Chapter 1 Introduction

1. The nicotinic acetylcholine receptor

Acetylcholine receptors, receptors for the neurotransmitter acetylcholine (ACh), exist in two forms: *muscarinic* and *nicotinic.* This was deduced from the way that the mushroom alkaloid toxin muscarine from the fungus *Amanita muscaria* and nicotine from *nicotiana tabaccum,* both mimic the action of acetylcholine at these receptors (Rang *et al*., 1999).

Muscarinic acetylcholine receptors (mAChRs) are 7 transmembrane G protein coupled receptors (Aidley, 1998), which deliver slow synaptic transmissions. They are found on smooth and cardiac muscles and in endocrine glands. mAChRs have also been detected in epithelial, endothelial and immune cells (Wessler *et al*., 1998; Kawashima and Fujii, 2000; Wessler and Kirkpatrick, 2001).

In contrast, nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels located in the plasma membranes of cells that mediate rapid synaptic transmission throughout the nervous system, particularly at vertebrate neuromuscular junctions (and also in the electric organs of the electric fish *Torpedo* and *Electrophorus*). nAChRs are present in many tissues in the body in addition to nerve and muscle. There are two forms of nAChRs - muscle and neuronal. Muscle type nAChRs are found in the neuromuscular junctions of somatic muscles (and also in the electric organs of the electric fish *Torpedo*

and *Electrophorus*). Neuronal type nAChRs are found in nerves of the central and peripheral nervous systems. Molecular biological and pharmacological studies have subsequently shown that nAChRs are also present in non-neuronal cells including human immune cells. Lymphocytes and the thymus express messenger RNA for nAChR subunits (Hiemke *et al*., 1996; Mihovilovic *et al*., 1998; Sato *et al*., 1999; Kawashima and Fuji, 2000; Lustig *et al*., 2001). Other non-neuronal sources of nAChRs include human and rodent bronchial epithelial cells; human epithelial cells from the alimentary tract and epidermal keratinocytes (Grando *et al*., 1995, 1996; Maus *et al*., 1998; Wessler *et al*., 1998; Wessler and Kirkpatrick, 2001). mRNA coding for the nAChR subunits has also been found in adult vertebrate muscle (Corriveau *et al*., 1995; Romano *et al*., 1997; Fischer *et al*., 1999).

Ligand-gated ion channels are pentamers that share certain structural features (Stroud *et al*., 1990; Cooper *et al*., 1991; Arias, 1997). The subunits of these receptors form agonist-binding sites in their extracellular domain and gated ion-conducting channels with the cell membrane (Karlin, 1993; Arias, 1997). Each protein comprises an oligomeric complex of homologous, four membrane spanning subunits. These have a common arrangement with a 15-residue, cysteine-loop between amino acids 128 and 142, which corresponds with the nAChR α subunit in *Torpedo* (Karlin, 1993; Sargent, 1993; Arias, 1997). A large number of genes have been cloned that encode subunits of nAChRs. It has been proposed that these subunits may be divided into subfamilies on the basis of both gene structure and mature protein sequence (Cordero-Erausquin *et al*., 2000; Corringer *et al*., 2000) and are summarised in Table 1.1.

nAChR subunits	Tribes	Sub-families	Composition	Physiology	Locations
α9	I	I	Homopentamers	: α-BTX sensitive : α7, α8: very fast desensitisation	α9: cochlea, olfactory bulb α7: limbic system, cortex α8: only found in chick
α7	II	II			
α8					
α5	III-3	III	Heteropentamers: α from III-1 β from III-2 β from III-2 or III-3	: α-BTX insensitive : β2-containing nAChR: Cyt<nic, DMPP; fast desensitisation : β4 containing: Cyt>nic, DMPP; slow desensitisation	α5: cortex, monoamine containing nuclei α3 β4: PNS, habenulo-interpedunuclaer system α4β2: thalamus, cortex α2: interpeduncular nucleus
β3					
α4	III-1				
α2					
α6					
α3					
β4	III-2				
β2					
α1	IV-1	IV	Heteropentamers: α1 γ α1 δ β1 Clock wise	: α-BTX sensitive	Skeletal muscle
β1	IV-3				
δ	IV-2				
γ					
ε					

Table 1.1 The nAChR known 16 subunits are classified into subfamilies and groups on the basis of both gene structure and mature protein sequence.

Members of subfamilies I and II (such as α7, α8 and α9) when expressed alone, are able to form functional homopentameric receptors (Couturier *et al*., 1990; Schoepfer *et al*., 1990; Elgoyhen *et al*., 1994; Gerzanich *et al*., 1994). In contrast, members of other subfamilies do not form functional homopentameric receptors and require subunits from other subfamilies to form functional receptors (e.g. α3β4) (Anand *et al*., 1991; Cooper *et al*., 1991; Karlin, 1993; Karlin and Akabas, 1995; Machold *et al*., 1995; Ramirez-Latorre *et al*., 1996; Wang *et al*., 1996; Groot-Kormelink *et al*., 1998).

1.1 Structure of nicotinic acetylcholine receptors

The most concentrated source of nAChRs is found in electric fish, such as *Torpedo* and *Electrophorus*, where the structure of these receptors was first determined. In *Torpedo* voltage-generating cells, are rich in cholinergic post-synaptic membrane containing nAChR (Aidley, 1998). The molecular mass of nAChR is 285-290 KDa. Sodium-dodecyl sulphate polyacrylamide gel (SDS-gel) electrophoresis shows the receptor to be a pentamer comprising four different polypeptides whose molecular masses are 40, 50, 60 and 65 KDa respectively. These are known as the α, β, γ and δ subunits (Aidley, 1998).

Muscle type nAChR is a glycoprotein membrane with five subunits (two α1, one β, one γ and one δ ($\alpha_2\beta\gamma\delta$) (Karlin, 1991). Each α-chain contains one binding site for ACh, so that there are two binding sites with the neighbouring cysteines at amino acid position 192-193 on α-subunit per receptor (Figure 1.1) (Raftery *et al.*, 1980; Popot and

Changeux, 1984; Changeux, 1995; Karlin and Akabas, 1995). Both sites interact in a positive co-operative manner and both sites must be occupied by ACh, or a nicotinic agonist, to induce channel activation. Binding sites on α subunits accounts for the majority of agonist binding sites since non-α subunits do not bind ACh. However, binding sites for ACh are located in the cleft between each α-subunit and its neighbouring γ or δ subunit (Karlin and Akabas, 1995). In the muscle type nAChR of mammalian adults the γ subunit is replaced by an ε subunit (Mishina *et al*., 1986; Gu *et al*., 1990; Camacho *et al*., 1993).

Unwin (1993, 1995) identified the detailed structure of nAChRs in the electric organ of *Torpedo*. In the receptor's resting state, five subunits are located at equidistant points on the circumference of the central axis (Figure 1.1). Thus, the receptor is cylindrical with a diameter of about 65 Å. The receptor projects about 60Å on the synaptic (extracellular) side of the membrane and to about 20 Å on the cytosolic (intracellular) side. The pore for the channel runs symmetrically along its axis. (Figure 1.1)

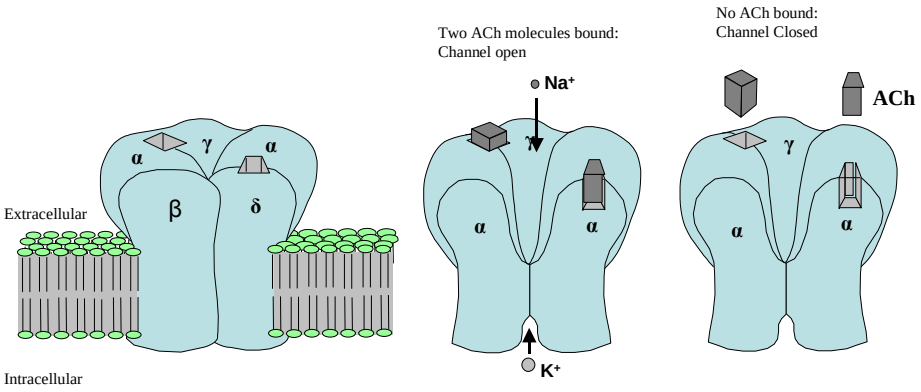


Figure 1.1 The muscle type nAChR channel comprises of five subunits, of which two α subunits are separated by the γ subunit. When two molecules of ACh bind to α subunits on the membrane surface, the receptor channel changes shape. A pore opens in that part of the channel, which is embedded in the lipid bilayer and cations (e.g. K^+, Na^+) flow through the opening channel (Adapted from Kandel *et al.*, 2000).

Nicotinic acetylcholine receptors found in the nervous system are referred to as neuronal nAChRs. Neuronal nAChRs share the same overall structure and functional features of muscle nAChRs, but generally comprise two α, and three β subunits, and also involve different subunits to those expressed in muscle. Nine α subunits (2α-α10) and three β subunits (β2-β4) have been identified in neuronal type nAChRs (Cooper *et al*., 1991; Deneris *et al*., 1991; Elgoyhen *et al*., 1994; Arias, 1997; Gotti *et al*., 1997; Lustig *et al*.,

2001). The α subunits are characterised by the presence of adjacent cysteines, which would be located only a few residues before the start of the M1 segment (this corresponds to cysteine 192 and cysteine 193 in the *Torpedo* α subunit). These pairs of cysteines residues are held to indicate the presence of an acetylcholine-binding site. Hence α subunits have also been referred to as "agonist binding subunits"; whereas β subunits have been called "structural subunits" on the presumption that they contribute to the formation of the nAChR channel but play no role in agonist recognition (Deneris *et al*., 1991; Arias, 1997; Corringer *et al*., 2000). However, the α5 subunit appears to be incapable of binding agonist because it lacks another key residue, tyrosine, found in all other α subunits upstream of the pair of cysteine residues mentioned above and is only found in nAChR containing other α subunits (Conroy *et al*., 1992; Wang *et al*., 1996; Corringer *et al*., 2000; Paterson and Nordberg, 2000;) (Figure 1.2).

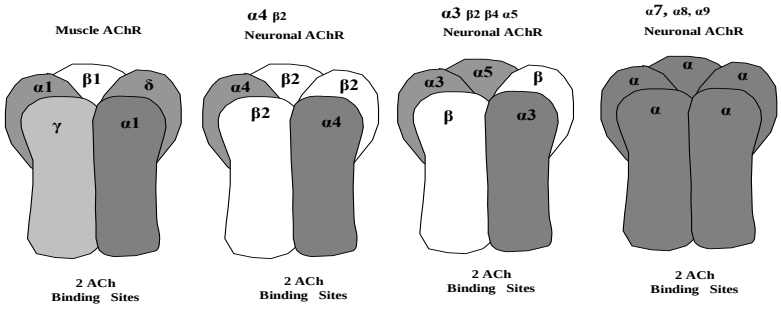


Figure 1.2 A pentameric structure of nAChR, subunit stoichiometry and a number of binding sites of muscle type, neuronal heteromeric α4β2, and α3β2β4α5 types and homomeric α7, α8, and α9 nAChRs (Adapted from Paterson and Nordberg, 2000).

α2, α3, or α4 subunits produce functional nAChRs with β2 or β4 subunits (Anand *et al*., 1991; Cooper *et al*., 1991; Arias, 1997; Paterson and Nordberg, 2000). In contrast, the α5 and β3 subunits are generally unable to combine successfully in pairwise combinations, but they have been expressed in heteromeric combinations with at least two other subunits types (Conti-Tronconi *et al*., 1994; Galzi and Changuex, 1995; McGehee and Role, 1995; Ramirez-Latorre *et al*., 1996; Wang *et al*., 1996; Groot-Kormelink *et al*., 1998; Lukas *et al*., 1999).

The α6 subunit has been shown to form a functional nAChR in combination with β4 subunits (Gerzanich *et al*., 1997). However, when α6 subunits are expressed in more complex combinations of α6α3β2, α6α4β2, or α6β4β3, they form functional channels, but the significance of these receptors is not clear (Gerzanich *et al*., 1997; Fucile *et al*., 1998; Kuryatov *et al*., 2000; Champtiaux *et al*., 2002).

A major subtype of neuronal nAChRs is the combination of α7 subunits, which are thought to form homomeric nAChRs in the CNS and peripheral nervous system (Couturier *et al*., 1990; Keyser *et al*., 1993; Sharples and Wonnacott, 2001). Palma *et al*., (1999) provided evidence for an α7β3 hetero-oligomer assembly in gene transfection experiments in *Xenopus oocytes*. In chick retina the α7 subunit forms heteromeric nAChRs with α8 and may combine with other subunits such as α5 in chick sympathetic neurones (Keyser *et al*., 1993; Gotti *et al*., 1997; Yu and Role, 1998). α8 and α9

subtypes have been identified in avian brain tissue and in the mammalian epithelium respectively (Schoepfer *et al*., 1990; Elgoyhen *et al*., 1994). To date, no such combinations have been found in mammalian cells.

In conclusion, nAChRs can be divided into neuronal and muscle type receptors based on their distribution. Furthermore, molecular studies reveal that these receptors can be further subdivided based on the subunits found in each receptor.

1.2 The Transmembrane structure of nAChR subunits

Binding of ACh to subunits of nAChR induces conformational changes that lead to opening of the channel and membrane depolarisation. An analysis of the amino acid sequence of nAChR has uncovered significant subunit homology between the neuronal and muscle types (Changeux and Eldstein, 1998). The ion channel has three parts. It has an extracellular N-terminal domain that consists of the neurotransmitter binding site. This is the main immunogenic region and is also a glycosylation site. There is a transmembrane domain comprising four membrane-spanning segments (M1-M4), of these, at least one of the M2 segments lines is the ion channel. Lastly, there is either a cytosolic entrance domain or another hydrophilic domain of variable length between M3-M4 (this is exposed to the cytoplasm) and it contains a number of functional phosphorylation sites (Figure 1.3) (Devillers-Thiery, 1993; Galzi and Changeux, 1995; Changeux and Edestein, 1998).

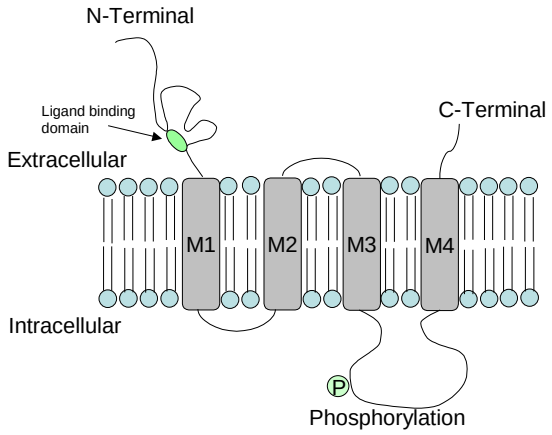


Figure 1. 3 The nAChR sequence showing the hydrophilic extracellular domain containing the ACh binding site, four transmembrane segments M1-M4, the intracellular hydrophobic domain and the small C terminal domain. The hydrophilic domain separating M3 from M4 faces the cytoplasm. It contains functional phosphorylation sites (Adapted from Paterson and Nordberg, 2000).

The inner lining of the receptor (M2 helices) comprises a ring of hydrophilic leucine amino acid side chains near the centre of the bilayer. These are positioned so close together that they prevent ions from passing through the channel. When the two acetylcholine receptor molecules bind to α subunits the allosteric conformational structure is changed. This leads to a slight twisting of the M2 helices, which draw the hydrophobic side chains away from the outer end of the channel and opens it up for the

passage of ions (Figure 1.4A and 1.4B) (Revah *et al*., 1990; Changeux *et al*., 1992; Galzi and Changeux, 1995; Changeux and Edestein, 1998).

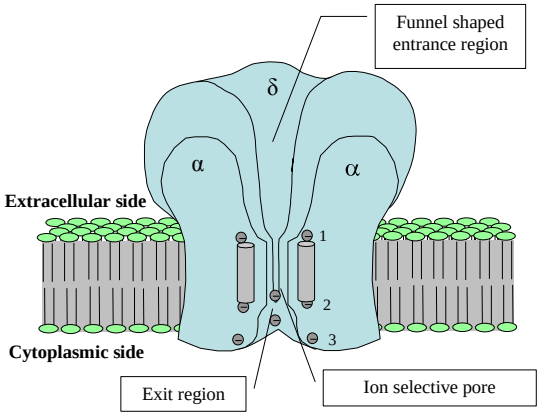


Figure 1.4A Functional model of nAChR (Adapted from Kandel *et al*., 2000).

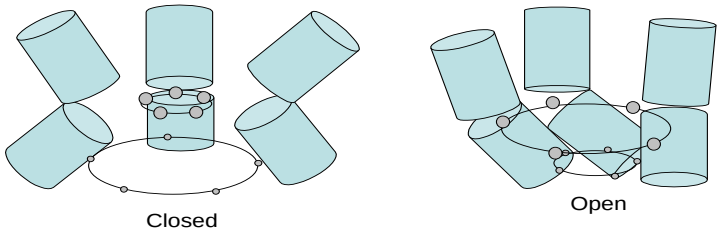


Figure 1.4B Model of the structure of the channel gating (Adapted from Kandel *et al*., 2000).

1.3 The ligand binding sites

As well as being subdivided by structure, nAChRs can also be subdivided by ligands that bind specifically to nAChRs containing particular combinations of subunits.

nAChR ligands can be classified into three main classes:

(1) Agonists such as acetylcholine (ACh), Nicotine (Nic), Epibatidine (Epi), ABT-418, Cytisine (Cyt), (+)-Anatoxin, anabaseine, RJR-2403, SIB-1765F, dimethyphenylpeperazinium (DMPP).

(2) Antagonists such as d-Tubocurarine, α-bungarotoxin (α-BTX), neuronal bungarotoxin (n-BTX), dihydro-β-Erythoidine (DHβE), Methyllcoconitine

(MLA), Mecamylamine (Mec); Clorisondamine (Chl), Strychnine, α-conotoxin IMI, α-Conotoxin MII.

(3) Allosteric ligands. These compounds may either stimulate or inhibit nAChR function by binding to regulatory site (allosteric site) other than the ACh binding site (Gotti *et al*., 1997; Paterson and Nordberg, 2000). These ligands produce their effect by acting at one of a number of ligand binding sites.

1.3.1 Acetylcholine (ACh) binding site

A number of studies, performed with *Torpedo*, muscle, and neuronal nAChRs identified ACh binding sites in the receptor structure (Galzi and Changeux, 1995; Karlin and Akabas, 1995). In hetero-oligomeric neuronal nicotinic receptors, two ACh binding sites are thought to exist at the interface between α and β subunits (Figure 1.5A) (Alkondon and Albuquerque, 1993; Corringer *et al*., 2000). However, in the homo-oligomeric α7, α8, α9 receptors, five identical ACh binding sites are formed due to the identical nature of α subunits making up the receptor protein (Figure 1.5A) (Bertrand *et al*., 1992; Conti-Fine *et al*., 1994; Corringer *et al*., 2000; Paterson and Nordberg, 2000).

ACh binds to amino acids on three loops (A, B and C Figure 1.5), from the α subunit and at least three loops (D, E and F) which are identified as a complementary component and are present on the β, γ or δ subunits. These loops are also found on the α subunit of homopentameric neuronal receptors and on β-subunits of heteromeric neuronal nAChRs

(Figure 1.5B) (Corringer *et al*., 1995; Galzi and Changeux, 1995; Chiara and Cohen 1997; Martin and Karlin, 1997; Changeux and Edelstein, 1998; Changeux and Edelstein, 2001; Corringer *et al*., 2000). These residues, from loop A, B and C, are a highly conserved from subunits α1 to α8 (except for α5, which has a higher sequence homology with the β subunit than with any α subunit. This subunit also lacks the principal component competitive binding) (Le Novére and Changeux, 1995; Changeux and Edelstein, 1998; Corringer *et al*., 1998). Therefore, α5 subunits do not form functional nAChRs as homomers or in pair with β1, β2, or β4 (Wang *et al*., 1996). In contrast, the amino acids in loops D, E and F may vary (Changeux and Edelstein, 1998; Corringer *et al*., 1998) and may explain ligand subunit selectivity.

In muscle type nAChR both α subunits are identical in amino acid sequence and the high and low affinity binding of agonists to the receptor is conditioned by the interaction between the α subunit and the γ or δ subunits. The high affinity site for ACh and the d-tubocurarine low affinity binding site are both located in the α subunit with the δ subunit (α/δ) (Arias, 1997). In contrast, the ACh low-affinity binding site and the d-tubocurarine high affinity-binding site are located at the interface between the α subunit and the γ subunit (α/γ) (Kubalex *et al*., 1987; Toyashima and Unwin 1990; Chiara and Cohen, 1992; Unwin 1993, 1995; Arias, 1997).

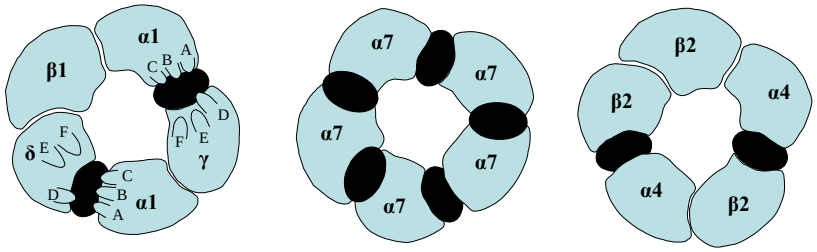


Figure 1.5A Subunit arrangement of muscle type nAChRs (left), and homomeric α7 and heteromeric of neuronal type nAChRs with 2 or 5 subunits (middle and right). The agonist binding sites are indicated by black shaded pockets, which contributed by loops A, B and C of the "principal component" and loops D, E, and F of the "complementary component" (Modified from Corringer *et al*., 2000).

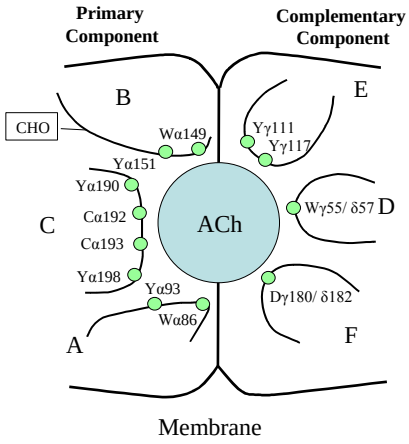


Figure 1.5B Ligand binding sites of nAChR. The principal component that consists of three loops A, B and C on the α subunit; the complementary component, consisting of loops D, E and F which are present on the γ or δ subunits of muscle type receptor - an α subunits of homopentameric or β subunits of heteropentameric neuronal nAChRs (Modified from Changeux and Edelstein, 2001).

1.3.2 Allosteric binding sites

Physostigmine, steroids, ethanol and Ca^{+2} ion channel blockers modulate the activity of neuronal nAChRs by modifying the properties of nAChRs via interactions with sites (allosteric sites) other than classical binding sites, which are insensitive to ACh (Paterson and Nordberg, 2000). These binding sites and the compounds, which activate them, have

not been studied in this project since they do not distinguish between different nAChR subtypes. The location of these allosteric sites are summarised diagrammatically in Figure 1.6.

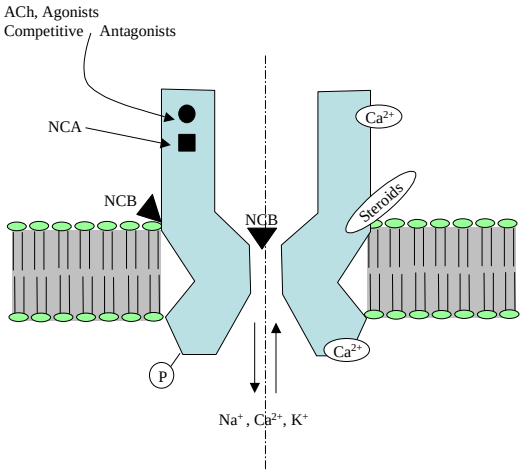


Figure 1. 6 Cross-section of nAChR. The ion channel, ACh binding site and multiple allosteric sites are located throughout the N - terminal extracellular domain. These allosteric sites include the non- competitive allosteric activator site (NCA); non-competitive sites (NCB); binding sites for Ca^{+2} and steroids and phosphorylation (P) sites (modified from Lena and Changeux, 1993).

1.3.3 Receptors desensitisation by phosphorylation

The phosphorylation of defined residues within the cytoplasmic loop of nAChRs by protein kinase A, protein kinase C or tyrosine kinase results in a desensitisation of nAChR (Huganir and Greengard, 1990; Hoffman *et al*., 1994). This desensitisation is further enhanced indirectly because of the intracellular changes in the concentration of Ca^{2+} and activation of Ca^{2+} sensitive protein kinases by these enzymes (Paterson and Nordberg, 2000).

1.4 Transition states of nAChRs

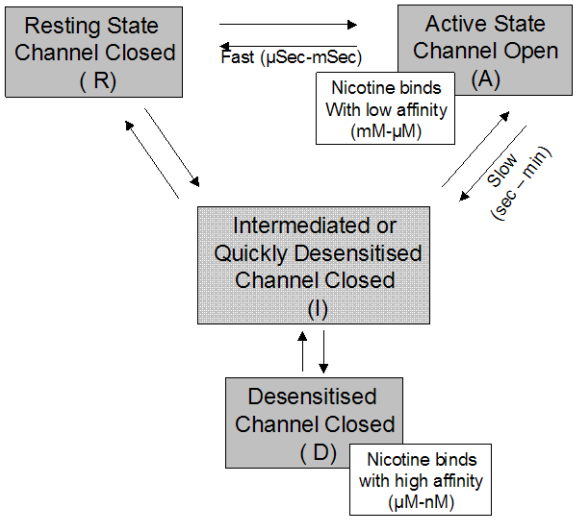


Figure 1. 7 Simplified scheme of converting states of nAChRs

The nAChRs are said to be allosteric because they regulate by means of chemical signals involving specialised proteins, which carry at least two topographically distinct sites. One is the binding site that carries the regulatory signal and other is the biologically active site (Monod *et al*., 1963; Galzi and Changeux, 1995).

The nAChRs may exist in several conformational states (Figure 1.7). These are a resting (R), or closed state, where the agonist is absent; and an activated state A, where the channel is opened (on a microsecond to millisecond time scale) which has a low affinity for ACh (K_d = 60-100 μM). There are also two desensitised refractory states I and D that are refractory to activation on a millisecond to minute time scale but which exhibit high affinity for ACh (K_d for ACh ranging from about 10 μM for the I state to 10nM for the D state) and nicotinic ligands (Galzi and Changeux, 1995; Changeux and Edelstein, 1998). Binding of ligands to the nAChR structure either at the ACh site or any of the allosteric sites can modify the equilibrium between the different conformational states of the receptor at any one time. Additionally, ligands binding to the nAChR can be considered to differentially stabilise the conformational state to which they preferentially bind (Lena and Changeux, 1993; Galzi and Changeux, 1995). Agonists initially stabilise the activated (open) state whereas competitive antagonists preferentially stabilise the nAChR in closed state, either the resting or desensitised configuration (Figure 1.7) (Galzi and Changeux, 1995; Bertrand *et al*., 1992).

Both muscle and neuronal nAChRs generally exist in one of three functional states: resting (or closed), open, or desensitised. Each functional state may reflect many discrete

conformational states with different pharmacological properties. The nAChRs in the resting state will, in the presence of agonist, undergo a fast transition to open state, called gating, and for most agonists also a transition to desensitised state. Because the desensitised state often exhibits higher affinity than the open state, most of the receptors will be in the desensitised state after prolonged agonist exposure. In the desensitised state, although the channel is closed and the receptor refractory, nAChRs still display a higher affinity for agonist binding (Sharples and Wonnacott, 2001). Activation and desensitisation of nAChRs correspond to transition between a small number of discrete structural states with distinct binding properties and ion channel conductance (Lena and Changeux, 1998; Changeux and Edelstein, 2001; 2005). The rates of desensitisation and recovery differ between nAChR subtypes; for example, the homo-oligomers α7 and α8 nAChRs display very rapid desensitisation (Couturier *et al*., 1990; Revah *et al*., 1991; Gerzanich *et al*., 1994; Gotti *et al*., 1994; McGehee and Role, 1995). In contrast, hetero-oligomeric nAChRs exhibit activation and desensitisation transitions with kinetics that vary, depending on the subunit composition (Galzi and Changeux, 1995). Prolonged agonist exposure may produce an inactivated state, from which recovery is very slow; the α4β2 neuronal nAChR is prone to inactivation on chronic nicotine treatment (Kuryatov *et al*., 2000; Sharples and Wonnacott, 2001).

Since desensitised nAChRs still bind ligands such desensitisation is more likely to be a factor in experiments where a functional response is measured as opposed to experiments where the binding of ligands to a receptor is measured.

1.5 The pharmacological diversity of nicotinic acetylcholine receptors

The pharmacological and functional properties of nAChRs vary depending on the subunit composition of the receptor and the species studied (Deneris *et al*., 1991; Role 1992; Sargent 1993; McGehee and Role, 1995; Corringer *et al*., 2000). This has led to the identification of selective ligands that are invaluable in identifying and characterising nAChRs on tissues. Many of functional experiments conducted with nAChRs have used artificial systems where receptors are expressed in oocytes or have studies cell lines.

Increased Ca^{+2} permeability induced by activation of nAChRs is of particular physiological importance to regulation of muscle activity. Neuronal nAChRs are more permeable to calcium ions than muscle nAChRs (Bertrand *et al*., 1993*a*; 1993*b*; Galzi and Changeux, 1995; McGehee and Role, 1995; Role and Berg, 1996). Among the neuronal nAChRs, α7 nAChRs are even more permeable to Ca^{+2} (McGehee and Role, 1995). The heteromeric nAChRs containing combinations of α and β subunits show greatest affinity for nicotinic agonists such as epibatidine, nicotine and cytisine, whereas homomeric α7, α8 and α9 receptors showed high affinity for α-BTX (McGehee and Role, 1995; Alexander and Peters 1999; Paterson and Nordberg, 2000). The desensitisation of nAChRs are also different depending on the subunit composition, for example, α3β2, α7, α8 heteromeric, and α7, α8 homomeric nAChRs desensitise very quickly, but α3β4 nAChRs desensitise slowly (McGehee and Role, 1995).

Nicotine binds to α4β2 nAChRs with high affinity (K_i=1-11 x 10^{-9}M) whereas it has a low affinity for α7 nAChRs (Ki= 400-8900x10^{-9}M). α8 nAChRs are more sensitive than α7 nAChRs to nicotine and other agonists (Gotti *et al*., 1997). Nicotine activates α4β2 nAChRs with an EC_{50} of 1 x10^{-6} M, compared with 500x10^{-6} M for α7 nAChRs and 200 x 10^{-6}M for α7/α8 nAChRs (Gotti *et al*., 1994; 1997). The α9 and α9/α10 nAChRs are unique in that nicotine is antagonist at these subtypes (IC_{50} = 31x10^{-6} M and 4 x10^{-6} M respectively) (Elgoyhen *et al*., 1994; 2001).

Epibatidine is the most potent nicotinic agonist at nAChRs and is a useful ligand for characterising receptors. It is the most potent nicotinic agonist for nAChRs and binds to nAChRs in brain tissue with an affinity of about 10-100x10^{-12}M (Houghting *et al*., 1995). Epibatidine has a particularly high affinity for α4β2 nAChRs (K_i=1.9x10^{-11}M) and about 10-100 fold lower affinity for α3β2 nAChRs (Ki =230 x10^{-12}M), and α3β4 nAChRs (Ki=380 x10^{-12}M) (Gerzanich *et al*., 1995; Sharples and Wonnacott, 2001). Binding affinity for epibatidine at α7 nAChRs is about 10,000-fold lower than at α4β2 nAChRs (Ki ~200 x10^{-9}M). The functional potency of epibatidine is also particularly high, with sub-micromolar, EC_{50} values for α4β2, α3β2, α3β4 and α8 neuronal nAChRs (Gerzanich *et al*., 1995).

Cytisine, a nicotinic ligand, is a potent agonist for all β4 containing nAChRs; but has a low efficacy at all β2-containing nAChRs. Thus, cytisine behaves as a partial agonist at α4β2 and α3β2 nAChRs (Luetje and Patrick, 1991). This emphasises the importance of the β subunit in determining agonist interactions with neuronal nAChRs, and cytisine can

antagonise the interaction of ACh with β2 containing nAChRs (Papke and Heinemann, 1993) reflecting its partial agonist activity.

The snake toxin α-bungarotoxin (α-BTX) is a specific and almost irreversible antagonist of mammalian α7 nAChRs. It binds specially to α7 nAChRs with an affinity of about 1 x 10^{-9}M (Couturier *et al*., 1990; Keyser *et al*., 1993; Sharples and Wonnacott, 2001). Low nanomolar concentrations ($10x10^{-9}$ M) of α-BTX effectively block ACh-induced responses mediated by α7 nAChR. This blockade is reversible only very slowly, but the size and kinetics of α-BTX can be a disadvantage in practice. However, α-BTX does not block other α/β heteromeric neuronal nAChRs making it a useful tool for receptor characterisation.

nAChR composed of α4β2 subunits are predominantly found in the CNS and bind nicotine and ACh with high affinity (Nordberg, 1993; Holladay *et al*., 1997). It has been reported that chick α4β2α5 nAChRs expressed in oocytes have about 125 times lower EC_{50} for ACh and twice the conductance of α4β2 nAChR (Ramirez-Latorre *et al*., 1996). So nAChR α4β2 subunits with α5 subunits are more sensitive to nicotine and ACh than α4β2 without α5 subunits. Thus, in functional studies nicotine can be used to distinguish α4β2 nAChRs containing α5 subunits.

nAChRs containing α4 subunits are more sensitive to activation by nicotine and acetylcholine than α3 subunit containing receptors. α4 containing receptors were also

more sensitive to desensitising levels of nicotine than α3 containing receptors. The presence of α4 subunits also influenced the rate of recovery from desensitisation; in that the rate was inversely proportional to the apparent affinity of nicotine for the desensitised state (Deneris *et al*., 1991; Fenster *et al*., 1997; 1999). nAChRs containing β2 subunits are desensitised by low concentrations of nicotine achieved near-maximal desensitisation more rapidly than β4 containing receptors. α3β2 containing receptors can be distinguished by their sensitivity to the neuronal bungarotoxin (e.g. K-bungarotoxin, toxin F and bungarotoxin 3.1) (Deneris *et al*., 1991). Nicotine is a full agonist for α3 neuronal nAChR that includes α5 subunits (Conti-Tronconi *et al*., 1994; Galzi and Changeux, 1995). In contrast, nicotine is only a partial agonist for α3 neuronal nAChRs that does not include the α5 subunits (Wang *et al*., 1996). Thus, in functional studies nicotine can be used to distinguish α3 nAChRs containing α5 subunits.

The α5 subunits are closely related in sequence to β3 subunits (Wang *et al*., 1996). The α5 subunits do not form functional nAChRs as homomers or in pair with β1, β2 or β4 (Wang *et al*., 1996). Thus, like β1 subunit α5 subunits may not be able to form ACh-binding sites by assembling with an appropriate interface (Wang *et al*., 1996). The presence of α5 subunits increases the rate of desensitisation of both nAChRs containing α3β2 and α2β4 (Wang *et al*., 1996).

α-conotoxin MII, a peptide with nicotinic antagonist activities, binds specifically to the α3β2 receptor (Harvey *et al*., 1997; Corringer *et al*., 2000). (±) Epibatidine also binds to

α3 containing nAChRs including, α3β2, α3α5β2 and α3β4 (Wang *et al*., 1996; Flores *et al*., 1992). However, α3 and α6 subunits show considerable sequence identity (80% in the ligand-binding extracellular domain). Thus, designing ligands to distinguish between α3 and α6 is particularly challenging and α-Conotoxin MII binds potently with high affinity for both α3β2 and α6 containing nAChRs. Thus, this toxin may not distinguish well between some subtypes containing α3 and α6 nAChR subunits (McIntosh *et al*., 2004).

α3 nAChR expressed in SH-SY5Y (human neuroblastoma cells) can be divided into 2 groups. Those with β2 subunit combinations (α3β2, α3β2α5, α3β2β4, and α3β2β4α5) has a higher apparent affinity for epibatidine than those without β2 subunits (α3β4, α3β4α5) (Wang *et al*., 1996). Making epibatidine a useful ligand to distinguish β2 containing nAChRs.

Receptors composed of α7 subunits or combination of α7 and α8, and α9 are sensitive to α- BTX (Anand *et al*., 1993; McGehee and Role, 1995; Luka *et al*., 1999; Paterson and Norberg, 2000). α7 nAChRs form homo-oligomeric nAChR that regulate cellular permeability to calcium in contrast to nAChR subtypes that regulate permeability to the other ions (Seguela *et al*., 1993; Castro and Albuquerque, 1995; McGehee and Role, 1995; Papke *et al*., 1996; Delbono *et al*., 1997). Thus, these receptors may be involved in many cellular functions that are dependent on the intracellular level of Ca^{2+} (Delbono *et al*., 1997). Changes in intracellular Ca^{2+} concentration results in a variety of metabolic

effects. However, α7 nAChRs desensitise very quickly (Peng *et al*., 1994). Using whole cell and outside out configurations of the patch-clamp technique Mike *et al*., (2000) examined the kinetic properties of α7 nAChR-gated currents. They compared the currents evoked by two endogenous agonists, ACh and choline. Kinetic modelling suggested that α7 nAChR had at least two non-equivalent paths to desensitised states and that choline dissociated faster than ACh from the receptor.

