, most of the open time of bursts is spent in the diliganded open state, as shown by the equilibrium occupancies of the open states; 0.22 for the diliganded open state, \( 0.76 \times 10^{-3} \) for the monoliganded \( a \) open state, and \( 0.76 \times 10^{-2} \) for the monoliganded \( b \) open state.

### 5.2.4 Predicted Effects of the eL78P Mutation on Synaptic Currents

The values obtained for the microscopic rate constants can be used to simulate the synaptic current that would be produced at the muscle endplate. The Scalcs program was used to simulate the synaptic current. The rate constant estimates from table 5.4 were used, assuming the receptor experiences exposure to a 0.1 ms pulse of 1 mM ACh. The predicted current is shown in figure 5.7, along with the current predicted from the wild type receptor rates from figure 4.3 (rescaled). The wild type macroscopic current has a predominant decay time constant of 2.1 ms, the eL78P receptors have a predominant decay time constant of 23.6 ms, 11-fold slower than the wild type receptors.

The predominant time constant of decay of a macroscopic current should be the same as the slowest time constant of the burst length distribution. However this holds only when agonist is at assumed to be at zero concentration after the initiation of a burst. Simulations of single channel burst length distributions with more than one open period at 100 nM ACh predict that the predominant time constant of the burst length distribution is 49.4 ms, which is slower than the decay time constant of the synaptic current of 23.6 ms. However when the simulation is repeated but with ACh at 1 nM then the predominant time constant of the burst length distribution with more than one open period is 23.9 ms, very close to the synaptic current decay time constant. Thus it would seem that in the case of the eL78P receptor which has increased activity
Figure 5.7. The predicted synaptic currents in response to a 0.1 ms pulse of 1 mM ACh (green line) for wild type (red) and εL78P nAChRs (blue). The currents were calculated from the estimates of the microscopic rate constants obtained from maximum likelihood fitting to scheme 1 at 100 nM ACh. The rates predict a macroscopic time constant of decay of 2.1 ms for the wild type receptors and 23.6 ms for the εL78P receptors.

Numbering AChbp

<table>
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<th>g Human ε</th>
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<td>PDQV</td>
<td>S V P I S S L W</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>* *</td>
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Figure 5.8. Alignment of the AChbp and human ε and α1, and the Torpedo α1 nAChR subunits. The site of the point mutation εL78P which causes a form of slow channel congenital myasthenia aligns with V74 of the AChbp, (shown framed).
compared with wild type receptors 100 nM is not sufficiently 'close to zero concentration' to accurately predict the decay of the synaptic current from the burst length distribution with more than one opening. At wild type receptors the predicted time constant of synaptic current decay is 2.1 ms (from section 4.2.1). This compares well with the predominant time constant of the burst length distribution of 2.9 ms (table 3.3, section 3.2.4), and therefore for wild type receptors 100 nM ACh is 'sufficiently close to zero' to predict the predominant decay time constant of the synaptic current.

5.3 Conclusions

The principal effect of the εL78P mutation is to increase the activity of the receptor. This occurs due to a decrease in the rate of channel closing when diliganded, and a decrease in the rate of ACh dissociation from the diliganded closed channel. When work was started on determining the effects of the εL78P mutation there was little information regarding the location of this residue in the nAChR. All that was known was that it must be within the large extracellular N-terminal domain. The L78 position had not been identified in any studies that attempted, some more successfully than others, to determine the residues that line the actual ACh binding sites. However, following the publication of the crystal structure of the Lymnaea ACh binding protein it became possible to predict the location of residue 78 in the ε subunit. Alignment of the human ε subunit and the AChbp shows that position εL78 aligns with V74 in the AChbp, shown in figure 5.8. In the structure of AChbp, this position is on the β3 strand which forms part of one of the two twisted β-sheets characteristic of AChbp and the Torpedo nAChR, shown in figure 5.9. This position is not in the ACh binding site, and not close to the pore region of the channel. However, the β3 strand is adjacent in the β-sheet to the β5' strand, which does line the binding site. The mutation of a leucine residue to a proline residue in this position would be expected to have several structural effects. First, as proline has a cyclic side chain attached at two points to the peptide backbone it introduces constraints on the conformation of the backbone. It can be said in a broad but accurate way that the substitution of a proline at this position will reduce the flexibility of this β strand. In addition, because proline is an imino acid it lacks a nitrogen-bonded backbone hydrogen which is present on all 'other' amino acids. According to the structure of AChbp, the nitrogen-bonded hydrogen of the valine...
The homologous residue to eL78P in the Achbp is V74 on strand β3. The backbone of this residue is hydrogen bonded to strand β5', which lines the Ach binding pocket. Loops lining the 'plus' side of the binding pocket are coloured orange, the 'minus' face of the binding site is coloured purple. Hepes is shown in yellow. Below is a schematic diagram showing how the replacement of the residue at position 74 with a proline residue will disrupt the hydrogen bonding between the β3 and β5' strands.
residue at position 74 is hydrogen bonded to V105 in the β5' strand. Therefore the substitution of a proline residue into this position will result in the absence of this hydrogen bond, shown in the schematic in figure 5.9. It may be that the absence of this hydrogen bond significantly reduces the stability of the twisted β sheet, or it may be that an alternative arrangement of hydrogen bonds linking the β3 and β5' strands occurs. In either scenario the changes induced by substitution of a single residue will extend beyond the position of this residue. Kinetically the mutation has effects on both binding and gating. It is therefore believed that there is a large change in the structure of the nAChR. It is entirely possible that a mutation far from the pore can affect gating, as gating does not only include the opening of the pore but will also include the kinetics of transducing the binding signal from the N-terminal domain to the pore. This transduction is believed to involve the whole of the N-terminal, the β sheets are believed to rotate in a synchronised fashion following the binding of agonist. Therefore disruption to one of these β sheets can be expected to affect gating.

It could be hypothesised that the location of the mutation in the ε subunit could lead to effects on only one binding site. However effects on both binding sites are seen; dissociation rates are decreased eight-fold at the α site and four-fold at the β site. This is entirely possible as the ε subunit lies between two α subunits in the arrangement of subunits around the pore. Thus seen from the extracellular face of the membrane, changes in the ε subunit could be transmitted clockwise to the neighbouring α subunit forming the α-ε binding site, or anticlockwise to the neighbouring α subunit forming the α-δ binding site. Unless a mutation is directly in the ε binding site of the ε subunit it is impossible to predict with any accuracy whether the mutation affects the αε or the αδ binding sites or associated gating steps.

The discovery that many of the long-lived bursts originate in one of the monoliganded states in the εL78P nAChR, and to a much lesser extent in the wild type nAChR has important consequences. It is usually stated that the missed events problem does not substantially alter the measurement of the duration of bursts. This is because by definition the missed events are brief and will thus add little to the overall burst length. However, in the case of the εL78P mutation, missing the initial monoliganded opening in a long burst will affect the burst length. This is because omission of the fast monoliganded open event will also result in the omission of all of the shut times prior to
the first detected open event, which will most likely be from the diliganded state. An estimate of the amount of time that a burst length will be underestimated by can be calculated simply by summing the mean lifetimes of the brief monoliganded open state, and the two shut states that have to be visited prior to the first diliganded opening of the burst. For wild type nAChRs missing a monoliganded opening with a mean lifetime of 13 μs will result in underestimating the burst length by 0.15 ms. For the eL78P mutation nAChRs missing a monoliganded opening with a mean lifetime of 6 μs will result in underestimating the burst length by 0.13 ms. These values are underestimates of the underestimation of burst lengths as they do not take into account bursts that are initiated in a monoliganded open state and then have multiple transitions between shut states before entering the diliganded open state.
6. Using EC50 as a Constraint during Fitting

6.1 Introduction

During the fits in section 4 one of the forward association rates was fixed at $1 \times 10^8 \text{M}^{-1} \text{s}^{-1}$. In theory it would be preferable to allow this parameter to remain free but to do this information regarding the absolute frequency of channel openings is required. This cannot be determined from analysing bursts of single channel activations in a patch with an unknown number of channels. However, the EC50 of a given channel can be used to supply this information. Therefore maximum likelihood fitting to scheme 1 was carried out but with one of the forward association rates constrained to give an EC50 of 14 μM, obtained previously by Chris Hatton (Hatton et al., 2003). This value is very similar to the value obtained in section 4 (whole cell dose response curve from U-tube) in which an EC50 value of 12.8 μM was obtained. This was sufficiently close to that used in the fits constrained by EC50 at a single concentration that it was decided to keep the EC50 constrained to 14 μM rather than 12.8 μM.

6.2 Results

It was found the fewer patches could be fitted adequately when using an EC50 constraint as opposed to fixing one of the forward association rates as in section 4. Histograms from six out of thirty patches were not accurately described by the resulting HJC distributions. The six patches that did not fit were at several different concentrations. Estimates of the rate constants obtained are shown in table 6.1, they are compared to those obtained in section 4 in which the EC50 was a free parameter and one of the association rates was fixed. Example histograms overlaid with HJC distributions are shown in figure 6.1. Good agreement was obtained for many of the parameters using the two methods of fitting. Constraining the rate constants to give an EC50 of 14 μM had no effect on the diliganded channel opening and shutting rates, they were still estimated to be approximately 50000 s$^{-1}$ and 2000 s$^{-1}$ respectively, and thus the diliganded gating efficacy was not different between the two fitting methods. In addition the rate of total agonist dissociation was unchanged, at approximately 16000 s$^{-1}$. The moniliganded gating parameters showed several differences, although in both
Figure 6.1. Open (top) and shut (bottom) time histograms from a patch in the presence of 100 nM ACh. Overlaid onto the histograms are the HJC distributions derived from maximum likelihood fitting to scheme 2. The blue line is corresponds to the HJC distribution with an imposed resolution of 30 μs (the same as the data shown). It matches well with the histograms of the data. The red dotted line corresponds to the HJC distribution with perfect resolution.
methods of fitting these parameters were highly variable. The estimated binding free parameters were essentially similar to those estimated using a fixed association rate.

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<td>0.019 ± 69 %</td>
<td>9770 ± 37 %</td>
<td>1030 ± 56 %</td>
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<th>$k_{-2a}$ (s$^{-1}$)</th>
<th>$K_a$ (µM)</th>
<th>$k_{+2b}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{-2b}$ (s$^{-1}$)</th>
<th>$K_b$ (µM)</th>
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<td>7590 ± 13 %</td>
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<td>8610 ± 16 %</td>
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Tables 6.1. Comparison of the estimates of the rate constants from fitting single channel data either with the forward association rate $k_{+2a}$ fixed, or by constraining the entire scheme to give an EC50 value of 14 µM. The diliganded parameters ($\alpha_2$, $\beta_2$, $E_2$), and total dissociation rate are very similar in both sets of fits. * = rate fixed to $1 \times 10^8$ M$^{-1}$ s$^{-1}$ or to give an EC50 of 14 µM.

However, when the association rate $k_{+2a}$ was fixed, the estimate of the associated dissociation equilibrium constant $K_a$ cannot be considered a true result and thus the affinities of the two binding sites cannot be justifiably compared. Fitting using a constrained EC50 allows this comparison. The previously fixed association rate $k_{+2a}$ gave a value of $1.09 \times 10^9$ M$^{-1}$ s$^{-1}$ for association at the $a$ site, this is around ten-fold
higher than it was originally fixed at when EC50 data wasn’t used. This gives the \(a\) site an affinity of 229 \(\mu\)M, which is around 2-fold lower than the affinity of the \(b\) site which had an affinity of 118 \(\mu\)M. However it should be noted that the values obtained for the affinities of the two sites are highly variable across the range of concentrations tested.

The difference in log likelihood values obtained for fitting with either a fixed ACh association rate or a constrained EC50 were compared across ACh concentrations, shown in figure 6.2. This difference was found to be both large and variable at 10 \(\mu\)M ACh, and even more variable at 50 \(\mu\)M ACh. Although the differences between the log likelihood values were variable at high ACh concentrations, greater values were consistently obtained at 50 \(\mu\)M ACh for the fitting process in which the EC50 was constrained. Thus it seems only at this concentration of ACh is it preferable to use an EC50 constraint rather than fixing an ACh association rate.