α9 containing nAChRs are further distinguished by their unusual pharmacology, including antagonism by strychnine, reversible block by α-BTX, and failure to respond to nicotine (Elgoyhen *et al*., 1994; Gerzanich *et al*., 1994; Gotti *et al*., 1997; Kart *et al*., 2000; Verbitsky *et al*., 2000). Functional expression studies have shown that the α9 receptor produces a physiologic response very similar to that of the cholinergic receptor that mediates the inhibitory efferent on cochlear hair cells (Yamamoto *et al*., 1997). α9 is characterised by its high calcium permeability relative to that of other nAChRs (Gerzanich *et al*., 1994; Kart *et al*., 2000) and it is also blocked by α- BTX. Verbitsky *et al*., (2000) showed that α9 nAChR displays the mixed nicotine-muscarinic properties of nicotinic cholinergic receptors.

The use of specific ligands to characterise nAChR functionally can provide insight into their structure. Using cultured rat hippocampal neurons, Alkondon and Albuquerque (1993) provided pharmacological and functional evidence for the presence of at least three distinct nAChR subtypes. They described four current types named 1A, 1B, II and III which are distinguished from one another on the basis of their kinetic and

pharmacological properties. Type 1A currents, the most commonly observed, are fast desensitising and have high sensitivity to blockade by α-BTX, κ-BTX and MLA (Methyllycacotinine). Thus, type 1A nAChR may be the same as of α7 homomeric receptors, which bind these ligands. In contrast, Type II and III currents desensitised very slowly. These were observed to be present on a small population of hippocampal neurons (Alkondon and Albuquerque, 1993; 1995; Albuquerque *et al*., 1997). Type II currents are present in 10% of neurons and were inhibited by DHβE (10 nM) but not by α-BTX. Type III currents were inhibits by Mecamylamine (1μM). A number of the agonists were useful in discriminating between the currents elicited; ACh, carbarchol, nicotine and were particularly effective in producing Type II currents, while cytisine appeared to be specific for Type III.

Zoli *et al*., (1998) expanded this functional classification of the nAChRs subtypes into four classes (I-IV) from autoradiographic and patch clamp studies of nAChRs. Type I nAChRs bind to α- BTX and had a low affinity for nicotinic agonists. Their distribution was not different in wild type and β2 knock out mice. Thus, these receptors did not contain a β2 subunit. The receptor producing type I responses corresponded to α7-bearing nAChR and corresponds to the receptors producing type IA current as described by Alkondon and Albuquerque (1993). Type II nAChRs contained the β2 subunit. Zoli *et al*., (1998) found that the rank order of potency for nicotinic agonists at this receptor was nicotine > cytisine and represent the vast majority at nAChRs in rodent brain. This rank order of agonist potency at these receptors is consistent with that of recombinant α4β2 receptors expressed in oocytes (Luetje and Patrick, 1991; Zoli *et al*., 1998). An

example of type II receptors is α4β2 and it is likely that other subunits such as α2, α3, α5, and β4 may co-assemble with β2 to form a range of functional receptors that contribute to this type II high affinity nAChR activity.

Epibatidine, bound with high affinity to type III nAChRs that did not contain β2 subunits. Electrophysiological experiments showed that the rank order of agonist potency for this nAChR was consistent with a subunit composition of α3β4 nAChRs (Luetje and Patrick, 1991; Zoli *et al*., 1998). Cytisine and epibatidine bound to type IV nAChRs with high affinity. Agonist potencies determined in electrophysiological experiments were consistent with the presence of β4 containing receptors (Luetje and Patrick, 1991; Zoli *et al*., 1998). Type IV receptors also exhibited faster desensitisation than type III nAChRs (Zoli *et al*., 1998). Choline is an essential nutrient and a precursor of neurotransmitter acetylcholine. It is produced at synapses during depolarisation and also upon hydrolysis of ACh via acetylcholineesterase. The findings of Alkondon and Albuquerque (2006) showed that choline inhibits type III (α3β4) nAChRs in hippocampal and dorsal striatal slices from rat brain. Previous reports demonstrated that choline is an efficacious agonist and desensitising agent at Type IA (α7) nAChRs in native neurons (Alkondon *et al*., 1997; 1999). Choline is a weak agonist of type III ($\alpha3\beta4^{*}$) nAChRs (Alkondon *et al*., 2003), but does not activate type II ($\alpha4\beta2^{*}$) nAChRs (Alkondon *et al*., 1997). (According to the recent nomenclature for nAChRs and their subunits, the asterisk next to nAChRs subunits is meant to indicate that exact subunit composition is not known; Lukas *et al*., 1999).

Taken together, the pharmacological and functional properties of nAChRs vary depending on the subunit composition and species. nAChRs can be divided in to at least three distinct subtypes according to their pharmacological and functional properties. These are type IA (α7), type II (α4β2), type III (α3β4) nAChRs.

1.6 Evidence for non-neuronal cholinergic nicotine receptors

Non-neuronal cells containing nAChRs have been reported in a number of studies. Potentially these cells can be studied by exogenous ligands such as nicotine. Conti-Fine and colleagues (2000) demonstrated that functional nAChRs are expressed in non-neuronal cells and particularly in the tegumental cells that line the external and internal body surfaces (bronchial epithelial and endothelial cells of blood vessels and skin keratinocytes) (Macklin *et al*., 1998; Maus *et al*., 1998; Conti-Fine *et al*., 2000; Wang *et al*., 2001). The nAChRs have also been shown to be widely prevalent in human airways (Wessler *et al*., 1998; Wessler and Kirkpatrick, 1999; Conti-Fine *et al*., 2000; Wang *et al*., 2001; Wessler and Kirkpatrick, 2001; West *et al*., 2003; Gahring and Rogers, 2006). Wang *et al.*, (2001) reported that cultured human bronchial epithelial (BE) cells have specific binding sites for ^{125}I-α-bungarotoxin (α-BTX). Patch-clamp studies showed the presence of a fast-desensitising current. It was activated by choline and nicotine and it was blocked reversibly by methyllycocotinine and irreversibly by α-BTX suggesting that α7 nAChR subunits were present on human BE cells. Similarly, Sekhon *et al*., (1999) found α7 transcripts and an α-BTX binding sites in the cells surrounding the large

airways and the blood vessels in monkey foetal lung. Nicotine administration to mother had the effect of increasing the expression of these receptors in the foetal lung. Extensive α7 nAChR in human lung cells has been demonstrated (Plummer *et al*., 2005). Plummer and colleagues (2005) identified the expression of α7 nAChR in human lung cancer cell lines as well as for healthy lung cells.

Other nAChR mRNA subunits included: α3, α5, α6, β2, and β4 as well as the other homomeric receptors composed of α7, α9, the muscle type α1, β1, δ and ε nAChR subunits, have also been found on human bronchial epithelial cells and airway fibroblasts using RT-PCR (Carlisle *et al*., 2004). Previous studies in human airways used RT-PCR and binding studies to show that α3, α5, α6, β2, and β4 nAChR subunits were present on human and rodent bronchial cells. These cells exhibit functional nAChR neuronal type, analogous to those expressed in parasympathetic ganglia, activated by nicotine (Maus *et al*., 1998). The pharmacological and ion gating properties of these cells (from patch clamp experiments) provided to be similar in that the most abundant type of neuronal nAChR expressed in sympathetic ganglia, contained α3, α5 and β4 and perhaps, β2 subunits (Boulter *et al*., 1987; Listerud *et al*., 1991; Vernallis *et al*., 1993).

α3, α5, β2, and β4 mRNA nAChR subunits have also been detected on vascular endothelial cells by *in situ* hybridisation (Macklin *et al*., 1998). The receptors were functional and had ion gating properties similar to those in ganglionic nAChRs formed by α3, α5, β2, and β4 nAChR subunits (Papke, 1993; Macklin *et al*., 1998). The structural

and functional properties of endothelial cell nAChRs appeared similar to those of nAChRs expressed by the other tegumental cells such as skin keratinocytes and bronchial cells (Grando *et al*., 1995; Maus *et al*., 1998).

α7 nAChRs also exists in human aortic endothelial cells (Wang *et al*., 2001). They were found to be responsible for cell proliferation (Trombino *et al*., 2004). However, the role of α7 nAChRs in the proliferation of vascular endothelial cells remains unproven. Recently, Hsu *et al*., (2005) reported that the α7 nAChR proteins are expressed on murine and porcine aorta endothelial cells. They showed that α7 nAChR proteins were expressed on the membrane and modulated survival of the vascular endothelial cells.

The expression of α2, α3, α4, α5, α7, α10, β2, and β4 mRNA of nAChR subunits were detected on rat endothelial cells by means of RT-PCR and immunohistochemistry (Brüggmann *et al.,* 2002, 2003; Moccia *et al*., 2004). These nAChRs inhibited expression of endothelial adhesion molecules for leukocytes, suggesting that the effect of nicotine on these cells may be anti-inflammatory in nature (Saeed *et al*., 2005).

Studies on the non-neuronal cholinergic system in normal epidermis using RT-PCR have demonstrated the presence of α3, α5, α7, α9, α10, β2, and β4 nAChR subunits in human keratinocytes (Grando *et al*., 1995; 1996; Nguyen *et al*., 2000; Hagforsen *et al*., 2002; Sgard *et al*., 2002), indicating that human keratinocytes express both heteromeric and homomeric nAChRs. α7-nAChR expression by keratinocytes was implicated in delayed

wound healing since nicotine inhibited keratinocyte migration (Zia *et al*., 1997, 2000; Chernyavski *et al*., 2004). The α7 nAChR appears to play an important role in cell cycle apoptosis progression and differentiation (Arredondo *et al*., 2002). Furthermore, nAChRs have been reported in oral keratinocytes (Nguyen *et al*., 2000; Arredondo *et al*., 2001, 2005), in human and rat placenta (Lips *et al*., 2005) and adipose tissue (Liu *et al*., 2004). Liu *et al*., 2004 showed that adipocytes have functional nAChRs and suggested that nicotine reduces TNF-alpha protein production in adipocytes through the activation of nAChRs.

1.7 Evidence of nAChRs in lymphocytes and immune cells

The widely documented expression of neurotransmitter receptors on the surface of immune cells is thought to be indicative of an interaction between the nervous and immune systems (Battaglioli *et al*., 1998; Kawashima and Fujii 2000; 2003; Fujii, 2004). The presence of nAChRs on immune cells is of interest for many reasons. First, many lymphoid organs are innerved with cholinergic fibres and nAChRs may mediate neuro-immune interactions (Bellinger *et al*., 1993; Singh and Fatani, 1988; Artico *et al*., 2002). Second, lymphocytes produce endogenous acetylcholine (ACh), which may be an autocrine/paracrine functional regulator (Kawashima and Fujii, 2003). Finally, nAChRs may mediate an immunomodulatory effect of nicotine, which may be relevant to tobacco toxicity (Conti-Fine *et al*., 2000).

Lymphocytes contain most of the essential components needed for them to constitute have an independent, non-neuronal cholinergic system. Acetylcholine (ACh) is the classical neurotransmitter of the parasympathetic nervous system and it is a possible neuro-immune modulator (Maslinski, 1989; Rinner *et al*., 1995). ACh synthesized and released from lymphocytes acts as an immunomodulator via muscarinic and nicotinic AChRs (Rinner *et al*., 1995; Kawashima and Fujii, 2003). ACh influences T cell dependent immune responses. However there is some evidence, which suggests that B-lymphocytes may also serve as a target for cholinergic stimulation (Maslinski, 1989).

Cholinergic agents mostly serve to concentrate enhancement of T cell dependent immune responses such as cytoxicity (Strom *et al*., 1972; 1974). Other effects include cell mobility, antibody production, and proliferation (Maslinski, 1989; Shok *et al*., 2003; 2005; 2006) and apoptosis (De Rosa *et al*., 2005). It has also been suggested that non-neuronal ACh acts as a local signalling molecule, since it is widely distributed in biological systems (Wessler *et al*., 1998). Blood ACh is mostly found in mononuclear leukocytes (MNLs) and there is a significant correlation between the ACh content of MNLs and of the whole blood (Kawashima *et al*., 1993; 1998; Kawashima and Fujii 2000; 2003). Both T and B-lymphocytes were shown to produce ACh when activated (Rinner *et al*., 1998; 1999). Thus, these findings suggest that ACh originating from MNLs could play a role in the modulation of function and metabolism of various blood cells. It would be likely that ACh has an important role in the regulation of the immune system via act on nAChRs on MNLs (Kawashima *et al*., 1993; Fujii *et al*., 1995, 1998; Kawashima and Fujii 2000; 2003).

Much less is known about nAChR on lymphocytes. There is evidence for a functionally distinct nAChR on human lymphocytes. Richman and Arnason (1979) studied the effect of carbachol on cell human lymphocyte proliferation and showed the presence of three distinct cholinergic receptors on human lymphocytes. Menard and Rola-Pleszcynski (1987) also found that nicotine had a biphasic suppressive effect on human peripheral lymphocytes proliferation suggesting that nAChRs on lymphocytes exert a functional effect.

A number of different techniques have been used in studying the presence of nAChRs in lymphocytes. These techniques include; analysis of specific ligand binding; analysis of RNA expression using RT-PCR or Northern blot analysis by radio and fluorescent labelling by immunocytochemical staining; and analysis by using specific antisera against subunits of nAChRs. In functional studies, stimulation of T and B-lymphocytes by nicotinic agonists produced a variety of biological effects (Maslinski, 1989; Kawashima and Fujii, 2000; 2003; De Rosa *et al*., 2005; Shok *et al*., 2006). Thus, nAChRs modulate the function of lymphocytes. Immunological activation *in vivo* appears to enhance cholinergic signal transmission between T-lymphocytes and target cells, including B-lymphocytes, by enhancing the synthesis and release of ACh from T lymphocytes (Kawashima and Fujii, 2000; 2003). Furthermore, stimulation with ACh and carbarchol increased the cytotoxic action of lymphocytes (Strom *et al*., 1972; 1973; 1974; 1981).

The detection of nicotine receptors in lymphocytes has also been investigated by measuring binding of radiolabelled cholinergic ligands to cells (Maslinski *et al*., 1980; Adem *et al.,* 1986; Grabczewska *et al*., 1990; Maslinski *et al*., 1992). Adem *et al*., (1986) showed ^{3}H-nicotine specific binding to intact lymphocytes and lymphocyte membranes in cells from patients with Alzheimer's disease and senile dementia of the Alzheimer's type. They found a number of nicotine binding sites in both intact lymphocytes and lymphocyte cell membrane; however, it is significant, that these studies failed to saturate [^{3}H] nicotine-binding sites in intact lymphocytes (Adem *et al*., 1986). It has also been reported that d-tubocurarine (a nicotinic antagonist) inhibited radiolabeled ACh binding by lymphocytes (Maslinski *et al*., 1980) suggesting that ligand binding sites on lymphocytes were receptors. Maslinski *et al*., (1992) also studied nicotine receptors on intact rat lymphocytes and thymocytes. They reported that the number of nicotine binding sites on rat T lymphocytes (1500 sites/cell) was similar to the value obtained by Adem *et al*., (1986) for human lymphocyte membranes and by Grabczewska *et al*., (1990) for intact human lymphocytes (2000 sites/cell).

Some published data demonstrated that the density of nicotine receptors was higher on granulocytes than on blood mononuclear cells with a *Kd* of 36 x10^{-9} M and 8.7x10^{4} sites per neutrophil (Davies *et al*., 1982). In addition, Hiemke *et al*., (1986) identified nAChR subunits in human circulating lymphocytes from 10 healthy volunteers. *In situ* hybridisation revealed that α4 subunits genes of nAChRs were expressed in lymphocytes from all subjects studied. α4 containing nAChRs appeared more abundant than were α3 containing nAChRs, since the staining was more pronounced (Hiemke *et al*., 1986).

The expression of mRNAs encoding the presence of nAChRs on immune cells has been studied using Northern blot and RT-PCR analyses by many investigators. Mihovilovic and Roses (1991; 1993; 1997; 1998) showed the expression of mRNAs encoding the α3, α5, and β4 subunit in human thymocytes and thymic epithelial cells. RT-PCR analysis of thymocytes subset indicated that immature thymocytes expressed higher levels of α3 and β4 subunits than more mature thymocytes. Compared to freshly isolated thymocytes, peripheral blood lymphocytes did not express α3, and β4 mRNA subunits. The α3, and β4 subunit gene is downregulated during the process of thymocyte maturation and that expression of α3, and β4 subunits depends on the integrity of the thymic microenvironment as thymocytes rapidly lose these transcripts upon removal from the thymic environment. The data obtained by molecular biological techniques have shown that the α3 mRNA subunit is expressed in human thymus, on thymocytes, but not in peripheral lymphocytes, suggesting negative transcriptional regulation of this subunit during T cell lineage maturation (Mihovilovic *et al*., 1997; 1998). In contrast, Hiemke *et al*., (1996) showed that circulating T lymphocytes could express low levels of the α3 subunit but only in eight out of 10 blood donors. These data imply that there is intersubject variability involved in the expression of this subunit. Battaglioli *et al*., (1998) also managed to demonstrate the expression and transcriptional regulation of human α3 nAChR subunit in T-lymphocyte cell lines (MOLT-4 and Jurkat cells). But they found no α3 nAChR subunit expression in human lymphoblast, promyelocyte or monocytic cell lines, suggesting that the distribution of the α3 nAChR subunit is restricted to specific subsets of immunocompetent cells. The α1 subunit in human

thymocytes (lymphocytic cell lines and α1, β1 and ε subunits in human thymic epithelial cells (non-lymphocytic cell lines) was reported by Wakkach *et al*., (1996). By using fluorescently labelled α-BTX Toyabe *et al*., (1997) also found α1 nAChR subunit expression on murine lymphocytes.

Variation in subunit expression between donors was reported by Sato *et al*., (1999) who analysed the expression of mRNA encoding nAChR subunits in human mononuclear leukocytes (MNLs) from seven healthy donors and in eight leukemic cell lines. They reported that all donor MNLs expressed α2, α5, and α7 subunits, but no MNLs showed α3, α4, β3, and β4 subunit expression. α4, and β3 subunit expression was not seen in any of the cell lines tested, nor was there any expression of three skeletal muscle type subunits (α1, β1 and ε subunits). Some neuronal nAChR subunits (α2, α3, α5, α6, α7, β2 and β4) have been found from three or more cell lines. The results of Sato *et al*., (1999) differ from those Wakkach *et al*., (1996), and Toyabe *et al*., (1997) who reported that the expression of the α1 subunit in human thymocytes and murine lymphocytes, respectively. They are likely indicative of the diversity of expression of nAChR subunit among individual subjects and species.

Benhammou *et al*., (2000) reported the expression of mRNA and the protein of a subset of α4β2, and α3β4 nAChRs in human lymphocytes and polymorphonuclear cells. Morgan *et al*., (2001) also found α2 and α7 mRNA subunits of nAChR in mononuclear cell line (THP-1) using RT-PCR. Peng *et al*., (2004) demonstrated the expression of both α9 and α10 mRNA of nAChR subunits in purified populations of human lymphocyte

subsets. However, single cell electrophysiologic recording techniques failed to identify an ionic current in response to applied Ach in both T and B cells suggesting that no functional properties of α9 and α10 have been found (Peng *et al*., 2004).

De Rosa *et al*., (2005) also showed the expression of muscle (α1, β, δ, ε and γ) and α9 nAChR subunits in human lymphocytes from healthy donors. They found that lymphocytes from all tested samples continued at least one muscle nAChR subunit. Again the mRNAs patterns varied between individuals as well as between samples from the same individual obtained at different times. Surprisingly, mRNAs encoding for different muscle subunits were not all present in the same individual at a given time. No γ and α9 mRNA subunits of nAChRs were detected in freshly isolated lymphocytes from all donors. The expression pattern of the muscle subunits varied in rat lymphocytes. Again, not all muscle subunits were present in the same sample of freshly isolated lymphocytes. In contrast to the mixed results from De Rosa *et al*., (2005), α9 mRNA subunits were detected in peripheral blood lymphocytes by Lustig *et al*., (2001), and Peng *et al*., (2004). Taken together, these results are indicative of a great diversity in the expression of nAChR subunits among individuals and among species.

De Rosa *et al*., (2005) also demonstrated that mRNA of α7 nAChR in peripheral blood lymphocytes by RT-PCR and determined that its expression is highly variable among individuals and within the same individual at different times. Up regulation of α7 mRNA of nAChR subunit can be systemically observed after incubation of lymphocytes with nicotine or α-BTX. No α7 mRNA nAChR subunit was expressed in rat lymphocytes.

Moreover, α7 was not detected after incubation of the rat lymphocytes with nicotine or α-BTX (De Rosa *et al*., 2005).

The role of nAChRs was identified mainly from work on T lymphocytes. Recently, some studies on B-lymphocyte derived cell lines and mouse B-lymphocytes demonstrated that nAChRs are expressed in these cells (Sato *et al*., 1999; Lustig *et al*., 2001; Shok *et al*., 2003; Shok *et al*., 2005) and are involved in regulating cell proliferation and antibody production (Shok *et al*., 2003). Shok *et al*., (2003) reported the presence of α4/β2 and of the α7 mRNA nAChR subunits in B lymphocytes-derived cell lines. The cell lines tested found nAChR α4 and α7 nAChR subunits, but not α3 and α5 subunits (Shok *et al*., 2003). Subsequently, Shok and colleagues (2005) using a radioligand binding assay and cell ELISA, showed that α4, β2 and/or α7 mRNA nAChR subunits are expressed in normal mouse B-lymphocytes. Recently, Shok *et al*., (2006) investigated the role of nAChR in lymphocytes and showed that nicotinic receptors are involved in regulating lymphocyte development of both T and B-lymphocytes (Shok *et al*., 2006).

Taken together, nAChRs are expressed on the surface of immune cells is generally accepted as evidence from the reviewed above. It is possible that the nervous system may influence immune responses on immune cells through nAChRs, even though many aspects of these interactions remain unknown. The expressions of nAChRs on immune cells are likely to have a diversity of nAChR subunits among individual subjects and species and between laboratories.

In spite of this evidence the existence of nAChRs on lymphocytes remains controversial due to discrepancies between published studies (Hiemke *et al*., 1996; Battaglioli *et al*., 1998; Sato *et al*., 1999; Villiger *et al*., 2002; Lustig *et al*., 2001, Peng *et al*., 2004). In studies using binding assays and cell proliferation assays, lymphocytes have been observed to carry nAChRs (Fucus *et al*., 1980; Richman and Arnason, 1979; Strom *et al*., 1981). Other studies using binding assays on cells from rats have been found that only T-lymphocytes and thymocytes express nAChRs, but neither macrophages nor B-lymphocytes bear nAChRs (Maslinski *et al*., 1992). In contrast, Grabczewska *et al*., (1990) demonstrated that leukemic-B-lymphocytes contained nAChR binding sites. In addition, Shok *et al*., (2003; 2005) reported nAChRs subunits on B-leukemic cell lines and B-lymphocytes of C57B1/6J mice. Some experiments showed that only thymic epithelial cells expressed α3, α5, and β4 mRNA of nAChR and freshly peripheral blood lymphocytes did not express those mRNA subunits (Mihovilovic *et al*., 1997, 1998). In contrast, Toyabe *et al*., (1997) showed that mouse peripheral blood lymphocytes expressed nAChRs on their surface as well as in the thymus. Some studies demonstrated the expression of α3 and α4 nAChRs subunits in lymphocytes using immunohistochemistry and *in situ* hybridisation (Hiemke *et al*., 1996). Benhammou *et al*., (2000) suggested that both mRNA and protein in lymphocytes contain both α4β2 and α3β4 nAChRs. However, other groups failed to find either α3 or α4 mRNA subunits in lymphocytes (Sato *et al*., 1999; Mihovilovic *et al*., 1997; 1998). The results of Sato *et al*., (1999) differ from those of Wakkach *et al*., (1996), and Toyabe *et al*., (1997), who reported the presence of the α1 subunit. The results are therefore equivocal and a more definitive identification of nAChRs on lymphocytes has yet to be achieved.

In summary**,** the immune function is not only regulated by the cytokine system, but may also be, at least in part, under the control of an independent non-neuronal lymphocytic cholinergic system. A large amount of data suggesting that nAChRs exist on T and B lymphocytes has been published. The function of these receptors is not entirely clear although it appears to involve a role in the proliferation of T-cells and also possibly to have an inhibitory effect on mediator release. Functional data from studies with monocytic cells and macrophages is limited and further experiments are required to demonstrate the presence and function of nAChRs, it needs to be determined whether these binding sites are non-cholinergic in nature.

1.8 Pharmacology and pharmacokinetics of nicotine

Nicotine is an alkaloid contained in the leaves of several species of tobacco plants (*Nicotiana*). The main commercial source of nicotine is the dried leaves of *Nicotiana tabaccum* and *N. rustica*. Nicotine is a tertiary amine composed of a pyrimidine and pyrrolydine ring. The chemical formula for nicotine is $C_{10}H_{14}N_2$ giving it a molecular mass of 162.23; nicotine is *3-(1-methyl-2-pyrrolidinyl) pyridine*. There are two different stereoisomers (-)-nicotine and (+)-nicotine. (-)-Nicotine is a highly potent pharmacologically active form, whereas (+)-nicotine is less potent (Jarvik, 1970; Armitage *et al*., 1978; Pool *et al*., 1985; Benowitz, 1986; Benowitz and Jacob, 1997; Benowitz *et al*., 1997).

Nicotine is a weak base, with a pKa of 8.0. The absorption of nicotine through cell membranes depends on the pH (Armitage and Turner 1970; Schievelbein *et al*., 1973). If the environment is acidic, nicotine is ionised and does not easily pass through membranes. At physiological pH (pH 7.4) non-ionised nicotine permeates membranes easily (Benowitz, 1996). Smokers maintain a nicotine plasma concentration of approximately 10^{-7}-10^{-6}M (Benowitz, 1996). However, the exact value depends upon the strength of tobacco and the extent of inhalation of the smoke (Benowitz, 1996; Rang *et al*, 1999). The half-life of nicotine in the body is approximately 2 hours, and nicotine accumulates over 6-8 hours then fall progressively at night (Benowitz *et al*., 1982*a*; 1982*b*; Benowitz, 1996). Thus in studies with nicotine concentrations in the range of $1x10^{-8}$-$1x10^{-6}$ M are most relevant to its use in man. Nicotine mimics ACh by binding stereo-selectively to nAChRs at the autonomic ganglia, in the adrenal medulla, at the neuromuscular junctions, and in the brain (Changeux *et al*., 1992; Changeux and Eldelstein, 1998).

Nicotine is mainly metabolised by liver, but also by the lungs and kidney. This occurs within1-2 hours of administration (Rang *et al*., 1999) and is summarised in Figure 1.8. Approximately 70-80% of nicotine is metabolised to cotinine which may be pharmacologically active and about 4 % to nicotine –N'-oxide (Benowitz *et al*., 1994; Benowitz and Jacob, 1994; Benowitz and Jacob, 2000).

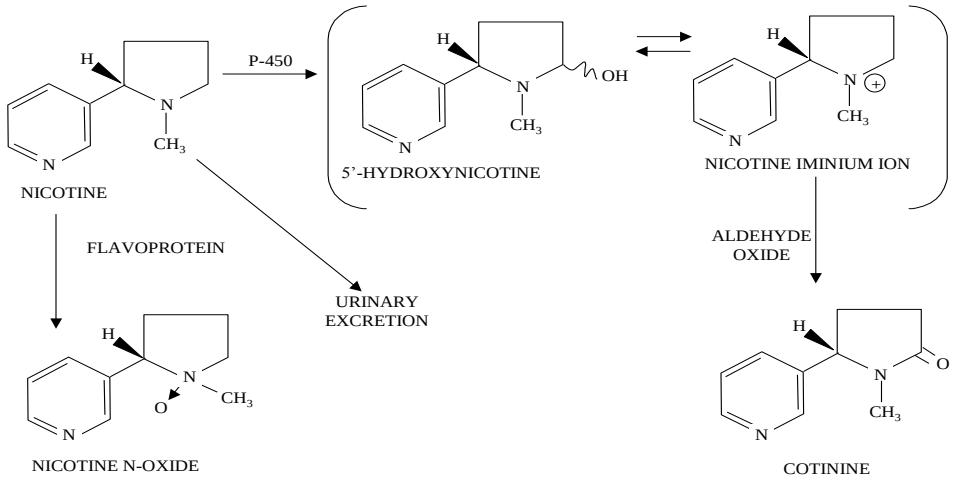


Figure 1. 8. Major pathways of nicotine metabolism. Adapted from Zevin *et al*., 1998

Nicotine clearance is the strongest predictor of an individual's plasma nicotine content. Cotinine levels are most strongly correlated with nicotine dose and, to a lesser extent, fractional conversion of nicotine to cotinine and cotinine clearance (Benowitz *et al*., 1997). The metabolic clearance of nicotine is slower in smokers than in non-smokers (Benowitz and Jacob, 1993, 2000).

Nicotine has inhibitory actions on immune/inflammatory cells and is being investigated for therapy of ulcerative colitis, Alzheimer's disease, Parkinson's diseases, Tourette's syndrome, sleep apnoea, and attention deficit disorder (Benowitz, 1996; de Jonge and Ulloa, 2007; Gallowitsch-Puerta and Pavlov, 2007; Tracey, 2007).

1.9 Effects of nicotine on nAChRs

Nicotine mimics ACh by binding to nAChR. Binding results in opening of the ion channel which briefly permits the passage of cations (Na^+, K^+ and Ca^{2+}) ions. This is followed by a series of conformational changes that produces a desensitised state of the receptor. In this configuration, the channel is closed and the receptor has become refractory to the agonist, although the agonist can still bind to the receptor with enhanced affinity. Low concentrations of agonist can push the receptor into the desensitised stated without going through the open state. From this refractory state, the receptor will normally return to its resting state where it is closed and sensitive to agonists. When the receptor experiences prolonged exposure to an agonist (even to low concentrations) the desensitised state will last much longer. These properties have implication for the functional effects of nicotine in therapeutic solutions and for experiments involving radioligand binding.

Interestingly, nicotine and epibatidine binding sites were found to be up regulated in polymorphonuclear leukocytes of tobacco smokers (Benwell *et al*., 1988; Lebargy *et al*., 1996; Perry *et al*., 1999; Benhammou *et al*., 2000). A study on peripheral blood lymphocytes and polymorphonuclear cells (PMNs) showed that nicotine receptors increase in tobacco smokers but decreased when the subject stops smoking (Lebargy *et el*., 1996). The rise in [^{3}H]-nicotine binding sites was correlated with tobacco use (Benhammou *et al*., 2000). Similarity, Shok and colleagues reported that long-term

nicotine exposure resulted in an increase of α4 and α7 specific antibody binding in hybridoma cells (Shok *et al*., 2003). Some reports, however, have indicated that nicotine down-regulates α7-containing nAChRs in autonomic neurons (Kawai and Berg, 2001).

A similar alteration in nicotine receptors has been reported in a post mortem study of smoker's brain (Benwell *et al*., 1988; Perry *et al*., 1999). They reported that the number of [^{3}H]-nicotine binding sites was higher in the post-mortem brains of human smokers than in non-smokers. Fenster *et al*., (1997, 1999) showed that nAChRs up-regulation may involve the desensitisation of α4β2 nAChRs expressed in *Xenopus laevis* oocytes, thus preventing any ion influx. Another study similarly reported that the number of [^{3}H] nicotine binding sites is correlated with the number of cigarettes smoked per day (Breese *et al*., 1997*a*; 1997*b*). Cormier *et al*., (2004) found that long-term exposure to nicotine caused specific-up-regulation of epibatidine binding sites (which correspond to an increase in heteromeric nAChR subtypes) but not α-BTX binding sites (which corresponds to the homomeric α7 nAChR) in polymorphonuclear leukocytes. Long-term exposure to nicotine induces a progressive loss of nAChR function (Peng *et al*., 1994; Fenster *et al*., 1999; Gentry and Lukas, 2002; Gentry *et al*., 2003; Jia *et al*., 2003; Lai *et al*., 2005; Vann *et al*., 2006).

In summary, from the studies reviewed above, long term consumption of tobacco by smokers increases the level of neuronal nAChRs in the brain, as well as, in immune cells. Thus, the number of [^{3}H]-(-)-nicotine binding sites from human peripheral lymphocytes from tobacco smokers should be higher than lymphocyte from non tobacco smokers.

1.10 The effect of nicotine on the immune /inflammatory system

Nicotine has been reported to affect both humoral and cell-mediated immune responses in rodents, monkeys, and humans (Neher, 1974; Gerrard *et al*., 1980; Burows *et al*., 1981; Miller *et al*., 1982; Tollerud *et al*., 1989; Nair *et al* 1990; Geng *et al*., 1995, 1996; Mills, 1998; Soropi *et al*., 1998; Kalra *et al*., 2000; Singh *et al*., 2000; Kalra *et al*., 2002; Middlebrook *et al*., 2002; Soropi, 2002; Kalra *et al*., 2004; Quik, 2004). In the last 10-20 years it has become increasingly recognised that cigarette smoking may have beneficial effects on chronic diseases such as ulcerative colitis (Harries *et al*., 1982; Logan *et al*., 1984; Van Dijk *et al*., 1995; 1998). Menard and Rola-Pleszcynski (1987) found that nicotine had a biphasic suppressor effect on lymphocyte proliferation. They hypothesised that suppressor T-lymphocytes might carry functional nAChR. Zhang and Petro (1996) also showed that exposure of murine T-lymphocytes to the concentration of nicotine found in the blood of smokers can alter the cell expression of CD28 and CTLA-4, and CD4 T cell cytokine expression pattern. Similarly, Bhatti and Hodgson (1997) found that nicotine and crude tobacco extracts inhibited the release of IL-8 from whole blood and mononuclear cells. However, Le Cam *et al*., (1996) found that nicotine had no effect on the *in vitro* release of IL-4 and IFN-γ from peripheral blood mononuclear cells and T cell clones. Geng *et al*., (1995, 1996) also suggested that chronic *in vivo* nicotine exposure led to T cell anergy.

Madretsma *et al*., (1996) also claimed that the immunomodulatory actions of nicotine were mediated by inhibition of cytokine release. Madretsma's finding are in agreement with an observed inhibition of cytokine production in mouse colonic mucosal after treatment with nicotine (Van Dijk *et al*., 1995, 1998). However, Ouyang *et al*., (2000) demonstrated that nicotine, even at the highest concentrations tested (1.4×10^{-3}M), had little effect on the production of the proinflamatory cytokines. It has been reported that the α7 nAChR subunits are required for ACh inhibition of the release of TNF-α from macrophages (Borovikova *et al*., 2000; Wang *et al*., 2003). Tissue culture studies have also identified interactions between nicotine (acting through α7 nAChR) and TNF-α release. These studies collectively suggest an interaction between the signalling pathways for acetylcholine activity and for TNF-α release (Carlson *et al*., 1998; Borovikova *et al*., 2000; Wang *et al*., 2003; Gahring and Rogers, 2006). Sykes *et al*., (2000) have demonstrated that nicotine reduced inflammation in the trinitrobenzene-sulphonic acid (TNBS) model of colonic damage in inflammatory bowel disease in comparison with sulphasalazine, a drug commonly used in treatment of inflammatory bowel disease (IBD). Nicotine also caused a significant reduction in TNF-α release from THP-1 cells.

Nicotine has many effects throughout the body; one of the earliest noted effects of nicotine on a peripheral tissue was the protective effect it has in ulcerative colitis (UC), a form of inflammatory bowel disease (IBD) (Logan *et al*., 1984; Motley *et al*., 1988; Person *et al*., 1990; Pullan *et al*., 1994). However nicotine exacerbates the other from of

IBD, Crohn's disease (Bures *et al*., 1982; Harries *et al*., 1982; Motley *et al*., 1987; Calkins, 1989).

Both UC and Crohn's diseases are characterised by macrophages and lymphocyte activation, subsequently resulting in a cascade of pro-inflammatory cytokines, such as IL-2, IL-1β, TNF-α and IFN-γ (Both *et al*., 1983; Radford-Smith, 1997; Moses *et al*., 1998; Van Dijk *et al*., 1998). Sartor (1991) showed that inflammatory bowel disease is characterised by an increased number of intestinal T-cells. The cytokines produced by these cells play an important immunoregulatory and effector's role in IBD. Early reports described patients with ulcerative colitis who experienced more severe disease progression upon cessation of smoking, which was ameliorated by returning to smoking (Birtwistle and Hall, 1996; Wolf and Lashner, 2000). In contrast, patients with Crohn's disease experienced severe disease when smoking, requiring the immediate and complete cessation of any tobacco use (Rubin and Hanauer, 2000; Hilsden *et al*., 2000).

Clinical studies indicate that nicotine administration can be efficacious in reducing the severity of UC (Pullan *et al*., 1994; Sandborn *et al*., 1997; 1999). Nicotine has been shown to be effective as a chemical therapy when used as a gum or transdermal patch (Lashner *et al*., 1990; Pullan *et al*., 1994; Thomas *et al*., 1994; 1995; 1996; 1998; Coulie *et al*., 2001; McGrath *et al*., 2004). In an animal model of IBD, low doses of nicotine reduced the inflammation whereas a high dose exacerbated inflammation (Qiu *et al*., 1997; Sykes *et al*., 2000).

The mechanism underlying these beneficial effects of nicotine is unknown. There is some evidence to show that smoking influences the cellular and the humoral immune system (Srivastana *et al*., 1991; Zijlstra *et al.,* 1997; 1998 and reviewed above). The concentration of pro-inflammatory cytokines are significantly decreased in the colonic mucosal of smokers with IBD (Sher *et al*., 1999). Accordingly, Borovikova *et al*., (2000) and Tracey (2002) reasoned that the parasympathetic nervous system might modulate the systemic inflammatory response. The mechanism through which nicotine acts in either of these diseases has not been resolved, and while both diseases are considered autoimmune in origin and thought to be related to the overproduction of inflammatory cytokines, nicotine has opposite actions on the two conditions. One question to be answered is which nAChRs, or possibly their respective expression levels, might participate in the modulation of these disease states and the differential response to nicotine.

Recently, Orr-Urtreger *et al*., (2005) found that mice deficient in the α5 nAChR subunit are more susceptible to experimentally induced IBD than their wild type controls. The absence of the α5 nAChR subunit increases susceptibility to disease initiation and the presence of α5 nAChR subunit in the wild type animal appears to enhance sensitivity to therapeutically administered nicotine (Orr-Urtreger *et al*., 2005). This raises the question as to whether nAChRs composition or function may differ between Crohn's and UC patients and may account for subject variability in response to nicotine. Richardson *et al*., (2001) who found the absence of α3 nAChR subunits is associated with a rare intestinal disease of childhood. Further studies by Xu *et al*., (1999) demonstrated that mice lacking α3 or both β2, and β4 nAChR subunits have similar autonomic dysfunctions

in the bowel. Whether indirect interaction with nAChRs expressed by epithelium or receptor expression by ganglia contributes to these disease processes remains to be determined (Xu *et al*., 1999).

The existence of non-cholinergic nAChRs on lymphocytes has also been studied (Maslinski, 1989; Morgan *et al*., 2001; Shok *et al*., 2003; De Rosa *et al*., 2005) and any effects of nicotine mediated through these receptors need to be fully determined and the presence of such receptors confirmed.

1.11 Aims of thesis

The aims of this thesis are to characterise the nicotinic acetylcholine receptors on human peripheral blood lymphocytes using binding assays, pharmacological and molecular biological techniques. The research undertaken is based on the studies of:

- Thomas *et al*., (1995, 1996), who showed a beneficial effect of nicotine in ulcerative colitis (UC).
- Sykes *et al*., (2000) who established that nicotine is a potent inhibitor of lipopolysacharide (LPS)-induced tumour necrosis factor α (TNF-α) release from THP-1 cells, a monocytic cell line, *in vitro.*
- Morgan *et al*., (2001) who found two binding sites for nicotine on THP-1 cells - one with the properties of a nAChR and a second receptor, which appeared to be non-cholinergic.
- Madretsma *et al*., (1996), who showed that nicotine at concentrations likely to be found in the blood of smokers inhibited interleukin-2 (IL-2) release from human lymphocytes *in vitro*.

The experiments described in this thesis were undertaken using radioligand binding assays, pharmacological and molecular biological techniques to characterise the nicotinic acetylcholine receptors present on human peripheral blood lymphocytes. The sensitivity of lymphocytes to nicotine and other nAChR-specific nicotinic agonists and antagonists was determined.

The characterisation of nicotine binding sites on lymphocytes, their signal transduction may give insight into how nicotine modulates ulcerative colitis. Data generated may also indicate how nicotine may be exploited to identify and a selective ligand that could be used to treat inflammatory diseases such as ulcerative colitis without having the undesirable adverse effects associated with nicotine. Such a molecule may have therapeutic utility.

Chapter 2 Nicotine receptor binding studies

2.1 Introduction

Evidence from functional experiments suggests that nicotine may have modulatory effects on immune cells such as decreased proliferation of human peripheral blood lymphocytes (PBMC) (Neher *et al*., 1974) and inhibition of apoptosis (Aoshiba *et al*., 1996). Similarly, Zhang and Petro (1996) showed that nicotine could decrease the production of IFN-γ and IL-2 by human PBMC *in vitro* but increased the production of IL-4 and IL-10.

However, these functional studies have provided conflicting data in that some workers showed that nicotine inhibited the production of cytokines from human lymphocytes (Madretsma *et al*., 1996) and from human leukemic monocytes (THP-1 cells) (Sykes *et al*., 2000), others found that nicotine only had a small inhibitory effect on cytokine release from blood monocytes (Ouyang *et al*., 2000; Nomura *et al*., 2003).

Attempts to characterise the receptor mediating these effects of nicotine with specific receptor antagonists have provided evidence for the existence of nAChRs on immune cells. Richman and Arnason (1979) and Richman *et al*., 1981 showed that ACh induced lymphocyte proliferation was blocked by the nicotine antagonist α-BTX ($1x10^{-6}$M) and d-

tubocurarine ($1x10^{-6}$M), suggesting that lymphocyte function can be modulated by α7 nAChR. Similarly, Menard and Rola-Pleszczynski (1987) showed that nicotine enhanced T-suppressor cell activity, an action that was abolished by nicotinic antagonists (d-tubocurarine) suggesting that nAChRs are involved in this process. Recently, Wang *et al*., (2003) demonstrated that nAChR the α7 subunit on immune cells is mediated inhibition of cytokine release from immune cells (Wang *et al*., 2003).

However, receptor binding studies on immune cells suggest the presence of an additional receptor for nicotine that was not competed for by conventional nicotinic ligands and only a single binding site was found (Davies *et al*., 1982; Hoss *et al*., 1986; Morgan *et al*., 2001). In contrast, Lebargy and colleagues (1996) showed that the nicotine-binding site on human granulocyte (polymorphonuclear cell) membranes had an affinity profile for nAChR ligands that is similar to that found in the brain. Interestingly, Morgan *et al*., (2001) reported the presence of two nicotine binding sites on THP-1 cells. These two binding sites had different binding characteristics, in that one site appeared to be cholinergic, whereas the second, high-affinity site did not bind a range of nAChR ligands other than nicotine (Morgan *et al*., 2001). However, it is uncertain whether or not this nicotine binding site exists on other cells or whether it constitutes a functional receptor.

In order extend the work of Morgan *et al*., (2001) the present study sought to identify nicotine receptors on human peripheral blood lymphocytes using radioligand binding assays and to characterise properties of any receptor subunits by competition studies with a range of nicotinic cholinergic receptor ligands. Because published studies have

identified binding sites for nicotine that are non-cholinergic in nature (Davies *et al*., 1982; Hoss *et al*., 1986; Morgan *et al*., 2001), [^{3}H]-(-)-nicotine was used as a ligand rather than other ligands, such as epibatidine, that may not interact with any non-cholinergic receptors present on lymphocytes.

2.2 Materials and methods

2.2.1 Materials

Sterile disposable plastics, sterile medium, PBS, and glass wares were used in all tissue culture work. Tissue culture flasks (75 ml^3, with canted neck and 0.2 μm vented blue plug seal) were purchased from Becton Dickinson UK LTD., Oxford. All other tissue culture and general plastics were purchased from Greiner Labortecknik, Norththampton, unless otherwise stated. Sterile tissue culture growth media (RPMI-1640), HISTOPAQUE® -1077, PBS tablets, Trypan blue, Whatman GF/B filters were purchased from Sigma-Aldrich Ltd, Poole, UK. Foetal Bovine Serum (FBS), Penicillin 50 IU/ml, Streptomycin 50 μg/ml and L-glutamine 2 mM were purchased from Life Technologies, Paisley, UK.

The radioisotope, [^{3}H]-(-)- nicotine, was purchased from NEN Life Sciences, Zaventem, Belgium and had a specificity of 69-81 Ci/mmol. This radioligand was > 97% pure with respect to (-)- nicotine. Liquiscint scintillation fluid was purchased from National Diagnostics, UK. Skatron 12 well micro tubes were purchased from Camo Ltd, Suffolk, UK. Other ligands used in competition assays namely nicotine, carbachol, α-bungarotoxin, atropine, cytisine, and hexamethonium were purchased from Sigma-

Aldrich, Poole, UK. Epibatidine was purchased from Torcris, St. Albans, UK. Structures of these agents are shown in Figure 2.1

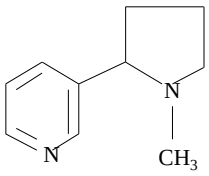
RPMI-1640 medium used in tissue culture work was supplemented with 10% Foetal Bovine Serum (FBS), Penicillin 50 IU/ml, Streptomycin 50 μg/ml and L-glutamine 2 mM.

Sterile Phosphate Buffered Saline (PBS) was prepared by dissolving 5 PBS tablets in 1L of distilled water and the solution was mixed using a magnetic stirrer. PBS solution was then autoclaved and stored at room temperature until required.

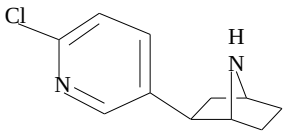
Hanks' Balanced Salt Solution (HBSS) without Ca^{+2} and $Mg^{+2}$was prepared by dissolving the agent listed below.

Component	**(1xLiquid)**	**g/L**
Inorganic salts		
KCl (Fisher Scientific International Company)	0.40	
KH_2PO_4 (BDH)	0.06	
NaCl (BDH)	8.00	
Na_2HPO_4 (BDH)	0.048	
Other components		
D-glucose (Sigma)	1.00	
EDTA (BDH)	1.00	
HEPES (BDH)	2.38	

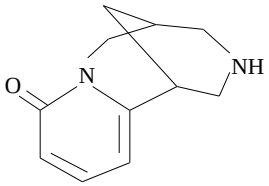
These contents were dissolved in distilled water and mixed using a magnetic stirrer. The pH was adjusted to 7.4 with NaOH solution (0.25M or 5.0M) using a pH meter, and then adjusted to a final volume of 1 litre. The solution was stored at 4ºC and used within two weeks of preparation. Final concentration of HBSS were 5 mM KCl, 0.5 mM KH_2PO_4, 0.13 M NaCl, 0.3 mM Na_2PO_4, and 5 mM D-glucose without Ca^{+2} or Mg^{+2} plus 20mM HEPES and 1 nM EDTA, and pH 7.4.



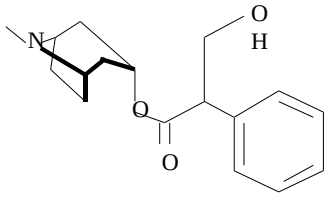
Nicotine



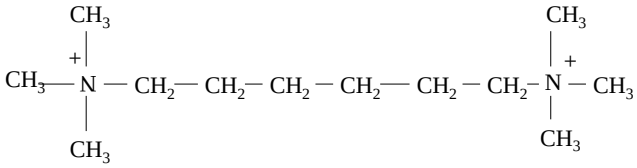
Epibatidine



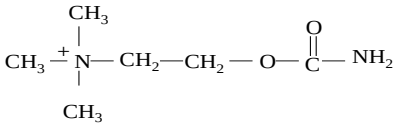
Cytisine



Atropine



Hexamethonium



Carbachol

Figure 2.1 Structures of ligands used for competition studies.

2.2.2 Methods

2.2.2.1 Isolation of human lymphocytes

All procedures were carried out in a class II laminar flow cabinet (Envair, Lancashire, UK). All media, PBS, glass wares, and disposable plastics were sterilised before being used. Human blood cells (Buffy coats) from healthy individual donors were obtained from the National Blood Transfusion Services (NBTS) (Colindale, London, UK). Blood was collected from donors using EDTA or heparin as anti-coagulant and all procedures were performed within 24 hours of sample collection from the National Blood Transfusion Services (Colindale). All human blood samples were tested, by NBTS, and found to be negative for anti-HIV, anti-HCV, and HbsAg and Syphilis. However, these products cannot be assumed to be free from infectious agents. Blood samples were handled carefully and discarded products were autoclaved. Lymphocytes and mononuclear cells were separated by density-gradient centrifugation through HISTOPAQUE® -1077 (Sigma Ltd., UK) with a density of 1.077g/ml. This technique was modified from the original method described by Boyum (1968).

HISTOPAQUE®-1077 (Sigma Ltd., UK) was brought to room temperature before starting the process. An aliquot of a single buffy coat (NBTS, Colindale, UK) was diluted by making a dilution of 1: 20 in Turk's solution (2% acetic acid, crystal violet,

gentian violet or azure blue). Then the leukocytes present were counted on all samples, using a haemacytometer (Improved Neubauer, Weber Scientific International Ltd, UK) as described in section 2.2.2.2.1. From, this count, the rest of the buffy coat was diluted with sterile Phosphate Buffered Saline (PBS) (Sigma Ltd., UK) in a sterile beaker to give a white blood cell density of approximately 1-10 x 10^6 cells/ml. 25 ml of diluted blood was then layered carefully over an equal volume (25:25 ml) of HISTOPAQUE® -1077 in a 50 ml sterile plastic centrifuge tube (Greiner Laboratechnik, UK) Figure2.2. The gradient was then centrifuged (Labofuge 400, Sorvall Heraeus, Kendro Laboratory Products Ltd., Hertfordshire, UK) at 1,000 g for 25 minutes at room temperature. After centrifugation, the white ring (mononuclear cells), found at the PBS/ HISTOPAQUE interface was collected (Figure 2.2) and washed twice with sterile PBS by centrifugation (Sorvall RT ™ 6000 D, UK) at 500 g for 7 minutes at 4°C and mononuclear cells were counted as described in section 2.2.2.2.1.

Finally, the cell pellet was resuspended in sterile RPMI-1640, supplemented with 10% Foetal Bovine Serum (FBS) (Life Technologies, Paisley, UK), Penicillin 50 IU/ml (Life Technologies, Paisley, UK), Streptomycin 50 μg/ml (Life Technologies, Paisley, UK) and L-glutamine 2 mM (Life Technologies, Paisley, UK), at a density of 5-20 x 10^6 cells/ml. The mononuclear cell suspensions were transferred to 75 ml^3 tissue culture flasks (Becton Dickinson UK Ltd., Oxford, UK) and incubated in humidified, atmosphere at 37°C with 5 % CO_2 for 12-24 hours (or overnight) to allow the adherence of mononuclear cells (monocytes). The non-adherent mononuclear cells (lymphocytes) were transferred to sterile 50 ml centrifuge tubes and were centrifuged at 1,700 rpm for

10 minutes at 4°C. The pellet was collected and resuspended in Hanks' Balanced Salt Solution (HBSS: 5 mM KCl, 0.5 mM KH_2PO_4, 0.13 M NaCl, 0.3 mM Na_2PO_4, and 5 mM D-glucose without Ca^{+2} or Mg^{+2} plus 20mM HEPES and 1 nM EDTA, pH 7.4) to a density of $1x10^7$ cells/ml. A final lymphocyte count was made as described in section 2.2.2.2.1. Cell viability, determined by Trypan blue exclusion, was usually in excess of 95%. The lymphocytes were then ready for further studies.

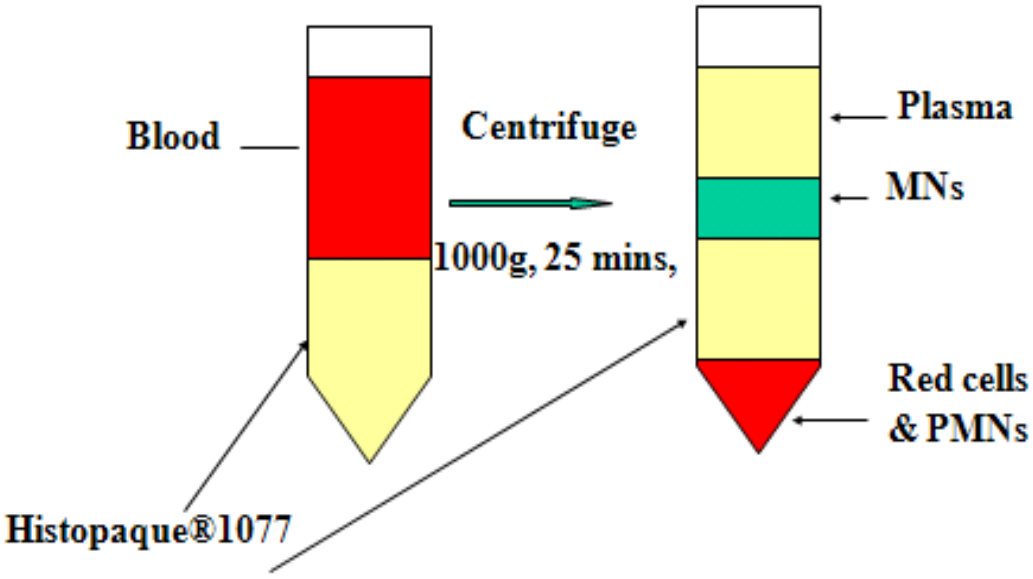


Figure 2.2 A diagrammatic representation of isolation of human lymphocytes using HISTOPAQUE® -1077 (MNs = Mononuclear cells, PMNs = Polymorphonuclear cells)

2.2.2.2 Leukocyte counting and viability checking

2.2.2.2.1 Leukocyte counting

Lymphocytes were counted by diluting the cell suspensions (1:20) in Trypan Blue or (in Turk's solution for leukocytes) and adding 10 µl to each chamber (A and B) of a haemacytometer (Improved Neubauer, Weber Scientific International Ltd, UK) with a chamber volume of 0.1 mm^3, containing a marked counting grid 1 mm^2 in area. The cells were allowed to settle for 1 minute and the grid was checked under the microscope (x20 objective). The grid is divided into 9 large squares covering a total area of 3 mm^2. Each 1 mm x 1mm square is marked by triple lines. Each large square is further divided into 25 medium squares (Figure 2.3). The fundamental measurement is the average number of cells per mm square, so the centre large square is usually counted. To obtain the total number of cells in this large square, the number of cells in each of the 25 medium squares was counted as shown in Figure 2.3. When counting cells bordering on triple ruling, the convention is to count only those cells touching the top and left hand side ruling of each square. Cell counts from the two chambers were recorded and added. To obtain the cell density, the numbers of cells were calculated the average cell count and multiply by the chamber conversion factor (ccf) for Improved Neubauer = x 10^4. The actual cell number was then calculated using the formula below:

Mean X =$\sum$Xi/n, where Xi=total no., $\sum$Xi = sum of cell totals, and n = no. of counts

Cell count (cells/ml) = X x ccf (chamber conversion factor =10^4) x dilution factor (1:20)

Sample cell count:

No of cells (X) = No. of cells from chamber A (25 grids) + No. of cells from chamber B (25 grids)/2

No. of cells/ml = X (average count/square) x dilution factor x chamber conversion factor (10^4) cells/ml

For example: (X) = No of cells from A (200) + No. of cells from B (220)/2=210

Average cell count (X) = 210

No. of cells/ml = 210 x 20 (dilution 1:20) x 10^4 cells/ml

= 4200 x 10^4 cells/ml

=4.2 x 10^7 cells/ml

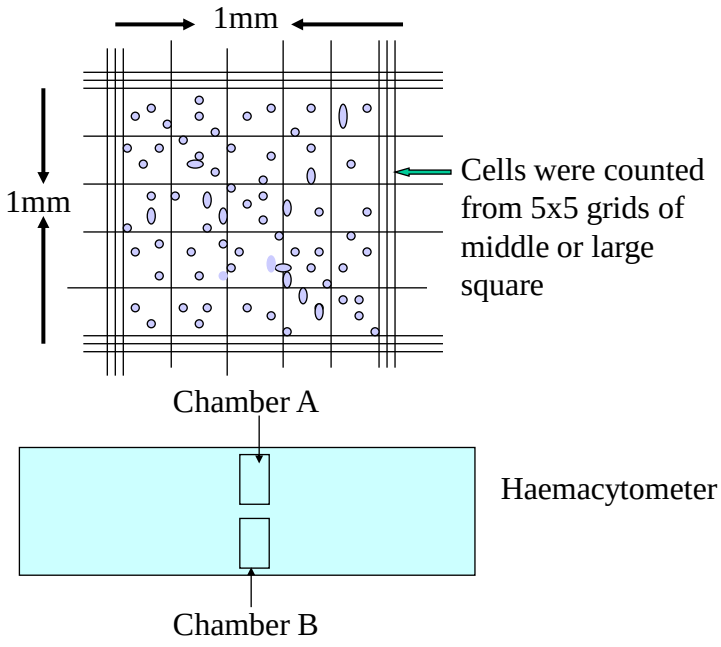


Figure 2.3 A diagram showing how to count cells using a haemacytometer

2.2.2.2.2 Cell viability checking by Trypan Blue dye exclusion method

The viability of the human lymphocytes was determined using the Trypan Blue exclusion method. Human lymphocytes were counted by making a dilution of 1:20 in Trypan Blue concentration (0.4 %) in 0.81% sodium chloride and 0.06% potassium phosphate dibasic (Sigma Ltd., UK). The suspension was incubated at room temperature for 30 seconds. A haemacytometer (Improved Neubauer, Weber Scientific International Ltd, UK) was used to determine the percentage viability by counting the number of stained and unstained cells in the haemacytometer grid. All counts were performed in duplicate as described in section 2.2.2.2.1. Cell viability was expressed as a percentage of unstained cells in the cell expression as follows.

Cell Viability (%) = (total viable / total viable and non viable) x 100

2.2.2.3 Cytospin slide preparation

100 μl of the non-adherent cell suspension was pipetted onto a microscope slide mounted in a cytospin holder and centrifuged (Labofuge 400, Sorvall Heraeus, Kendro Laboratory Products Ltd., Hertfordshire, UK) at 250 g for 5 minutes at room temperature. The slide was dried and was immersed in absolute ethanol for 5 minutes. Finally the slide was dried again at room temperature. This was ready for staining process.

2.2.2.4 Slide staining (May Grunwald-Geimsa stain)

The slide was stained using May Grunwald-Giemsa stain according to a standard procedure (Dacie and Lewis, 1991). Firstly, the cytospin preparation was fixed in absolute ethanol for 5 minutes, the cytospin was then stained for 5 minutes with May Grunwald stain diluted with an equal volume of phosphate buffer pH 6.8. Without washing, the film was then stained with Giemsa stain (diluted 1/9 V/V in phosphate buffer pH 6.8) for 15 minutes. Finally, slides were washed thoroughly with phosphate buffer (pH 6.8) for 5 minutes to complete differentiation. Slides were air dried and examined under an oil immersion objective lens (1000 x magnification). At least 5 microscope fields, or 100 cells per slide, were identified and counted. From these data, the % of lymphocytes in each preparation was determined using the formula below.

% lymphocytes = (number of lymphocytes / total number of leukocytes) x 100

2.2.2.5 Radioligand binding studies

[^{3}H]- (-)-Nicotine binding studies

Binding experiments were performed as previously described (Morgan *et al*., 2001), using a filtration assay with the aid of a Skatron cell harvester. [^{3}H] - (-)-nicotine was

used as the radioligand and was kept frozen (-20ºC) in aliquots of ethanol to avoid degradation. Radiolabeled nicotine was used within 2 months of its synthesis (or supply).

Three kinds of experimental binding protocols were used. These were kinetic binding experiments, saturation binding experiments, and competitive binding experiments.

Human lymphocytes (1 x 10^7 cells/ml) were resuspended in Hanks' Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}, pH 7.4). The cells were incubated in a final volume of 250 μl of assay buffer (HBSS). Cells were incubated with 25 μl of [^{3}H]-(-)-nicotine in microtube wells, at 4°C (Camo Ltd, Suffolk, U K). The assays were performed at 4°C in order to reduce the degradation of radioligand and/or receptor. The incubation time was varied depending on the type of experiment. For the single point of each experiment, the assay was completed in triplicate. Non-specific binding was determined by incubating cells in the reaction mixture in the presence of 1 x 10^{-5} M unlabeled-(-)-nicotine.

Incubations were terminated by filtration using a cell harvester, and filters were washed at least three times with 5 ml of ice-cold assay buffer (HBSS). Finally, filters were removed from the harvester and placed into Scintillation vials containing 4 ml of Liquiscint (National Diagnostics, UK). The radiation in each filter was measured by a Packard 2500 Liquid Scintillation counter. Specific binding was determined by subtracting the non-specific binding from the total binding (Specific binding = Total binding - nonspecific binding).

Kinetic studies

Kinetic binding experiments, measure binding at various times to determine the rate constants for radioligand association and dissociation. A concentration of 2 x 10^{-8}M (20 nM) [^{3}H]-(-)-nicotine was used in these kinetic binding studies. In these experiments incubation times of 0, 1, 5, 10, 15, 30, 60, and 90, 120 minutes at 4°C were used. For each incubation time, assays were performed in triplicate for each donor (one sample) and lymphocytes from 4 different donors (4 samples) were used in these kinetic experiments and the data obtained expressed as means ± S.E.M. Specific binding at each time point was determined by subtracting non specific binding from total binding.

Saturation studies

Saturation binding studies measure the equilibrium binding of increasing concentration of [^{3}H]-(-)-nicotine for the receptor. Saturation experiments were used to determine the affinity (K*d*) of [^{3}H]-(-)-nicotine for the receptor and the density (B*max*) of a specific receptor on human lymphocytes. Fourteen [^{3}H]-(-)-nicotine concentrations ranging from 0.9 nM to 40 nM were used. The incubation time for these experiments was 50 minutes at 4°C. In each experiment, all [^{3}H]-(-)-nicotine concentrations were replicated in triplicate. Specific binding at each concentration can be determined by subtracting nonspecific binding from total binding. For each study, 5 lymphocyte samples from different donors were used and the data obtained expressed as means ± S.E.M. The K*d*

and B*max* were analysed by non-linear regression analysis from Prism Graphad software by fitting the data to the equation for saturation curve.

$Y = B_{max\ x}\ X\ /K_d+X$, Where X was the radioligand concentration used in nM, and Y was specific binding.

Competitive studies

Competitive binding studies measure equilibrium binding of a single concentration of [^{3}H]-(-)-nicotine at various concentration of unlabeled ligands (competitors) that compete for the receptor present, as the concentration of unlabeled ligands were increased, the amount of radioligand bound to the receptor decreased. Competitive binding studies were used to determine the affinity of unlabeled ligands for the receptor indirectly by measuring its ability to compete with a radioligand. In these experiments, a range of nicotinic cholinergic receptor ligands, listed earlier in this chapter (section 2.2.1) and α-bungarotoxin were used. Various concentrations of each unlabelled ligands (1×10^{-5}M – 1×10^{-13}M) were used. We chose to study these ligands because (-)-nicotine, epibatidine, and cytisine, (nAChR agonists) have a high affinity for the heteromeric nAChRs containing combinations of α and β subunits (α2, α3, α4, and α6) (Gotti *et al*., 2000) while α-bungarotoxin (nAChR antagonist) has a high affinity for α7 homomeric nAChRs (Paterson and Nordberg, 2000), and hexamethonium is antagonist of neuronal nAChRs. Carbachol is an agonist for nicotinic and muscarinic acetylcholine receptors while atropine is antagonist for the muscarinic acetylcholine receptors. For these studies, A single concentration of [^{3}H] - (-) -nicotine of 1.5×10^{-8}M (15 nM) was used. Incubations

were performed at 4°C for 35 minutes. In each experiment, a given concentration of unlabeled ligand was repeated in triplicate. As described above, in each concentration of unlabeled ligand, lymphocyte samples from 4-5 different donors were used. Specific binding at each concentration of unlabeled ligand can be determined by subtracting nonspecific binding from total binding.

2.2.2.5.1 Data analysis

The data from binding assays were analysed by Prism (GraphPad; San Diego, USA). All values including B*max*, K*d*, half-life, and K*i* values were determined from data using non-linear curve fitting and analysis procedures contained in the software. These programs fit non-linear regressions to data using an iterative procedure. Values were calculated from individual experiment and that the data presented are means ± SEM of the number of experiments stated.

2.3 Results

2.3.1 Characterisation of human lymphocytes

Yield of lymphocytes

The number of lymphocytes recovered from a sample of blood differed from donor to donor and was dependent, to an extent, on the initial volume of the Buffy coat provided. However, usually approximately 30 x 10^7 cells were recovered from a single Buffy coat with initial volume of 60 ml and giving final volume of 30 ml of cells when suspended at 1 x 10^7cells/ml. This, in turn limited the number of incubations that could be performed on cells from a single donor.

For example in competition studies about 20 single points of concentrations (from 1 x 10^{-5}M – 1 x 10^{-13}M) in triplicate for each unlabeled ligand have to be done for both total binding and non specific binding. Thus the total number of lymphocytes used in the competition studies, calculated by this formula (20 concentrations x 6 tubes for each single point x 200 µl of lymphocyte suspension containing 1 x 10^7 cells/ml) = 24,000 µl (24 ml) so that at least 24 ml. of 1 x 10^7 cells/ml was needed for each unlabeled ligand used in the competition experiments.

Sometimes the yield of lymphocytes recovered from a donor was not sufficient to a full experiment. This comprised approximately 20% of the Buffy coats used and these samples were processed to provide lymphocyte membrane protein for use in future work.

2.3.1.1 Viability of cellular preparation

The viability of the cells was routinely checked at the beginning of each experiment. All lymphocyte cell suspensions used in these experiments had viability greater than 95 % as determined by Trypan Blue exclusion tests. The lymphocyte purity was greater than 99% as determined staining cytospin preparations followed by microscopic examination (Figure 2.4).

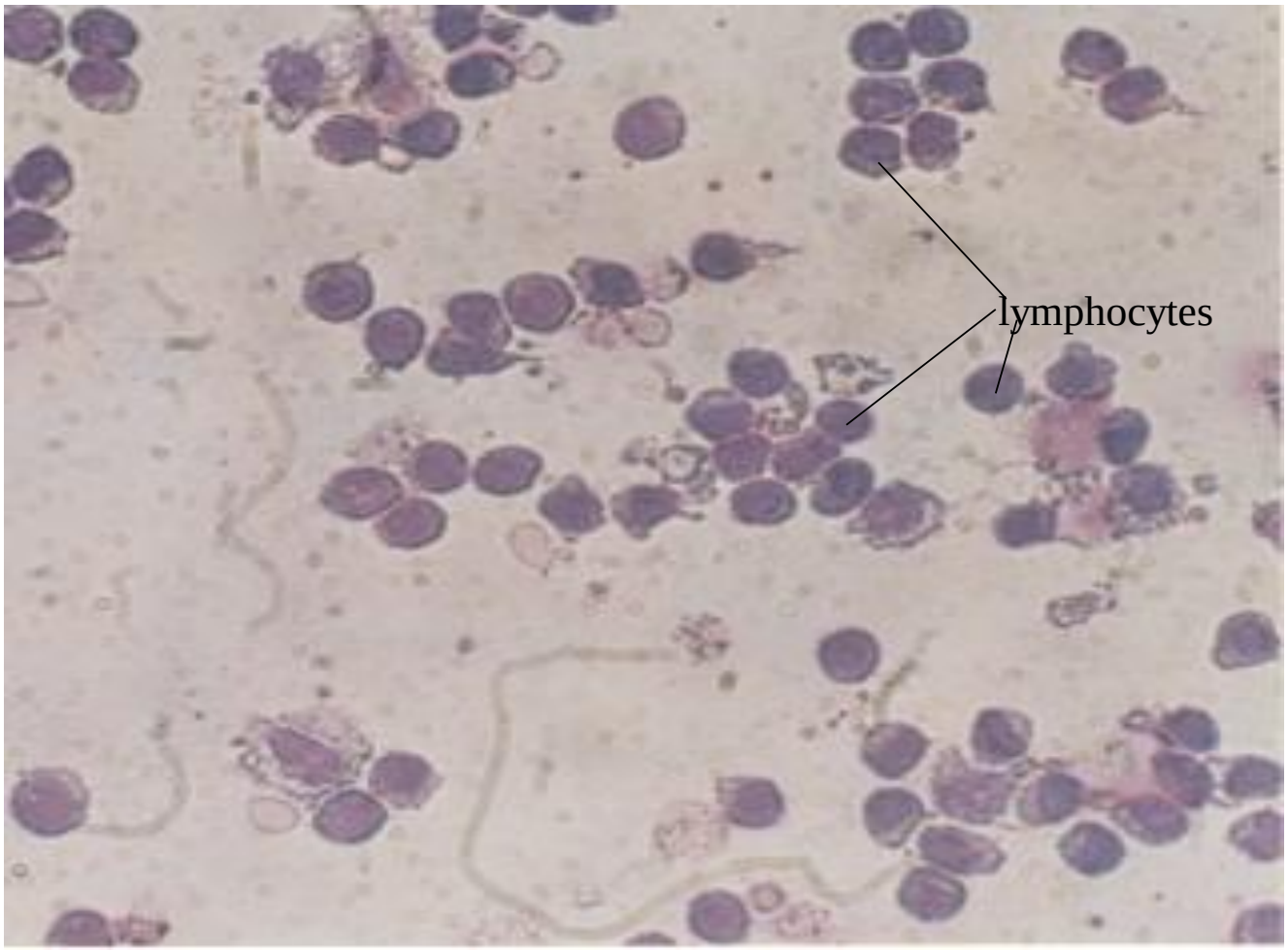


Figure 2.4 A photograph of a typical cytospin preparations of purified human peripheral blood lymphocytes used in the present study.

2.3.2 Radioligand binding experiments

2.3.2.1 Kinetic binding experiments

The binding of [^{3}H]-(-)-nicotine (2 x 10^{-8}M or 20 nM) to human peripheral blood lymphocytes was performed at 4˚C as preliminary binding studies on lymphocyte membranes and THP-1 cells showed that there was a large level of non-specific binding recorded in samples incubated at 37ºC compared to those incubated at 4 ºC (Morgan, 2001, PhD Thesis). The ratio of non-specific binding and specific binding was calculated at 37ºC the ratio was 5.3 and at 4 ºC was 3.1 (Morgan, 2001, PhD Thesis). As a result these subsequent binding experiments were performed at 4ºC. The assays were also performed at 4°C in order to reduce the degradation of radioligand and/or receptor. The specific binding represents the difference between the total and non-specific counts (the specific binding = total binding - non specific binding). Prism subtracted nonspecific binding from total binding to give specific binding at each time point. The total, non-specific and specific binding over time is shown in Figure 2.5.

From the data shown in Figure 2.6 [^{3}H]-(-)-nicotine binding at 4˚C displayed a half time of association of 12.02 ± 5.15 minutes and reached a plateau after 25 minutes (Figure 2.6). This steady state continued for a further 35 minutes. At approximately 60 minutes, the specific binding began to decline rapidly (Figure 2.6).

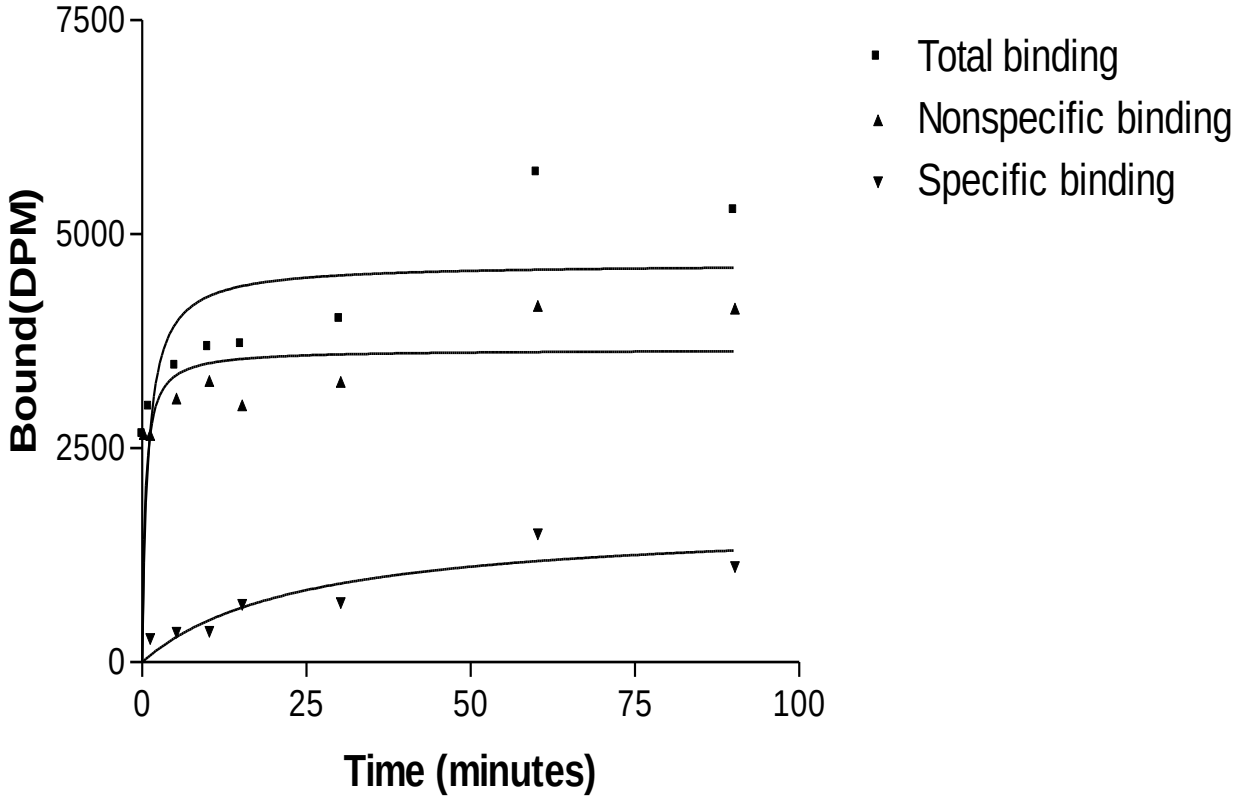


Figure 2.5 Time course for equilibration of [^{3}H]-(-)-nicotine with human lymphocytes. Each point represents the mean ± SEM of data from 4 donors performed in triplicate. The non-specific binding was determined by the use of 10^{-5}M cold unlabeled-(-)-nicotine. Specific binding is the difference between total and non-specific binding. [^{3}H]-(-)-Nicotine $2x10^{-8}$M (20nM) bound to human lymphocytes in a time-specific manner. From these data half time values of association were calculated by Prism software.