### 6.2.1 Concentration Dependence of Rate Constants

The concentration-dependencies of the kinetic parameters were investigated, shown in figures 6.3 and 6.4. Ideally none of the parameters should be dependent on concentration. However, \(\alpha_2\) shows a clear concentration dependence, a log-linear fit gives a significant slope of 317 \(\ln (x)\), \(P<0.01\), \(R = 0.96\). This leads to a concentration dependence of the diliganded efficacy, \(E_2\). The log-linear fit of the \(E_2\) data gives a slope of \(-2.7 \ln (x)\), which is significantly different from zero, \(R = -0.92\), \(P<0.05\). The slope of the log-linear fit to \(\beta_2\) was not significantly different from zero. As with fitting using a fixed association rate, the graphs of the moniliganded parameters showed a large amount of scatter and thus no empirical effects of agonist concentration could be determined. Most binding parameters although showing some variability did not show any concentration dependence. In particular the concentration dependence of \(k_{+2b}\) seen when using a fixed forward association rate was not apparent when using the EC50 constraint. The sum of the two dissociation rates from the diliganded open state, the total dissociation rate, did show some concentration dependence with the log-linear fit giving a slope of \(-2590 \ln (x)\), \(R = -0.94\), \(P<0.05\). However if the points measured at 50 \(\mu\)M ACh are omitted, as these are much lower than the rates measured at lower concentrations then the \(R\) value falls to 0.61 and the slope is not significantly different from zero, \(P>0.3\). Thus it is unclear from these data whether the total dissociation rate is concentration dependent throughout the whole range of ACh concentrations tested,
Figure 6.2. Log likelihood ratios for scheme 1 fitted either with a fixed ACh association rate (L1) or the EC50 constrained to be 14 μM (L2). There was much greater difference and variability in the log likelihood values at high ACh concentrations. Numbers in brackets refer to the number of patches at each ACh concentration.
Figure 6.3. The effects of ACh concentration on diliganded gating kinetic parameters when rates were constrained to give an EC50 of 14 μM. There was a significant positive correlation between $\alpha_2$ and ACh concentration (A), which is also reflected in the graph showing the effects of concentration on diliganded efficacy (C). There was no significant correlation between $\beta_2$ and ACh concentration (B).
Figure 6.4. The effects of ACh concentration on binding parameters when rates were constrained to give an EC50 of 14 μM. Although there was variability in the results there does not appear to be any concentration dependence of the individual rate constants. There is some concentration dependence of the total rate of dissociation from the diliganded shut state. This is a tentative finding as this dependence is due entirely to values from patches in the presence of a single concentration, 50 μM.
but the rate is not concentration dependent over the range of 50 nM ACh to 10 µM ACh.

6.3 Conclusions

Using an EC50 constraint rather than fixing a forward ACh association rate has little effect on the estimates of the diliganded parameters. It also has little effect on the free parameters of ACh binding and dissociation. However, errors in the value at which the EC50 is constrained have been shown to alter the reliability of the rate constant estimates (Colquhoun et al, 2003). For example a two-fold error in the EC50 can alter the estimate of the rate varied to obey the EC50 by 4-fold. Therefore it seems preferable to use a fixed forward association rate, which leaves no doubt that the fixed rate is incorrect. Concentration dependence of $\alpha_2$ seen when using a fixed forward association rate was also seen when the rate constant were constrained to a specified EC50 value. There also appeared to be some concentration dependence of the total rate of ACh dissociation from the diliganded shut state. This was not seen when using a fixed forward association rate.
7. A Scheme that incorporates Block of the Ion Channel by ACh

7.1 Introduction

It is known that ACh itself can physically occlude the open pore of nAChRs. This appears to be strengthened considerably as a result of the positive charge on ACh, as the phenomenon is much reduced at positive membrane potentials. The demonstration of pore block is a reduction in apparent amplitude of the single channel currents at very high ACh concentrations, and an increase in the open channel noise. The increase in noise is due to the rapid rate of dissociation of pore-blocking ACh coupled with the high frequency of ACh association with the pore at high ACh concentrations ([ACh] \times k_{+\text{Block}}). This was seen in patches recorded in the presence of 500 µM ACh, shown in figure 7.1. This would lead to a mean lifetime of the diliganded open state of 2.5 µs (using figures from table 7.1), and therefore this is manifested as an increase in the open channel noise. Due to the high level of open channel noise at 500 µM ACh, the resolution obtained is too low to make meaningful measurements of the short-lived pore blocked state, therefore only patches recorded with an ACh concentration of 50 µM or less were used in the kinetic analysis. At this concentration and lower although there is no visible decrease in apparent amplitude there is a reduction in the mean length of open periods of the diliganded openings (see table 3.1 and figure 3.5B), due to the blocking actions of ACh. To investigate the extent of pore blockage by the agonist scheme 1 was extended to include a blocked pore state with two agonists already bound to the ACh binding sites (scheme 2). This scheme is of interest not only to determine the rate constants for ACh binding and dissociation to the open channel pore, but also to see if it can account for the ‘concentration-dependence’ of the channel closing rate seen in previous sections. It was hypothesised that by adding a concentration-dependent state to the scheme this would prevent the rate of \( \alpha_2 \) increasing at high ACh concentrations as there would be a further ‘escape route’ from the diliganded open state.
Figure 7.1. Single channel traces showing the difference in the open channel noise between a 50 μM ACh and 500 μM ACh patch. At 500 μM ACh the open channel noise is much greater, indicating rapid block of the pore by ACh. This leads to a decrease in the apparent amplitude of the openings. The traces in the left column are 1 s long, those in the right column are 100 ms long. Channel openings are shown as downward deflections.
7.2 Results

Maximum likelihood fitting of the rate constants from scheme 2 to individual single channel records was carried out. As in section 4, a fixed forward association rate was used and the ACh binding sites were assumed to be independent of each other. Out of thirty patches, six could not be fitted to scheme 2. A further patch was excluded from the final results as although the HJC distributions described the open time and shut time histograms, the values of some of the rate constants were unfeasibly high to represent true rate constants. Of the remaining twenty three patches it was found that the estimates of the diliganded parameters were essentially unchanged when compared to those obtained fitting to scheme 1, shown in table 7.1. Example open and shut time duration histograms overlaid with HJC distributions are shown in figure 7.2. The rate of channel opening ($\beta_2$) was estimated at 44500 s$^{-1}$, little changed from that determined from scheme 1 of 50500 s$^{-1}$. The rate of channel shutting ($\alpha_2$) was also similar, 1470 s$^{-1}$ for scheme 2 and 1950 s$^{-1}$ for scheme 1. As both $\alpha_2$ and $\beta_2$ were similar, the diliganded efficacy was also essentially unchanged. The total rate of ACh dissociation from the diliganded shut state was slightly higher when using scheme 2, although the spread of
Figure 7.2. Open time duration (top) and shut time duration (bottom) histograms overlaid with their respective HJC distributions (blue continuous lines). In both cases the HJC distributions accurately describe the data of the histograms. The patch presented here was in the presence of 1 μM ACh. The red dotted lines show the expected HJC distributions given ideal resolution.
the data was much larger (4 % c.v.m. for scheme 1 compared to 32 % c.v.m. for scheme 2).

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Table 7.1. Estimates of the gating rate constants from fitting to a scheme with a pore blocked state (scheme 2) compared with estimates of rate constants from a scheme lacking the pore blocked state (scheme 1, results from section 4). $\alpha_2$ and $\beta_2$ were essentially unchanged, as therefore was $E_2$. The monoliganded gating parameters showed a high degree of variability from both schemes. The association rate constant was poorly defined when patches at the whole range of ACh concentrations was included. Figure 6.5 shows that the estimates for both $k_{+block}$ and $k_{-block}$ had c.v.m.s that were negatively correlated with increasing ACh concentration. The estimates for the rates at only the highest ACh concentrations show much less variability.
7.2.1 Effects of ACh concentration on the rate constants

The effect of ACh concentration on the estimates of the diliganded parameters is shown in figure 7.3. The estimates of both $\alpha_2$ and $\beta_2$ were seen to decrease at higher ACh concentrations. As in all schemes tested, the moniliganded gating parameters showed a great deal of variability. The estimate of the rate of ACh binding and to the pore was not well defined, giving a c.v.m. of 54%. However the rate of dissociation of ACh from the pore (rate of unblock) was well defined, giving a rate of approximately $63000 \text{ s}^{-1}$. From this the lifetime of the blocked state can be calculated to be $16 \mu\text{s}$. The effect of ACh concentration on the estimates of the ACh blocking parameters are shown in figure 7.4. There were possible correlations between the on and off rates of ACh and ACh concentration although these should be viewed tenuously due to the correlation of estimate variability with ACh concentration, discussed below.

Since the amount of variability in the diliganded parameters, usually robustly estimated, was higher when using scheme 2, the amount of variation as a function of ACh concentration was examined. The graphs in figure 7.5 show that the c.v.m.s of the agonist blocking parameters were negatively correlated with increasing ACh concentration. This makes perfect post hoc sense, as at the lower concentrations there will be fewer blocking events occurring on which to base the estimates of the rates. There were no significant correlations between the c.v.m.s of $\alpha_2$ and the total rate of dissociation with ACh concentration. However a positive correlation between the c.v.m. of the $\beta_2$ estimates and ACh concentration was seen. Due to the high variability in the estimates of the ACh blocking rate constants at low concentrations, the rates estimated only from patches at $10 \mu\text{M}$ and $50 \mu\text{M}$ ACh were examined. The mean rate of $k_{\text{on Block}}$ was $123 \pm 23.9 \text{ M}^{-1} \text{ s}^{-1}$, the mean rate of $k_{\text{Block}}$ was $93600 \pm 6910 \text{ s}^{-1}$, $n = 11$ in both cases. These figures predict that the blocked state has a lifetime of $11 \mu\text{s}$.

To compare suitability of schemes 1 and 2 in describing the single channel data, the percentage change in the log likelihood values were calculated. This was calculated by subtracting the log likelihood value obtained when fitting with scheme 1 from the log likelihood value obtained when fitting to scheme 2, for each patch. Most patches at low ACh concentrations showed no large differences in the log likelihood values for the two schemes (a range of $-32$ to $68$ for patches at $50 \text{nM}$, $100 \text{nM}$, and $1 \text{ M ACh}$). However there were large and variable differences at $10 \mu\text{M}$ ACh (range of $1$ to $16900$)
Figure 7.3. Concentration dependence of the diliganded rate constants derived from scheme 2. $\alpha_2$ and $\beta_2$ both showed a slight decrease at higher ACh concentrations, ($R = -0.90, P<0.05$ and $R = -0.90, P<0.05$ respectively). These combine to give $E_2$ a slight concentration dependence ($R = -0.88, P<0.05$). For the total rate of ACh dissociation from the diliganded state there was a large amount of variability at 10 µM ACh, although there was no concentration dependence of this rate.
Figure 7.4. The effect of ACh concentration on the rates of ACh association, ACh dissociation, and the equilibrium constant for ACh block. The rates of ACh association and dissociation seem to be correlated with ACh concentration, but these are unreliable results as the variability in the estimates of these rate constants decreases with increasing ACh concentration, see figure 7.5. The estimates of the equilibrium constant are varied but not correlated with ACh concentration.
Figure 7.5. The variation in the c.v. ms of the main rate constants at different ACh concentrations. Left column, the variability of both the rates of ACh binding and unbinding to the pore were strongly negatively correlated with ACh concentration, ($R = -0.88$, $P<0.05$ for $k_{\text{Block}}$ and $R = -0.98$, $P<0.01$ for $k_{\text{Block}}$). Right column, the variability in the estimates of $\alpha_2$ and the total dissociation rates are not significantly correlated with ACh concentration. The positive correlation seen in the case of $\beta_2$ gives a slope that is significantly different from zero, ($P=0.04$).
and at 50 μM ACh (range of 3850 to 95600). Thus it seems that at high ACh concentrations the blocked state of the receptor should be taken into account.

7.3 Conclusions

The use of scheme 2 demonstrates that the estimates of the diliganded parameters are not affected by either the inclusion or exclusion of an ACh blocked pore state. In addition it predicts that blockages of the pore by ACh have a lifetime of around 11 μs. The inclusion of the blocked state eliminates the positive correlation between ACh concentration and α₂ seen when fitting to scheme 1 (sections 4 and 5). However there is now a negative correlation, and the introduction of a negative correlation between β₂ and ACh concentration. These relationships indicate that scheme 2 is not entirely adequate to describe the nAChR.
8. A Scheme that incorporates a Desensitised State

8.1 Introduction

To examine whether the inclusion of a desensitised shut state would improve the likelihood of the kinetic parameters or eliminate the concentration dependence of some of the rate constants seen in section 4, a diliganded desensitised state was added to scheme 1. This state was added as desensitisation becomes apparent at high concentrations of ACh and therefore it is assumed that at least one of the desensitised states of the receptor is diliganded. This state has also been included in studies of the mouse nAChR to account for shut times of around 1 ms seen in these receptors (Salamone et al., 1999).

Scheme 3

8.2 Results

Maximum likelihood fitting of the rate constants from scheme 3 to individual single channel records was carried out. As in section 4, a fixed forward association rate was used and the ACh binding sites were assumed to be independent of each other. Out of thirty patches, three could not be fitted to scheme 3. Of the remaining twenty seven
patches it was found that the estimates of the diliganded parameters were essentially unchanged when compared to those obtained fitting to scheme 1, shown in table 8.1, example histograms are shown in figure 8.1.

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Table 8.1. Estimates of the gating rate constants from fitting to a scheme with a diliganded desensitised state (scheme 3) compared with estimates of rate constants from a scheme lacking the pre blocked state (scheme 1, results from section 4). There inclusion of the desensitised state had little effect on the estimates of the diliganded gating parameters. As before, the moniliganded gating parameters showed a high degree of variability, more so when the desensitised state was included.

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<td>± 22 %</td>
<td>± 10 %</td>
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<tr>
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Table 8.2. Estimates of the rate constants for binding and unbinding of ACh from fitting to scheme 3 (which includes a desensitised state) and scheme 1 (which lacks a desensitised state, results from section 4). * = fixed parameter and therefore no c.v.m. is given.
Figure 8.1. Open time durations (top) and shut time durations (bottom) for wild type nAChRs in the presence of 50 nM ACh. The solid blue line shows the superimposed HJC distributions, which describe the data well. The dotted red line is the HJC distribution given ideal resolution.
In both schemes 1 and 3 $\alpha_2$ was around $2000 \, \text{s}^{-1}$ and $\beta_2$ was around $55000 \, \text{s}^{-1}$. Therefore the efficacy of diliganded channel gating was unchanged between the two schemes at around 27. As in all the other schemes tested most of the monoliganded parameters had a large amount of variability in their estimates. There were some slight changes in the rates of agonist binding, shown in table 8.2, most notably of $k_{+2b}$ which was increased from $3.3 \, \text{mM}^{-1} \, \text{s}^{-1}$ to $6.2 \, \text{mM}^{-1} \, \text{s}^{-1}$ when the desensitised state was included in the scheme being fitted to. The differences in the rates of dissociation of ACh from the two different sites was less marked when a desensitised state was included in the scheme. However, the total rate of dissociation remained little changed at $14100 \, \text{s}^{-1}$, compared to $16300 \, \text{s}^{-1}$ when the desensitised state was not included, shown in table 8.2. The estimated rates of the desensitised parameters were $3930 \, \text{s}^{-1}$ for entry into desensitisation and $1860 \, \text{s}^{-1}$ for exit from desensitisation, shown in table 8.3. These values predict a lifetime of 0.5 ms for the desensitised state, and when taken together with the diliganded parameters they predict a lifetime of 0.17 ms for the diliganded open state. The predicted lifetime of the diliganded open state when the desensitised state is not included is 0.51 ms.

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<tbody>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>With desensitisation</td>
<td>27</td>
<td>1860 ±96%</td>
<td>3930 ±34%</td>
<td>61.2 ±37%</td>
</tr>
</tbody>
</table>

Table 8.3. The rate constants describing entry and exit from the desensitised state. $\alpha_D$, the rate of exit from the desensitised state is poorly defined.

The log likelihood values obtained from fitting the data to scheme 1 and scheme 3 were compared. There was little difference in the values at low ACh concentrations, (range of $-153$ to $62$ at $50 \, \text{nM}$, $100 \, \text{nM}$, and $1 \, \mu\text{M ACh}$). However greater differences were seen at $10 \, \mu\text{M ACh}$ (range of $-4$ to $16900$) and at $50 \, \mu\text{M ACh}$ (range of $3830$ to $93500$). This suggests that a scheme including a desensitised state is a better description of nAChRs at high ACh concentration. However there are few changes in the well estimated parameters when schemes with and without the desensitised state are compared, and it is known that a single desensitised state leading from the diliganded open state is not an accurate description of desensitisation.
8.2.1 Effects of ACh concentration on the rate constants

As with the other schemes tested, the concentration dependence (of which there should be none) of the rate constants was examined. None of the diliganded gating parameters ($\alpha_2$, $\beta_2$, and $E_2$) were found to be dependent on concentration, shown in figure 8.2.

When the diliganded parameters were fitted with log linear lines of best fit none of the slopes were significantly different from zero. Thus it appears that the inclusion of a desensitised state abolishes the aberrant concentration dependence of $\alpha_2$ and $E_2$ seen when the desensitised state was not included (see sections 4, 6, and 7). The rates describing desensitisation ($\alpha_D$ and $\beta_D$) were variable across the range of concentrations tested shown in figure 8.2. There appeared to be a non-linear correlation between ACh concentration and $\beta_D$. The overall mean value was $3930 \text{ s}^{-1} \pm 34 \%$ whereas when separated into low concentrations (50nM to 1 $\mu$M) and high concentrations (10 $\mu$M to 50$n$M) values of $6610 \text{ s}^{-1} \pm 31 \%$ and $30.1 \text{ s}^{-1} \pm 44 \%$ respectively were obtained. This could be because inclusion of an ACh-blocked state is required at high ACh concentrations. The estimates for the individual ACh association and dissociation rates were variable across the range of ACh concentrations tested, shown in figure 8.3. The only clear correlation seen was with $k_{+2b}$, which decreased with increasing ACh concentration. The source of this correlation is unknown. There was no concentration dependence of the robustly estimated total rate of ACh dissociation.

8.3 Conclusions

The inclusion of a desensitised state was originally thought to be necessary to account for an increase in shut times around 1 ms seen at high ACh concentrations (Salamone et al., 1999). However, the omission or inclusion of a diliganded desensitised state has little effect on the mean estimates of the diliganded parameters. The estimates of the desensitisation gating rate constants were found to be extremely variable. This is in contrast to the findings presented previously (Hatton et al., 2003) in which the values of $\alpha_D$ and $\beta_D$ were given as $689 \text{ s}^{-1} \pm 51 \%$ and $65.3 \text{ s}^{-1} \pm 33 \%$ respectively. These numbers predicts a mean length of sojourns into the desensitised state of 1.45 ms, compared to this study which predicts the mean length to be 0.54 ms. However, due to the large standard errors in the estimation of $\alpha_D$ in this study, the previous value of $\alpha_D$ falls well within the range of these errors.
Figure 8.2. Estimates of rate constants at a range of ACh concentrations. None of the diliganded gating parameters ($\alpha_1$, $\beta_2$, $E_3$) show any dependence on concentration, when fitted with log-linear straight lines none of the slopes were significantly different from zero. The desensitisation parameters showed a large amount of variability. In particular the estimates of $\beta_1$, showed a non-linear negative correlation with ACh concentration. This led to a negative correlation between ACh concentration and the efficacy of desensitisation ($E_D$).
Figure 8.3. The estimates for the ACh association and dissociation were variable across ACh concentrations. $k_{a2b}$ was seen to decrease with increasing ACh concentration, and this leads to a positive correlation between $K_b$ and ACh concentration. The total rate of ACh dissociation was independent of ACh concentration.
It is certain that there are several desensitised states within the complete kinetic
description of nAChRs. If it is assumed that the rates between the diliganded open state
and the diliganded shut state are not orders of magnitude greater than rates of entry and
exit into other desensitised states then the rate constants describing entry and exit into
the desensitised state used in scheme 3 can have little physical meaning. This is
because these rates represent the convolution of all the rates describing exit and reentry
into the diliganded open state as it is known that there is more than one desensitised
state, and that desensitisation is part of a cyclical process that can return the receptor to
the unliganded state without reopening. Therefore the rates describing desensitisation
are seen as being present merely to help to estimate the other rate constants more
accurately, allowing for the fact there are concentration-independent rates leading to and
from the diliganded open state. Indeed the inclusion of the desensitised state abolished
the nonsensical concentration dependence of $\alpha_2$ seen in sections 4, 6, and 7.
9. Dissociation of Acetylcholine from Diliganded Open Channels

9.1 Introduction

One of the most obvious omissions in scheme 1 is the lack of pathways connecting monoliganded open states to the diliganded open state. There are no data to suggest that once a ligand has bound to one state and opened the channel, that another ligand cannot bind to the other site. Adding two pathways (one for each binding site) in this way adds considerably to the complexity of the model and is illustrated in scheme 4. These additional rates introduces four extra parameters. However introduction of the extra pathways also introduce two new cycles into the scheme, all of which should obey microscopic reversibility, and therefore the number of free parameters is increased only by two. Calculations based on the data from scheme 1 were used to characterise these extra rates. The results of these calculations were confirmed by using maximum likelihood fitting to a scheme which allowed direct transitions between monoliganded open and diliganded open states.

Scheme 4

\[ \begin{align*}
\text{AR}^* & \quad \text{AR} \quad \text{AR}^* \\
\alpha_{1a} & \quad \beta_{1a} \quad k_{2b(o)} \quad k_{2b(o)} \\
& \quad k_{+1a} \quad k_{-1a} \quad k_{+2b} \quad k_{-2b} \\
& \quad k_{+1b} \quad k_{-1b} \quad k_{+2a} \quad k_{-2a} \\
& \quad \alpha_{1b} \quad \beta_{1b} \quad k_{+2a(o)} \quad k_{-2a(o)} \\
\end{align*} \]
9.2 Results

9.2.1 Predicted properties of nAChRs when agonist can associate and disassociate from open channels (scheme 4)

Because of the restraints of microscopic reversibility the equilibrium dissociation rates for these new steps can be calculated from the other three equilibrium constants in the rest of the cycles. Defining $K_{2\alpha(o)}$ as $k_{-2\alpha(o)}/k_{+2\alpha(o)}$ and using the rate constant values obtained from scheme 1 from table 4.1 then,

$$K_{2\alpha(o)} = \frac{\alpha_2 k_{-2\alpha} \beta_{1\beta}}{\alpha_{1\beta} k_{+2\alpha} \beta_2} \quad K_{2\alpha(o)} = 0.047 \text{ } \mu\text{M}$$

(9.1)

$$K_{2\beta(o)} = \frac{\alpha_2 k_{-2\beta} \beta_{1\alpha}}{\alpha_{1\alpha} k_{+2\beta} \beta_2} \quad K_{2\beta(o)} = 0.0059 \text{ } \mu\text{M}$$

(9.2)

If it is assumed that the rate of association of ACh is unaffected by whether the pore is open or closed (i.e. $k_{+2\beta(o)} = k_{+2\beta}$), the dissociation rates from the doubly-liganded open to the monoliganded open states can be simply calculated. With this assumption and using the association rate values from section 4,

$$k_{-2\alpha(o)} = K_{2\alpha(o)} \times k_{+2\alpha(o)} \quad k_{-2\alpha(o)} = 4.7 \text{ s}^{-1}$$