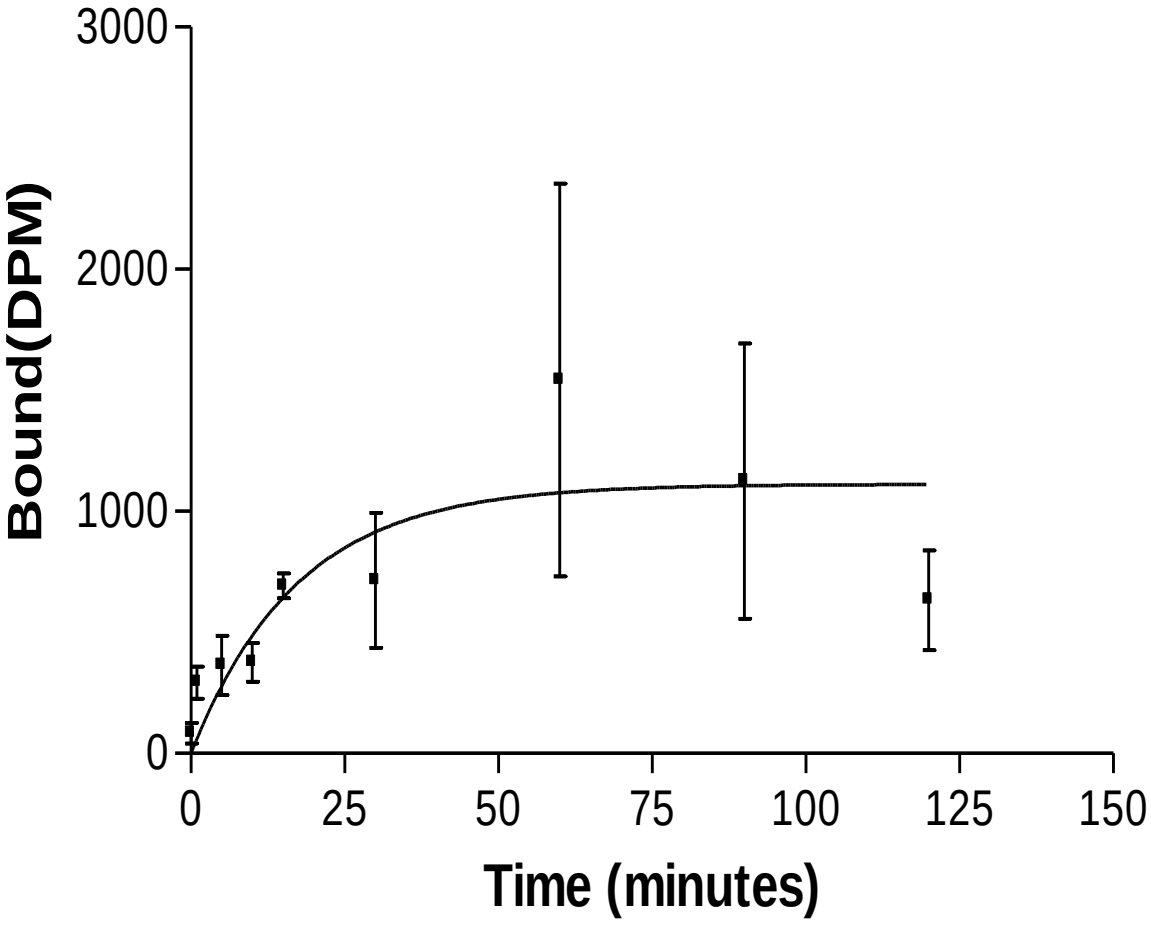


Figure 2.6. Time course for equilibration of binding of [^{3}H]-(-)-nicotine (2x10^{-8}M or 20 nM) to intact human lymphocytes at 4˚C. Specific binding data were analysed by Prism and the half-life of association was obtained. The half-time for association (T½) was 12.02 ± 5.15 minutes. Each point represents the mean ± SEM of data from 4 donors.

When an excess of unlabelled nicotine was added to cell suspensions that had been preincubated with 2.0 x 10^{-8}M or 20nM [^{3}H]-(-)-nicotine for sufficient time for equilibrium of specific binding to be reached (25 minutes), the specific binding of the radioligand decreased in a time-dependent fashion. From these data a half-life of dissociation for [^{3}H]-(-)-nicotine from human lymphocytes was analysed by Prism. It was 20.61 ± 6.96 minutes (Figure 2.7)

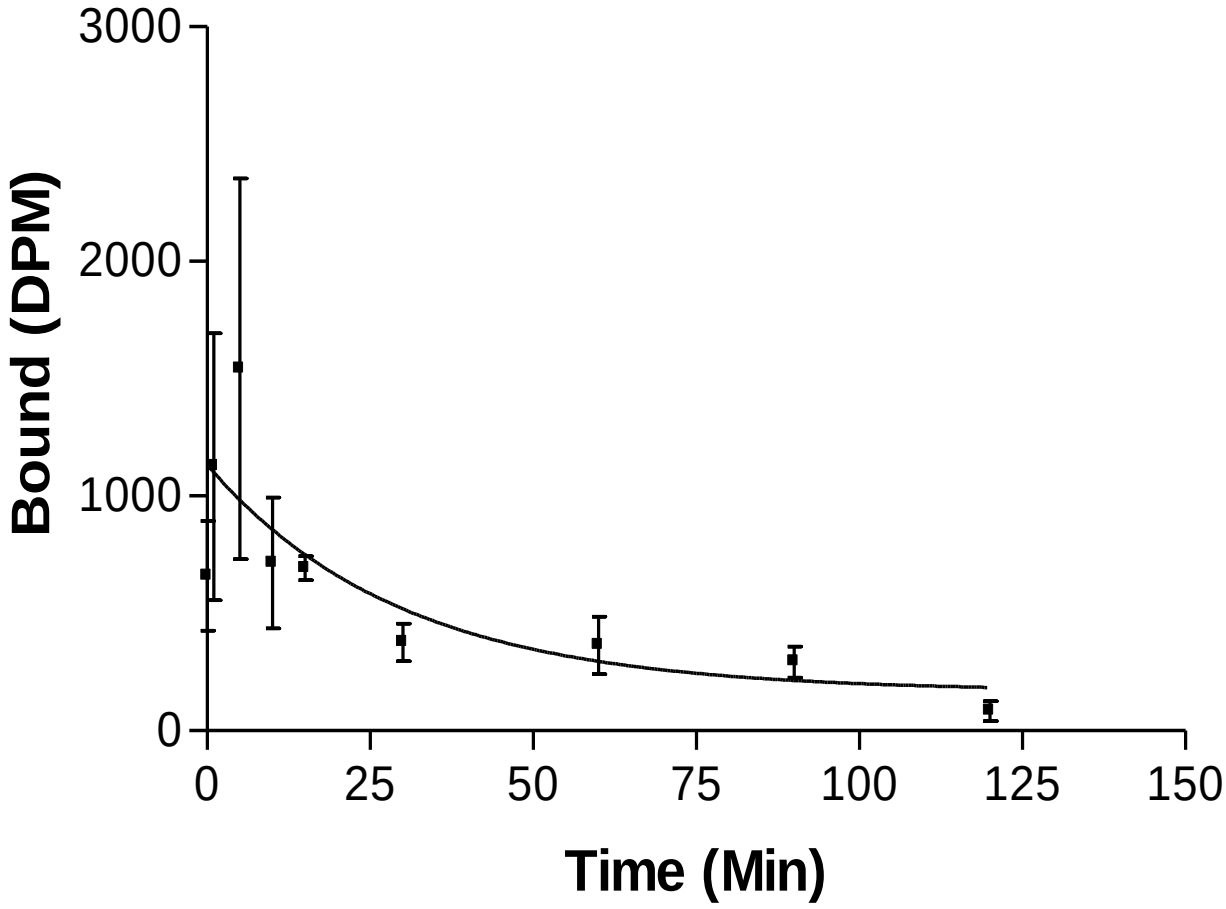


Figure 2.7 A dissociation time course for binding of [^{3}H]-(-)-nicotine (2x 10^{-8}M or 20nM) to human lymphocytes. Dissociation was determined by the reduction of nicotine bound at different time points after addition of an excess of cold unlabeled-(-)-nicotine (1x10^{-5}M). Data shown are from a representative experiment performed in triplicate from 4 different donors. T½ was 20.61 ± 6.96 minutes.

2.3.2.2 Saturation binding experiments

Saturation binding experiments were undertaken to measure the specific binding, at equilibrium, of various concentration of [^{3}H]-(-)-nicotine so that the number of binding sites (B*max*), and the ligand affinity (K*d*), could be determined. Concentrations of [^{3}H]-(-)-nicotine from 0.9 nM-40 nM (0.9×10^{-9}M - 4.0×10^{-8}M) were used in these studies on intact human lymphocytes. From these data the presence of a single binding site for nicotine was identified on human lymphocytes (Figure 2.8) with a K*d* of 15.00 ± 5.759 nM ($1.5 \pm 5.759 \times 10^{-8}$M) and a B*max* of 2253 ± 409.00 sites/cell. From Figure 2.8 it can also be seen that at concentrations of [^{3}H]-(-)-nicotine greater than 30 nM (3.0×10^{-8}M), the specific binding started to decline. K*d* and B*max* values were analysed by non-linear regression analysis from GraphPad Prism.

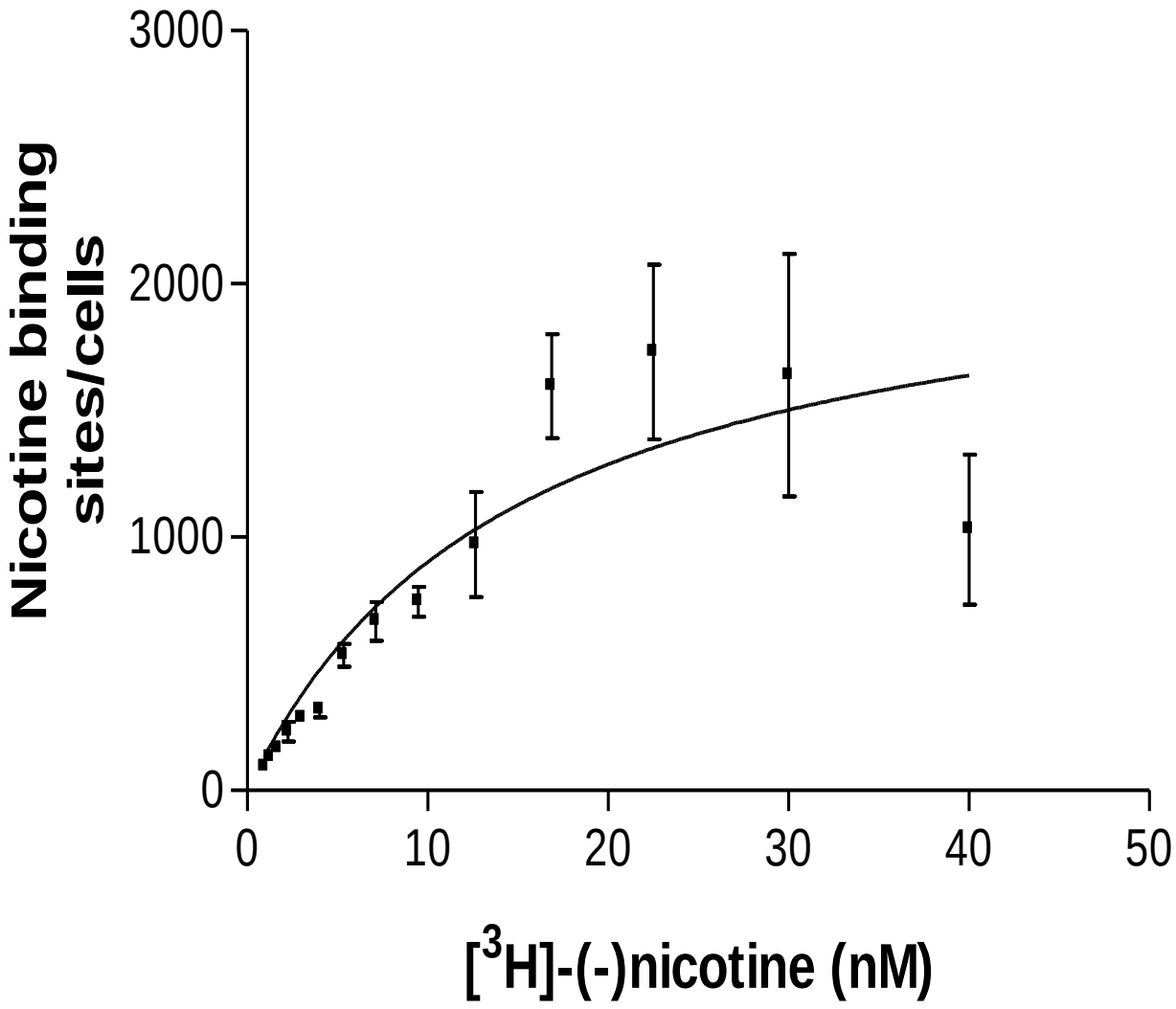


Figure 2.8 Saturation binding of [^{3}H]-(-)-nicotine to human lymphocytes. The graph shows specific binding of [^{3}H]-(-)-nicotine to human lymphocytes. The results were obtained from a filtration assay conducted at 4 ºC for 50 minutes. Data represented are the mean ± SEM of 5 experiments and each experiment was performed in triplicate. The B*max* of binding site was 2253 ± 409.00 sites/cells and K*d* was 15.00 ± 5.759 nM (1.5 ± 5.759 x 10^{-8}M).

2.3.2.3 Competition binding studies

Competition binding experiments were undertaken to measure the specific binding, at equilibrium, of a single concentration of [^{3}H]-(-)-nicotine (1.5 x 10^{-8}M or15nM), in the presence of various concentrations of an unlabeled compound so that the affinity of the compound for the receptor could be determined. A number of compounds such as (-) -nicotine, α-bungarotoxin, cytisine, epibatidine, hexamethonium, atropine, and carbachol were used to investigate the nature of nicotine binding sites on human lymphocytes. Each compound was studied in 5-6 donors and each determination performed in triplicate. IC50 values were calculated from 5-6 replicate experiments performed in triplicate. All data were analysed by monophasic competition curves by GraphPad Prism. The data are presented in Table 2.1 and summarised the displacement of [^{3}H]-(-)-nicotine from human lymphocytes by a range of nAChR ligands. However, close inspection of Figures 2.9-2.15 shows that many of the ligands did not displace [^{3}H]-(-)-nicotine in a consistent manner and IC50 values may be misleading. Thus, ligands could be classified as those that displace [^{3}H]-(-)-nicotine and those that do not. These data summarised in Table 2.2. Figures 2.9-2.15 illustrates typical experiments in graphical form for each ligand to show the pattern of competition of these ligands with [^{3}H]-(-)-nicotine binding to human lymphocytes.

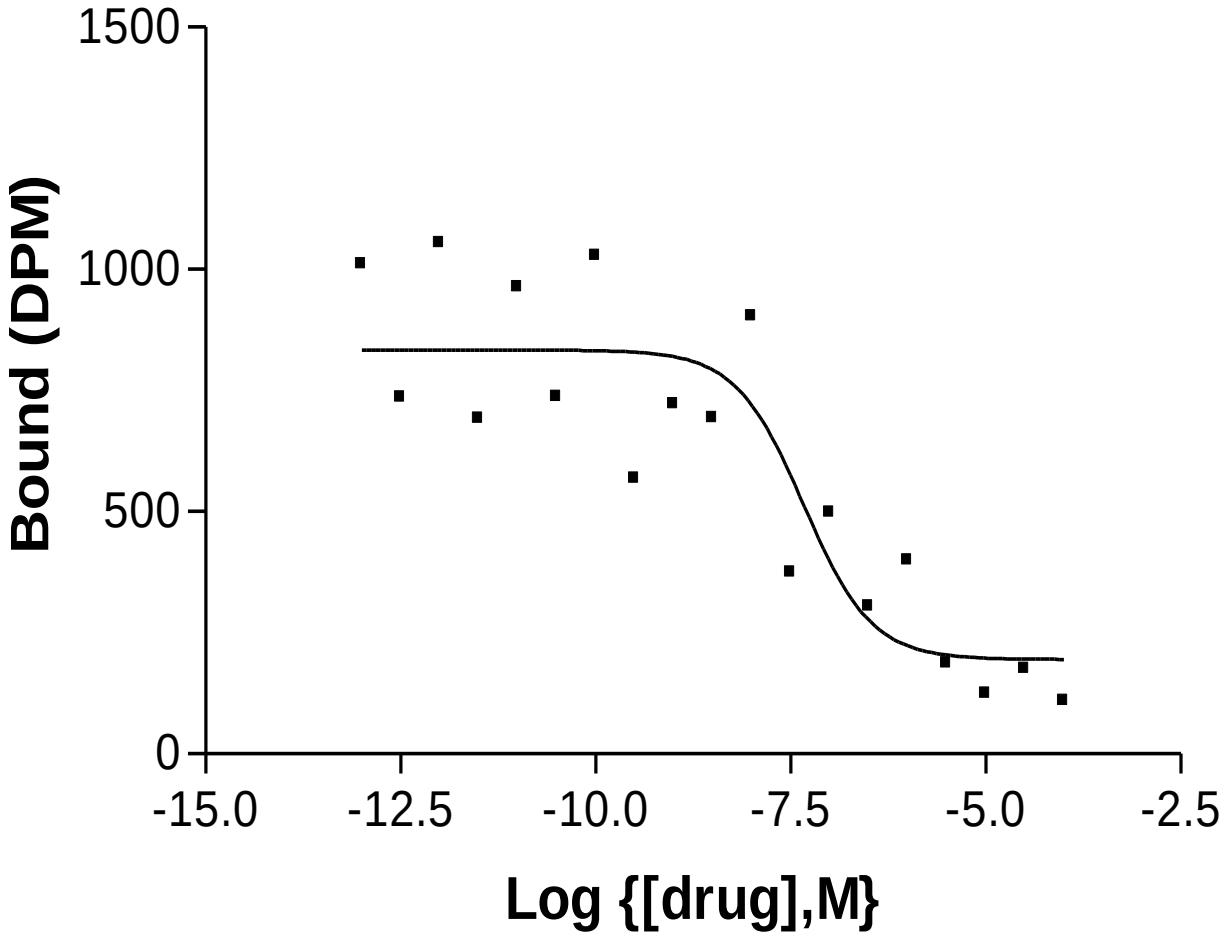


Figure 2.9 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by unlabeled-(-)-Nicotine. Cells were incubated with 15 nM (1.5 x 10^{-8}M) [^{3}H]-(-)-nicotine in the presence of a serial dilution of (-)-nicotine for 35 minutes, at 4ºC. This is a single experiment in graphical form for nicotine. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean S.E.M calculated. Log {[drug], M}=Log drug concentration (M).

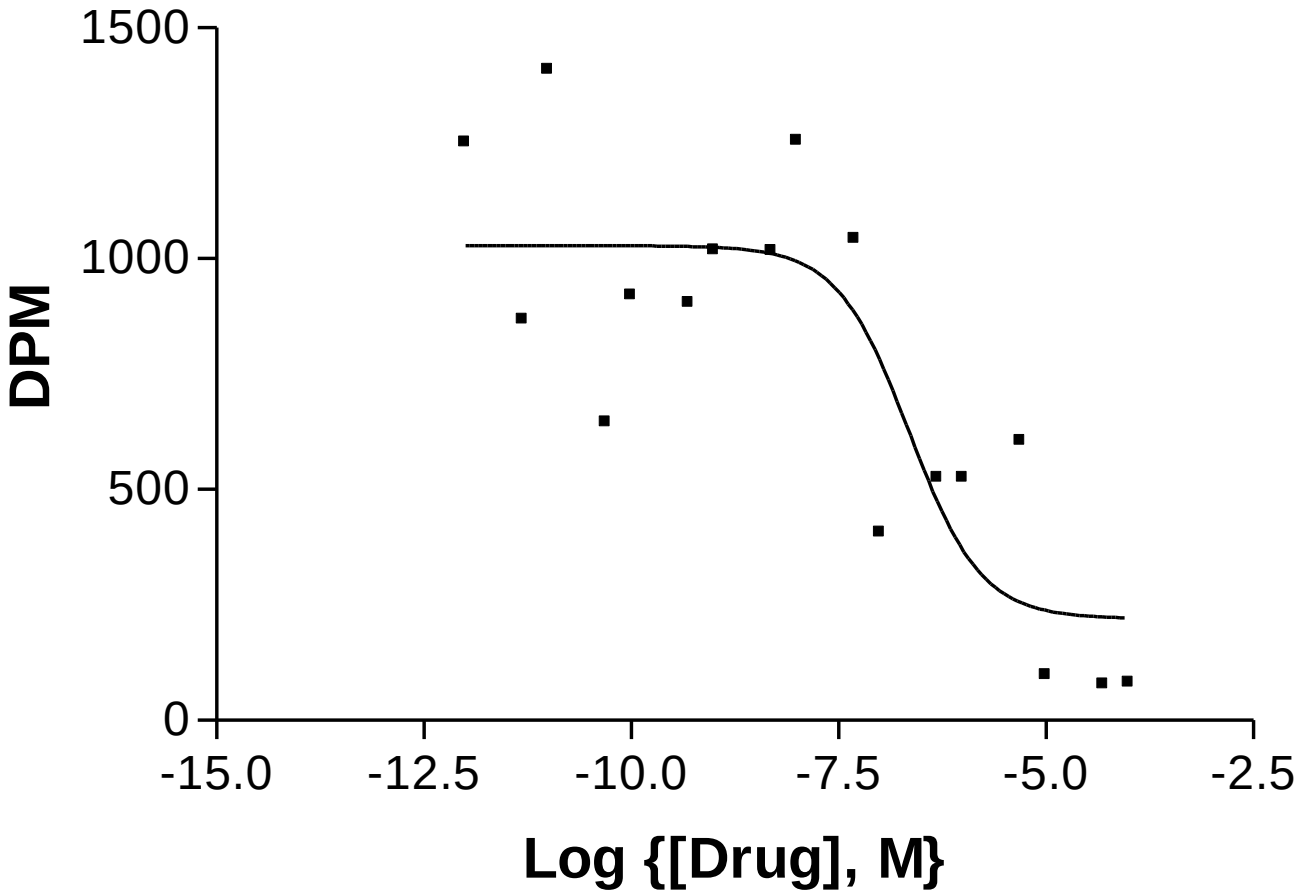


Figure 2.10 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by hexamethonium. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of hexamethonium for 35 minutes at 4ºC. This is a single experiment in graphical form for hexamethonium. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean S.E.M calculated. Log {[drug], M} = Log drug concentration (M).

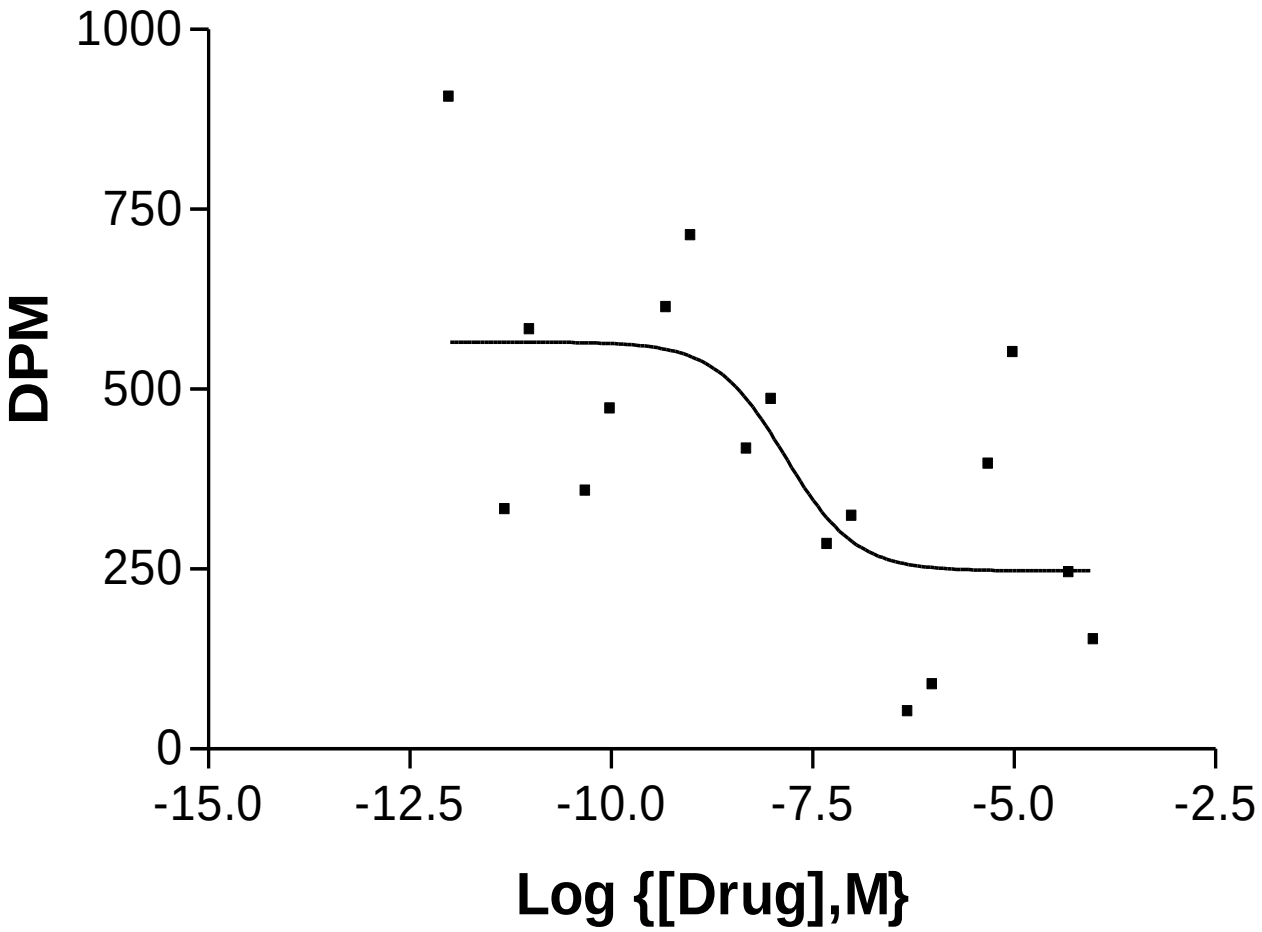


Figure 2.11 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by carbachol. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of carbachol for 35 minutes at 4ºC. This is a single experiment in graphical form for carbachol. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean S.E.M calculated. Log {[drug], M} = Log drug concentration (M).

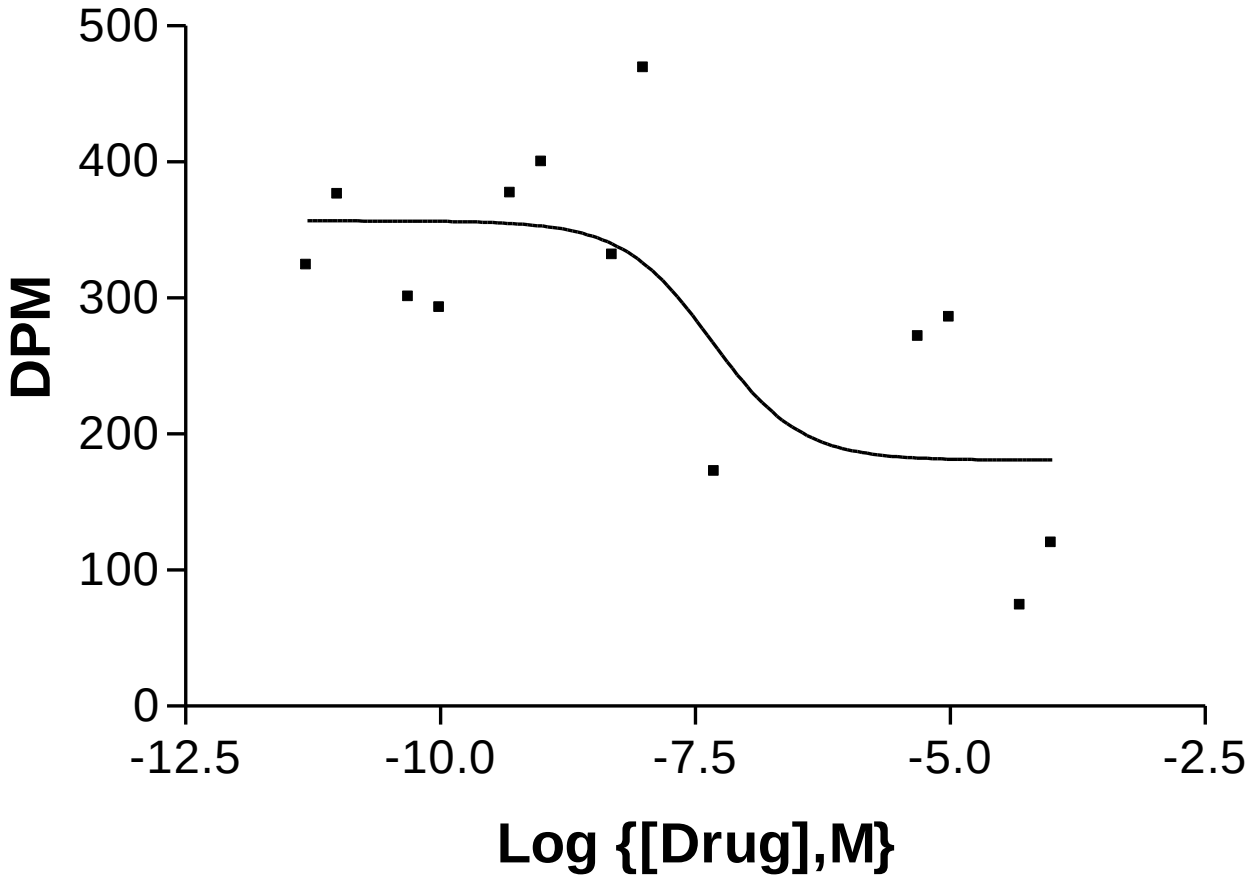


Figure 2.12 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by cytisine. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of cytisine for 35 minutes at 4ºC. This is a single experiment in graphical form for cytisine. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean and S.E.M calculated. Log {[drug], M} = Log drug concentration (M).

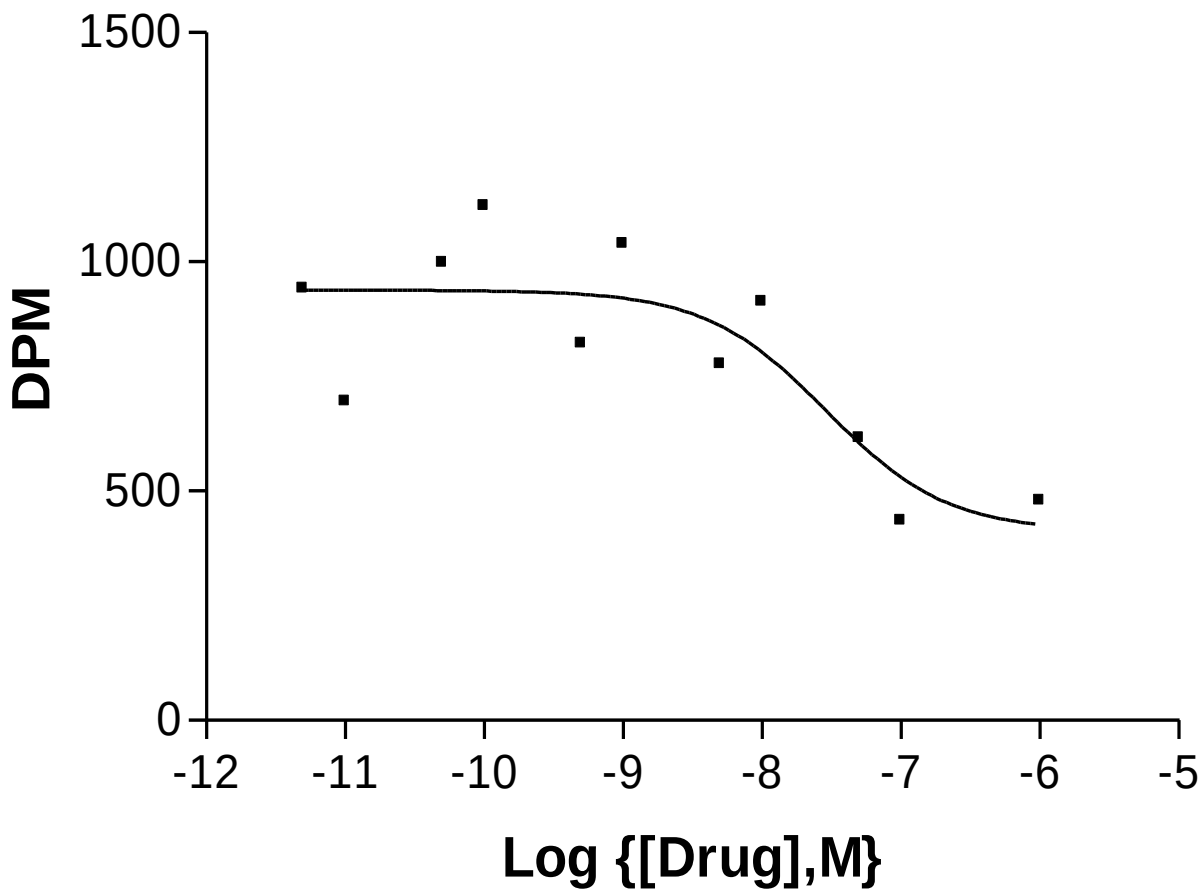


Figure 2.13 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by epibatidine. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of epibatidine for 35 minutes at 4ºC. This is a single experiment in graphical form for epibatidine. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean and S.E.M calculated. Log {[drug], M} = Log drug concentration (M).