(9.3)

$$k_{-2\beta(o)} = K_{2\beta(o)} \times k_{+2\beta(o)} \quad k_{-2\beta(o)} = 19.2 \text{ s}^{-1}$$

(9.4)

These dissociation rates are very slow compared with all of the other rates in the scheme. They are also in the same range as the value of 24 s$^{-1}$ obtained in a study of mouse nAChRs (in which it was assumed that both sites were identical, so the dissociation rate at each site would be 12 s$^{-1}$) obtained for dissociation of ACh from open channels, (Grosman & Auerbach, 2001). By combining the rates obtained above with $\alpha_2$ (the diliganded channel closing rate constant) it can be calculated that once in the diliganded open state, the probability of an agonist molecule dissociating from either site before the channel closes is 0.012, calculated below.
\[
\text{Prob}[\text{leave } A_2R^* \text{ for } A_2R] = \frac{\alpha_2}{(k_{-2a(o)} + k_{-2b(o)} + \alpha_2)} = 0.988 \tag{9.5}
\]

\[
\text{Prob}[\text{leave } A_2R^* \text{ for RA}^*] = \frac{k_{-2a(o)}}{(k_{-2a(o)} + k_{-2b(o)} + \alpha_2)} = 9.72 \times 10^{-3} \tag{9.6}
\]

\[
\text{Prob}[\text{leave } A_2R^* \text{ for AR}^*] = \frac{k_{-2b(o)}}{(k_{-2a(o)} + k_{-2b(o)} + \alpha_2)} = 2.36 \times 10^{-3} \tag{9.7}
\]

\[
\text{Prob}[\text{leave } A_2R^* \text{ for either RA}^* \text{ or AR}^*] = 9.72 \times 10^{-3} + 2.36 \times 10^{-3} = 0.012 \tag{9.8}
\]

Thus leaving the diliganded open state for a moniliganded open state would be very infrequent.

Introduction of rates linking the moniliganded and diliganded open states means that some of the properties of the moniliganded open states are now concentration dependent. The mean open times of moniliganded openings elicited from the \(a\) and the \(b\) sites are 13 \(\mu s\) and 36 \(\mu s\) respectively when transitions between the open states are prohibited. However when agonist associations to open channels are included, the lifetimes of the moniliganded open states decrease with increasing agonist concentration as shown in figure 9.1A. At 1 mM ACh the mean lifetimes of the moniliganded states are reduced to 0.3 \(\mu s\) (at the \(a\) site) and 5.7 \(\mu s\) (at the \(b\) site). In addition the probability of reaching the \(A_2R^*\) state from the open moniliganded state is now also concentration dependent, (equations 9.9 and 9.10) and shown graphically in figure 9.1B.

\[
P[\text{leaves } AR^* \text{ for } A_2R^* \text{ in } AR^*] = \frac{k_{+2b(o)}[ACh]}{k_{+2b(o)}[ACh] + \alpha_{1a}} \tag{9.9}
\]

\[
P[\text{leaves } RA^* \text{ for } A_2R^* \text{ in } RA^*] = \frac{k_{+2a(o)}[ACh]}{k_{+2a(o)}[ACh] + \alpha_{1b}} \tag{9.10}
\]

What figure 9.1B does not show is that the probability of reaching the moniliganded open state from the moniliganded shut state is reduced at increased concentrations of
Figure 9.1. Theoretical relationships between ACh concentration and (A) the lifetime of the moniliganded open states, (B) the probability of the receptor going from a moniliganded open state directly to the diliganded open state, (C) the probability of reaching the diliganded open state from a moniliganded shut state via a moniliganded open state. These relationships all assume that direct transitions between the moniliganded open states and the diliganded state are possible.
ACh as there will be increased agonist association to the monoliganded shut states at high ACh concentrations. These two factors combine to give a concentration at which there is a maximum probability of reaching the $A_2R^*$ state via a monoliganded open state given the channel is monoliganded and closed. Figure 9.1C shows the probability of reaching the diliganded open state via the monoliganded open state as opposed to via the diliganded closed state calculated from equations 9.11 and 9.12.

\[
P(\text{leave } AR \text{ for } A_2R^* \text{ via } AR^* \text{ in } AR) = \frac{k_{+2b(o)}[ACh]}{k_{+2b(o)}[ACh] + \alpha_{1a}} \times \frac{\beta_{ib}}{\beta_{ib} + k_{-1a} + k_{+2b}[ACh]}
\]

(9.11)

\[
P(\text{leave } RA \text{ for } A_2R^* \text{ via } RA^* \text{ in } RA) = \frac{k_{+2a(o)}[ACh]}{k_{+2a(o)}[ACh] + \alpha_{1b}} \times \frac{\beta_{1a}}{\beta_{1a} + k_{-1b} + k_{+2a}[ACh]}
\]

(9.12)

As can be seen from figure 9.1C, the probability of reaching the diliganded open state via the monoliganded open state reaches a plateau at low ACh concentrations. However the probability never rises above 0.05. Of course these calculations assume similar association rates for ACh whether the channel is open or closed. However this may not be the case.

All of the previous theoretical considerations based in data obtained from fitting to scheme 1 suggest that transitions between the monoliganded open and the diliganded open states are infrequent.

9.2.2 Estimates of the rate constants of a scheme incorporating ACh dissociation from diliganded open channels (scheme 4).

As described previously for scheme 1, the data were fitted using the maximum likelihood technique to scheme 4, which now incorporates transitions between monoliganded open states and diliganded open states. Unfortunately not all of the HJC distributions described the data accurately. Of thirty patches (at a range of ACh concentrations), seven did not show matches between the HJC distributions and the open and / or shut time histograms. Of these seven patches, no pattern regarding ACh concentration, temperature, date of the experiments or $t_{\text{crit}}$ values could be determined. A further patch that did have good fits was omitted from further data analysis due to the
very high values of \( \alpha_2 \) and \( \beta_2 \) obtained, (33000 s\(^{-1}\) and 212000 s\(^{-1}\) respectively.) It is believed that these results correspond to the 'high' solution that has equal likelihood as the much more common 'low' solution of around 2000 s\(^{-1}\) and 50000 s\(^{-1}\) respectively. This phenomenon is analogous to the existence of two solutions (for purely mathematical reasons) to some simultaneous equations. It arises in even the simplest kinetic scheme containing a single open state and a single shut state, (Colquhoun et al, 2003). Figure 9.2 shows example fits showing good comparisons between the overlaid HJC distributions and the open and shut time histograms. The values of the kinetic parameters determined are compared with the values obtained from fits to scheme 1 in tables 9.1, 9.2, and 9.3.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>n</th>
<th>( \alpha_2 ) (s(^{-1}))</th>
<th>( \beta_2 ) (s(^{-1}))</th>
<th>( E_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>1950 ± 5%</td>
<td>50500 ± 4%</td>
<td>26.8 ± 5%</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>2130 ± 7%</td>
<td>56500 ± 4%</td>
<td>29.0 ± 6%</td>
</tr>
</tbody>
</table>

Fold change - - -
P value - nd nd nd

<table>
<thead>
<tr>
<th>Scheme</th>
<th>n</th>
<th>( \alpha_{1a} )</th>
<th>( \beta_{1a} )</th>
<th>( E_{1a} )</th>
<th>( \alpha_{1b} )</th>
<th>( \beta_{1b} )</th>
<th>( E_{1b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>76400 ± 25%</td>
<td>3420 ± 51%</td>
<td>0.318 ± 57%</td>
<td>27800 ± 62%</td>
<td>642 ± 62%</td>
<td>0.179 ± 69%</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>149000 ± 95%</td>
<td>2740 ± 37%</td>
<td>0.953 ± 39%</td>
<td>707000 ± 88%</td>
<td>23200 ± 89%</td>
<td>0.385 ± 73%</td>
</tr>
</tbody>
</table>

Fold change - 2 - 3 25 36 2

Table 9.1 The gating rate constants and efficacy values obtained for the rate constants when fitting patches with either scheme 1 or scheme 4. The diliganded parameters (\( \alpha_2 \), \( \beta_2 \), and \( E_2 \)) are unchanged. The moniliganded gating rate constants show a high degree of variability and differences between the schemes.
Figure 9.2. Open (A) and shut (B) time histograms from a patch in the presence of 10 μM ACh. Overlaid onto the histograms are the HJC distributions derived from maximum likelihood fitting to scheme 4. The blue line corresponds to the HJC distribution with an imposed resolution of 30 μs (the same as the data shown). The red dotted line corresponds to the HJC distribution with perfect resolution.
Table 9.2 The binding rate constants and efficacy values obtained for the rate constants when fitting patches with either scheme 1 or scheme 4. Most parameters are unchanged, curiously the dissociation rates appear to swapped between the $a$ and $b$ sites between the two schemes.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>$n$</th>
<th>$k_{+a}$</th>
<th>$k_{-a}$</th>
<th>$K_a$</th>
<th>$k_{+b}$</th>
<th>$k_{-b}$</th>
<th>$K_b$</th>
<th>Total Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>100</td>
<td>5230</td>
<td>52.3</td>
<td>3270</td>
<td>11100</td>
<td>76.3</td>
<td>16300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 15%</td>
<td>± 15%</td>
<td>± 15%</td>
<td>± 10%</td>
<td>± 27%</td>
<td>± 4%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>100</td>
<td>9480</td>
<td>94.8</td>
<td>8200</td>
<td>5050</td>
<td>83.8</td>
<td>14500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 15%</td>
<td>± 15%</td>
<td>± 56%</td>
<td>± 20%</td>
<td>± 33%</td>
<td>± 7%</td>
<td></td>
</tr>
</tbody>
</table>

Table 9.3 The rate constants describing transitions between monoliganded open states and diliganded open states (which are absent in scheme 1). These values show that transitions between the monoliganded and diliganded open states are infrequent.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>$n$</th>
<th>$k_{+a(0)}$</th>
<th>$k_{-a(0)}$</th>
<th>$K_{a(0)}$</th>
<th>$k_{+b(0)}$</th>
<th>$k_{-b(0)}$</th>
<th>$K_{b(0)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>7710</td>
<td>39.1</td>
<td>2.65</td>
<td>9210</td>
<td>63.7</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 64%</td>
<td>± 56%</td>
<td>± 92%</td>
<td>± 63%</td>
<td>± 45%</td>
<td>± 81%</td>
</tr>
</tbody>
</table>

The figures from table 9.1 highlight the robustness of the diliganded parameters and variability of the monoliganded parameters. The diliganded gating parameters ($\alpha_2$, $\beta_2$, and $E_2$) show little variation between the two schemes. The dissociation rates of ACh from the diliganded closed receptor seem to have been swapped in scheme 4, that is that the $a$ site is very much similar to the $b$ site of scheme 1 and vice versa. This may reflect the difficulty in assigning a particular dissociation rate to a particular site. However it is encouraging that the actual values are similar; around 5000 s$^{-1}$ for one site and around 10000 s$^{-1}$ for the other site.

The values obtained for the rates of dissociation from the open channel are both low, 39 s$^{-1}$ and 64 s$^{-1}$. This means that from the open diliganded state, 95% of the transitions will involve closure of the pore, rather than dissociation of an agonist molecule. Also, compared to the closed channel, the open channels have a 79 to 243-fold decrease in the rate of agonist dissociation, depending on which site the ACh dissociates from.
Therefore agonist dissociation from open channels does occur, but infrequently. The association rates for ACh binding to the monoliganded open channel are greater or similar to those estimated for the closed channel (depending on which ACh site). However, these transitions will still be infrequent due to the very low occupancy of the monoliganded open states.

As with scheme 1 the effects of ACh concentration on the rate constants was examined (ideally the rate constants should be concentration independent), shown in figures 9.3 and 9.4. There were no significant correlations between the diliganded gating parameters and ACh concentrations. This is in contrast to the fits to scheme 1, in which there was a concentration dependence of $\alpha_2$ increased with increasing ACh concentration. Of the binding parameters, the only significant correlation with ACh concentration was seen with $k_{+12b}$ (which subsequently gives $K_b$ a correlation with ACh concentration). The reasons for these observed changes in the kinetic parameters with concentration are unknown. There was no correlation between $\alpha_{1b}$ and concentration as seen with fits to scheme 1, however as with scheme 1 there were large variations in the estimates of the monoliganded gating rate constants.

From examining likelihood values obtained for each patch using either scheme 1 or scheme 4 it is not clear which (if any) of the two schemes describes the data more favourably. The differences in the log likelihood values for each scheme obtained are plotted on a one-dimensional graph in figure 9.5. Most of the patches examined had very similar log likelihoods, however there were at least three patches in which a much greater log-likelihood value was obtained when fitting with scheme 4 compared to scheme 1, and one patch had a lower log likelihood when fitting with scheme 4. Scheme 4 seemed to be a much better description of the data at 50μM ACh; the three largest percentage changes in log likelihood values were seen at 50μM ACh. This may be caused by the lack of any desensitised or ACh-blocked states in the scheme. These states are known to be visited at high ACh concentrations, the transitions from the diliganded open state to the monoliganded open states may substitute for the absence of desensitised and blocked states by acting as 'exit routes' from the diliganded open state.
9.3 Conclusions

Schemes 1 and 4 compare well to each other. They both give good agreement regarding the values of the diliganded parameters, and although the individual dissociation rates from the diliganded shut channel appear to be the opposite in the two schemes, the actual values are very similar. In addition it is worth noting that only the monoliganded gating parameters were significantly different, and these are known to be unreliably estimated anyway. The values obtained for dissociation from the diliganded open channel confirm the conclusions of the theoretical calculations; that dissociation from the open channel is an infrequent event. This section shows that it is reasonable to omit connections between the monoliganded and diliganded open states. This finding is relevant not only in the context of scheme 4, but is a simplifying fact that can be used in more complicated schemes.
Figure 9.3. Effects of ACh concentration on diliganded gating kinetic parameters when including transitions between open moniliganded states and the open diliganded state (scheme 4). There was a slight negative correlation between $E_2$ and ACh concentration, although the slope of a log-linear line of best fit was not significantly different from zero ($P>0.05$).
Figure 9.4. Effects of ACh concentration on binding kinetic parameters when including transitions between open moniliganded states and the open diliganded state. All with the exception of total dissociation rate show some concentration dependence.
Figure 9.5. For most patches there was very little difference in the log likelihood values obtained from fitting with scheme 1 or with scheme 4 (which includes transitions from the monoliganded open state to the diliganded open state). Positive values indicate that scheme 4 had a greater log likelihood value than scheme 1. All of the points refer to a single patch except for the point indicated which represents fourteen patches.
10. Effects of α-conotoxin GI wild type muscle nAChRs

10.1 Introduction

α-conotoxin GI is a thirteen amino acid peptide found in the venom of the cone snail *Conus geographus* (Gray *et al*, 1981), it is a high affinity competitive antagonist at muscle nAChRs (Sine *et al*, 1995). Very little work has been published on examining the effects of competitive antagonists on single channel properties. There is evidence that a κ-conotoxin (termed confusingly κ-BtX) increases the open probability of Ca^{2+}- and voltage-sensitive BK channels (Fan *et al*, 2003). It has also been demonstrated that α-conotoxin GI has different affinities for the two different binding sites in adult human (Quiram & Sine, 1998) and for *Torpedo* and mouse nAChRs (Groebe *et al*, 1997).

It is not known whether singly liganded openings can occur whilst one of the two ACh binding sites is blocked with α-conotoxin-GI. This may provide evidence for how the receptor changes conformation during monoliganded openings, i.e. can a monoliganded opening occur through the movement of a single binding site and associated gate, or does the movement require a concerted conformational change of the whole protein complex?

10.2 Results

Most of the experiments using α-conotoxin GI were carried out in the presence of 10 μM ACh. A suitable concentration of ACh had to be used that was great enough to provide enough channel activations in the presence of a potent competitive antagonist, but also low enough so that there would be sufficient single-liganded events detected as a total proportion of all of the activations seen. The only previous study using α-conotoxin-GI in combination with adult human muscle nAChRs (i.e. containing the ε subunit) examined affinity of the toxin for the receptor by measuring competition against the initial rate of ^{125}I-α-Bgt binding (Quiram & Sine, 1998). In that study fitting the inhibition data with a two site model gave individual dissociation rate constants of 2 nM and 140 nM, and an apparent affinity of 20 nM. To determine a suitable range of concentrations of α-conotoxin GI to use in single channel experiments, inhibition of whole cell currents was briefly examined. Figure 10.1 shows the responses...
Figure 10.1. Two examples of whole cells responses to pulses of 10 μM ACh. The pulses of ACh were applied manually through a U-tube every 30 seconds (A) or every 60 seconds (B). α-conotoxin GI inhibits the ACh-elicited currents in a dose-dependent manner. Points are averaged in overlapping groups of three for clarity, error bars are the s.d.m. In both cells as well as inhibition by α-conotoxin GI there appears to be some ‘run up’ of current. A inhibition dose response curve is shown for the lower cell, the IC50 value is 12.5 nM.
of two cells to pulses of 10 μM ACh in the presence of a range of α-conotoxin GI concentrations, and a inhibition dose response curve for one of the cells. The IC50 value of 12.5 nM obtained is of the same order of the apparent α-conotoxin GI affinity found in the binding studies.

Cell-attached patches were obtained with both ACh and α-conotoxin-GI within the pipette. Example traces are shown in figure 10.2. By eye there appeared to be little difference in the patches obtained with and without various concentrations of α-conotoxin-GI.

Under nearly all conditions tested (various ACh and α-conotoxin GI concentrations) visual inspection of the open period histograms revealed an increased proportion of very short openings compared to patches without α-conotoxin GI, shown in figure 10.3. The one exception was with patches recorded in the presence of 10 μM ACh and 1 nM α-conotoxin GI (the highest and lowest concentrations used of ACh and α-conotoxin GI respectively). These patches did not seem to have an increased proportion of short openings. However from the predicted IC50 for α-conotoxin GI on adult human receptors, an effect should be detected. One possible explanation for the lack of effect at this low toxin concentration is that the toxin may be absorbed on the walls of the glass pipette, thus reducing the free concentration in the pipette. Fitting the open time histograms with three exponential components indicated that increasing concentrations of α-conotoxin GI increased the area of this time constant. There were little detectable changes in the values and relative areas of the two slower open time components, shown in table 10.1, and figure 10.5.

Shut time histograms were fitted with five exponential components, shown in figure 10.4. The values obtained for the shut time constants and their relative areas at a range of α-conotoxin GI concentrations were similar to those in the absence of the toxin, shown in table 10.2 and figure 10.5. However the longest shut time component, which represents shut times between the activation of individual channels had a slightly larger area at high concentrations of α-conotoxin-GI than at low concentrations.

Maximum likelihood fitting of the idealised records in the presence of α-conotoxin GI to scheme 1 was attempted. This scheme lacks any aspect of competitive antagonist
Figure 10.2. Single channel traces of muscle nAChRs in the presence of 10 μM ACh with and without 100 nM α-conotoxin GI. No clear difference could be detected by eye between single channel records under these two conditions.
Figure 10.3. Open time duration histograms obtained in the presence of 10 μM ACh and various concentrations of α-conotoxin-GI. At high concentrations of the toxin there is a higher proportion of short openings.
Withou\(\text{t }\alpha\text{-conotoxin-GI}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{without_conotoxin.png}
\caption{Shut time duration histograms obtained in the presence of 10 \(\mu\text{M ACh}\) and various concentrations of \(\alpha\text{-conotoxin-GI}\). At high concentrations of the toxin there is a higher proportion of very long shut times.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{1_nM_conotoxin.png}
\caption{1 nM \(\alpha\text{-conotoxin-GI}\)}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{10_nM_conotoxin.png}
\caption{10 nM \(\alpha\text{-conotoxin-GI}\)}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{100_nM_conotoxin.png}
\caption{100 nM \(\alpha\text{-conotoxin-GI}\)}
\end{figure}
Figure 10.5. The open time and shut time duration parameters of nAChR in the presence of 10 μM ACh and a range of α-conotoxin-Gl concentrations. Open time duration histograms were fitted with three exponential components. The main effect of increasing the α-conotoxin-GI concentration is the increase in area of the fastest component, and a decrease in the value of this fastest component. The shut time duration histograms were fitted with five exponential components. The first four of these are shown in the graphs, the fifth component is excluded as it is influenced by the number of channels in each patch, which although unknown, varies from patch to patch.
binding and thus it was thought that the rate constants obtained would not represent true values of physical processes, but may show some asymmetric site differences to patches in the presence of ACh alone, reflecting the differing affinity of α-conotoxin GI for the

<table>
<thead>
<tr>
<th>[ACh] (µM)</th>
<th>[α-CTX GI] (nM)</th>
<th>n</th>
<th>Mean open period (ms)</th>
<th>$\tau_1$ (ms)</th>
<th>$a_1$ (%)</th>
<th>$\tau_2$ (ms)</th>
<th>$a_2$ (%)</th>
<th>$\tau_3$ (ms)</th>
<th>$a_3$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>7</td>
<td>1.12 ± 0.05</td>
<td>0.044 ± 0.017</td>
<td>9.7 ± 1.4</td>
<td>0.556 ± 0.076</td>
<td>27.4 ± 4.7</td>
<td>1.626 ± 0.036</td>
<td>62.9 ± 4.6</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4</td>
<td>1.32 ± 0.10</td>
<td>0.020 ± 0.002</td>
<td>12.5 ± 1.3</td>
<td>0.261 ± 0.028</td>
<td>13.6 ± 4.8</td>
<td>1.730 ± 0.1090</td>
<td>74.0 ± 4.9</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>3</td>
<td>1.26 ± 0.10</td>
<td>0.017 ± 0.001</td>
<td>19.9 ± 0.31</td>
<td>0.522 ± 0.117</td>
<td>20.6 ± 8.43</td>
<td>1.943 ± 0.201</td>
<td>59.5 ± 8.6</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>2</td>
<td>0.86 ± 0.36</td>
<td>0.014 ± 0.008</td>
<td>43.0 ± 26.2</td>
<td>0.505 ± 0.250</td>
<td>16.7 ± 1.2</td>
<td>2.130 ± 0.330</td>
<td>40.4 ± 25.0</td>
</tr>
</tbody>
</table>

Table 10.1. Time constants and their relative areas fitted to the open time histograms of nAChRs at a range of α-conotoxin GI concentrations and in the presence of 10 µM ACh. There was an increase in area of the fastest component. The values obtained in the absence of α-conotoxin-GI vary slightly from those given in section 4 as here they include more recent patches that were recorded on the same day as some of the patches in the presence of α-conotoxin-GI.