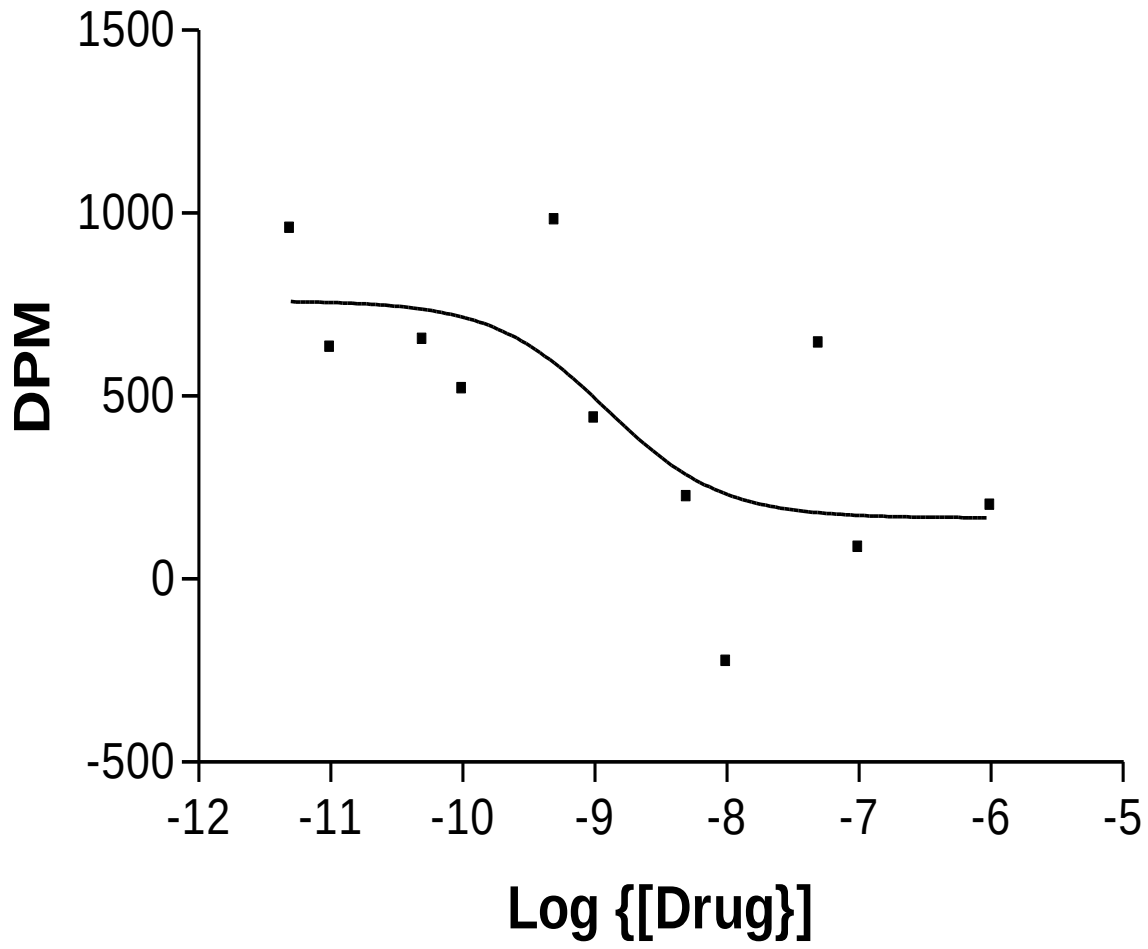


Figure 2.14 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by α bungarotoxin. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of alpha bungarotoxin for 35 minutes at 4ºC. This is a single experiment in graphical form for α bungarotoxin. Each point is the mean of a single concentration performed in triplicate. IC50 values were calculated from 5 replicate experiments and the mean and S.E.M calculated. Log {[drug], M} = Log drug concentration (M).

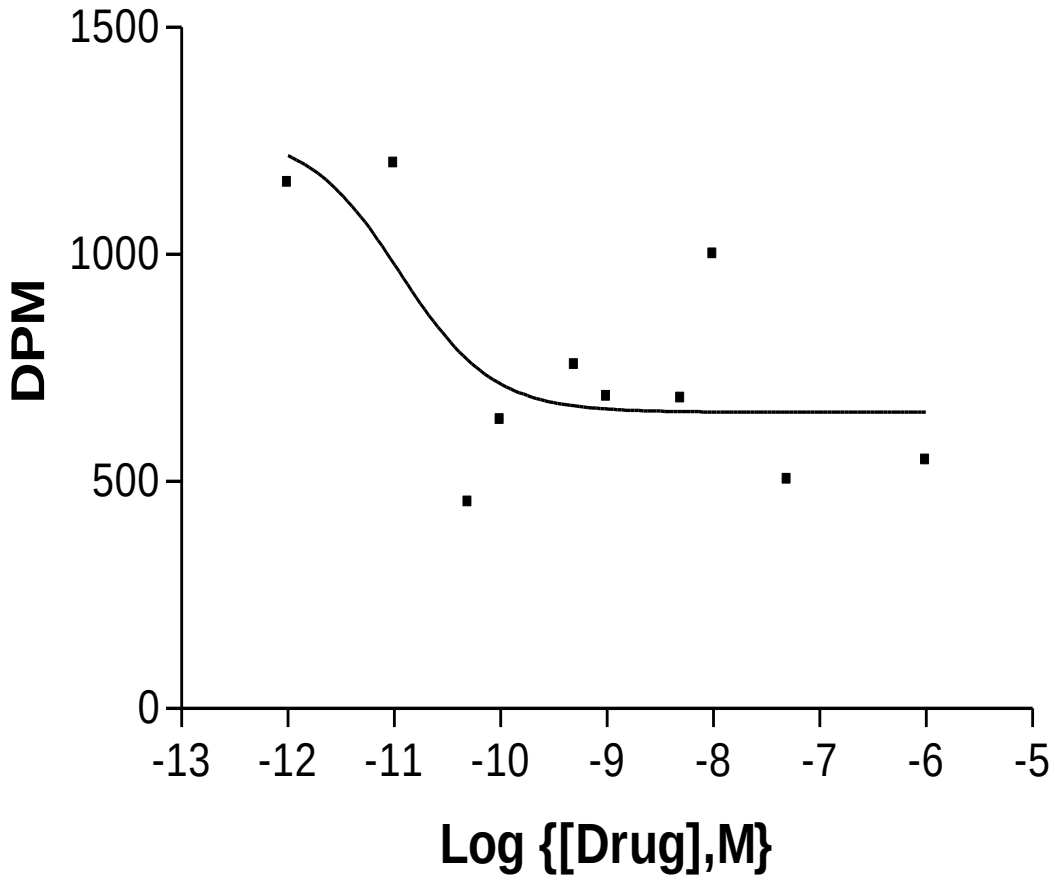


Figure 2.15 Competition binding of [^{3}H]-(-)-nicotine to human lymphocytes by atropine. Cells were incubated with 15 nM [^{3}H]-(-)-nicotine in the presence of a serial dilution of atropine for 20 minutes. This is a single experiment in graphical form for atropine. IC50 values were calculated from 5 replicate experiments and the mean and S.E.M calculated. Each point is the mean of a single concentration performed in triplicate. Log {[drug], M} = Log drug concentration (M).

Competing ligand	IC50 (nM)
Nicotine	**2.30 ± 5.65 nM**
Hexamethonium	**3.88 ± 1.566 nM**
Carbachol	**75.65 ± 0.69 nM**
Cytisine	**45.63 ± 2.60 nM**
Epibatidine	**10.48 ± 2.14 nM**
α-BTX	**13.30 ± 2.83 nM**
Atropine	**35.00 ± 6.6 nM**

Table 2.1 A table showing the IC50 for the different ligands used in the competitive assay. All values were calculated from computer analysis from individual experiment and then the mean SEM of at least 4-5 experiments.

Competing ligand	Displacement
Nicotine	Displacement
Hexamethonium	Partial displacement (Variable SPB)
Carbachol	No displacement
Cytisine	Partial displacement
Epibatidine	Displacement
α-BTX	Displacement
Atropine	Partial displacement

Table 2.2 A table showing the range of ligands used in the competitive assay that displaced [^{3}H]-(-)-nicotine from human peripheral blood lymphocytes. SPB = Specific binding

2.4 Discussion

Data described in the present chapter show the presence of a single binding site for nicotine on human peripheral blood lymphocytes. The presence of nicotinic acetylcholine receptors on intact rat lymphocytes has been documented previously (Maslinski *et al*., 1992) on human lymphocytes isolated from patients with Parkinson's and Alzheimers diseases (Adem *et al.*, 1986). While Davies *et al*., (1982) reported that nicotine bound specifically to lymphocytes. However, little evidence exists on the characterisation of the nicotine binding site on lymphocytes.

The present study will increase knowledge of specific binding site for nicotine on human peripheral blood lymphocytes. By showing that nicotinic acetylcholine receptors exist on human lymphocytes and by describing the pharmacologic properties of the nAChR subunits.

The results presented in this chapter (radioligand binding experiments) show that there are specific binding sites for nicotine present on intact human peripheral blood lymphocytes. These binding sites have a half-life of association for nicotine of 12.02 ± 5.15 minutes and an equilibration time for binding of 25 minutes. These findings are similar to those obtained from experiments on [^{3}H]-(-)-nicotine binding to human peripheral blood lymphocytes (PBL) where binding peaked at 30 minutes and the half-life for binding was 8 minutes (Grabczewska *et al*., 1990). Maslinski *et al*., (1992) also obtained an association time of 30 minutes at 4ºC for [^{3}H]-(-)-nicotine binding to rat

whole lymphocytes. Thus, the data obtained in the present study are consistent with previously published data on human and rat lymphocytes and suggest that the binding measured is likely to be a nicotinic acetylcholine receptor.

The equilibration time obtained in the present study is faster than that reported for lymphocyte membranes by Morgan (2001, PhD Thesis), which showed an equilibrium time by 90 minutes and a half time association of 40.1 minutes. The equilibration values obtained from the present study are also faster than those previously published on granulocyte membranes, where an equilibration time of 60 minutes was obtained (Lebargy *et al*., 1996). These data suggest that in a given cell type, the association times may be faster when using whole cells, than in experiments using membrane preparations. It is possible that in membrane preparations, a larger number of receptors bound [^{3}H]-(-)-nicotine thus causing the membranes to take longer to reach equilibrium state because membrane preparations prevent the receptor from being internalized.

However, the association time obtained in the present study is slower than those reported on granulocyte preparations, which achieved maximum binding in 8-10 minutes (Davies *et al*., 1982). The association time was also slower than that obtained for nicotine binding sites on THP-1 (monocytic cell line) whole cells which reported an association time for nicotine binding of 5 minutes (Morgan *et al*., 2001). It is possible that different nAChRs subunits were present on these cells or that in the present study the number of cells (1×10^7 cells/ml) used in the binding studies was less than Davies *et al*., (1982) and Morgan *et al*., (2001) (2×10^7 cells/ml).

The specific binding site of nicotine on intact human lymphocytes used in the present study remained at peak levels for about 60 minutes, after which the specific binding fell to approximately one-third of the maximum level. The change of specific binding from this study differs from the results obtained by a previous study (Morgan *et al*., 2001), which found that after 60 minutes period, the specific binding declined by approximately two-thirds of the maximum level. Conformational changes in nAChRs have been shown to alter the affinities of ligands for binding sites, and this may be the reason for the reduction in specific binding over time (Lena and Changeux, 1993, 1998; Wonnacott, 1990) and could explain the differences between published studies.

Nicotine binding to intact human peripheral blood lymphocytes was found to be reversible, as specific binding was reversed fully over time when an excess of unlabeled nicotine was added (half-time of dissociation = 20.61 ± 6.69 minutes). These dissociation kinetics of binding were similar to that reported from nicotine binding sites performed on human polymorphonuclear cells, (half-life of dissociation of 20 minutes) (Davies *et al*., 1982). This value is slower than the dissociation kinetics of binding found for nicotine binding to THP-1 cells (1.14 ± 0.56 minutes) (Morgan, 2001, PhD Thesis).

The specific binding site of [^{3}H]-(-)- nicotine to intact human lymphocytes was saturable and was best fitted by monophasic exponential binding curve suggesting the presence of a single binding site for nicotine. Using non-linear regression analysis, a one-site model for analysis of the saturation curve resulted in *Kd* and B*max* values of 15.00 ± 5.759 nM

(1.5 ± 5.759 x 10^{-8}M) and 2253 ± 409.00 sites/cell. The *Kd* value obtained from this study which used [^{3}H]-(-)- nicotine binding to human lymphocytes, falls between the *Kd* values reported for nicotine binding on other leukocytes (Davies *et al*., 1982; Grabczewska *et al*., 1990; Maslinski *et al*., 1992; Lebargy *et al*., 1996; Benhammou *et al*., 2000;). Lebargy *et al*., (1996) reported a *Kd* of 1.08 nM (1.08 x 10^{-9}M) for granulocytes, while Benhammou *et al*., (2000) reported a *Kd* of 3.47 nM (3.47 x 10^{-9} M) for peripheral human polymorphonuclear (PMN) leukocytes. Grabczewska *et al*., (1990) reported a *Kd* of 4 nM (4 x 10^{-9}M) for human peripheral lymphocytes while Maslinski *et al*., (1992) reported a *Kd* of 10 (1 x 10^{-8}M) and 7.5 nM (7.5 x 10^{-9}M) from rat lymphocytes and rat thymocytes, respectively. Davies *et al*., (1982) reported a *Kd* of 36 nM (3.6 x 10^{-8}M) for granulocytes and 30 nM (3.0 x 10^{-8}M) for human lymphocytes. The *Kd* obtained in the present study was similar to that obtained by Morgan *et al*., (2001) for THP-1 cells (12.1 ± 1.6 nM or 1.2 x 10^{-8}M). Because non specific binding was high in all published studies that used [^{3}H]-(-)-nicotine as a radioligand the variation in *Kd* values not unexpected. Furthermore, variations of this magnitude have been reported when measuring nicotine binding in the brain (Wonnacott, 1987).

The B*max* value obtained in the present study was 2253 ± 409.00 sites/cell which is similar to that obtained by Grabczewska *et al*., (1990) for human peripheral lymphocytes (2000 sites per cell) and also similar to that obtained from rat lymphocytes (B*max* 1500 sites/ cell) (Maslinski *et al*., 1992). However, the number of binding sites/cell obtained in the present study differs from that of Morgan (2001, PhD thesis) (6174 sites per cell). The B*max* obtained on human lymphocytes by Davies *et al*., (1982) (18000 sites/cell)

was also greater than the B*max* in the present study. It is unclear whether or not this variation reflects differences in experimental methodology and further experiments are required to establish why this variation exists.

The value for receptor numbers is usually calculated assuming equal receptor distribution on each cell, while the real number of binding sites on the surface may depend on its function, stage of maturation (Maslinski *et al*. 1992) or activation (Grabczewska *et al*., 1990) and diseases (Adem *et al*., 1986). The nicotinic binding sites in malignant T-lymphocytes have a significantly higher density of nicotine binding sites compared to normal peripheral blood lymphocytes, or normal T cells which derived from normal lymphocytes (Grabczewska *et al*., 1990). The number of binding sites in polymorphonuclear cells (PMN) was an increased in tobacco smokers and correlated with the number of cigarettes smoked per day (Benhammou *et al*., 2000; Lebargy *et al*., 1996). The number of [^{3}H]-(-)-nicotine binding sites to different constituents of the human peripheral leukocytes population also showed a different number of binding sites (Davies *et al*., 1982) for example, neutrophils and monocytes had an average of 87,000 and 74, 000 binding sites per cell respectively while lymphocytes had even fewer binding sites for nicotine (Davies *et al*., 1982).

In the present study [^{3}H]-(-)- nicotine binding to human peripheral lymphocytes showed the presence of a single of nicotine binding site. However, competition studies with other ligands may enable this binding site to be further subdivided. These findings are similar to the results for [^{3}H]-(-)- nicotine binding to rat and human lymphocytes published by

others (Maslinski *et al*., 1992; Grabczewska *et al*., 1990). Benhammou *et al*., (2000) also found one nicotine-binding site on [^{3}H]-(-)- nicotine binding to human peripheral polymorphonuclear cells. Davies *et al*., (1982) suggested that the non-cholinergic receptor on human phagocytic leukocytes was a single, novel class of receptor. Morgan *et al*., (2001) also identified two nicotine binding sites on THP-1 cells one of which was non-cholinergic in nature. This non cholinergic site appeared to bind (+)-nicotine with an apparent high affinity. However, the identity of the second site may have resulted from the presence of a contaminant in the radioligand (Morgan *et al*., 2001) and further studies with radioligand of known isomeric purity would necessary to resolve this question.

In saturation experiments, binding of [^{3}H]-(-)-nicotine to human lymphocytes (for 50 minutes) at concentrations, greater than 30 nM (3.0×10^{-8}M) rapidly declined. Because of this, the incubation time was reduced from 50 minutes to 25 minutes in order to prevent desensitisation of nAChRs occurring. However the data obtained still showed the same decline for the binding of [^{3}H]-(-)-nicotine to human lymphocytes with time at concentrations greater than 30 nM (3.0×10^{-8}M). It is possible that the conformational changes in nAChRs may be the reason for the reduction in specific binding seen when the receptor is exposed to high concentration of [^{3}H]-(-)-nicotine (Lena and Changeux, 1993, 1998). Interestingly, none of any previous studies in which [^{3}H]-(-)-nicotine was used as a ligand for saturation study used concentrations greater than 30 nM (Davies *et al*., 1982; Adem *et al*., 1986; Grabczewska *et al*., 1990; Maslinski *et al*., 1992; Benhammou *et al*., 2000). Thus for, saturation studies, nicotine binding to leukocytes seems to be consistent up to a ligand concentration of 10 nM (1×10^{-9}M) and then becomes more variable. This

could be due to the receptor desensitization and/or internalisation associated with using higher ligands concentrations or the other factor involved such as lipophilicity of nicotine.

In order to determine whether [^{3}H]-(-)-nicotine binding to lymphocytes was cholinergic in nature; a range of classical cholinergic nicotinic and muscarinic ligands were tested for their ability to displace [^{3}H]-(-)-nicotine binding. (-)-Nicotine, epibatidine, and α BTX fully displaced [^{3}H]-(-)-nicotine binding to human lymphocytes. These findings suggest that human peripheral blood lymphocytes may contain nAChR subunits that specifically bind these ligands. (-)-Nicotine and epibatidine have a high affinity for the heteromeric nAChRs containing combinations of α and β subunits, especially α 4 subunits (Gotti *et al*., 2000), and α BTX, an antagonist, has a high affinity for α7 homomeric nAChRs (Paterson and Nordberg, 2000). Thus the α7 nAChR subunit might be another subunit that is present on human peripheral lymphocytes. However, only unlabeled nicotine, epibatidine, and α BTX fully displaced nicotine from lymphocytes and all the other ligands studied did not fully displace nicotine and any displacement was variable. Thus, it appears that lymphocytes composed of at least α4 and α7 nAChR subunits rather the others are present on the cells studied.

Epibatidine has been shown to have a higher affinity than nicotine for nicotinic cholinergic binding sites (Gerzanich *et al*., 1995; Houghtling *et al*., 1995). In the present study, epibatidine had a lower affinity than nicotine for the binding site on human lymphocytes. This unexpected difference could be accounted for by the presence of

different nAChR subunits being present in receptors on human lymphocytes than those in the receptors studies by Gerzanich *et al*., (1995) and Houghtling *et al*., (1995).

From the data obtained in the competition studies, which showed that (-) - nicotine, epibatidine, and α-BTX competed with radiolabeled nicotine it can be concluded that human peripheral blood lymphocytes contain α4 and α7 of nAChR subunits. Data obtained from competition studies with other ligands also suggest the presence of a cholinergic binding site on human lymphocyte cells. In the present study, the IC50 value of 2.30 ± 5.56 nM (2.30 x 10^{-9} M) for (-)- nicotine, the IC value of 10.48 nM (1.048 x 10^{-8}M) for epibatidine, and the IC50 values of 13.3 ± 2.83 nM (1.33 x 10^{-8}M) for α-BTX fall between the IC50 values reported from other published data (Morgan *et al*., 2001; Sharples and Wonnacott, 2001).

Data obtained from the competition binding experiments were analysed using a monophasic competition curve. From these data an IC50 value of 2.30 ± 5.65 nM for nicotine was calculated for the five experiments performed using lymphocytes from different donors. The IC50 values for (-)-nicotine obtained from the present study appear to be lower than the concentration of radiolabeled nicotine (15 nM) incubated with intact human lymphocytes. Because this experiment puts unlabelled ligand against radiolabeled ligand in direct competition, the IC50 value should not be less than the concentration of the radiolabeled used. This finding suggests that the concentration of radioligand, present at the receptor, was less than that added to the incubation. Therefore, the IC50 values

derived from the competition studies may be associated with some unknown form of error. There are some possible explanations for this conclusion:

It is unlikely that the actual concentration of radioligand purchased from the manufacturer was less than that concentration stated. The radioligand could have been affected by degradation or radiolysis and we were unable to check for concentration and purity. However, several batches of radioligand were used and the same IC50 was seen throughout the present study.

The degradation of radioligand might have occurred during storage. When we received the radioligand the solution was divided into aliquots (10 μl) in an eppendorf tube to prevent evaporation of the solvent by repeating opening of the same container. It is possible that this increased the likelihood of degradation even though the aliquots were frozen (-20ºC). As a result of this, a lower concentration of [^{3}H]-(-)-nicotine was exposed to the binding site in the competition experiments.

Nicotine is a lipophilic compound by its nature and it is very sticky and difficult to work with. In all experiments the high values of non-specific binding and some of variation observed may be due in part to the binding of the radioligand to the glass fiber filters and cell membranes. If nicotine bound to the plastic eppendorf in which it was stored, this might have led to a lowering of the concentration of [^{3}H]-(-)-nicotine.

However, these factors alone are unlikely to account for the low IC50 values obtained. IC50 values for nicotine which are lower than expected have been reported by others (Davies *et al*., 1982; Morgan *et al*., 2001). Thus, any problem is likely to reside in the radioligand used. Interestingly, Morgan *et al*., (2001) found evidence for a second binding site for nicotine to which a range of nAChR ligands did not bind. In these experiments only the isomers of nicotine displaced the radioligand from this binding site and (+) - nicotine had a higher affinity than (-) - nicotine suggesting the site was non-cholinergic in nature. Because, Morgan *et al* (2001) also did not determine the isomeric purity of their radioligand it is possible that a small percentage of radiolabeled (+) - nicotine was responsible for their finding, including the low IC50 values obtained. The same radioligand was used in the present study. Clearly further studies are required in order to understand the underlying reason for the low IC50 value for nicotine obtained in the present and earlier studies.

Data described in radioligand binding studies has demonstrated the presence of a single specific nicotine binding site on human peripheral blood lymphocytes, although, the specific binding sites for [^{3}H]-(-)-nicotine on human peripheral lymphocytes is small by comparison to the non-specific binding. Interestingly, the small specific binding found in this study is similar to that found in other studies (Adem *et al*., 1986; Grabczewska *et al*., 1990; Maus *et al*., 1998; Morgan *et al*., 2001). One explanation for this could lie with the fact that different peripheral blood lymphocytes from different donors contain different subtypes of nAChRs. Also the number of nAChRs on immune cells is less than that in the brain.

The specific binding site on human peripheral blood lymphocytes obtained from the radioligand binding studies varies between samples (donors). We have no information, as to which donors were tobacco smokers, a factor that may influence the data obtained. With specific binding accounting for only small proportion of total activity in each tube, any small change in non-specific binding to, for example, the filter, could account for the marked variation seen.

In summary the present study has found a single nicotine-binding site on human lymphocytes with a K*d* 15 ± 5.759 nM and B*max* 2253± 409 sites/cell. The specific binding was small and showed marked variation between subjects. The lymphocytes used in this study were obtained from buffy coats provided by the National Blood Transfusion Services and information on tobacco consumption was unavailable. The work of Lebargy *et al*., (1996) showed that tobacco smoking up-regulated a high affinity-binding site for nicotine on granulocytes. Similarly, Benhammou *et al*., (2000) reported that [^{3}H]-nicotine binding in peripheral polymorphonuclear cells (PMN) was increased in smokers and this increase correlated with tobacco use. If the same were true of lymphocytes, the presence of such cells may have contributed to some of the unexpected data obtained in the present study.

As discussed earlier, the variation in specific binding between subjects observed in this study may be attributed to many factors other than smoking behavior. For example, particular cell sub-population differences (Maslinski, 1989; Mihovilovic and Roses,

1991, 1993; Kuo *et al*., 2002), stage of maturation (Maslinski *et al*., 1987; Grabczewska *et al*., 1990; Maslinski *et al*.,1992) and or/activation process (Maslinski *et al*., 1992). The quality of the incubation medium is another important factor (Maslinski *et al*., 1983). The number of nAChRs on lymphocytes may be dependent on cell function (Strom *et al*., 1971). The influence of cell maturation on nAChRs in lymphocytes has been described in many studies (Mihovilovic and Roses, 1993; Rinner *et al*., 1994; Kuo *et al*., 2002). There are changes in the aging process that are specific although not limited to lymphocytes. Such effects can also be observed in the brain (Zoli *et al*., 1995; Gotti *et al*., 1997). It is possible that these results are mirrored in the human brain and lymphocytes although this needs to be verified in further research.

Binding affinities for a range of ligands were calculated in order to characterise the nAChRs present on lymphocytes. However, full characterisation of the receptor from the radioligand data obtained in the present study was difficult. Of the ligands studied, (-)-nicotine, epibatidine and α bungarotoxin compete with [^{3}H]-(-)-nicotine radioligand, indicating the possible existence of α4 and α7 subunits of nAChR on human peripheral blood lymphocytes. Because epibatidine binds potently to heteromeric nAChRs especially, nAChRs containing α4β2, and α7 subunits (Gerzanich *et al*., 1995; Gotti *et al*., 2000), and α BTX binds specifically to nAChR containing α7 (Anand *et al*., 1993; Bertrand *et al*., 1992; Paterson and Nordberg, 2000). As these results, the presence of nAChR containing these subunits is indicated. However, more work needs to be done to identify the nicotine receptor subtypes on human lymphocytes using other techniques

such as reverse transcriptase polymerase chain reaction (RT-PCR) and immunoblotting using antibodies specific to nAChR subunits.

Chapter 3 Determining the presence of mRNA for the subunits of nAChR in human peripheral blood lymphocytes

3.1 Introduction

The evidence for expression of mRNAs for the subunits of nAChR in thymus, mature peripheral lymphocytes and lymphocyte-derived cell lines was previously reviewed in section 1.7 (Chapter 1). However, the existence of nicotinic acetylcholine receptors on lymphocytes remains controversial. The diversity of expression of nAChR subunits found in these studies may well reflect differences between individuals, species, and different sub- population of cells in each study.

Recently, Shok *et al*., (2003; 2005; 2006) reported the presence of α4β2 and α7 nAChR subunit mRNA in B-lymphocyte derived cell lines and mouse B-lymphocytes. They also showed that activation of α7 nAChR subunits with nicotine stimulated cell proliferation and prevented antibody production, whereas, blocking nAChRs by toxins (cobra toxin, or weak toxin) prevented proliferation and enhanced antibody production.

α7 nAChR mRNA was also detected in human peripheral lymphocytes (De Rosa *et al*., 2005). De Rosa *et al*., (2005) also found that nicotine induced the transcription of α7 mRNA after incubation of lymphocytes with nicotine**.** However, expression was variable as either the appearance or the disappearance of α7 mRNA subunit could be observed

among individuals and within the same individual at different times (De Rosa *et al*., 2005). De Rosa *et al*., (2005) also showed that the α7 mRNA subunit was not present in rat splenic and peripheral lymphocytes suggesting expression is restricted to human cells.

The data obtained from radioligand binding studies described in chapter 2 demonstrated the presence of a single nicotine-binding site on human peripheral blood lymphocytes with a *Kd* 15 ± 5.759 nM (1.5 ± 5.759 x 10^{-8}M) and B*max* 2253 ± 409 sites/cell. Competition studies showed that the ligands competing with [^{3}H]-(-)-nicotine were (-)-nicotine, epibatidine, and α-bungarotoxin suggesting that this binding site contains nAChR composed of α7 or α4and/or β2 subunits. Epibatidine and (-)-nicotine are known to bind heteromeric nAChRs containing α4β2 (Gerzanich *et al*., 1995; Sihver *et al*., 1999; Gotti *et al*., 2000), whereas α-bungarotoxin is specific to both muscle-type and homomeric nAChRs (Lindstrom *et al*., 1987; Couturier *et al*., 1990; McLane *et al*., 1991; Paterson and Nordberg, 2000). The results obtained from these binding studies suggested that human peripheral lymphocytes expressed both heteromeric and homomeric (or muscle-type) nAChRs. It was proposed that human lymphocytes carry α4 nAChR containing subunits for heteromeric nAChRs and/or any β2 subunits, and also α-BTX sensitive homomeric nAChRs α7 containing subunits. This may be deduced from the fact that lymphocytes express a composition of α subunits similar to that found in the brain (Paterson and Nordberg, 2000). In the present chapter the hypothesis for the subtypes of nAChRs expressed in human peripheral blood lymphocytes proposed from radioligand binding studies will be investigated. The RT-PCR (Reverse Transcriptase Polymerase

Chain Reaction) technique will be used to identify the presence of mRNA subunits of nAChR in human peripheral blood lymphocytes from different donors.

We expect that knowing the expression of acetylcholine receptor subunits in human peripheral lymphocytes, together with the data obtained from the competition of cholinergic ligands for nicotine on lymphocytes will contribute to knowledge of how different subunit compositions alter the ligand binding characteristics on nicotinic acetylcholine receptors. Thus, demonstration of nicotinic acetylcholine receptor subunit expression would provide evidence that the binding sites for nicotine on human lymphocytes found by radioligand binding might be cholinergic in nature.

Therefore experiments described in the present chapter attempt to demonstrate the presence of mRNA for nAChR subunits on human peripheral blood lymphocytes by RT-PCR.

3.2 Materials and methods

3.2.1 DEPC (diethyl pyrocarbonate) treatment

To remove RNases from glassware, eppendorf tubes, PCR- type polypropylene tubes, and plastics tips, they were soaked overnight in a 0.1 (v/v) diethyl pyrocabonate (DEPC) solution followed by autoclaving the following morning at 121ºC (15 psi) for 30 minutes.

3.2.2 RNA Isolation from human peripheral blood lymphocytes

To examine the expression of nicotinic acetylcholine receptor mRNA subunits in human peripheral blood lymphocytes, RT- PCR was performed on total RNA isolated from human peripheral blood lymphocytes. The human peripheral blood lymphocytes used in the present study were obtained from 10 Buffy coats collected from different donors by NBTS. We were given no information as to which donors were tobacco smokers or non-smokers.

Human peripheral blood lymphocytes were isolated as described in section 2.2.2.1 of chapter 2. Total RNA from human lymphocytes was isolated using a single-step method, which used the RNA STAT-60 ^{TM} reagent (Biogenesis, Poole, UK), containing phenol and guanidium thiocyanate in monophase solution. The modified RNA STAT-60

protocol was based on the methods described by Chomczynski and Sacchi (1987); Sambrook *et al*., (1989), and Kedzierski and Porter (1991). Hands and dust may be a major source of the RNase contamination. Therefore, gloves were worn, and sterile, disposable polypropylene tubes were used throughout the procedure. All work was carried out with diethyl pyrocarbonate (DEPC) (0.1% v/v) treated utensils, plastic tips, and glassware.

RNA isolation from human peripheral blood lymphocytes by the modified RNA STAT-60 ™ method includes the following steps:

1. Homogenisation RNA STAT-60 ™ (1ml per 5-10 x 10^6 cells)
2. RNA Extraction 1 volume of homogenate + 0.2 volume of chloroform
3. RNA Precipitation 0.5 volume of isopropanol
4. RNA Wash 75% ethanol

Unless stated otherwise the procedure is carried out at room temperature.

RNA extraction from human peripheral blood lymphocytes using a modified RNA STAT-60 ™ protocol

Excess media was removed from human lymphocyte cell suspensions; the excess media was removed by transferring the cell suspension to a 50 ml a sterile plastic centrifuge tube (Greiner Laboratecknik, UK). The suspension was then centrifuged (Labofuge 400,

Sorvall Heraeus, Kendro Laboratory Products Ltd., Hertfordshire, UK) at 1500 rpm for 10 minutes at room temperature to obtain the pellet. The supernatant was then removed by aspiration and the cell pellet was placed on ice immediately. The tube was gently agitated to loosen the pellet (still kept on ice). Then 10 ml of RNA STAT-60^{TM} reagent was added to loosen the pellet, and the mixture was mixed trituration until an even homogenate was obtained.

Following homogenisation, the homogenate was incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. The RNA extraction was then performed in a cabinet. The RNA was extracted by adding 2 ml (20%) Chloroform (ACS grade). The homogenate was shaken vigorously and kept at room temperature for 3 minutes. The homogenate was then centrifuged at 12,000 x g (SS-34 rotor, Sorvall, Kendro Laboratory Products PLC, UK) for 15 minutes at 4˚C. Following centrifugation, the colourless upper aqueous phase, which contained RNA, was collected into a fresh tube (the volume of the aqueous phase should be about 6 ml (60%) of the volume of RNA STAT-60^{TM} used for homogenization). 5 ml of isopropanol (ACS grade) (0.5 ml of isopropanol per 1 ml of RNA STAT-60^{TM} used for homogenisation) was added to the aqueous phase and mixed to precipitate RNA. The sample was incubated at -20ºC for 30 minutes. The sample was then centrifuged at 12,000 x g (SS-34 rotor, Sorvall, Kendro Laboratory Products PLC, UK) for 1 hour at 4ºC. A white precipitate (RNA) was formed (often visible before centrifugation) at the bottom of the tube. The supernatant was then removed and the RNA pellet was washed twice with 20 ml of cold 75% ethanol (ACS grade) by vortex to mix and subsequent

centrifugation at 7,500 x g (SS-34 rotor, Sorvall, Kendro Laboratory Products PLC, UK) for 45 minutes at 4 ˚C. The 75% ethanol supernatant was carefully removed (75% ethanol was prepared using diethyl pyrocarbonate (DEPC) treated RNase-free water).

The RNA pellet was air died at room temperature for 5-10 minutes. It was then dissolved in 100 µl of diethyl pyrocarbonate (DEPC) treated RNase-free water (0.1% v/v). A 10 µl aliquot was removed to check for yield and purity of total RNA. The remainder of RNA suspension was stored at –80˚C until required for RT-PCR assays.

3.2.2.1 Quantification and determination of quality of total RNA

The RNA, prepared as described above, was diluted (1:100) in DEPC treated RNase free water to give a final volume of 1 ml and the RNA concentration was then determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (Helios γ, Unicam, UK) using quartz cuvettes. An absorbance of 1 unit at 260 nm was equivalent to an RNA concentration of 40 µg per ml ($A_{260} = 1 \rightarrow 40$ µg/ml). The concentration of RNA was calculated by the following formula:

Concentration of RNA sample = Spectophotometric conversion (40 µg/ml) x A_{260} x dilution factor

An example

Dilution = 10µl +990 µl distilled water (1/100 dilution)

Measure absorbance of diluted sample in a quart cuvette

$A_{260} = 0.300$

Concentration of total RNA sample = 40 x 0.30 x 100

=1200 µg/ml or 1.2 µg/µl

The RNA purity was determined by the ratio of the readings of the absorbance at 260 and 280 nm (A_{260}/A_{280}). The RNA sample was diluted (1:100) in DEPC treated RNase free water to give a final volume of 1 ml and the absorbance was measured at 260 and 280 nm using a spectrophotometer. The A_{260}/A_{280} ratio was determined. The final preparation of pure total RNA was free of proteins and DNA and should have A_{260}/A_{280} ratio › 1.8.

The integrity and size distribution of total RNA was checked by 1% agarose gel electrophoresis and followed by ethidium bromide staining. For the most RNA species of interest, a concentration of 1.0-1.2% (w/v) agarose will give the best results. The degradation was determined by loading 5 µg RNA onto a 1% agarose gel and subjecting to electrophoresis at 100 V for 1 hour. The gel was stained with ethidium bromide (5µg/ml in gel running buffer) and destained in 1x TAE buffer as described in section 3.2.5. RNA bands were then visualized by examination of gels under ultraviolet light.

3.2.3 Design of the nAChR primers for RT-PCR

The specific primers for the RT-PCR analysis used were based on sequences described by Summers (2003, PhD Thesis). Primer design was conduced from the gene sequences published at the National Centre for Biotechnology Information (NCBI), excluding homogeneous regions between subunits, and the primer sequences were compared back to the NCBI nucleotide basic local alignment search tool (nBLAST), which confirmed the specificity for all primer set to their target mRNA.

Human nAChR mRNA sequences were obtained from the NCBI database. The Accession numbers for the mRNA sequences used in the primer design were

NM_000079 for α1,

NM_000742 for α2,

NM_000743 for α3,

NM_000744 for α4,

NM_000745 for α5,

NM_004198 for α6,

NM_000746 for α7,

NM_017581 for α9,

NM_020402 for α10,

NM_000747 for β1,

NM_000748 for β2,

NM_000749 for β3,

NM_000750 for β4

These sequences were compared to the human genome using the NCBI nucleotide basic local alignment search tool (nBLAST) facility, searching for sequence matches and for identification and conformation of exon-intron boundaries. Once the sequences were aligned and identified by the program matching the correct gene 98 to 100% identity, these sequences then formed the basis for the primer design.

The selected sequences were then aligned, each sequence with other sequence, as well as all sequences together, using the Clustal W program at the European Bioinformatics Institute (EBI). These multiple alignments allowed the identification of sequence similarities, which were excluded from the primer design. The sequences were subsequently pasted into a nucleotide file of the computer program Version 3.05 (Generunner). Primer pairs were designed incorporating exon-intron boundaries and with the exclusion of the sequence similarities identified by Clustal W program. Furthermore, the Generunner program was utilized for structural analysis, the identification of potential inter- and intra-cellular complex formation, and for the calculation of GC% content and melting temperature of the primer pairs.