<table>
<thead>
<tr>
<th>[ACh] (nM)</th>
<th>[α-conotoxin-GI] (nM)</th>
<th>n</th>
<th>$\beta_2$ (s$^{-1}$)</th>
<th>$\alpha_2$ (s$^{-1}$)</th>
<th>Total rate of dissociation (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>7</td>
<td>1620 ± 9.3 %</td>
<td>47900 ± 9.4 %</td>
<td>16100 ± 5.9 %</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>1</td>
<td>1960 ± 9.3 %</td>
<td>50200 ± 9.4 %</td>
<td>16200 ± 5.9 %</td>
</tr>
</tbody>
</table>

Table 10.3. For the single patch in the presence of α-conotoxin-GI that was well described by HJC distributions the diliganded parameters (those that are estimated with little error) were essentially unchanged.
<table>
<thead>
<tr>
<th>[ACh] (µM)</th>
<th>[α-CTX-GI] (nM)</th>
<th>n</th>
<th>( \tau_1 ) (ms)</th>
<th>( a_1 ) (%)</th>
<th>( \tau_2 ) (ms)</th>
<th>( a_2 ) (%)</th>
<th>( \tau_3 ) (ms)</th>
<th>( a_3 ) (%)</th>
<th>( \tau_4 ) (ms)</th>
<th>( a_4 ) (%)</th>
<th>( \tau_5 ) (ms)</th>
<th>( a_5 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>7</td>
<td>0.017 ± 0.001</td>
<td>63.0 ± 3.6</td>
<td>0.207 ± 0.057</td>
<td>5.8 ± 1.8</td>
<td>3.051 ± 0.303</td>
<td>26.7 ± 2.9</td>
<td>32.093 ± 7.753</td>
<td>3.2 ± 1.5</td>
<td>1478 ± 658</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4</td>
<td>0.014 ± 0.001</td>
<td>72.8 ± 2.9</td>
<td>0.124 ± 0.048</td>
<td>6.1 ± 0.9</td>
<td>2.110 ± 0.339</td>
<td>13.7 ± 3.9</td>
<td>21.04 ± 6.18</td>
<td>5.5 ± 3.4</td>
<td>316.25 ± 72.45</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>3</td>
<td>0.016 ± 0.002</td>
<td>69.0 ± 4.7</td>
<td>0.178 ± 0.078</td>
<td>4.9 ± 0.8</td>
<td>2.933 ± 0.352</td>
<td>21.3 ± 4.1</td>
<td>103.52 ± 69.08</td>
<td>2.5 ± 1.0</td>
<td>2063 ± 1702</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>2</td>
<td>0.014 ± 0.002</td>
<td>75.7 ± 8.1</td>
<td>0.090 ± 0.013</td>
<td>3.6 ± 0.6</td>
<td>2.295 ± 0.245</td>
<td>16.2 ± 5.1</td>
<td>77.75 ± 34.45</td>
<td>1.0 ± 0.5</td>
<td>3314 ± 2594</td>
<td>3.6 ± 1.9</td>
</tr>
</tbody>
</table>

Table 10.2. Time constants and their relative areas fitted to the shut time histograms of nAChRs at a range of α-conotoxin GI concentrations and in the presence of 10 µM ACh. There were little effects of increasing the concentration of α-conotoxin GI.
Figure 10.6. The HJC distributions obtained when fitting single channel data in the presence of α-conotoxin-GI to scheme 1 overlaid on to open time (left column) and shut time (right column) duration histograms. Of twelve patches only one patch with 100 nM ACh and 1 nM α-conotoxin-GI was adequately described by the HJC distributions. The open time durations were more often better described by the HJC distributions than the shut time durations.
As virtually all of the patches obtained in the presence of ACh and α-conotoxin GI did not produce HJC distributions that matched the histograms of open periods and shut times when fitted to scheme 1, a more complex scheme was introduced. Scheme 5 is an extension of scheme 1 that introduces two different binding sites for a competitive antagonist, and the possibility that monoliganded openings can occur when an antagonist molecule is bound to the site not occupied by ACh. In this scheme, A represents an ACh molecule and B represents a molecule of competitive antagonist, in this case, α-conotoxin GI.

Scheme 5

To reduce the number of free parameters, several constraints and fixed rates were used. It was assumed, as in all other cases in this work that the ACh binding sites were independent. It was also assumed that the α-conotoxin GI binding sites were independent. The two α-conotoxin GI association rate constants \( q_+ \) were fixed at \( 1 \times 10^7 \text{M}^{-1} \text{s}^{-1} \). This is ten-fold lower than the value of the \( k_{+1a} \) is fixed at (one of the ACh association rate constants). It is expected that α-conotoxin GI will have a lower association rate compared to ACh due to its much larger size. The α-conotoxin GI dissociation rates \( q_- \) were fixed at values to give equilibrium dissociation constants of 2 nM and 140 nM, these were the values obtained for α-conotoxin GI and adult human
nAChRs in binding experiments (Quiram & Sine, 1998). Initially site $a$ was fixed as the low affinity site for $\alpha$-conotoxin GI. These rate constants are no more than plausible guesses based on the available data from the literature, but rates of association and dissociation for a slow competitive blocker cannot be inferred from the data. Such a blocker would give rise to long shut periods that would be indistinguishable from desensitised states, and which would in any case, have to be counted as 'between bursts' in the absence of knowledge of the number of channels in the patch. However it is unlikely that the observable bursts depend strongly on the exact rates for conotoxin binding, and the important thing is that the numbers used do reflect selective binding to one of the two ACh binding sites.

Attempts to fit this single channel to this scheme were unsuccessful when it was assumed that channels with one molecule of ACh and one molecule of $\alpha$-conotoxin GI bound could open in the same way as channels with one molecule of ACh only bound. The shut time length histograms matched the HJC distributions, but the open time histograms not well-described by the fitted HJC distributions.

Good fits of the data could be obtained, however when it was assumed that the channel could not open when one molecule of ACh and one molecule of $\alpha$-conotoxin GI were bound at the same time (this was achieved by fixing the entry rates into the two open states with a molecule of ACh and a molecule of $\alpha$-conotoxin GI at 0.01 s$^{-1}$). The HJC distributions overlaid onto open period and shut time duration histograms are shown in figure 10.7. The estimates of the diliganded rate constants were similar to those found in the absence of $\alpha$-conotoxin GI, namely 2040 s$^{-1}$ for $\alpha_2$, and 60700 s$^{-1}$ for $\beta_2$. The dissociation rate from the $b$ site was also unaffected, a value of 11200 s$^{-1}$ was obtained, which compares well with that obtained with ACh alone (10100 s$^{-1}$). However the rate constant for dissociation of ACh from the $a$ site was noticeably slower than in the absence of $\alpha$-conotoxin GI, 887 s$^{-1}$ for this patch compared with 5440 s$^{-1}$ for patches in the presence of 10 $\mu$M ACh alone. A pure competitive antagonist should not change this rate constant, so it may be that the mechanism in scheme 5 is inadequate in some way.

To determine whether the assignment of the equilibrium dissociation constant of 2 nM and 140 nM for $\alpha$-conotoxin GI in the scheme had been assigned to the correct sites (the
Figure 10.7. Open and shut time duration histograms overlaid with HJC distributions. The distributions are of data fitted to scheme 5. This scheme accounts for binding of a competitive antagonist to the nAChr with different affinities at the two sites. The patch was obtained in the presence of 10 μM ACh and 100 nM α-conotoxin GI.
a or b sites), the values were swapped around to make site b the low affinity site for α-conotoxin GI. Fitting the same data set with these constraints to scheme 5 also yielded good fits to the data. The estimates obtained for the rate constants were similar to those with site a as the low affinity α-conotoxin GI binding site, but, contrary to the predictions for a pure competitive antagonist the value for $k_{-2 \alpha}$ was much reduced compared with patches in the absence of α-conotoxin GI, (752 s⁻¹, compared to the 5440 s⁻¹).

10.3 Conclusions

The presence of the competitive antagonist α-conotoxin-GI during activation of the muscle nAChR leads to an increase in the proportion of short openings seen. This indicates that the toxin is more efficient at blocking long openings (from diliganded receptors) than the very short openings, which are believed to represent monoliganded openings. Interestingly, the proportion of intermediate length openings was not correlated with α-conotoxin GI concentration. If the intermediate openings represent a second population of monoliganded openings then this indicates that α-conotoxin-GI only affects one of the two populations of monoliganded openings which ties in with the fact that the toxin has different affinities for the two ACh binding sites.

The fact that the HJC distributions did not match the shut time durations histograms was an interesting find. When fitting these distributions with exponential components there were no detectable trends in the shut time duration component parameters in the presence or absence of α-conotoxin GI (even at 100 nM, eight-fold greater than the IC50 value). Although there was a slight increase in the proportion of long shut time durations at high α-conotoxin-GI concentrations, this would not be detected in maximum likelihood fitting as these shut times would be excluded when defining the bursts. Although these experiments did not yield information regarding the differences between the two sites they do highlight the greater sensitivity of kinetic modelling compared to fitting histograms with exponential components. When fitting to the scheme describing antagonist binding, the dissociation rate of ACh from only the α site was reduced. This highlights binding site asymmetry of α-conotoxin properties.
11. Discussion

The muscle nAChR is responsible for transducing the chemical signals from motor neurones into a voltage change across the membrane of the muscle end plate. To fully understand fully how this process occurs, and how it is affected by different agonists, antagonists, and mutations in the receptor, it is necessary to describe the single channel properties of the receptor in terms of the underlying rate constants governing the receptors functions.

The properties of human muscle nAChR and ACh at a range of concentrations from 50 nM to 100 μM were investigated. This range was used because below 50 nM the number of events per record becomes very low and above 100 μM channel block and a large increase in open channel noise becomes apparent. Usable patches at 1 μM ACh were obtained less frequently than at other concentrations because for much of the time more than one channel was open at the same time. This is believed to be because the concentration of ACh was too high relative to the amount of desensitisation given the number of channels in the patch.

The human nAChR has been described in terms of open and shut times by fitting exponential components to open and shut time duration histograms. The overall mean open period of human muscle nAChRs is 1.2 ± 0.1 ms as measured from fitting exponential components to open period histograms at 30 μs. It should be remembered that due to brevity of short shuttings, many are missed so the apparent length of open periods is sensitive to the imposed resolution. For example when fitting with open period distributions with three exponential components (Parzefall et al, 1998) using ultra low noise recording obtained a value of 730 μs for the longest mean open period duration and 10 μs for the shortest mean open period duration (M. Heckmann, personal communication). The value of 10 μs supersedes that obtained by Heckmann and colleagues initially reported in mouse muscle (Parzefall et al, 1998). It is very close to the 13 μs estimated in this study, calculated from the rates in table 4.1. The ultra low noise recordings had resolution of about 6 μs, and were at – 200 mV. It has been shown that the effect of hyperpolarisation of the membrane can increase the open channel lifetime (Colquhoun & Ogden, 1988), so the value of 730 μs is probably an overestimate of the mean channel lifetime at – 100 mV.
At low concentrations of ACh three exponential components are needed to describe the open periods. As the area of the slowest component increases with increasing ACh concentration it is reasonable that these represent diliganded openings (Colquhoun & Sakmann, 1981; Colquhoun & Sakmann, 1985). The experiments with α-conotoxin GI showed that at 10 μM ACh there was an increased proportion of very short openings with increasing α-conotoxin GI concentration. This suggests that at least the very shortest openings are from a monoliganded state. It is not known whether α-conotoxin GI can competitively block monoliganded openings but it is certain that it can competitively block the diliganded openings.

The shut time duration histograms were described by three to five exponential components, depending on the concentration of ACh. The shut time duration histograms clearly show the existence of desensitised states which become apparent at high ACh concentrations.

The burst properties of the receptors were examined. Bursts are defined as groups of openings that are separated by shut times that are all shorter than a specified critical shut time, \( t_{crit} \). Bursts can be defined by choosing a \( t_{crit} \) that lies between any pair of well-separated components in the shut time distribution. There are two reasons for dividing openings up into groups. The first reason is to look at individual activations of the receptor, defined as the visible events that occur between the time an agonist molecule is bound to the receptor and the time when the receptor returns to the vacant state. This can be used to define the shape of synaptic currents (Wyllie et al, 1998). The second reason is to define groups of openings that all originate from the same individual channel (as required for fitting single channel data using the HJCFIT programme). At very low agonist concentrations, these two definitions of a 'burst of openings' are the same. The aim is to define a \( t_{crit} \) that includes within the burst only shut time durations when at least one agonist molecule is bound. At high agonist concentrations the individual activations are too close together for it be possible to distinguish where one ends and the next begins. In this case the aim is to define a \( t_{crit} \) value such that all of the many activations in the group (often referred to as a 'cluster') all come from one individual channel.

The main component of brief shut times 'within activations' was about 15 μs in this work (and in Hatton et al, 2003). This agrees well with the estimate of 16 μs obtained
by ultra-low noise patch clamping which allowed a resolution of 6 μs (Parzefall et al., 1998).

### 11.1 Maximum likelihood fitting of single channel data to kinetic schemes

The method of maximum likelihood fitting of single channel data to kinetic schemes is preferable to fitting exponential components to histograms of channel properties such as open period duration, shut time duration, and burst length duration. This is because the free parameters that are estimated are the rate constants in a mechanism, quantities that have a real physical significance, as opposed to the empirical time constants found by fitting distributions which have no direct physical interpretation. It is well known that the number of exponential components that can be fitted to an open or shut time duration histogram indicates the minimum number of open or shut. Maximum likelihood fitting can incorporate states that are known (or postulated) to exist but cannot be detected by fitting exponential components. An example is provided by the shut time durations of nAChRs. There must be at least four shut states of the muscle nAChR, corresponding to an unliganded, two different monoliganded, and a diliganded shut state. However, at low concentrations of ACh no more than three components can be fitted to the shut time duration histograms. Attempts to fit four components will result either in one component having zero area, or the time constants showing great variability between identical experiments. In addition, maximum likelihood fitting takes into account the correlations consistently seen between open periods durations and shut time durations. This information is also used in the fitting of bivariate distributions (Magleby & Song, 1992). However in these case only the correlation between adjacent intervals are considered. By fitting entire single channel records at once, correlations between all intervals (within sections of the record deemed to be from a single channel) are taken into account.

An accurate kinetic description of a protein complex is a useful tool. It is of interest for several reasons, possibly the most important of which is that it forms a quantitative description of the frequencies of the conformational changes that are occurring in the protein. This is information about the natural world that is of intrinsic interest. Furthermore this information can be used to determine the effects of natural and introduced amino acid mutations on the functioning of the protein. In addition the effects of pharmacological agents on the underlying rate constants describing a proteins activity can be determined. The usefulness of kinetic descriptions of proteins extends
far beyond the muscle nAChR studied here. The techniques of maximum likelihood fitting of raw (idealised) data can in theory be applied to any protein in which there are more than one clearly distinguishable state (although each clearly distinguishable state can be composed of several indistinguishable states). In the case of ion channels, the rapid and large change in current amplitude between the closed and open states is used.

In the case of synaptic physiology microscopic rate constants are much more useful parameters than their ratio for a particular reaction (the equilibrium constant). This is because ‘fast’ neurotransmitters such as ACh at the neuromuscular junction, and glutamate and GABA in the central nervous system rarely reach equilibrium with their respective receptors due to their rapid degradation or sequestration from the synaptic cleft.

Maximum likelihood fitting to kinetic schemes is not perfect however, and cannot be applied blindly. The main problem is to identify a reaction mechanism that describes physical reality with sufficient accuracy. Even with a realistic mechanism there will often be more free parameters than can be estimated with good precision. The most important limiting factors in estimation are (a) the lack of knowledge of the number of channels in the patch, (b) the frequent occurrence of heterogeneity which may prevent simultaneous fit of records over a wide range of agonist concentrations, and (c) the limited time resolution of the data (even with exact correction for missed events it is impossible to correct for things that are never seen). In addition it is important to consider the numbers of states, the connections between states, the physical plausibility of rate constant estimates, and the accuracy of the calculated distributions that are believed to describe the single channel data.

11.2 Estimates of microscopic rate constants
The estimates obtained for the diliganded channel opening rate constant, $\beta_2$, for the human receptor are similar to those found previously in human, and mouse muscle nAChRs. $\beta_2$ has been estimated in several studies to be 50000 to 60000 s$^{-1}$ at 20 °C at the mouse nAChR (Wang et al., 1997; Salamone et al., 1999) The estimate of $\beta_2$ is very similar to that found for frog nAChRs (30600 s$^{-1}$ at 11 °C; Colquhoun & Sakmann, 1985), bearing in mind the difference in temperature (the difference corresponds to a $Q_{10}$ of about 2, a common value for rate constants (Gutfreund, 1995)).
The rate constant for diliganded channel closing ($\alpha_2$) was estimated as approximately $2000 \text{ s}^{-1}$. This is again similar to the value of $714 \text{ s}^{-1}$ for frog muscle at 11 °C (Colquhoun & Sakmann, 1985), $8200 \text{ s}^{-1}$, if a $Q_{10}$ of about 2 is assumed. Mouse nAChRs also appear to be similar to human nAChRs. Auerbach and colleagues have estimated $\alpha_2$ for mouse nAChRs $\alpha_2$ as $1300 \text{ s}^{-1}$ (Akk et al., 1999), $1700 \text{ s}^{-1}$ (Wang et al., 1997), and $2600 \text{ s}^{-1}$ (Salamone et al., 1999). The equilibrium gating constant (or ‘efficacy’), the ratio of opening to shutting rate constants, $E_2 = \beta_2/\alpha_2$, for diliganded channel gating is 25 -30 in these and other recent studies.

The total rate of dissociation of ACh (which can be measured more accurately than the two individual rates of dissociation from each site) are different in human and mouse. This study estimates this total rate to be approximately $15000 \text{ s}^{-1}$ while estimates for the mouse receptor include $36000 \text{ s}^{-1}$ (Akk et al., 1999), $44000 \text{ s}^{-1}$ (Wang et al., 1997), $57000 \text{ s}^{-1}$ (Grosman & Auerbach, 2001), $61000 \text{ s}^{-1}$ (Salamone et al., 1999).

Individually the two sites in both human and mouse have different dissociation rates, values of $18000 \text{ s}^{-1}$ and $43000 \text{ s}^{-1}$ are given for the mouse receptor (Salamone et al., 1999). The justification for assuming that the two binding sites are different can be seen from the estimates of $k_{-a}$ and $k_{-b}$. In all of the schemes tested in which a fixed ACh association rate was utilised the dissociation rates of ACh are different, as shown in table 11.1. In the fits using scheme 1 with an EC50 constraint used they dissociation rates appear quite similar, but the equilibrium dissociation constants for the two sites still showed a difference, being 229 µM and 118 µM.

The equilibrium dissociation constant for the $b$ site was in the range of 50 µM to 120 µM for all of the schemes tested here. This compares reasonably well to the value of around 80 µM found for binding of ACh to the shut state in frog muscle (Colquhoun & Ogden, 1988) on the assumption of initially-equivalent binding sites. The equilibrium constant for binding of ACh to the $a$ site found here, about 230 µM this is roughly two to four-fold higher than at the $b$ site (depending on the scheme used). This is not a large difference, given the error in estimation of moniliganded rates. However it is not greatly different from the factor of about three between the equilibrium constants for binding of suberyldicholine (8 µM and 27 µM), estimated from scheme 1 by much simpler methods by (Colquhoun & Sakmann, 1985), note that suberyldicholine has a higher affinity than ACh, so the absolute numbers are smaller.
The blocking equilibrium constant was estimated as 1.3 mM in frog muscle (Colquhoun & Ogden, 1988), slightly higher than the 0.78 mM predicted here. However both of these values are lower than the 7.7 mM predicted for block of mouse nAChRs (Akk et al, 1999). In this case the rates of ACh dissociation from the pore are similar (94000 s$^{-1}$ for human nAChRs and 74000 s$^{-1}$ for mouse nAChRs), the differences arise in the estimates of the rate of ACh association to the pore. (Ohno et al., 1997) estimated 3.2 mM as the equilibrium constant on human nAChRs, the rate of block was 48 µM$^{-1}$s$^{-1}$ and the rate on unblock was 155000 s$^{-1}$. These were fitted (using a different implementation of the maximum likelihood method) to a scheme with identical binding sites with patches in the presence of 3 µM to 100 µM ACh.

Dissociation rates of ACh from diliganded open channels were estimated as 40 s$^{-1}$ and 64 s$^{-1}$ from the a and b sites respectively. These are in the same range as the 24 s$^{-1}$ estimated for mouse nAChRs estimated by (Grosman & Auerbach, 2001). These values indicate that dissociations from the diliganded open state are infrequent, especially when compared with the rate of channel shutting from the diliganded open state.

It has been demonstrated that the inclusion of an ACh blocked state, or a desensitised state, or transitions from the moniliganded open states to the diliganded open states, or using an EC50 as a constraint do not affect dramatically the estimates of the diliganded parameters, summarised in table 11.1. The concentration dependence of $\alpha_2$ was apparent when data were fitted to scheme 1 (with one fixed association rate or with rates constrained to obey an EC50 of 14 µM), or to scheme 2 (includes an ACh-pore blocked state). These relationships were not apparent when fitting to schemes 3 (including a desensitised state) or scheme 4 (including direct transitions between moniliganded open and diliganded open states). Of these two schemes it would seem preferable to use scheme 4. This is because this scheme incorporates connections and states that all have real physical significance. However the desensitised state in scheme 3 is known to be a vast oversimplification of desensitisation.
<table>
<thead>
<tr>
<th>Scheme</th>
<th>n</th>
<th>$\alpha_2$ (s$^{-1}$)</th>
<th>$\beta_2$ (s$^{-1}$)</th>
<th>$E_2$</th>
<th>Total dissociation (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 1 (fixed forward rate)</td>
<td>29</td>
<td>1950 ± 5 %</td>
<td>50500 ± 4 %</td>
<td>26.8</td>
<td>16300 ± 4 %</td>
</tr>
<tr>
<td>Scheme 1 (EC50 constraint)</td>
<td>24</td>
<td>1990 ± 6 %</td>
<td>53500 ± 7 %</td>
<td>27.7</td>
<td>16200 ± 6 %</td>
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<tr>
<td>Scheme 2 (incorporates pore block)</td>
<td>23</td>
<td>1470 ± 12 %</td>
<td>44500 ± 15 %</td>
<td>29.2</td>
<td>24400 ± 32 %</td>
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<tr>
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<td>27</td>
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<td>60300 ± 3 %</td>
<td>28.1</td>
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<tr>
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<td>30</td>
<td>2130 ± 7 %</td>
<td>56500 ± 4 %</td>
<td>29.0</td>
<td>14500 ± 7 %</td>
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<th>Scheme</th>
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<th>$k_{a_a}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{-a}$ (s$^{-1}$)</th>
<th>$K_a$ (µM)</th>
<th>$k_{i_b}$ (µM$^{-1}$ s$^{-1}$)</th>
<th>$k_{-b}$ (s$^{-1}$)</th>
<th>$K_b$ (µM)</th>
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<tr>
<td>Scheme 1 (fixed forward rate)</td>
<td>29</td>
<td>100 ± 15 %</td>
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<td>Scheme 2 (incorporates pore block)</td>
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<td>100 ± 38 %</td>
<td>21100 ± 38 %</td>
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<tr>
<td>Scheme 3 (incorporates desensitisation)</td>
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<th>$\beta_{i_a}$ (s$^{-1}$)</th>
<th>$E_{i_a}$</th>
<th>$\alpha_{i_b}$ (s$^{-1}$)</th>
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<td>27</td>
<td>536000 ± 97 %</td>
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Table 11.1. (previous page) Comparison of the rate constants estimated for wild type nAChRs using a variety of different kinetic schemes.

An assumption that has been included in all of the schemes tested in this text is that of independence between the ACh binding sites; that is that the ACh association and dissociation rates at site $a$ and independent as to whether site $b$ is occupied or not. This may well not be the case, however with this assumption good fits of HJC distributions to the single channel data are obtained. It has been shown previously (Colquhoun et al, 2003) that reliable estimates of the rate constants when the ACh binding sites are assumed to be non-independent cannot be obtained unless the number of channels in a patch obtained at low concentration is known. Furthermore it is desirable to describe the receptor with as few free parameters that still correspond to the functional and structural information available about the receptor.

It has been found that εL221F mutation in human nAChRs, near the start of the M1 region) seemed to have a considerably greater effect on the $a$ site than on the $b$ site, (Hatton et al, 2003) and quite good fits could be obtained for the mutant data could be obtained with the rate constants for the $b$ site fixed at their wild-type values. This allows the speculation that the $a$ (low affinity ) site corresponds to the αe ACh binding site. With the mutation investigated here, εL78P, the effects on monoliganded rates are not sufficiently clear to confirm or contradict this speculation.