The final products of the primer design as shown in Table 3.1 were finally compared against the human genome at NCBI using the nBLAST facility, to check the specificity of each primer.

nAChR Subunit	Forward primer 5´to3´	Reverse primer 5´to 3´	RT-PCR Product (bp)
α1	GAA GCT GGG CAC CTG GA	GCA TGA CGA AGT GGT AGG TG	190
α 2	GGT TCT TCT CAT CCA CAT CG	CCG CTC TCC CAG TAG TCC	379
α3	ACC GTC TAT TTG AGC GGC	GAG CCG ATC AGG ACC AGA	474
α4	GGC CCA CCT GTT CCA TG	TCT TCT CGC CAC ACT CGG	387
α5	CAA GAC TAC GAA AGA TGG GTT	GTG ACA TAC GGA TAC CAG CA	556
α6	GTG CAA CTG AGG AGA GGC T	GAG TCC AGG TTA TCA TGC CA	356
α7	AGA GTT CTT GCT ACA TCG ATG	GAA GTA CTG GGC TAT CAA TGG	451
α10	ACT CTG AAT GTG ACC CTG GA	ACG GGA AGG CTG CTA CAT	327
β 1	CAC CTA CTT CCC CTT CGA CT	GAA TGA AGA TCT GAC GGA CC	589
β2	TCA GGA TAC AGA GGA GCG G	CTC GTA CAT GCC GTC AGC	309
β3	GGA CAG ACC ACA AGT TAC GC	CGT GAT AAA GGG ATA GGA GTA C	418
β4	TCT GGT TGC CTG ACA TCG T	CAT GTC TAT CTC CGT GTG GTC	220

Table 3.1 Forward and reverse primers for mRNA of nAChR subunits and the expected RT-PCR product size in base pairs (bp)

3.2.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

Experiments were carried out using the Access RT-PCR (Promega, UK) kit and the protocol provided was adapted for the conditions required by each pair of primers. To avoid RNase contamination, gloves were worn, and sterile, disposable polypropylene tubes were used throughout the procedure. All preparation work was carried out on ice with diethyl pyrocarbonate (DEPC) (0.1% v/v) treated eppendorf tubes, plastic tips, and glassware.

Human lymphocytes total RNA was used as the experimental template. Total human brain RNA (Origene Technologies, Inc, UK) provided the positive control template. Primers used for nAChR mRNA were specific to α1 to α7, and α10, also β1 to β4 subunits (Invitrogen™, life technologies, UK).

One-step RT-PCR was carried out using a Thermal Cycler (Master Cycler Gradient, Eppendorf, UK). RT-was carried out in a final total volume of 25 µl. The reaction RT-PCR mixture consisted of:

1x AMV/Tfl 5x Reaction buffer

0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP)

1µM Forward primer

1 µM Reverse primer

1mM Magnesium sulphate ($MgSO_4$)

AMV Reverse transcriptase (5units/µl)

Tfl DNA Polymerase (5 units/µl)

RNA template

Nuclease free water was added to make up a final volume of 25 µl

3.2.4.1 RT-PCR reaction mixture and PCR cycling protocol

The reaction mixture was assembled in a 200 µl PCR-type polypropylene tube (Robbins Scientific), by adding 5 µl of AMV/Tfl 5x Reaction Buffer (final concentration 1x), 0.5 µl of dNTP mix (10 mM each dNTP) (final concentration 0.2 mM each), 0.25 µl (25pmol) of specific forward primer (final concentration 1µM), 0.25 µl (25pmol) of reverse primer (final concentration 1µM), and 1 µl of 25 mM Magnesium sulphate (MgSO4) (final concentration 1mM).

The components were gently mixed by pipetting. AMV Reverse Transcriptase (5U/µl) and Tfl DNA Polymerase (5U/µl) were subsequently added (to a final volume concentration of 0.1 U/µl, each), nuclease free water was added (to a final volume of 25 µl). The reaction was initiated by adding the relevant RNA template (to a final concentration of 1 µg). The RT-PCR tubes were capped firmly and were placed into a Thermal Cycler (Master Gradient, Effpendorf, UK). The PCR cycling profiles were set as the following protocol.

For the first strand cDNA synthesis, 1 cycle at 48ºC for 45 minutes (reverse transcription), and 1 cycle at 94ºC for 2 minutes (AMV RT-inactivation, RNA/cDNA/primer denaturation) were carried out.

For the second strand synthesis and PCR amplification, a further 35 cycles at 94ºC for 30 seconds (denaturation), 55º C for 1 minute (annealing) (varied depended on the pair of primers, temperatures varied from 50.2ºC - 60.0ºC), and the extension step at 68º C for 2 minutes. The final extension 1 cycle at 68º C for 7 minutes, and the last 1 cycle at 4º C (soak) were carried out.

3.2.5 Analysis of PCR products by gel electrophoresis

The reaction products were analysed by 1.5 % agarose gel electrophoresis. Electrophoresis gel was prepared by adding 1.5 g agarose (National Diagnostics) in 100 ml 1xTAE buffer (0.04M Tris base, 0.001M EDTA Sigma-Aldrich, UK and pH 8.0 with glacial acetic acid). The gel solution was boiled until the agarose dissolved and left until it was warm enough to pour into a gel mold and the comb was inserted. Once the gel had set it was placed in a gel tank and the tank filled with 1xTAE. The gel was then submerged in electrophoresis buffer (TAE) in electrophoresis tank prior to loading. 6 µl of reaction mixture was added to 2 µl of mini-gel loading buffer (Sigma-Aldrich, UK) and mixed; the samples were then loaded. Before loading the samples, the air bubbles from the wells were removed by rinsing them with electrophoresis buffer. The samples

were then loaded by inserting the plastic tip deep into the well and expelling the sample slowly. DNA molecular weight markers (100 bp DNA Ladder) (New England, BioLabs, Inc) (1µl of DNA marker + 2 of mini-gel loading buffer + 4µl TE buffer; 10mM Tris HCl, 1mM EDTA, pH 8.0), were loaded along with sample to a separate well in each gel. The electrodes of the electrophoresis apparatus (Biorad, UK) were connected so that the DNA will be migrated towards the anode or positive lead (usually red). The gel was run at a constant 50 volts for 3 hours until the bromophenol blue dye (in mini-gel loading buffer) has migrated approximately 2/3 of the way through the gel.

The gel was stained with ethidium bromide (1µg/ml in 1xTAE) for 15 minutes and destained by soaking in 1xTAE buffer for 10-15 minutes. The gel was then visualized under the UV light on a gel documenter using Gene snap program (Syngene). Ethidium bromide in the gel allowed visualization of DNA with UV light. The gel image was recorded by taking a photograph using the gel documentation system Gene snap program (Syngene). The sizes of the PCR products were estimated from the migration of DNA molecular weight markers (100 bp DNA Ladder) (New England, BioLabs, Inc) run concurrently in the same gel.

3.3 Results

3.3.1 Yield and purity of total RNA

The concentration and purity of RNA samples were measured as described in section 3.2.2.1. The yield of 10 total RNA samples isolated from human peripheral blood lymphocytes collected from 10 different donors was calculated. The reading of A_{260} in a spectrophotometer using quartz cuvette was between 0.15 and 1.0. The concentration of total RNA in the 10 samples obtained was between 1.00 - 1.60 µg/µl.

All the total RNA samples isolated from human peripheral blood lymphocytes had an A_{260}/A_{280} ratio › 1.8 (ratio of 1.8 - 2.3). Therefore, the total RNA samples were free of contamination by DNA and protein.

The integrity and size distribution of the RNA obtained is shown in Figures 3.1-3.3. The respective ribosomal bands were appeared as sharp bands on the stained gel. The 28S ribosomal RNA (rRNA) bands were seen at approximately twice the intensity of the 18S rRNA bands (Figures 3.1-3.4).

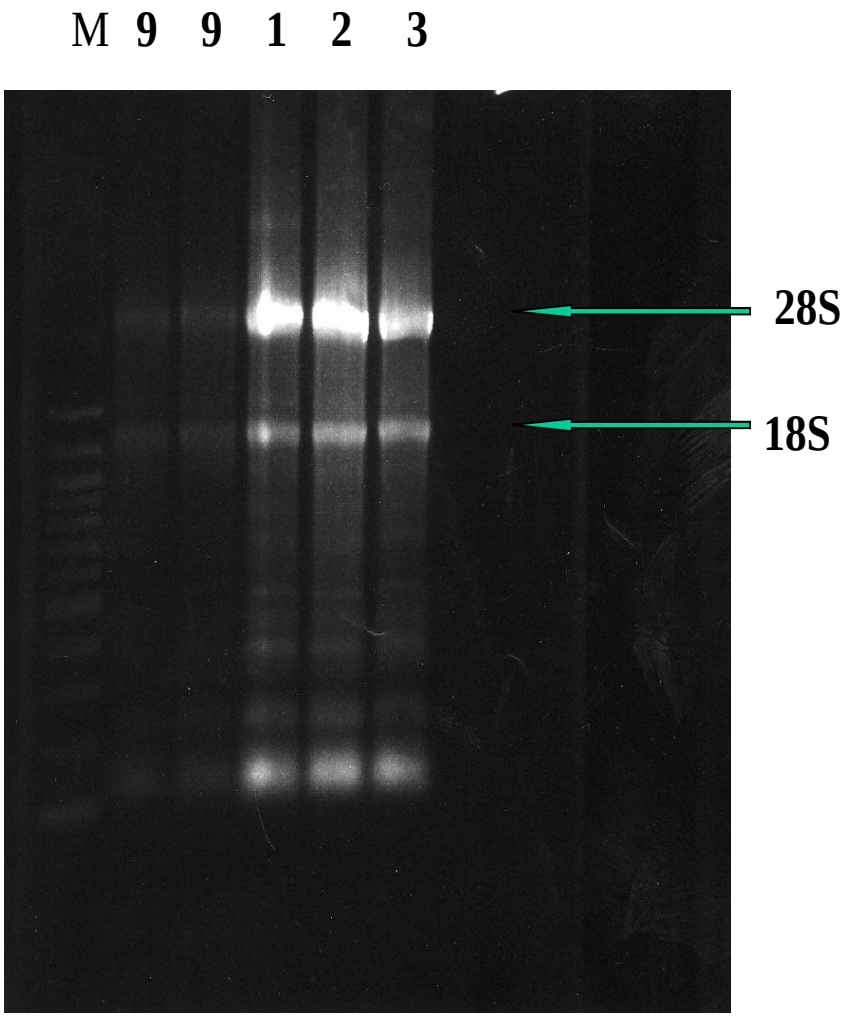


Figure 3.1 A photograph of total RNA isolated from human peripheral blood lymphocytes using RNA-STAT 60™ reagent and visualised by ethidium bromide staining on a 1.0 % agarose gel electrophoresis. Lane M = DNA Ladder, 1=sample no 1, 2=sample no 2, 3 = Sample no 3, 9 =Sample no 9. 28S = 28S ribosomal RNA (rRNA) bands, 18S = 18S ribosomal RNA

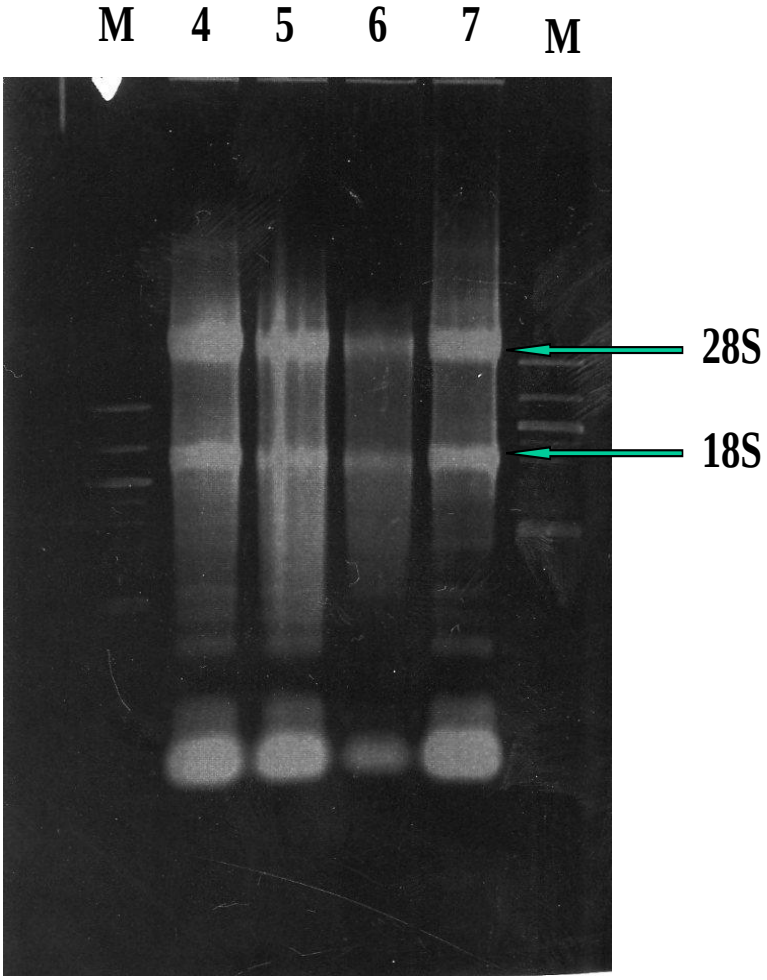


Figure 3.2 A photograph of total RNA isolated from human peripheral blood lymphocytes using RNA-STAT 60™ reagent and visualised by ethidium bromide staining on a 1.0 % agarose gel electrophoresis. Lane M = DNA Ladder, 4 = sample no 4, 5 = sample no 5, 6 = sample no 6, 7 =sample no 7. 28S = 28S ribosomal RNA (rRNA) bands, 18S = 18S ribosomal RNA

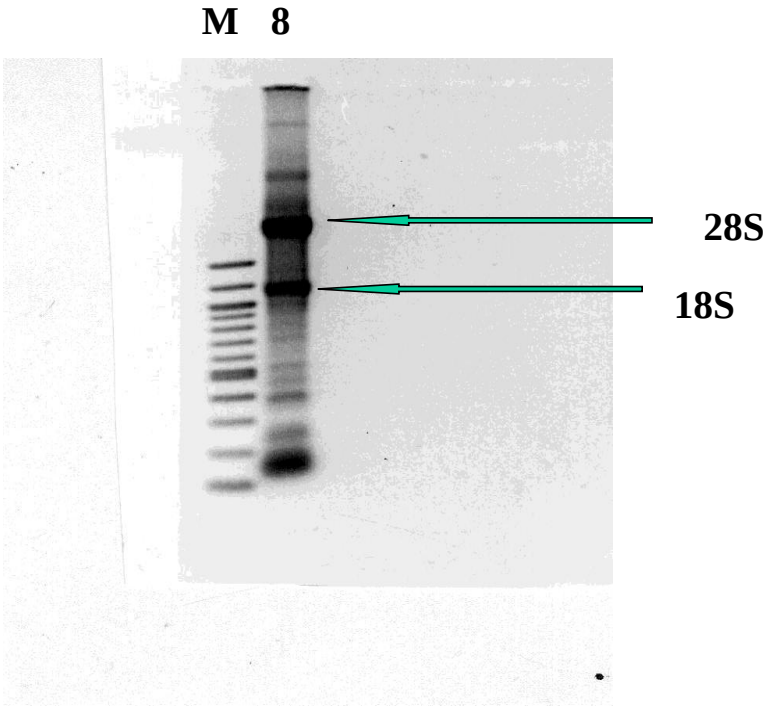


Figure 3.3 A photograph of total RNA isolated from human peripheral blood lymphocytes using RNA-STAT 60™ reagent and visualised by ethidium bromide staining on a 1.0 % agarose gel electrophoresis. Lane M = DNA Ladder, 8 = sample no 8. 28S = 28S ribosomal RNA (rRNA) bands, 18S = 18S ribosomal RNA.

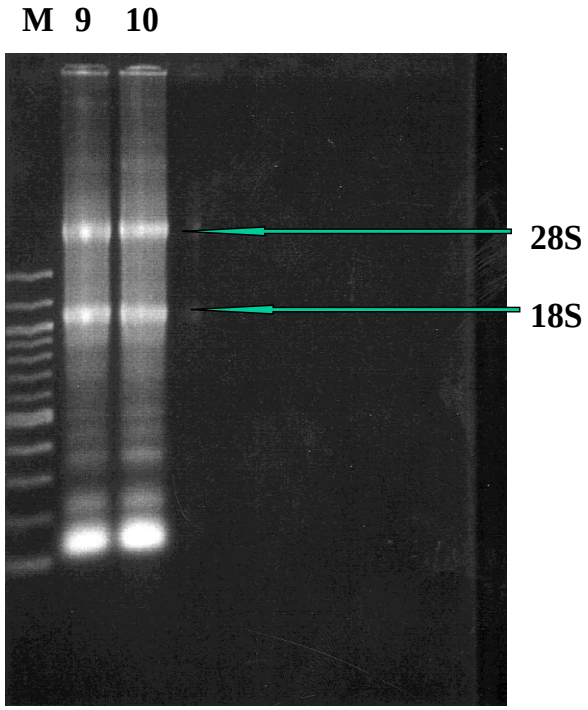


Figure 3.4 A photograph of total RNA isolated from human peripheral blood lymphocytes using RNA-STAT 60™ reagent and visualised by ethidium bromide staining on a 1.0 % agarose gel electrophoresis. Lane M = DNA Ladder, 9 = sample no 9, 10 = sample no 10. 28S = 28S ribosomal RNA (rRNA) bands, 18S = 18S ribosomal RNA.

3.3.2. RT-PCR

In each experiment, folllowing electrophoresis, and ethidium bromide staining a PCR product was observed in each lane with a molecular size that corresponded to the predicted size of PCR product if there was nAChR subunit mRNA present in the human peripheral blood lymphocyte samples tested (Figures 3.5-3.9). The predicted size of these products was 190 bp for α1, 349 bp for α2, 474 bp for α3, 387 bp for α4 (Figure 3.5), 556 bp for α5 (Figure 3.6), 356 bp for α6, 451 bp for α7 (Figure 3.7), 589 bp for β1 (Figure 3.8), 309 bp for β2 (Figure 3.9), 418 bp for β3, and 220 bp for β4.

All human peripheral blood lymphocytes tested expressed mRNA for α4, α5 α7, and β2 subunits of neuronal nAChRs and β1subunit for muscle nAChR. No mRNA for α1, α2, α3, α6, α10, and β3 or β4 subunits of nAChR was detected in any of the samples of total RNA from lymphocytes studied (Table 3.2) and (Figures 3.5-3.9). Expression of the α5 mRNA subunit of nAChR was observed in the lymphocytes in each sample of lymphocyte studied. The expression pattern of mRNA for α4, α7, β1, and β2 mRNAs subunits of nAChRs varied between individuals and did not appear to correlate with blood group (Table 3.2).

No	Blood group	Muscle type		Neuronal type									
		α1	β1	α2	α3	α 4	α 5	α6	α7	α10	β2	β3	β4
1	O+	-	+/-	-	-	+	+	-	+	-	-	-	-
2	A+	-	+	-	-	+	+	-	-	-	-	-	-
3	A+	-	+/-	-	-	+/-	+	-	+	-	-	-	-
4	AB+	-	+	-	-	+	+	-	+/-	-	+	-	-
5	AB+	-	+	-	-	+	+	-	+/-	-	+	-	-
6	AB-	-	+	-	-	+	+	-	-	-	-	-	-
7	AB+	-	+	-	-	+	+	-	+/-	-	+	-	-
8	A+	-	+	-	-	+	+	-	+	-	+	-	-
9	A+	-	+/-	-	-	+/-	+	-	+	-	+	-	-
10	O+	-	+	-	-	+	+	-	+	-	-	-	-

Table 3.2 The expression of mRNAs encoding nicotinic acetylcholine receptor subunits in human peripheral blood lymphocytes. The predicted products were observed by 1.5 % agarose gel electrophoresis

(+) = mRNAs were present in human lymphocytes

(-) = mRNA was not detected in human lymphocytes

(+/-) = mRNAs were detected, but the product bands were faint.

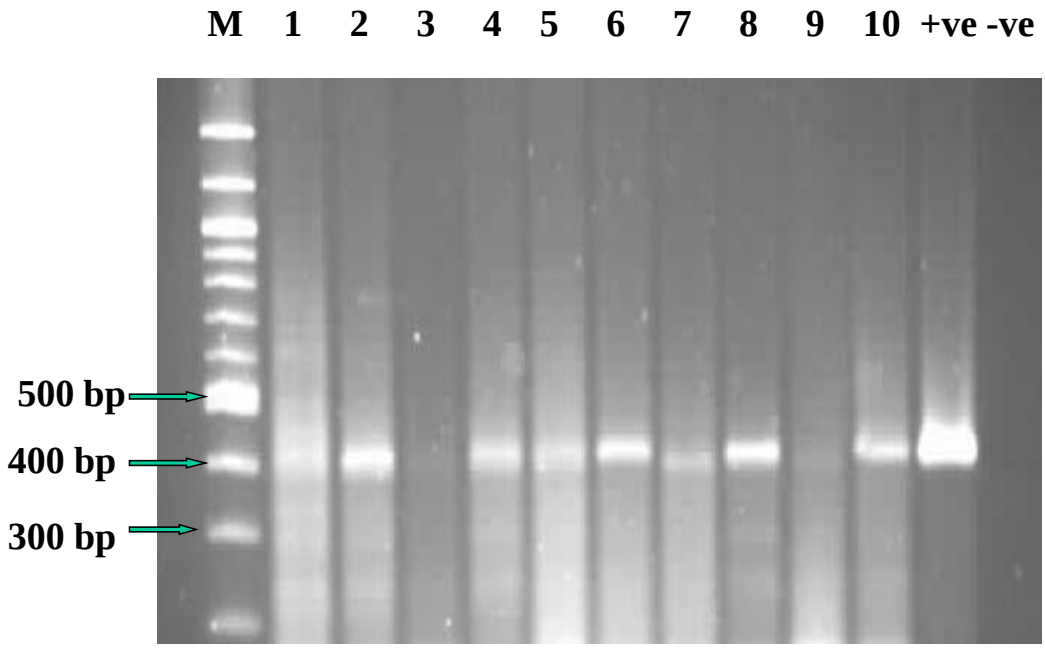


Figure 3.5 A photograph of a gel showing the product from RT-PCR amplification of α4 nAChR subunit mRNA from 1 µg of human peripheral blood lymphocyte total RNA using the Access RT-PCR system. The expected product length was 387 bp. Lane M = DNA Ladder (100bp DNA Ladder), Lanes 1-10 = samples number 1-10, Lane + ve = positive control (human brain total RNA), Lane -ve = no RNA template.

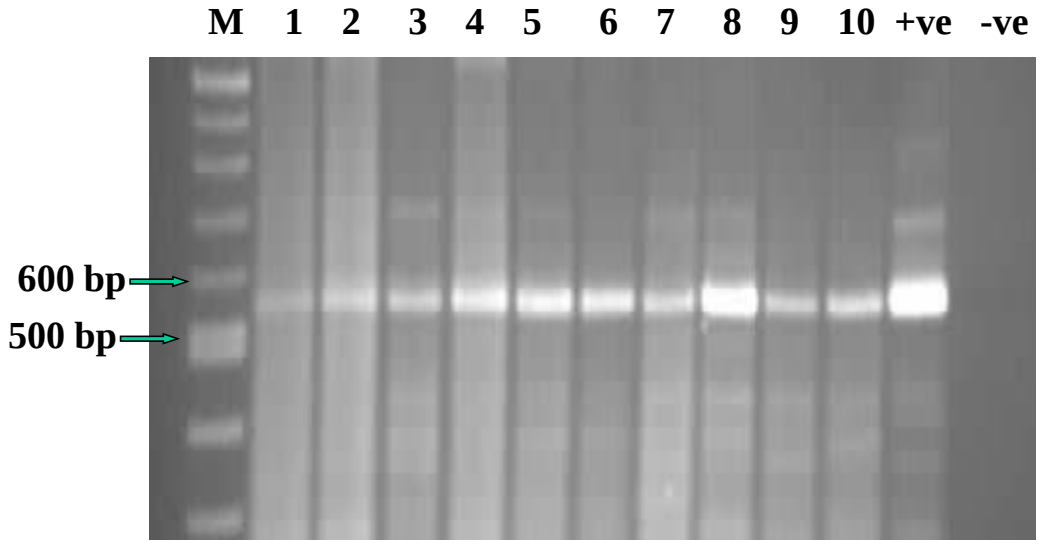


Figure 3.6 A **photograph of a gel showing the product from RT-PCR amplification of α5 nAChR subunit mRNA from 1 µg of human peripheral blood lymphocyte total RNA using the Access RT-PCR system. The expected product length was 556 bp. Lane M = DNA Ladder (100bp DNA Ladder), Lanes 1-10 = samples number 1-10, Lane +ve = positive control (human brain total RNA), Lane -ve = no RNA template.**

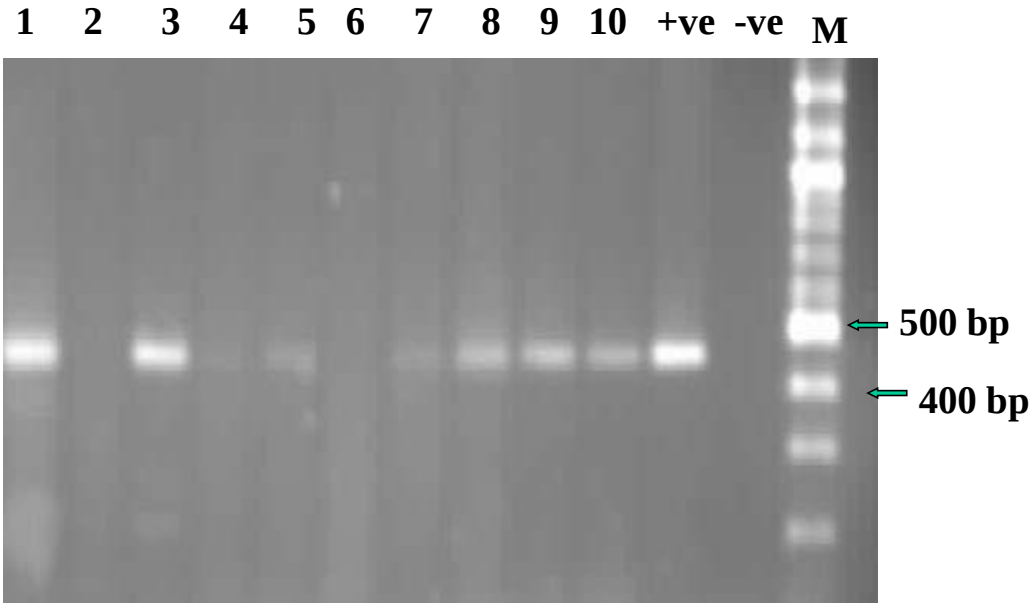


Figure 3.7 A **photograph of a gel showing the product from RT-PCR amplification of α7 nAChR subunit mRNA from 1 µg of human peripheral blood lymphocyte total RNA using the Access RT-PCR system. The expected product length was 451 bp. Lanes 1-10 = samples number 1-10, Lane +ve = positive control (human brain total RNA), Lane -ve = no RNA template, Lane M = DNA Ladder (100 bp DNA Ladder).**

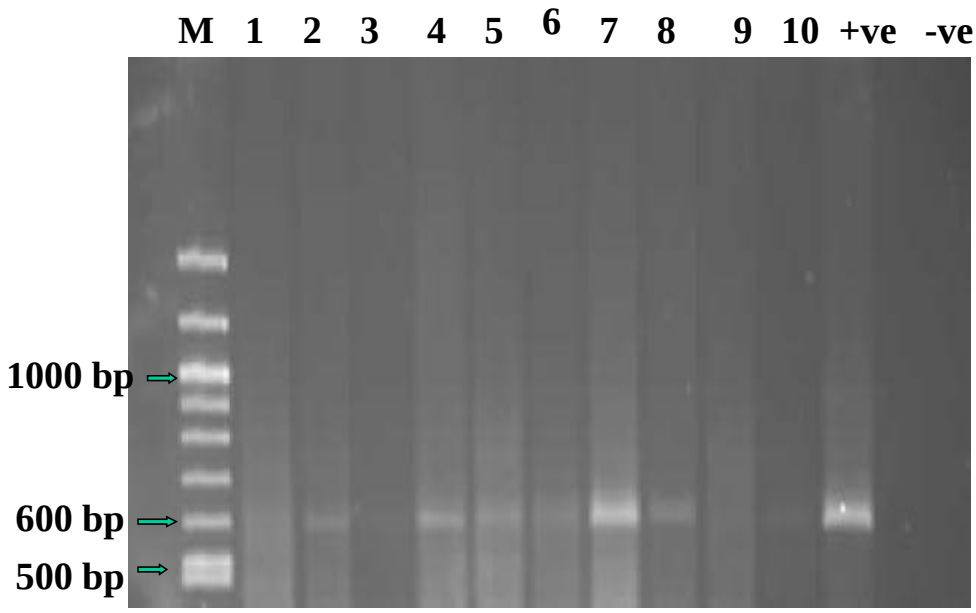


Figure 3.8 A **photograph of a gel showing the product from RT-PCR amplification of β1 nAChR subunit mRNA from 1 µg of human peripheral blood lymphocyte total RNA using the Access RT-PCR system. The expected product length was 589 bp. Lane M = DNA Ladder (100 bp DNA Ladder), Lanes 1-10 = samples number 1-10, Lane +ve = positive control (human brain total RNA), Lane -ve = no RNA template.**

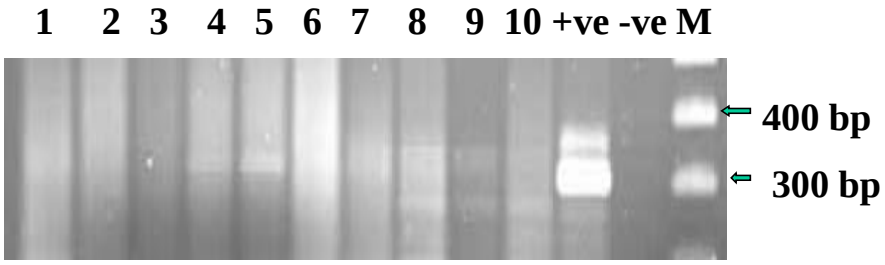


Figure 3.9 A **photograph of a gel showing the product from RT-PCR amplification of β2 nAChR subunit mRNA from 1 µg of human peripheral blood lymphocyte total RNA using the Access RT-PCR system. The expected product length was 309 bp. Lanes 1-10 = samples number 1-10, Lane +ve = positive control (human brain total RNA), Lane -ve = no RNA template, Lane M = DNA Ladder (100 bp DNA Ladder).**

3.4 Discussion

In the present study we have detected mRNAs encoding different subunits of nAChRs in human peripheral blood lymphocytes. We found that all human peripheral blood lymphocyte samples tested expressed mRNAs for α4, α5, α7, and β2 neuronal nAChRs subunits and β1 muscle nAChR subunit. The expression of mRNA for the α1, α2, α3, α6, α10, β3, and β4 subunits of nAChRs was not seen in the lymphocytes of any the samples studied (Table 3.2). Expression of mRNA for the α5 subunit of nAChR was observed in the lymphocytes in each sample of lymphocytes. In contrast, the expression pattern of mRNAs for α4, α7, β1, and β2 mRNAs subunits of nAChRs varied between individuals.

Although, identification of nAChRs from the radioligand binding data described in the chapter 2 was difficult. (-)-nicotine, epibatidine and α-bungarotoxin competed with [^{3}H]-(-)-nicotine, indicating the possible existence of α4 and α 7 subunit containing nAChRs on human lymphocytes. This conclusion was made because epibatidine is a potent agonist for α4 containing nAChRs (Gerzanich *et al*., 1995; Gotti *et al*., 2000; Sharples and Wonnacot, 2001), and α-BTX is very sensitive to nAChR containing α 7 subunits (Bertrand *et al*., 1992; Anand *et al*., 1993; Sharples and Wonnacot, 2001). Thus, the presence of nAChR containing these subunits is indicated.

The data obtained from the RT-PCR studies described in the present chapter (α4, α5, α7, β1, and β2 mRNA for the nAChR subunits) support and extend the radioligand binding

data from chapter 2 in that we proposed that human lymphocytes would express mRNA for α4, α7 nAChR subunits. As the data described in the present chapter, the RT-PCR data obtained from human peripheral blood lymphocytes show the expression of mRNA for the α4, and α7 nAChR subunits suggested by the radioligand binding data described in chapter 2. In addition, the data described in the present chapter also show the presence of mRNA for the α5, β1, and β2 nAChR subunits.

The presence of nAChRs on lymphocytes has been reported by others (Davies *et al*., 1982; Maslinski, 1989; Grabczewska *et al*., 1990) but the patterns of receptor expression at the molecular level as well as their functional roles remain unknown. The functional roles of nAChRs on lymphocytes have been suggested by pharmacological studies that showed that nAChRs agonists modulate the functions of lymphocytes (Richman and Arnason, 1979; Menard and Rola-Pleszcynski, 1987; Maslinski, 1989; Rinner *et al*., 1994; Toyabe *et al*., 1997). Kawashima *et al*., (1998) and Fuji *et al*., (1999) have shown that ACh in the blood originates from T lymphocytes. Although the level of cholinesterase in the blood is high, T lymphocytes often make direct contact with their targets, such as other lymphocytes, so even a small amount of ACh released by T lymphocytes should be able to interact with the receptors of the target cell prior to its hydrolysis by cholinesterase. Taken together, these findings suggest that blood ACh released from T lymphocytes can transduce signals to nAChRs on lymphocytes in an autocrine and/ or paracrine manner during immune responses.

We observed a high degree of variation in nAChR subunit expression pattern among individuals (Table 3.2). This variability in expression of mRNAs for α4, α7, β1, and β2 mRNA subunits of nAChRs suggested that the level of expression may not only be genetically determined but may also be regulated by environmental factors, such as infection, physiological stress or smoking behaviour. Such effects are also suggested by the work of Lebargy *et al*., (1996) who reported that tobacco smoking caused the expression of a high affinity-binding site for nicotine on granulocytes. Similarly, Benhammou *et al*., (2000) also reported that [^{3}H]-(-)-nicotine binding in peripheral polymorphonuclear cells (PMN) was increased in smokers and that this increase correlated with tobacco use. Moreover, Comier *et al*., (2004) also showed that long-term exposure to nicotine modulated the level and activity of nAChRs in white blood cells from tobacco smokers and mice. They found that epibatidine binding sites, which corresponded to α4β2 neuronal heteromeric nAChR subtypes, were detected in white blood cells of smokers but not in white blood cells of non smokers. They also concluded that the α4 and β2 subunits comprise the up-regulated nAChRs (Cormier *et al*., 2004). If the same were true of lymphocytes, a white cell subgroup, the presence of such cells may have contributed to some of variation seen in terms of nAChR subunit expression obtained in the present study. Interestingly, recent work from De Rosa *et al*., (2005) showed that such variation could be avoided by using human peripheral blood lymphocytes isolated from non-smoker volunteers. In these cells, De Rosa *et al*., (2005), who published their data after we finished the experiments described in this (postdate this thesis), found that the expression pattern of α7 mRNA subunit and muscle type nAChR

subunits still varied between individuals and within an individual studied at different times. However, we were unable to select volunteers to the same degree.

Controversial data as to the nature of nAChR subunits expressed in blood cells can be found throughout the literature (Hiemke *et al*.1996, Wakkach *et al*., 1996; Navaneetham *et al*., 1997; Toyabe *et al*., 1997; Sato *et al*., 1999; Kuo *et al*., 2002; De Rosa *et al*., 2005). Due to this high variability, the presence of nAChRs in lymphocytes has remained controversial and the identification of the role of this extra-neuronal cholinergic system turns out to be quite difficult.

From the data obtained by RT- PCR in the present chapter, we have observed the expression of mRNA for β1subunit of muscle nAChR but not mRNA for α1 in human peripheral blood lymphocytes. However, data have been published showing the expression of α1 mRNA subunit of nAChR (Wakkach *et al*., 1996; Toyabe *et al*., 1997). The data obtained from RT-PCR in the present study showing the absence of α1 mRNA subunit of nAChR is similar to that published by Sato *et al*., (1999). However, Sato *et al*., (1999) did not find β1 nAChR subunit mRNA. Although, mRNA for the β1 nAChR subunit has been detected in some lymphocytes used in the presennt study, not all samples expressed this subunit. The results obtained in the present study are similar to the findings of De Rosa *et al*., (2005) who demonstrated the expression of mRNA for α1, δ, ε, β1 of muscle nAChRs subunits in freshly isolated lymphocytes. De Rosa *et al*., (2005), who published their data after we finished the experiments described in this

(postdate this thesis), also observed a high variability in the expression pattern among individuals and within the same individual when examined at different times.

The data obtained from RT-PCR experiments in the present study showed the presence of α4, α5, α7, β1 and β2 mRNAs for nAChR subunits. All human peripheral blood lymphocyte samples tested expressed mRNAs encoding the α5 subunit but not all samples expressed α4, α7, β1 and β2 mRNAs subunits. The pattern of expression of α4, α7, β1 and β2 mRNA subunits varies between individuals. The expression pattern of nAChR subunits from the observations described above is similar to that of Sato *et al*., (1999), who reported the expression of mRNA subunits of nAChRs in human mononuclear leukocytes from seven healthy donors and in eight leukemic cell lines. They detected the expression of nAChR subunits in every sample, but the specific expression pattern varied between samples (Sato *et al*., 1999). In these experiments, expression of mRNAs encoding the α2, α5, and α7subunits were expressed in human mononuclear leukocytes from all donors, whereas no mononuclear leukocytes expressed the α3, α4, β3, and β4 subunits. The expression of the α6 and β2 subunits was observed in mononuclear leukocytes from two and three individuals out of seven donors (Sato *et al*., 1999). This may be indicative of diversity of expression of nicotine acetylcholine receptor subunit among individual subjects and species. Sato *et al*., (1999) found no mRNA expression for the α3, α4, β3, and β4 subunits in mononuclear leukocytes. This is consistent with the RT-PCR data obtained in the present study which showed that human lymphocytes do not express the α3, β3, and β4 subunits but contain α4 mRNA subunits.

The expression of mRNA encoding the α3, α5, and β4 subunits has also been reported in human thymocytes (Mihovilovic *et al*., 1991, 1993). Hiemke *et al*., (1996) confirmed α3, and α4 subunit expreesion in circulating lymphocytes, but in contrast, the experiments described in the present chapter did not detect any mRNA for α3, and β4 subunits. This may be related to T-cell maturation (Mihovilovic *et al*., 1991, 1993; Rinner *et al*., 1994) in that expression of these subunits ceases in mature cells.

The α7 subunit was detected in eight out of ten of the lymphocyte samples tested. This finding is similar to the expression of α7 subunit nAChR in human lymphocytes from healthy donors reported by De Rosa *et al*., (2005). De Rosa *et al*., (2005) showed that mRNA for the α7 nAChR subunit was expressed in 5 out of 8 in human peripheral blood lymphocyte samples tested. They concluded that the α7 subunit expression was highly variable among individuals and even within the same individual at different times (De Rosa *et al*., 2005). This observation contrasts with that reported by Sato *et al*., (1999) who found that human mononuclear leukocytes from all seven healthy donors expressed the α7 subunit.

The results obtained from the present study showing that the expression of α4, α7, β2, and β1 mRNA of nAChR subunits is indicative of diversity and variability within and between individual subjects. This is in contrast to α5 mRNA nAChR subunit which showed a consistent pattern of expression. It would be interesting to determine if the nAChR expression is restricted to a specific subset or/and the stage of maturation of lymphocytes.

The data described in this chapter provided evidence for nicotinic cholinergic receptors being actively expressed in human peripheral blood lymphocytes. These finding strengthen the possibility that nicotine binding site on human peripheral blood lymphocytes is cholinergic and that the cells respond directly to nicotine.

In summary, the results obtained from the present chapter show that human peripheral blood lymphocytes expressed mRNA for α4, α5, α7, and β2 nAChRs subunits and mRNA for the β1 nAChR subunit. The expression of mRNA for the α1, α2, α3, α6, α10, β3, and β4 subunits of nAChRs was not observed in the lymphocytes from all the samples. However, mRNA for the α5 nAChR subunit was detected in the lymphocytes from each sample. The expression pattern of mRNAs for α4, α7, β1, and β2 subunits of nAChRs varied between individuals.

From the data obtained, it is clear that there is an intersubject variation in terms of nAChR subunit expression on peripheral human lymphocytes. This variation could explain some of the variability seen in the radioligand binding studies described in chapter 2, although the two sets of experiments were done on cells from different donors.

The present chapter human peripheral blood lymphocytes express mRNA of nAChR subunits described above and to prove that these nAChRs subnits also express the protein of these subunits or not. Therefore, western blotting will be used to confirm the expression of protein in these subunits in the next chapter.

Chapter 4 Determination of nAChR subunit protein in human peripheral blood lymphocyte membranes by immunoblotting

4.1 Introduction

The expression of nAChRs in immune cells, including lymphocytes, has been described (Hiemke *et al*., 1996; Wakkach *et al*., 1996; Mihovilovic and Roses, 1991; 1993; Mihovilovic *et al*., 1997; Toyabe *et al*., 1997; Sato *et al*., 1999). The presence of nAChRs in lymphocytes has also been established by radioligand binding studies (Maslinski *et al*., 1980; Adem *et al*., 1986; Grabczewska *et al*., 1990; Maslinski *et al*., 1992). Binding studies with radioligand revealed nAChRs on human peripheral blood lymphocytes (Adem *et al*., 1986) but the data obtained were not conclusive. Most of the nAChR ligands used were non-specific and non-saturable kinetics were reported (Adem *et al*., 1986; Maslinski, 1989). Therefore, molecular biological techniques such as RT-PCR have been used to reveal the presence of mRNA for nAChRs subunits in lymphocytes. The existence of mRNAs for nAChRs using molecular and/or immunohistochemical approaches has also been reported in the literature (Hiemke *et al*., 1996; Mihovilovic and Roses, 1991; 1993; Mihovilovic *et al*., 1997; Toyabe *et al*., 1997; Sato *et al*., 1999; De Rosa *et al*., 2005; Shok *et al*., 2005). Furthermore, the presence of

nAChRs on lymphocytes has been shown by pharmacological studies (Richman *et al*., 1981).

In previous reports, Hiemke *et al*., (1996) reported the presence of α3, α4 nAChR subunits on human lymphocytes by *in situ hybridisation.* Mihovilovic and Roses (1993) and Mihovilovic *et al*., (1997) showed the presence of mRNA for α3, α5, and β4 nAChR subtypes found in both thymocytes and thymic epithelial cells. Sato *et al*., (1999) showed that mRNAs for α2, α5, and α7 nAChR subunits also found in neurons are predominately expressed in peripheral T lymphocytes. None of these studies confirmed that the subunit protein of nAChRs was expressed on cell membranes.

Radioligand binding studies, described in chapter 2, indicated the existence of α4, α7 and/or β2 containing nAChR on human peripheral blood lymphocytes. RT- PCR studies, described in chapter 3, provided data to support these finding by showing the presence of mRNA for α4, α7, and β2 nAChR subunits and for other nAChR subunits (α5, and β1) as well. However, in these experiments some intersubject variation was seen. In addition to variability in mRNA expression, variability in receptor protein expression in lymphocyte cell membranes may also exist, contributing to some of the variability seen in the radioligand binding studies (Chapter 2). Others have used radioligand binding and RT-PCR experiments to analyse nAChR expression in peripheral blood mononuclear leukocytes, thymus, thymocytes, leukemic T and B cell lines and lymphocytes (Davies, *et al*., 1982; Adem *et al*., 1986; Grabczewska *et al*., 1990; Wakkach *et al*., 1996; Maslinski *et al*., 1992; Mihovilovic and Roses, 1993; Lebargy *et al*., 1996; Toyabe *et al*.,

1997; Sato *et al*., 1999; Benhammou *et al*., 2000; De Rosa *et al*., 2005; Shok *et al*., 2003; 2005). However to date, it would appear that only one study has used immunoblotting techniques to show the presence of α4 nAChR subunit protein on polymorphonuclear cells (PMNs) (Benhammou *et al*., 2000).

To confirm the presence of α4, α5, α7 and β2 nAChR subunits protein in the human peripheral blood lymphocytes used by us. Western blotting was used to analyse nAChR subunit protein in membranes of these cells.

In this chapter Werstern blot analysis was conducted on human peripheral blood lymphocyte protein to confirm the presence of α4, α5, α7 and β2 nAChR subunit protein in human peripheral blood lymphocytes. We looked for α4, α5, α7 and β2 subunits nAChRs protein based on the findings obtained from our radioligand binding and RT-PCR studies. We did not confirm the presence of β1 subunit protein because this subunit could only form a functional nAChR in combination with α1 and other γ and δ subunits and we did not find α1 subunit nAChR mRNA by RT-PCR in the experiments described in chapter 2.

Data obtained from such experiments would provide evidence that nAChR subunits detected at the mRNA level by RT-PCR were expressed as protein, supporting data obtained from radioligand binding experiments. Thus, the data described in the present chapter will provide additional information supporting existing evidence for a functional action of for nicotine on immune cells mediated via nAChRs.

4.2 Materials and methods

4.2.1 Materials

12 samples of human blood cells (Buffy coats) from healthy individual donors were obtained from National Blood Transfusion Services (NTBS) (Conlindale, London, UK). Human peripheral blood lymphocytes were isolated as described in section 2.2.2.1 (Chapter 2). We were given no information as to which donors were tobacco smokers or non-smokers, nor was information provided as to the ethic background of donors.

Monoclonal antibodies against neuronal nicotinic acetylcholine receptor, α4, α5, and β2 subunits were purchased from Covance Research Products, Berkley, USA. The monoclonal antibody against neuronal nicotinic acetylcholine receptor α7 subunit was purchased from Research Diagnostic Inc, Flanders, NJ, USA.

The α4 monoclonal antibody was generated in rats against purified rat brain neuronal nAChR α4 subunit. The α5 monoclonal antibody was generated in rat against purified chicken brain neuronal nAChRs α5 subunit. The α7 monoclonal antibody was generated in mice against an epitope at the intracellular loop of the human and rat nAChR α7 subunit. The β2 monoclonal antibody was generated in rat raised against purified

neuronal nAChRs from chicken brain. The homogenate protein from rat brain was used as a positive control.

The anti-rat IgG heavy and light chains (Goat) alkaline phosphatase, the goat anti-mouse IgG were purchased from Merck Biosciences Ltd., Nottingham, UK. The second step antibody conjugates, peroxidase goat anti-rat IgG and peroxidase goat anti-mouse IgG were purchased from Merck Biosciences Ltd., Nottingham, UK. Prestained protein marker was purchased from Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK. Perfect Protein™ HRP Western blot Kit was purchased from Merck Biosciences Ltd., Nottingham, UK.

4.2.2 Methods

4.2.2.1 Preparation of protein from human peripheral blood lymphocytes

The protocol used was adapted from that described in Immonochemistry Protocols, Western blots, Covance Research Products (USA) (2003). Protein from human peripheral blood lymphocytes was prepared as follows. 0.5 ml of lysis buffer (Laemmli Sample Buffer, 10% glycerol, 2% Sodium dodecyl sulphate (SDS), 125mM Tris-HCl, pH 6.8) containing protease inhibitors (60 μg/ml aproptinin (Sigma Ltd., UK) and 10 μg/ml of leupeptin (Sigma Ltd., UK)) were added to approximately 10^7 human lymphocytes isolated from buffy coat cells as described in section 2.2.2.1 in chapter 2. The cells were incubated on ice for 5 minutes and then incubated at 95°C to reduce the viscosity of the lysate. The lysate was centrifuged at 14,000 x g (Hettich EBA 12, Bench Centrifuge, Sartorius Epsom, Surrey, UK) for 10 minutes to remove the cellular debris. The clarified cell lysate (supernatant) was carefully removed into clean eppendorf tubes and the protein concentration was determined using the Bradford assay (Bradford, 1976). An equal volume of 2x Laemmli sample buffer (125 mM Tris. pH 6.8, 20% glycerol, 4 % of 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol (DTT) or 2 % 2-mercaptoethanol) was added. The mixture was boiled at 100° C for 5-10 minutes. All eppendorf tubes were labelled with date as well as isolate identifier and extract type.

Samples were either used immediately or were stored at -20 °C until required. Each well of a mini SDS-polyacrylamide gel was loaded with 10-20 μg of cell lysate protein.

4.2.2.2 Preparation of membrane protein from rat brain

The method used was based on the Synaptic Plasma Membrane Protein Extraction protocol published by BD Biosciences, Pharmingen (UK) (2003). All the procedures were carried out at 4°C using precooled reagents. The brain was removed from the cranium of a rat killed by exposure to carbon dioxide and immediately placed into ice-cold dissection buffer (50 mM Tris-acetate (TA), pH 7.4, 10% sucrose, 5mM EDTA) containing freshly protease inhibitors (60 μg/ml aproptinin (Sigma, UK) and 10 μg/ml of leupeptin (Sigma, UK)).

A further nine volumes of dissecting buffer was added and the brain was homogenised in a motor-driven glass Teflon homogeniser (B.Braun, F.T. glass Teflon homogeniser c/o Sartorius, Epsom, Surrey, UK) at 900 rpm for 12-15 strokes. The homogenate was centrifuged at 800 x g at 4°C (SS-34 rotor, Sorvall, UK) for 20 minutes. The supernatant was discarded and the pellet was homogenised in a motor-driven glass Teflon homogeniser (B.Braun, F.T. glass Teflon homogeniser c/o Sartorius, Epsom, Surrey, UK) and centrifuged at 16,000 x g at 4°C (SS-34 rotor, Sorvall, Kendro Laboratory Products PLC, Hertfordshire, UK) for 30 minutes. The resulting pellets were resuspended in 10 ml 320 mM sucrose by pipetting up and down and centrifuged at 9,200 x g at 4°C (SS-34

rotor, Sorvall, Kendro Laboratory Products PLC, Hertfordshire, UK) for 15 minutes. The pellet was resuspended in a total volume of 15 ml 320 mM sucrose and a further nine volumes of water were added and the mixture homogenised (B.Braun, F.T. glass Teflon homogeniser c/o Sartorius, Epsom, Surrey, UK) for 3 strokes at 1500 rpm. The protease inhibitors were added and incubated on ice for 15-30 minutes. The homogenate was centrifuged (SS-34 rotor, Sorvall, Kendro Laboratory Products Ltd, Hertfordshire, UK) at 25,000 x g at 4°C for 20 minutes. The pellet was resuspended in 5 ml water and sucrose and water added to give a final volume of 45 ml 1.2 M sucrose. HEPES was added to a final concentration of 5 mM and the protease inhibitor described added as above. The sample was homogenised for 3 strokes at 1000 rpm (B.Braun, F.T. glass Teflon homogeniser c/o Sartorius, Epsom, Surrey, UK) and centrifuged at 9,200 x g at 4°C (SS-34 rotor, Sorvall, Kendro Laboratory Products Ltd, Hertfordshire, UK) for 20 minutes. The pellet was resuspended in water or TA (50 mM Tris-acetate, pH 7.4). The protein concentration was determined using the Bradford reagent (Bio-Rad, Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK). The protein was stored at –80 ° C until required for further study. 20-50 μg of protein was added to each lane of a mini SDS-polyacrlamide gel.

4.2.2.3 Protein assay

The protein concentration of all samples was determined using the Bradford assay (Bradford, 1976). The Bradford method is a quantitative protein assay method, based on

the binding of a dye, Coomassie Brilliant Blue G-250 dye, to a protein sample, and comparing this binding to a standard curve generated by reaction of known amounts of a standard protein Bovine Serum Albumin (BSA). Bovine Serum Albumin (BSA; Sigma Ltd, UK) was used as a standard protein and was prepared at a concentration of 0.6 mg/ml in distilled water. This solution was further diluted in distilled water to give final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4; 0.5 mg/ml of standard protein, respectively (see Appendix 1). Then a 10 μl aliquot of each concentration of protein solution and samples of lymphocyte protein was transferred into a 96 well plate (Falcon, UK). Distilled water was used as a control blank. Bradford reagent containing dye, phosphoric acid, and methanol (Bio-Rad, Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) was diluted 1:5 with distilled water and 200 μl of the resulting solution was added to each well. The plate was then incubated at room temperature for 5-30 minutes and absorbance measured in a multi well plate reader (Labsystem Multiskan RC, UK) at 590 nm. The protein concentrations from samples were determined from the protein (BSA) standard curve.

4.2.2.4 Western blotting

4.2.2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel (SDS-PAGE) preparation

SDS-PAGE was performed using the Laemmli (1970) method, with a discontinuous Tris-glycine buffer system. The stacking and resolving gels were 5 and 10 % SDS-PAGE

respectively. The SDS-PAGE glass plates were 10 x 12 cm in size. The acrylamide, buffers, SDS and water were carefully measured out into two side-arm flasks, one for resolving gel and one for stacking gel as listed in Table 4.1, and 4.2 using 30% 37.5:1 acrylamide.

The Mini-gel apparatus (Bio-Rad, UK) was prepared and assembled as follows. The glass plates were cleaned with acetone and then with 70 % IMS to remove any grease that may inhibit the polymerisation of the acrylamide. When the glass plates had dried and one plate was wrapped with a casting strap, 1 mm spacers were placed on each edge. Matched glasses were then placed on the top to form the cassette. The assembled glasses were clamped. It was carefully clamped just above casting strap and stood in vertical position. The glass plates were marked about 0.5–1.0 cm below the comb, and then the comb was removed. 1.5% ammonium persulphate was prepared by dissolving 0.075g ammonium persulphate (pre-weighed in a bottle) in 5 ml distilled water and the bottle was mixed to dissolve, then the required volume of gel mix. The acrylamide resolving gel solution was prepared in a 250 Buchner flask (for two mini-gels; 6.7 ml 30% acrylamide/0.8% N, N´-methylene bis acrylamide mixture (BDH Ltd, UK), 10 ml resolving gel buffer (Tris-HCl pH 8.8), 0.2 ml 10% SDS solution, 1.0 ml 1.5% ammonium persulphate, 2.1 ml distilled water to make a final volume of 20 ml) as shown in Table 4.1. The resolving gel mix was then de-gassed for 1 minute and 10 µl N, N, N´, N´-Tetramethylethylenediamine (TEMED) (Sigma, UK) was added to the gel mix, which was swirled 4-5 times and taken up into a pipette gently so as not as produce bubbles.

Immediately the gel was poured into the assembled glasses to the level of mark, and carefully covered with a small amount of distilled water (> 1 ml) to exclude air.

Reagent	**Volume (ml)**	**Final Concentration**
30% acrylamide solution (30% acrylamide, 0.8% bis acrylamide)	6.7	10%
0.75 M Resolving gel buffer	10.0	0.375 M
10% SDS	0.2	0.1%
Distilled Water	2.1	
1.5% Ammonium persulphate	1.0	0.75%
Final Volume	20	
TEMED	10 µl	0.05%

Table 4.1 Reagents and volumes required for preparation of 10% SDS-PAGE resolving gel for 2 mini-gels.

The 5% stacking gel mix was carefully measured out into a 250 side-arm flask (for 2 gels; 1.7 ml 30% acrylamide/0.8% N, N´-methylene bis acrylamide mixture (BDH Ltd, UK), 2.5 ml 0.75 M resolving gel buffer (Tris-HCl pH 8.8), 0.1 ml 10% SDS solution, 0.5 ml 1.5% ammonium persulphate, 5.2 ml distilled water to make a final volume of 10

ml) as listed in Table 4.2 The stacking gel mix was then de-gassed for 1 minute, by which time of the resolving gel should have set. A visible gelatin of resolving gel appeared as a clear refractive index change between the polymerised gel and overlaying water. The water was poured off and blotted dry. TEMED (Sigma, UK) was added to the stacking gel mix and swirled 4-5 times. The stacking gel was poured into mould and the comb was pushed in at an angle so that no air bubbles were trapped. The gel cassettes were left to set at room temperature for at least one hour after pouring the stacking gel. The reservoir buffer (0.025 M Tris.HCl, 0.192 M Glycine, 0.1% SDS) was prepared for running gel electrophoresis.

Reagent	**Volume (ml)**	**Final Concentration**
30% acrylamide solution (30% acrylamide, 0.8% bis acrylamide)	1.7	5%
0.75 M Resolving gel buffer	2.5	0.125 M
10% SDS	0.1	0.1%
Distilled Water	5.2	
1.5% Ammonium persulphate	0.5	0.75%
Final Volume	**10**	
TEMED	10 µl	0.05%

Table 4.2 Reagents and volumes required for preparation of 5% SDS-PGAGE stacking gel for 2 mini-gels

4.2.2.4.2 Gel electrophoresis

Samples were removed and fully thawed before use. The clamped gels were removed from the stand and assembled in a Protean II vertical electrophoresis tank (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK). Samples of lymphocyte protein were loaded at a concentration of approximately 20 μg/well and the gel was subjected to electrophoresis at a constant voltage of 200 V for 30-40 minutes at room temperature. The power pack was switched off when the dye front was part way down the orange bar of the mini gel. The equipment was disassembled. The gels were either transferred to a blotting buffer sandwich for electrophoretic transfer to nitrocellulose membrane for immunoblotting, or carefully transferred to a solution of Coomaassie blue (0.1% Coomaassie brilliant blue R250 in 50% methanol, 10% Glacial acetic acid) in a plastic box to stain the protein in the gel.

4.2.2.4.3 Protein staining with Coomassie Blue

Protein bands were detected using Coomassie blue. The gels were submerged in Coomassie blue stain (0.1% Coomaassie brilliant blue R250 in 50% methanol, 10% Glacial acetic acid) and shaken gently for at least 30 minutes (or left overnight) on a rocker platform at room temperature. The stain was poured off and the gels were rinsed in small volume of destaining solution (30% Methanol, 10 % Glacial acetic acid). The

gels were then further destained by gently shaking at room temperature until protein bands could be seen clearly and the surrounding gel was almost clear. The gels were rinsed with distilled water and photographs were taken.

4.2.2.4.4 Transfer of proteins to nitrocellulose membranes

The electrophoretic transfer of protein from SDS-polyacrylamide gels onto nitrocellulose membranes was performed using the method described by Towbin *et al*., (1979). The gel cassettes were disassembled and the gels were carefully transferred to the Western blotting buffer prior to blotting. This was done to facilitate the removal of electrophoresis buffer salts and detergents. For each gel to be transferred, one piece of blotting membrane nitrocellulose (0.45 μM) (BDH Laboratory Supplies, UK) and two pieces of 3 mm filter paper (Whatman, UK) were cut to a size that is slightly larger than the gel. The blotting membrane was always handled by using forceps and gloves to avoid contamination. The transfer sandwiches were prepared using two pieces of Whatman filter paper and two Scotch brite pads (Filter pads). The membrane, 2 pieces of Whatman filter paper and Western blot apparatus were soaked in Western blotting buffer (0.025 M Tris, 0.192 M Glycine, 20% Methanol) for 15-30 minutes. The transfer sandwiches were then prepared as shown in Figure 4.1, ensuring that no bubbles were trapped between the layers. Any air bubbles were moved by gently rolling a Pasteur pipette over each layer in the sandwich. On the black plates of cassette (Negative electrode), a Scotch brite pad (filter pad) was placed followed by a piece of Whatman filter paper and then followed by the gel. Nitrocellulose membrane was placed on top of the gel and followed by placing

another piece of Whatman filter paper and then followed by Scoth brite pad (filter pad) as shown in Figure 4.1.

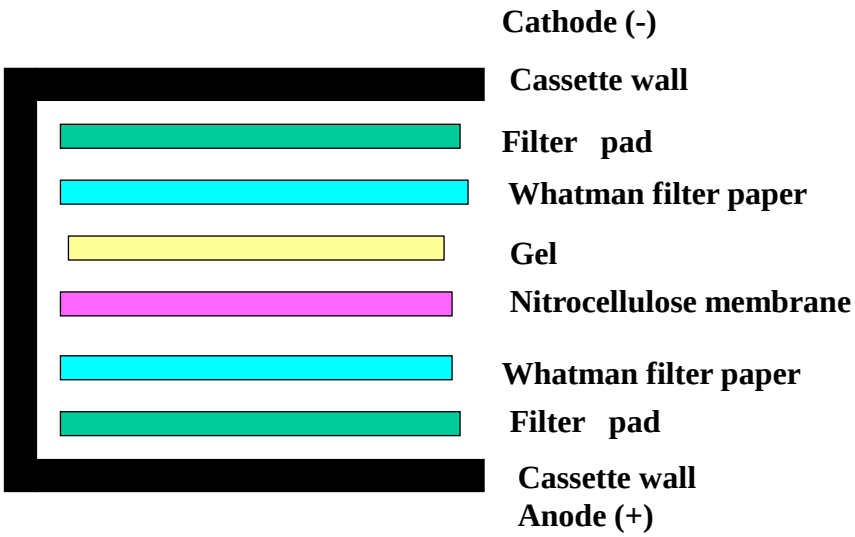


Figure 4.1 A photograph showing schematic of tank blotting method.

The cassette was closed and placed in a Biorad Transblot tank (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) and subjected to electrophoresis at 200 mA at room temperature for 1-1½ hours. After transfer, the cassette was opened and the top pads were removed. The Whatman paper was carefully peeled back and the nitrocellulose paper was trimmed using a gel as guide. The nitrocellulose membranes either were developed immediately or dried and stored at room temperature until required for further study.

4.2.2.4.5 Immunoblotting

After transferring protein from an SDS-PAGE gel to a membrane, as described in section 4.2.2.4.1, 4.2.2.4.2, and 4.2.2.4.4, membranes were subjected to immunoblotting. In order to prevent the remaining protein-free binding sites on the membrane from binding directly to primary or secondary antibody and giving rise a high background signal. Blots were blocked by immersing the nitrocellulose membrane in 5% semi skimmed milk (non-fat dried milk)(Marvell, UK) in phosphate buffered saline (PBS) containing 0.05% Tween 20, V/V (PBS-Tween 20) (Sigma Ltd, UK) and shaking gently for 2 hours at room temperature. Blots were then incubated with a monoclonal antibody (primary antibody) against a neuronal nicotinic acetylcholine receptor subunit (final dilution of 1:1,000 in PBS-Tween 20 for monoclonal antibody against α4, and a final dilution of 1: 500 for monoclonal antibodies against α7, α5 and β2) overnight at 4 °C on a rocking platform. The membranes were then washed twice with distilled water and four times with PBS-Tween 20 with 5 minutes shaking for each wash. A goat anti-rat IgG horseradish peroxidase (secondary antibody) for monoclonal antibodies against α 4, α5 and β2, and a goat anti-mouse IgG horseradish peroxidise for α7 (Merck Bioscienes Ltd, Nottingham, UK) was added (dilution 1:5,000 in PBS-Tween 20 plus 5 % semi skimmed milk) and incubated with shaking for 2 hours. After rinsing and washing as described above, the presence or absence of expected bands were detected using an Enhanced Chemiluminescence (ECL) method.

4.2.2.4.6 Detection protocol for Enhanced Chemiluminescence (ECL)

A detection system used in the present study to detect horseradish peroxidase linked antibodies was the method of enhanced chemiluminescence (ECL). Equal volumes (0.5 ml each reagent per blot) of ECL reagent (Amersham Life Science, UK) No.1 and No.2 were mixed. The membrane was placed on a piece of cling film with the protein side up, detection reagent was added to cover the membrane and incubated for 1 minute at room temperature without agitation. The excess reagent was removed using absorbent paper and the membrane was then wrapped with cling film, making sure that no air was trapped between the blot and cling film.

The following processes were done in a dark room. The membrane, with protein side up, was placed in a film cassette as quickly as possible and the light was the switched off. A sheet of hyperfilm-ECL (Amersham, Life Science, UK) was cut to the size of the membrane and the film was placed directly onto the membrane. The cassette was closed and the film was exposed to record protein bands. The precise exposure time used depended on the monoclonal antibody used as primary antibody (α4 for 5-10 minutes, α 5 for 30 minutes, α7 for 15 minutes and β2 for 20-25 minutes). The film was removed and placed into the developer solution for 10 seconds or until the protein bands were developed and then in water. Finally, the film was fixed in the fixer reagent for 5 minutes and washed again in water. The film was then dried in the air.

4.3 Results

Western blot analysis was used to analyse the presence of α4, α5, α7 and β2 nAChR subunit protein in membranes of the human peripheral blood lymphocytes using monoclonal antibodies raised against each nAChR subunit as described in section 4.2.1.

Figures 4.2 and 4.3 show the protein profiles from rat brain and human peripheral blood lymphocytes on a Coomassie Brilliant Blue stained SDS-PAGE gel. As shown in Figures 4.2 and 4.3, protein bands from human peripheral blood lymphocytes showed a range of bands with molecular sizes from 10 to 250 kD. Rat brain protein, which we used as positive control, also showed the same range of protein bands as did protein bands from human peripheral blood lymphocytes. The expected protein bands for the various nAChR subunits were, α4 molecular size of ≈70 kD, α5 approximate molecular weight of ≈53 kD, α7 approximate molecular weight of ≈56.499 kD, and β2 with a molecular weight of ≈57 kD.

Protein isolated from human peripheral blood lymphocytes on a Coomassie Brilliant Blue stained SDS-PAGE gel, samples No1-3 showed faint protein bands with an approximate molecular weight of ≈75 kD but showed an intensive band with molecular size of about 25-37, and 50 kD (Figure 4.2). In contrast, protein from human lymphocyte samples No 4-5 showed intensive bands with molecular weights of ≈ 25-37, 50, and 75 kD, respectively (Figure 4.2). Sample No 6 showed a very intensive band with an

approximate molecular size of ≈50 and 75 kD but showed a faint band with molecular weight of ≈25-37 kD (Figure 4.2).

Data shown in Figure 4.3 also depicts protein from human peripheral blood lymphocytes samples isolated from samples 7-12 on a Coomassie Brilliant Blue stained SDS-PAGE gel. In these samples, proteins with molecular weights of ≈25-37, and 50 kD were observed with intensive bands in all samples, but a protein with a molecular size of 75 kD was observed with faint bands in samples no 8-12. Sample No 7 showed a very intensive band with an approximate molecular size of 25-37, 50 and 75 kD (Figure 4.3).

Western blot analysis using monoclonal antibodies raised against neuronal acetylcholine receptor, α4, α5, α7 and β2 subunits were carried out to determine the presence of these nAChR protein subunit. As shown in Figures 4.4 - 4.11, each of the antibodies reacted with the protein isolated from human peripheral blood lymphocyte samples tested. The bands with an of the appropriate size for the presence of α4 (≈70 kD) (Figures 4.4 - 4.5), α5 (≈53 kD) (Figures 4.6 - 4.7), α7 (≈56.449 kD) (Figures 4.8 - 4.9), and β2 (≈57 kD) (Figures 4.10 - 4.11) nAChR subunit protein were identified in human peripheral blood lymphocyte samples tested.

In the present study, most of the human peripheral blood lymphocyte samples tested expressed protein for α4 (Figures 4.4 and 4.5). In these blots obtained, a band was seen with a molecular size of ≈ 70 kD in most of the samples tested (Figures 4.4 and 4.5), but the intensity varied from subject to subject (Figures 4.4 and 4.5). Figures 4.6 and 4.7

demonstrate the bands with an approximate molecular size of ≈ 53 kD for the presence of α5 nAChR protein subunit (Figures 4.6 and 4.7). Again the densities of bands varied from subject to subject. Figure 4.8 and 4.9 show the bands with appropriate size for α7 nAChR subunits. The protein bands for α7 nAChR protein subunits showed in both blots obtained, a band was seen with a molecular size of ≈ 56.449 kD in all samples tested (Figures 4.8 and 4.9). In these blots, bands with the predicted molecular size were apparent on blots and the bands varied in intensity from subject to subject.

In contrast, expression of β2 nAChR protein subunit was seen in a few samples tested (Figures 4.10 and 4.11). The protein bands from β2 nAChR protein subunits were not very clear, a band was seen with a molecular size of ≈ 57 kD in samples 3,5 (with very faint bands that could see on the blots obtained but could not photograph), and samples 7-12 (Figure 4.10 and 4.11).

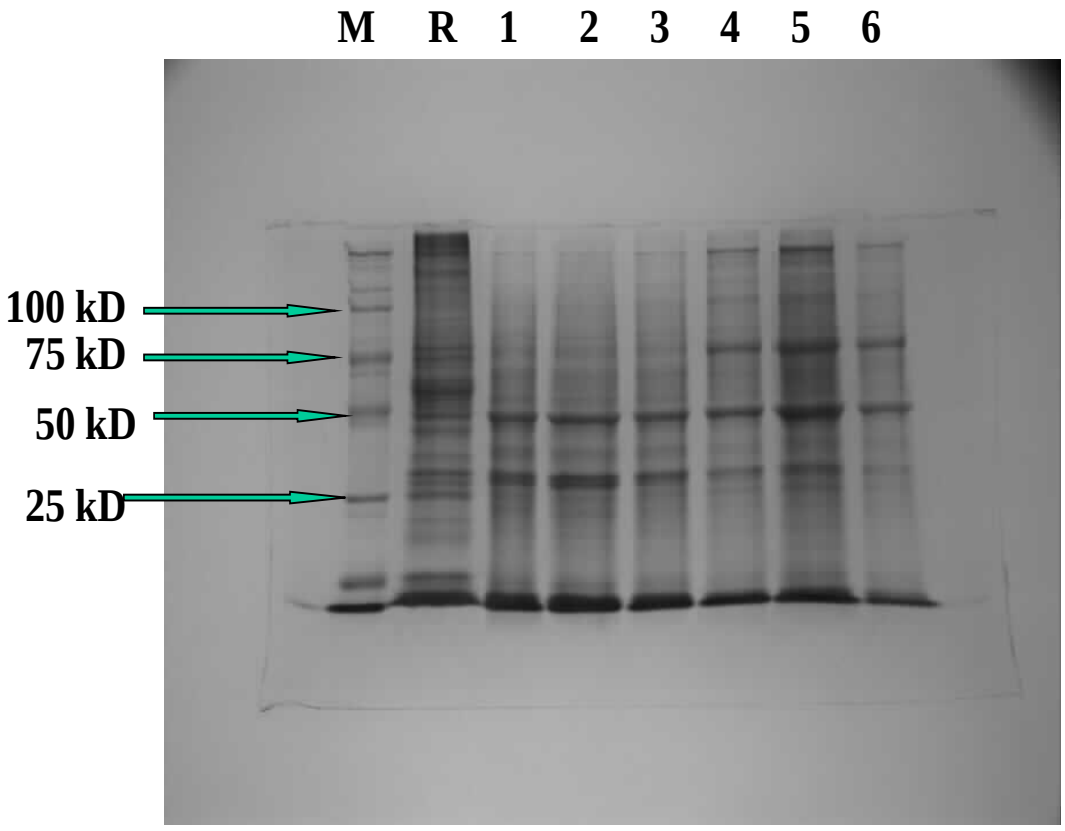


Figure 4.2 Protein from rat brain and human peripheral blood lymphocytes samples (20 µg/lane). The gel was stained with Coomassie Brilliant Blue. Lane M = Precision molecular weight marker (Bio-Rad), Lane R = rat brain protein, Lane No 1-6 = protein from human peripheral blood lymphocyte samples No 1-6.

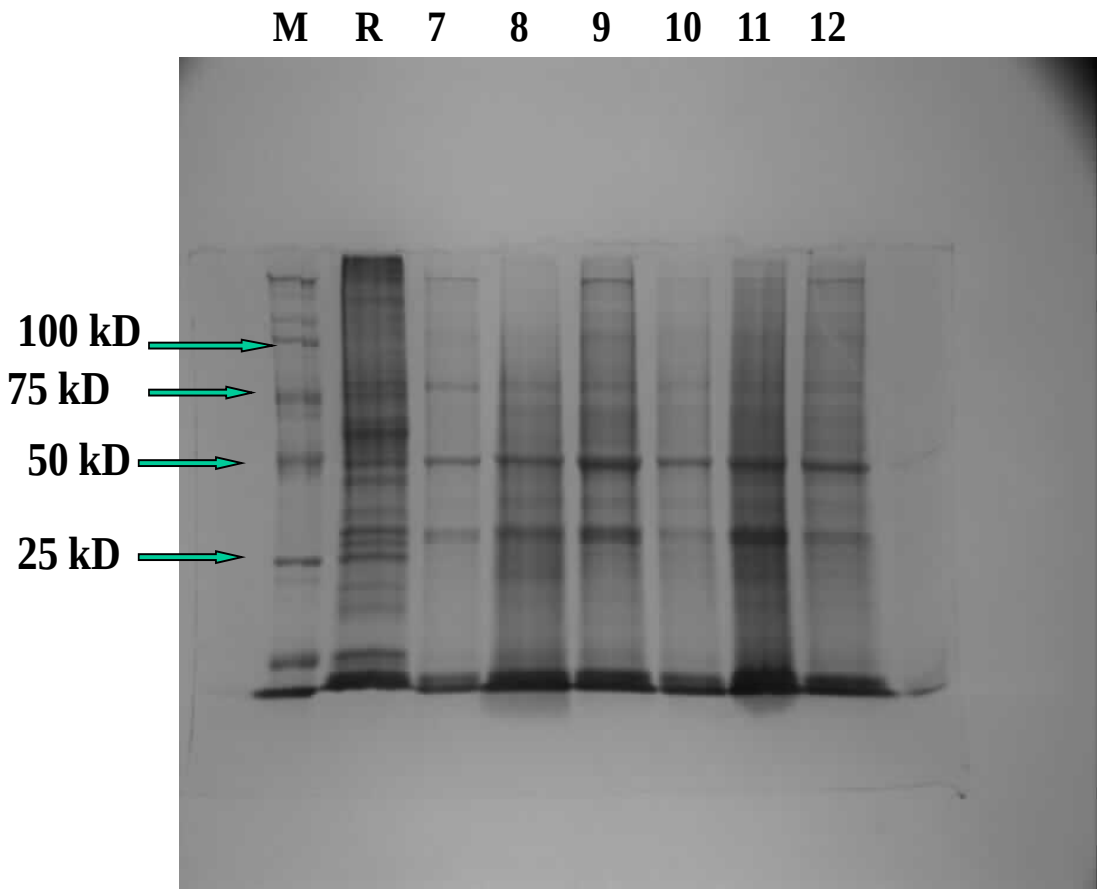


Figure 4.3 Protein from rat brain and human peripheral blood lymphocytes samples (20 µg/lane). The gel was stained with Coomassie Brilliant Blue. Lane M = Precision molecular weight marker (Bio-Rad), Lane R = rat brain protein, Lane No 7-12 = protein from human peripheral blood lymphocyte samples No 7-12.