It has been suggested that the αδ site corresponds to the site with higher ACh affinity based on studies of the CMS mutation εN182Y and the homologous mutation in the δ subunit, δN187Y (Sine et al, 2002b). However these studies relied on using maximum likelihood fitting to a sequential scheme, and this is inappropriate to assess the two different binding sites as outlined below.

```
R  k+1 → AR  k+2 → A2 R  β2 → A2 R*
  k-1   k-2   α2
```

The above simple linear scheme is the simplest kinetic scheme that can be used for the muscle nicotinic acetylcholine receptor (nAChR). It takes into account the presence of two ACh binding sites and a distinct gating step, though it does not account for
monoliganded openings or correlations. It is sometimes mistakenly supposed that the two steps, with equilibrium constants $K_1$ and $K_2$, describe the binding of ACh at the two different binding sites. This is not the case. At rest the nAChR has two vacant ACh binding sites. The above scheme allows only a single sort the initial binding step with association rate constant $k_{+1}$ describing association of the first molecule of ACh whichever of the two sites it binds to (so the actual rate that appears in the Q matrix is $2k_{+1}$ multiplied by agonist concentration). This describes binding to a single class of binding site. As there is only one possible ACh association rate leaving the R state this scheme necessitates that at rest the two binding sites are identical. Likewise there is only one rate constant, $k_{-2}$, for leaving the diliganded state. Once in the AR state a second ACh association (with rate $k_{+2}$) must occur at the vacant site for efficient activation of the receptor. If $k_{+1} = k_{+2}$ and $k_{-1} = k_{-2}$ then the ACh binding sites are independent of each other and behave exactly like two entirely separate and identical binding sites. If $k_{+1} \neq k_{+2}$ and $k_{-1} \neq k_{-2}$ then the usual interpretation would be that the binding sites show cooperativity, that is that there is an interaction between the two binding sites. This term should not be confused with the cooperativity of the response which will be seen because of the concerted conformational change, even when $k_{+1} = k_{+2}$ and $k_{-1} = k_{-2}$. When $k_{+1} \neq k_{+2}$ and $k_{-1} \neq k_{-2}$ this means that the two binding sites, although initially identical, are changed in some way by the first binding so the rates for the second binding are changed. That is, the binding rates for the second binding depend on whether the other site is occupied or not. This still does not imply any differences between the two sites because the scheme is symmetrical; binding to site $a$ is affected by the occupancy of site $b$ to exactly the same extent as the binding to site $b$ is affected by the occupancy of site $a$. The mechanisms of such interactions if they occur implies some substantial change in the receptor conformation after the first binding while the channel is still shut, as the binding sites are some 45 Å apart.

In terms of microscopic rate constants the simple linear scheme is unsuitable to describe muscle nAChRs (Wang et al., 1997; Salamone et al., 1999; Hatton et al., 2003). What is clear however is that the dissociation rates from the two sites are different, this is the case for most muscle nAChRs studied and highlights the unsuitability of using sequential kinetic schemes. The two ACh binding sites of mammalian nAChRs are also structurally different. Each site is composed of a 'plus' face from an $\alpha$ subunit and a 'minus' face from either a $\delta$ or an $\varepsilon$ subunit in the adult receptors ($\varepsilon$ is substituted with $\gamma$ in the fetal form of the receptor). As well as the differences in the 'minus' face of the
binding sites it is still possible due to the pseudosymmetry of the receptor that the two \( \alpha \) subunits (with identical amino acid sequences) could be in different conformations due to their different subunit neighbours in the resting state (Unwin et al., 2002).

Functional data can also define the differences in the binding properties between the two ACh binding sites, though the distinction is on the margin of resolvability, and this may account for the contradictory reports in the literature. Although the two sites of mouse nAChRs have been reported to have the same equilibrium affinity for ACh, they have different ACh dissociation rates (Salamone et al., 1999). In terms of physiological activity it is these dissociation rates (and the diliganded gating parameters) that determine the time course of the endplate current decay. ACh does not reach equilibrium with muscle nAChRs in the body.

This study and others (Milone et al., 1997; Ohno et al., 1997; Hatton et al., 2003) demonstrate that the ACh dissociation rates are different in the human nAChR. Scheme 1 is required once this is acknowledged. This scheme allows for initial ACh binding at either site. In this scheme the subscript number refers to the sequential binding of ACh (first or second ACh binding event) and the subscript letters \( a \) and \( b \) refer to the two different sites. If \( k_{+1a} = k_{+1b} \) and \( k_{-1a} = k_{-1b} \) then the two binding sites would be identical and in this case it would be logical to collapse scheme 2 to the simple linear scheme shown on page 194. When \( k_{+1a} = k_{+2a} \) and \( k_{-1a} = k_{-2a} \) then this dictates that there is no interaction between the two binding sites as described previously.

The addition of the competitive antagonist \( \alpha \)-conotoxin GI to nAChRs led to an increase in the proportion of the shortest, presumably moniliganded, openings, showing that it was blocking the diliganded openings. Good fits could be obtained only if it was assumed that the mixed moniliganded complexes (with one ACh and one conotoxin molecule bound) could not open. This alone is sufficient to indicate that it is unlikely that the experiment will tell us whether the \( a \) site or the \( b \) site is the one with higher affinity for conotoxin. Good fits could be obtained when it was assumed that mixed complexes could not open, but contrary to the predictions for a pure competitive antagonist, the estimate of the value for \( k_{-2a} \) (the dissociation rate for the \( a \) site) was much reduced compared with patches in the absence of \( \alpha \)-conotoxin GI, (752 s\(^{-1}\), compared with 5440 s\(^{-1}\)). This may itself be sufficient to indicate some sort asymmetrical effects on the ACh binding sites, but the fits were insensitive to which of
the binding sites was set to have the greater affinity for α-conotoxin GI, which confirms that the experiment could not distinguish whether the a site or the b site is the one with higher affinity for α-conotoxin GI.

Other studies with α-conotoxin GI highlight the importance of considering species differences when studying nAChRs. It has been shown to have two different IC50 values for the nAChR. In human adult receptors, these have been calculated as 2 nM and 140 nM using a I^{125}-α-bungarotoxin competition assays (Quiram & Sine 1998). No judgment as to which of these corresponds to the αδ site and which to the αε site can be made from this data. Competition assays were also used to estimate the IC50s of α-conotoxin GI at both Torpedo and mouse nAChRs. α-conotoxin GI has lower affinity at the αδ site on Torpedo nAChRs, but lower affinity at the αγ site of fetal mouse receptors (Groebe et al, 1997). From the amino acid residue sequences of the binding site regions (shown in figure 1.8) it is unclear whether the human αε site should be more similar to mouse or Torpedo receptors.

There are few explanations for the structural rearrangements that occur during monoliganded openings of nAChRs. The amplitude of the currents of monoliganded openings appear to be identical to that of the diliganded open channels. Therefore it is expected that the immediate local structure of the open pore around the middle of M2 is essentially identical in the case of both mono- and diliganded openings. The durations of the monoliganded openings are both much shorter than those elicited from diliganded receptors. This means that the structure of the entire receptor cannot be the same in these different types of states. Interestingly, evidence has also been presented that shows the two monoliganded open states themselves are different to each other. Due to the asymmetry in the positioning of the binding sites it is not altogether surprising the activation via one site should elicit different responses to activation via the other site. What is not known is whether a single liganded opening involves the concerted changes in conformation of all the subunits that are expected during diliganded openings. If there is a concerted conformation change, the question remains as to why to these lead to much less stable (although with similar conductance) open states when only one agonist is bound.
11.3 The slow channel congenital myasthenic mutation εL78P

The over activity of nAChRs containing the SCCMS mutation εL78P is thought to be the key event in the pathology of the disease. The muscle weakness observed is due to degradation of the end plate region. This is thought to be caused by excess Ca$^{2+}$ entry into the muscle cytosol which may activate the apoptotic machinery. The source of the Ca$^{2+}$ is probably from the sarcoplasmic reticulum stores rather than through direct entry through the nAChRs.

Many different mutations can cause congenital slow channel myasthenic syndromes, but there appears to be no direct correlation between the severity of the mutation on channel function, and the severity of the symptoms experienced, e.g. (Croxen et al., 1997; Milone et al., 1997) The εL78P mutation is responsible for very long bursts of activation and synaptic current decay is predicted to be slowed 11-fold compared to that seen with wild type receptors, but the symptoms are mild compared to other cases of SCCMS. It is a reasonable hypothesis that in patients with the εL78P mutation there is an upregulation in the expression of the fetal γ subunit. This would explain why a severe mutation in the ε subunit does not lead to severe symptoms. It in other cases SCCMS due to ε subunit mutations this upregulation of the γ subunit has been detected (Engel et al., 1996; Ohno et al., 1997).

An interesting finding to come from the analysis of the εL78P nAChR is that most of the long pathogenic activations actually originate in the monoliganded α state. This highlights the importance of considering the monoliganded open and monoliganded shut states even when examining events that primarily involve only long bursts. This find also highlights the importance of taking into account missed events in the HJC distributions. As the monoliganded openings are brief, many will be omitted from the idealisation of the single channel record. As a result, the time spent in the shut states between the end of the monoliganded opening and the entry into the diliganded open state will be excised from the burst length durations.

11.4 Further work

The complete description of the kinetic scheme of the nAChR is turning out to be considerably more complex then that envisaged by Katz and colleagues in the early fifties. Much of this complexity arises due to the fact that the nAChR is a heteromeric protein, with two different ACh binding sites. However it is possible to describe
features that should be included in the complete scheme and some of the rate constants can be estimated quite precisely.

In a full scheme, desensitisation should be accounted for. Although not physiologically important in the context of wild type human muscle nAChRs, the phenomenon could be important in the cases of certain congenital myasthenic mutations e.g. (Milone et al., 1997). In these cases it would be useful to have a kinetic framework in which to examine the effects of the mutation on desensitisation. A full kinetic scheme for the muscle nAChR is shown in below, open states are indicated with a *, desensitised states are indicated by a D.

This scheme accounts for the possible occupancies of the two ACh binding sites in the resting state, and their associated open and desensitised states. It is a logical extension of the scheme first proposed by Katz, though it is clear that incorporation of only one desensitized state is still too simple (Elenes & Auerbach, 2002). For clarity the equilibrium arrows have been drawn as single lines. The central blue cycle highlights the resting state in various degrees of agonist ligation. The upper red cycle highlights the open states. The lower green cycle highlights the desensitised states. This scheme is based simply on the different ligation states of the nAChR and the fact that there are resting, open, and desensitised states.

In the form shown, there are too many rates constants to be estimated without applying constraints on some of the rate constants. Some states (like R*, the unliganded open
state) are visited so rarely that there is little or no information about them. So it seems that the above scheme could be simplified.
12. Appendix

The following is a list of schemes used for the kinetic analysis in this text.

Key:
- R  Receptor
- A  Agonist molecule
- B  Antagonist molecule
- *  Open state
- $\alpha$, $\beta$  Rate constants describing protein conformational changes.
- $k_+$  Agonist association rate constant (multiplied by agonist concentration during fitting).
- $k_-$  Agonist dissociation rate constant.
- $q_+$  Antagonist association rate constant (multiplied by concentration during fitting).
- $q_-$  Antagonist disassociation rate constant.

Scheme 1:

\[
\begin{align*}
AR^* & \xrightarrow{\beta_1} A_R \xrightarrow{\alpha_1} AR^* \\
AR & \xrightarrow{k_{+1a}} A_{2R} \xrightarrow{\beta_2} A_{2R}^* \\
R & \xrightarrow{k_{-1b}} RA \xrightarrow{k_{+2a}} RA^* \\
A_{2R} & \xrightarrow{\alpha_2} A_{2R}^* \\
RA & \xrightarrow{k_{-a}} RA^* \xrightarrow{k_{-b}} RA \\
RA^* & \xrightarrow{\beta_{1b}} RA \xrightarrow{\alpha_{1b}} RA^*
\end{align*}
\]
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