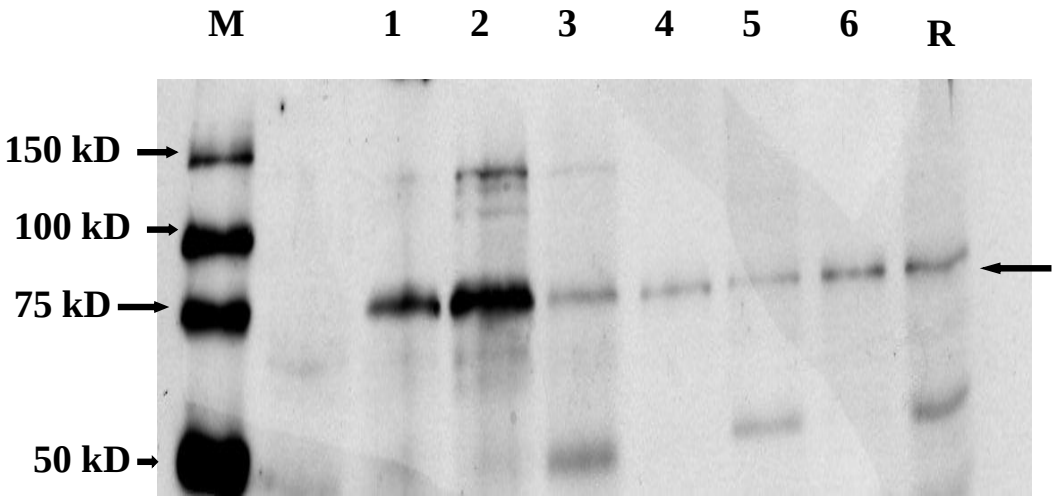


Figure 4.4 Western blot analysis of human lymphocytes was screened for the presence of nAChR α4 subunit protein using a monoclonal antibody against α4 nAChR protein subunit. The predicted molecular weight of the α4 nAChR subunit is ≈70 kD. The protein products were visualised by ECL. Lane 1 = Perfect Protein HRP Western Makers (Novagen), Lane R = rat brain membrane protein, Lane No 1-6 = protein from human lymphocytes (samples No 1-6) (← = predicted molecular weight).

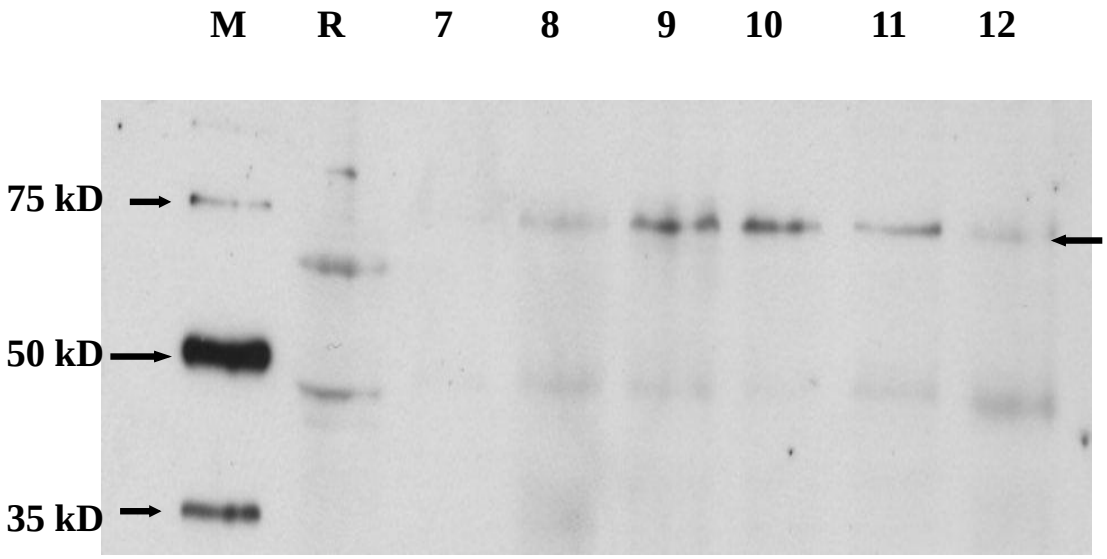


Figure 4.5 Western blot analysis of human lymphocytes was screened for the presence of nAChR α4 using a monoclonal antibody against the α4 nAChR subunit. The predicted molecular weight of the α4 nAChR subunit is ≈70 kD. The protein products were visualised by ECL. Lane 1 = Prefect Protein HRP Western Markers (Novagen), Lane R = rat brain membrane protein, Lane No 7-12= protein from human lymphocytes (samples No 7-12) (← = predicted molecular weight).

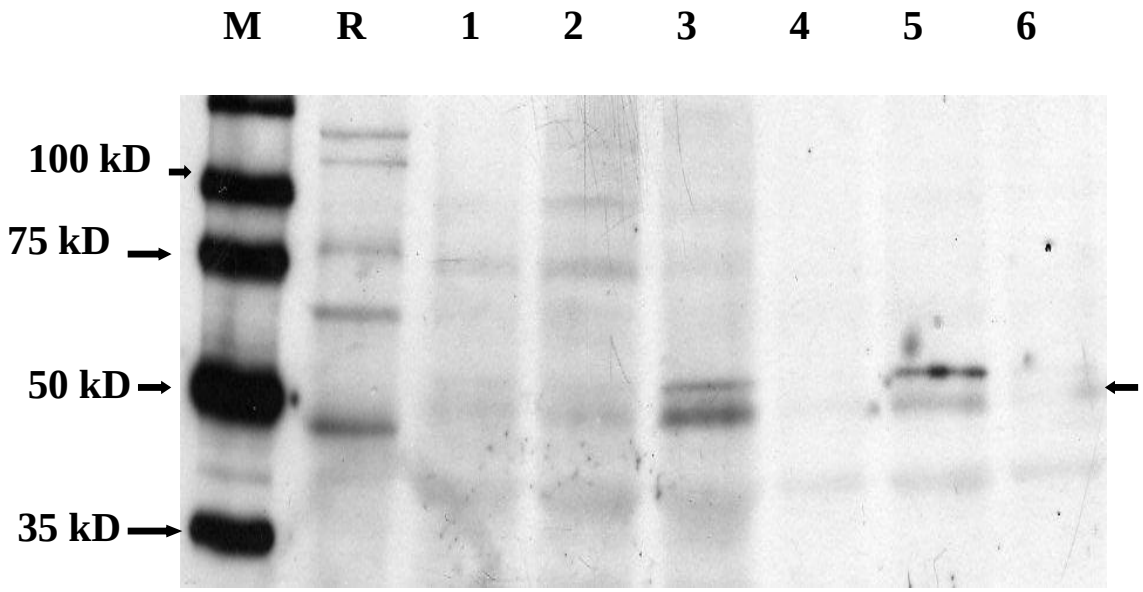


Figure 4.6 Western blot analysis of human lymphocytes was screened for the presence of nAChR α5 subunit protein using a monoclonal antibody against the α5 nAChR subunit. The predicted molecular weight is ≈ 53 kD. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1= Perfect Protein HRP Western Markers (Novagen), Lane 2 = rat brain protein membrane, Lane 1-6 =samples from human lymphocytes (Samples No1 - 6) (← = predicted molecular weight).

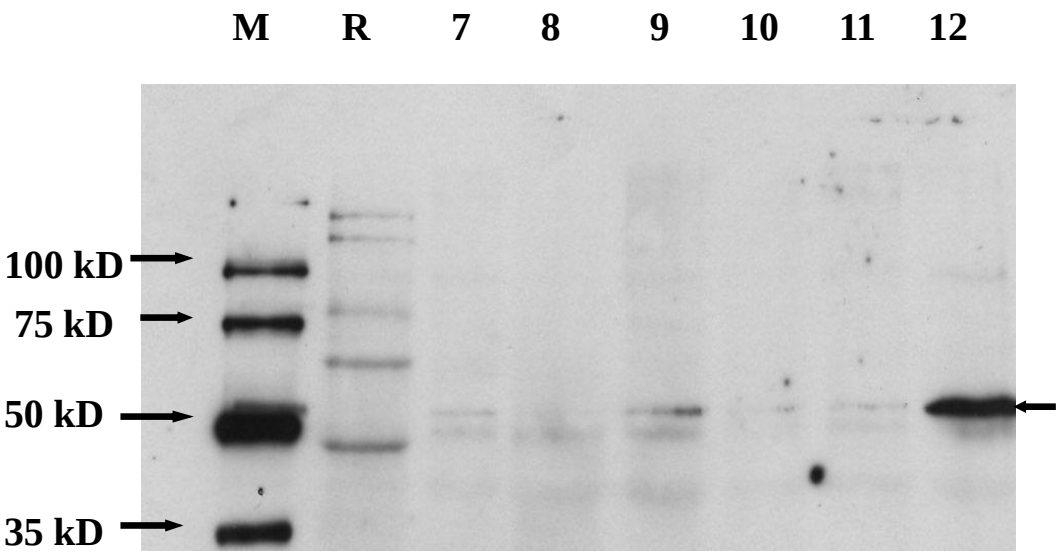


Figure 4.7 Western blot analysis of human lymphocytes was screened for the presence of nAChR *α*5 subunit protein using a monoclonal antibody against the *α*5 nAChR subunit. The predicted molecular weight is 53 kD. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1=Perfect Protein HRP Western Markers (Novagen), Lane 2=rat brain protein membrane, Lane No 7-12 = human lymphocytes (Samples No7 - 12) (← = predicted molecular weight).

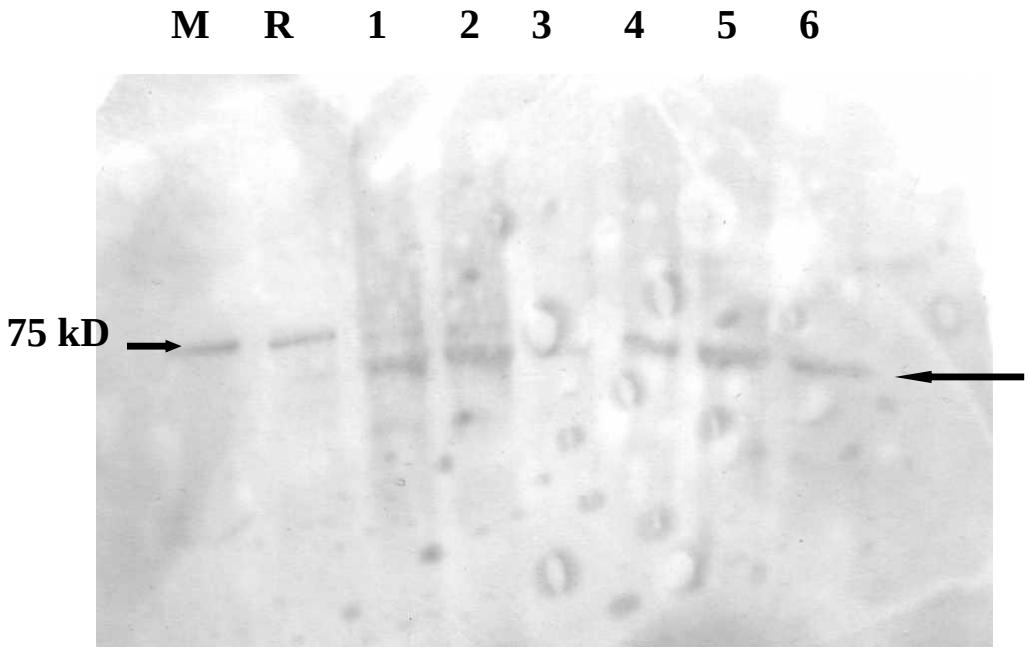


Figure 4.8 Western blot analysis of human lymphocytes was screened for the presence of nAChR α7 subunit protein using a monoclonal antibody against α7 subunit. The predicted molecular weight is ≈56.449 kD. This antibody was specific to both human and rat α7 subunit protein nAChR. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1= Perfect Protein HRP Western Markers (Novagen), Lane 2=rat brain protein membrane, Lane No 1-6 = human lymphocytes (Samples No1- 6) (← = predicted molecular weight).

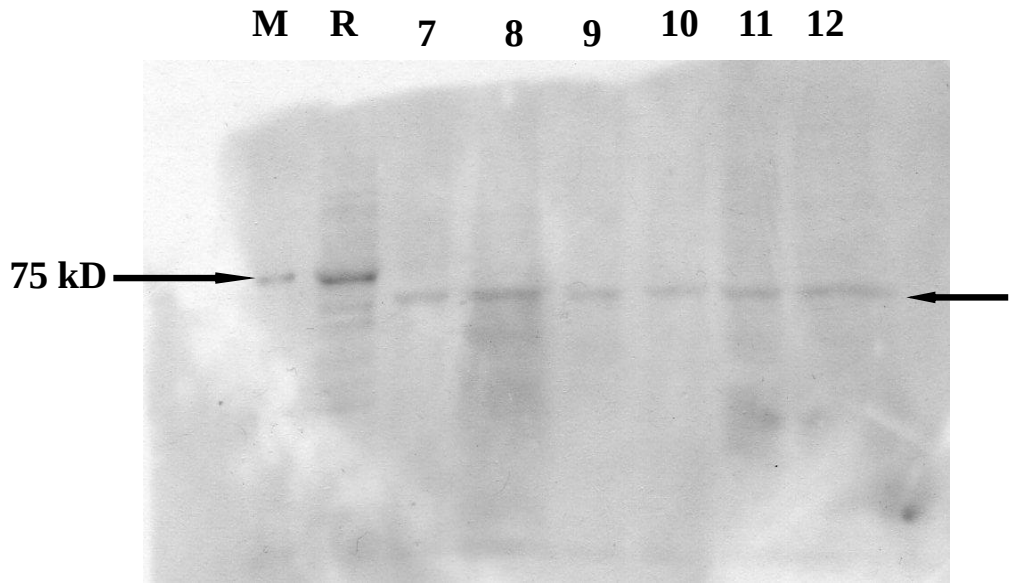


Figure 4.9 Western blot analysis of human lymphocytes was screened for the presence of nAChR α7 subunit protein using a monoclonal antibody against the α7 nAChR subunit. The predicted molecular weight is ≈56.449 kD. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1 = Perfect Protein HRP Western Makers (Novagen), Lane 2 = rat brain protein membrane, Lane No 7-12 = human lymphocytes (Samples No7 - 12) (← = predicted molecular weight).

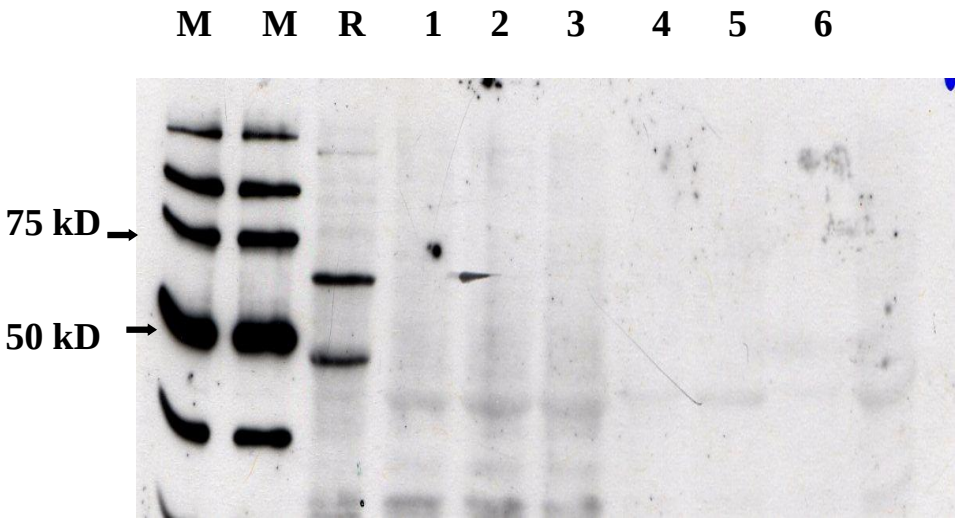


Figure 4.10 Western blot analysis of human lymphocytes was screened for the presence of nAChR β2 subunit protein using a monoclonal antibody against the β2 nAChR subunit. The predicted molecular weight is ≈57 kD. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1 = Perfect Protein HRP Western Markers (Novagen), Lane 2 = rat brain protein membrane, Lane No 1-6 = human lymphocytes (Samples No1 - 6).

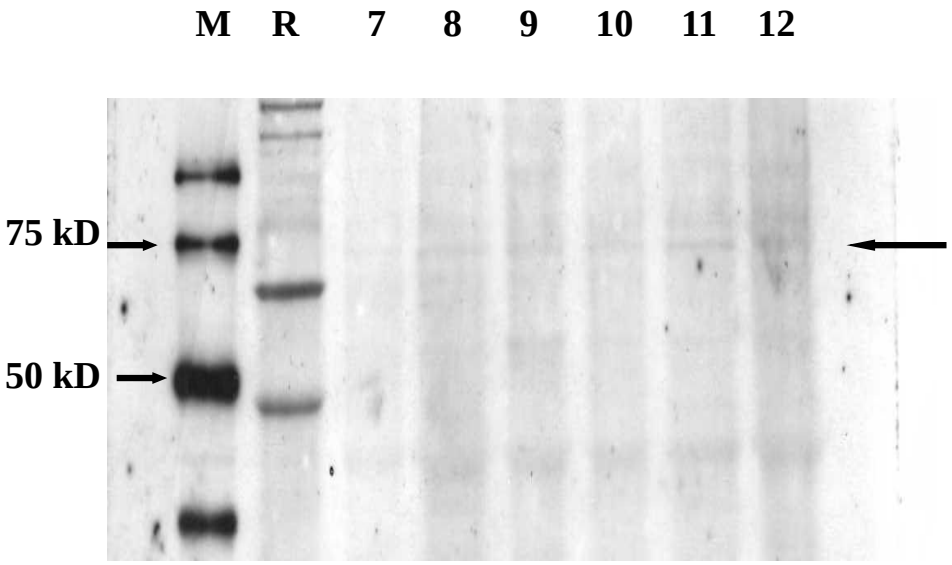


Figure 4.11 Western blot analysis of human lymphocytes was screened for the presence of nAChR β2 subunit protein using a monoclonal antibody against the β2 nAChR subunit. The predicted molecular weight is ≈57 kD. In all samples, a band of the appropriate size was identified and the protein products were visualised by ELC. Lane 1 = Perfect Protein HRP Western Markers (Novagen), Lane 2=rat brain protein membrane, Lane No 7-12 = human peripheral blood lymphocytes (Samples No7 - 12). (← = predicted molecular weight).

4.4 Discussion

In the present study we have confirmed the protein expression of α4, α5, α7 and β2 nAChR subunits that were detected in human peripheral blood lymphocytes by RT-PCR experiments described in chapter 3.

The results obtained from the Western blot analysis show that protein for α4, α5 and α7 nAChR subunits was expressed in most, but not all of the human lymphocyte samples tested and some of the bands obtained were faint. In contrast, protein for the β2 nAChR subunit was observed in a few of samples tested and the bands were faint suggesting that expression of this nAChR subunit may be low or the β2 monoclonal antibody used, was not specific enough to detect the nAChR protein subunit from human peripheral blood lymphocytes.

To our knowledge this is the first report that has demonstrated the expression of nAChR protein subunits for α4, α5, α7, and β2 in human peripheral lymphocytes from a sample of different donors and shown intersubject variation in protein expression. Only one other study (Benhammou *et al*., 2000) showed the presence of α3, α4, α7, and β2 nAChRs protein on polymorphonuclear leukocytes (PMN) from 2 smokers, and 1 non-smoker.

Previous studies have revealed that the α3 and α4 nAChR subunit genes in immune cells such as lymphocytes could be detected by immunohistochemistry and *in situ* hybridisation (Hiemke *et al*., 1996). However, others failed to find either α3 or α4 nAChR subunit expression in lymphocytes (Sato *et al*., 1999; Mihovilovic and Roses, 1993; Mihovilovic *et al*., 1997; 1998). None of these studies showed the presence of the nAChR protein subunits. Experiments described in this thesis have used radioligand binding studies to characterise the presence and pharmacological properties of nAChR on human peripheral blood lymphocytes. RT-PCR and Western blot analysis were then used to examine mRNA and protein subunits of nAChR expressed on human peripheral blood lymphocytes.

Proteins from human peripheral blood lymphocytes (20 µg/lane) on a Coomassie Brilliant Blue stained SDS-PAGE gel were seen but the protein bands obtained varied in intensity from subject to subject and samples from some subjects produced a protein band that very faint although equal amounts of protein were loaded onto gels. A high degree of variability in the protein expression pattern was observed between individuals.

The results described in the present study, using Western blot analysis, show that most of the human peripheral blood lymphocytes tested expressed protein for α4 (Figure 4.4 and 4.5), α5 (Figures 4.6-4.7) and α7 (Figures 4.8-4.9) nAChR subunits. The expression of β2 nAChR subunit protein was seen in only a few sample tested (Figure 4.10-4.11). The protein bands for β2 nAChR protein subunits were not very clear.

The intersubject variation in nAChR subunit expression can be seen from the Western blot analysis data described as well as in the results obtained from radioligand binding studies in chapter 2 and RT-PCR in chapter 3. This intersubject variation may explain the differences seen between reports published in the literature and may suggest genetic variability or the induction of nAChR expression in response to factors such as exposure to nicotine in the environment.

In the present study, a monoclonal antibody against the α4 nAChR subunit bound to protein from human peripheral blood lymphocytes. This suggests that human peripheral blood lymphocytes contain the α4 nAChR subunit. The results obtained from the present study support the results obtained from radioligand binding in chapter 2 showing that epibatidine, a potent agonist for α4β2 nAChR, bound to α4 nAChR on human peripheral blood lymphocytes. The mRNA for the α4 nAChR subunit was also detecting by RT-PCR. Thus it seems likely that human peripheral blood lymphocytes express α4β2 nAChR and these receptors are responsible for nicotine binding to lymphocytes and for any effects of nicotine on cell function.

The results obtained in the present chapter support the finding of Hiemke *et al*., (1996) who demonstrated the expression of α4 mRNAs for nAChR subunit on human lymphocytes using immunohistochemistry and *in situ hybridisation.*

The bands obtained for human peripheral blood lymphocyte protein membrane that bind specifically to α4 monoclonal antibody, exhibit a higher molecular weight than do those for rat brain. This might be because the samples come from different species. Despite the small difference in molecular weight, they both bind to the same monoclonal antibody. These two proteins, from different species, therefore appear to be a homologous protein subunit (Whiting *et al*., 1987; Whiting and Lindstrom *et al*., 1988). Therefore, this might explain why proteins from rat brain and human peripheral blood lymphocytes have different sizes on blots while the proteins bound to the same antibody.

The monoclonal antibody against the α5 nAChR subunit bound to most of the samples tested suggesting that human lymphocytes contain the α5 nAChR protein subunit. Therefore, data obtained from Western blot analysis show that the protein of α5 nAChR subunits, exists on human peripheral blood lymphocytes. Again, this data supports the data obtained from RT-PCR experiments described in Chapter 3 which showed the presence of α5 mRNA for the nAChR subunit. All donors lymphocytes expressed mRNAs encoding the α5 subunit but not all the samples tested, expressed protein. Suggesting that in some subjects mRNA was not translated into protein.

The α5 subunit does not form a functional nAChR by itself or any β subunit (Conroy *et al*., 1992; Wang *et al*., 1996). However, α5 subunits can be incorporated into nAChR with three different subunits α3β2/4 and α4β2 subunits to form hetero-oligomers and produce changes in channel properties (Conroy *et al*., 1992; Conti-Tronconi *et al*., 1994; Galzi and Changuex, 1995; McGehee and Role, 1995; Ramirez-Latorre *et al*., 1996;

Wang *et al*., 1996; Gerzanich *et al*., 1998). It is possible to conclude from the data presented in chapter 3 and from the present chapter, that human peripheral blood lymphocytes do contain mRNA for the α5 nAChR subunit, although the radioligand binding data described in chapter 2 could not identify whether or not the receptors present contained α5 subunit containing nAChR. Furthermore, there seem to be no ligands available that are specific for α5 containing nAChR subunit. Alternatively, it is conceivable that human lymphocytes express α5 nAChR subunit protein but this does not form a functional receptor.

The monoclonal antibody raised against α7, bound to human lymphocyte protein. Most samples tested bound the antibody. Therefore the results obtained from Western blot analysis show that the protein of α7 nAChR subunits, exist on human peripheral blood lymphocytes. These data support the data obtained from radioligand binding experiments in Chapter 2 showing that alpha bungarotoxin (antagonist which binds specifically to nAChR containing α7) bound to human peripheral blood lymphocytes and data obtained from RT-PCR in Chapter 3 showed the presence of α7mRNA for the nAChR subunit.

The monoclonal antibody raised against the β2 nAChR subunit, exhibited binding to human peripheral blood lymphocytes and showed very faint bands. Few samples tested bound to the antibody however the bands observed were faint. This faint staining might be because the monoclonal antibody used in the present study was not as sensitive for the proteins expressed on human peripheral blood lymphocytes unlike the other antibodies used. This antibody was generated in rat against purified neuronal nicotinic acetylcholine

receptors from chicken brain. Therefore, this antibody was raised from a different species and cross reacts with chicken and rodent nAChRs as well as human, but it cross reacts with human with low affinity.

The bands obtained for human lymphocyte protein that bind specifically to α4, α7, and β2 monoclonal antibodies, exhibit a different molecular weight than do those for rat brain protein that was used as positive control. This might be because the samples come from different species. Despite the small difference in molecular weight, they both bind to the same monoclonal antibody. These two proteins, from different species, therefore appear to be a homologous protein subunit. Some studies have shown that the subunit structure of nAChR from rat brain contains two types of protein with *Mr* 51,000 and *Mr* 79,000 subunits (Whiting *et al*., 1987). The smaller subunit is homologous to the 49,000 *Mr* subunit of nAChR from chicken brain since both bind anti-sera to α subunits of nAChRs from the *Torpedo* electric organ monoclonal antibodies (Whiting *et al*., 1987).

The subunit structure of nAChR from bovine brain is similar to that of the nAChR subtype from chicken brain with ACh-binding site *Mr* 75,000 (Whiting *et al*., 1987). The structural subunit of nAChR from bovine brain is homologous to structural subunits of nAChRs from chicken and rat brains (Whiting and Lindstrom *et al*., 1988). Similarly, the 74,400 *Mr* ACh-binding subunit of nAChR from bovine brain is homologous to the 79,000 *Mr* subunit from rat brain (Whiting and Lindstrom, 1988) and to the 75,000 *Mr* ACh-binding subunit of nAChR subtype of chicken brain (Whiting *et al*., 1987). This might be the reason why proteins from rat brain which used as positive control did not

give the same molecular weight as human peripheral blood lymphocyte membrane protein did. Therefore proteins from rat brain and human peripheral blood lymphocytes have different molecular sizes on blots while the proteins bound to the same antibody.

We observed a high degree of variability in the protein expression pattern between individuals. Such variability pattern and varied in intensity of protein expression for α4, α5, α7 and β2 nAChR subunits suggests that the level of protein expression may not only be genetically determined, but may also be regulated by environmental factors such as infection, physiological stress or smoking behaviour. Such effects are suggested by the work of Lebargy *et al*., (1996) who reported that tobacco smoking caused the expression of a high affinity-binding site for nicotine on granulocytes. Similarly, Benhammou *et al*., (2000) also showed that [^{3}H]-(-)-nicotine binding in peripheral polymorphonuclear cells (PMN) was increased in smokers and this increase correlated with tobacco use.

The results obtained from the present study confirm the existence of nAChRs in human peripheral blood lymphocytes. Although the protein of nAChR recognised by these monoclonal antibodies remains to be further clarified and its structural and functional significance need to be determined.

In summary, the data obtained from Western blot analysis described in the present chapter (protein subunits of the nAChR have been found in human peripheral blood lymphocytes) show that protein for α4, α5, α7 and β2 nAChR subunits, are expressed on human peripheral blood lymphocytes. The data obtained from Western blot analysis

support and confirm the radioligand binding data from chapter 2 and RT-PCR studies in chapter 3. We assumed from the radioligand binding studies that human lymphocytes do show the presence of mRNA for α4, α7 and/or β2, nAChR subunits. As the results show, the RT-PCR data obtained from human peripheral blood lymphocytes do show the presence of mRNA for α4, α7 nAChR subunits suggested by the radioligand binding data described in chapter 2. In addition, the data described in chapter 3 also show the presence of mRNA for the α5, β1, and β2 nAChR subunits. The data obtained from Western blot analysis in the present chapter confirm that human peripheral blood lymphocytes contain α4, α5, α7 and β2 nAChR subunits, suggesting that human peripheral blood lymphocytes carry α4β2 or α4β2α5 and possibly the α7 nAChR subtypes.

Chapter 5 General discussion

The overall aim of the experiments described in the present thesis was to identify and characterise the nicotinic acetylcholine receptors (nAChRs) on human peripheral blood lymphocytes in terms of receptor subtype using radioligand binding assays, pharmacological and molecular biological techniques. The presence of nicotinic acetylcholine receptors (nAChRs) binding (-)- nicotine on human lymphocytes was identified by radioligand binding studies using [^{3}H]-(-)-nicotine as a radioligand. The pharmacological properties of nAChRs on human peripheral blood lymphocytes were studied by competition binding studies with a range of nicotinic cholinergic receptor ligands (nicotine and other nAChR-specific nicotinic agonists and antagonists). Molecular biological techniques were then used to demonstrate mRNA for the subunits of nAChRs found by radioligand binding studies. Finally, Western blot analysis was used to confirm whether the mRNA detected by PCR was translated to protein in the cell under study using the specific monoclonal antibodies specific for each subunit of nAChRs.

The data obtained from the binding studies suggested the presence of one binding site for (-)- nicotine on human peripheral blood lymphocytes with a *Kd* 15 ± 5.759 nM (1.5 ± 5.759 x 10^{-8}M) and B*max* 2253 ± 409 sites/cell. The competition studies showed that ligands competing with [^{3}H]-(-)-nicotine were (-)-nicotine, epibatidine and α-bungarotoxin. Thus, radioligand-binding experiments suggested that the presence of α7

and possibly α4 or/and β2 containing nAChR subunits. Furthermore, RT-PCR techniques demonstrated the presence of mRNAs encoding different subunits of nAChRs in human peripheral blood lymphocytes. We found that human peripheral blood lymphocytes tested expressed mRNAs for α4, α5, α7, β2 neuronal nAChRs subunits and β1 muscle nAChR subunit. Expression of mRNA for the α5 subunit of nAChR was observed in the lymphocytes in each sample of lymphocytes. In contrast, the expression pattern of mRNAs for α4, α7, β1, and β2 mRNAs subunits of nAChRs varied between individuals.

Finally**,** Western blot analysis was used to confirm that mRNA expression resulted in the expression of protein for nAChR subunits in human peripheral blood lymphocytes using the antibodies against α4, α5, α7, and β2 nAChR subunits**,** which had been detected by RT-PCR. The data obtained from Western blot analysis described in the present study showed that protein for α4, α5, α7, and β2 nAChR subunits, are expressed on human peripheral blood lymphocytes. From the results obtained in this study, it is possible to conclude that human peripheral blood lymphocytes contain nAChR with composition of α4β2, α4β2α5 subunits, and possibly the α7 homomeric nAChR.

The data obtained in the present study contributes to the evidence for a functional action of nicotine on the immune cells mediated through an interaction with nAChR.

The data described in the present study also adds to already existing evidence supporting a direct role for nicotine on immune cells. There is now evidence that nicotine may have

effects at sites other than the brain and nervous system. The published literature in the main supports this conclusion although there are caveats, already mentioned, which should induce a measure of caution when seeking to use the data from this study to support this interpretation.

Nicotine is known to have a number of effects on immune cell function. The first goal was to investigate the anti-inflammatory effect of nicotine on the immune system and to characterise this effect using cholinergic drugs. The knowledge of how nicotine binds to the nAChR binding sites on lymphocytes may show how nicotine modulates ulcerative colitis. Data generated may also indicate how nicotine can be exploited in order to identify a selective ligand for the nAChRs that are involved in inflammatory diseases such as ulcerative colitis. Such a molecule would have therapeutic utility.

There is a whole raft of research, which supports this hypothesis. Thomas *et al*., (1994; 1995; 1996; 1998) showed a beneficial effect of nicotine in ulcerative colitis. Sykes *et al*., (2000) showed that nicotine inhibited a rodent model of colitis. Petro *et al*., (1992) found that nicotine inhibited cytokine release from murine splenic lymphocytes. Sykes *et al*., (2000) also established that nicotine is a potent inhibitor of lipopolysacharide (LPS)-induced tumour necrosis factor α (TNF α) release from THP-1 cells, a monocytic cell line, *in vitro*. Further data suggest that the receptor mediating this action may be distinct from the nicotine cholinergic receptors found on the other cells. THP-1 cells also appear to contain two binding sites for nicotine. One binding site has the properties of a nicotinic acetylcholine receptor (nAChR) and the other receptor has a high affinity-

binding site that appears to be non-cholinergic in nature (Morgan *et al*., 2001). Further preliminary studies have shown that nicotine at concentrations likely to be found in the blood of smokers inhibits interleukin-2 (IL-2) release from human lymphocytes *in vitro* (Madretsma *et al*., 1996).

Building on these preliminary findings, receptor-binding studies were carried out to investigate the nature of the nicotinic acetylcholine receptors on human peripheral blood lymphocytes by studying the binding of radiolabeled (-)-nicotine to these cells. Non-cholinergic binding sites have been demonstrated on immune cells (Davies *et al*., 1982; Hoss *et al*., 1986; Morgan *et al*., 2001), which encouraged the belief that a non-cholinergic binding site might exist on human peripheral blood lymphocytes.

The data obtained from the binding studies described in chapter 2 suggested the presence of one binding site for (-)- nicotine on human peripheral blood lymphocytes with a *Kd* 15 ± 5.759 nM (15 ± 5.759 x 10^{-8}M) and B*max* 2253 ± 409 sites/cell. Furthermore, data described in chapter 3 used RT-PCR to identify mRNA for α4, α5, α7, β1, and β2 nAChR subunits in human peripheral blood lymphocytes. Finally data presented in chapter 4**,** used Western blot analysis to confirm protein expression of the nAChR subunits in human peripheral lymphocytes using the antibodies against α4, α5, α7, and β2 nAChR subunits**,** which had been detecting by RT-PCR. Thus the findings of chapters 2- 4 from the present study suggest the presence of one nAChR on human peripheral blood lymphocytes that accounts for all of the binding of nicotine to these cells and no evidence was obtained to suggest the presence of non-cholinergic nicotine receptor.

Radioligand binding studies are useful for quantifying and mapping the distribution of nAChRs, and determining pharmacological profiles. The data obtained from the present study by radioligand binding studies has found one possible nicotine-binding site on human lymphocytes with a *Kd* 15 ± 5.759 nM (1.5 ± 5.759 x 10^{-8}M) and B*max* 2253 ± 409 sites/cell. Because of the equivocal nature of the data obtained it is not possible to draw any definitive conclusions from the lymphocyte binding data with regard to the possible nature of this binding site. The specific binding of nicotine, observed in the present study was low and showed marked variation between subjects. One explanation for this could lie with the fact that peripheral blood lymphocytes from different donors contain different subtypes of nAChRs. Also the number of nAChRs on immune cells is less than that in the brain. In the present study we had no control over the subjects whose blood was used. The lymphocytes used in this study were obtained from buffy coats provided by the National Blood Transfusion Service and information on genetic background or tobacco consumption was unavailable.

Lebargy *et al*., (1996) reported that tobacco smoking up-regulated a high affinity-binding site for nicotine on granulocytes. Similarly, Benhammou *et al*., (2000) also reported that [^{3}H]-(-)-nicotine binding in peripheral polymorphonuclear cells (PMN) was increased in smokers and this increase correlated with tobacco use. If the same were true of lymphocytes, white cell groups, the presence of such cells may have contributed to some of unexpected data obtained in the present study.

Data obtained from radioligand binding studies showing the presence of a nicotine binding site on human peripheral blood lymphocytes did not provide a definitive answer as to nature of the nAChR present because of low specific binding and intersubject variability. Identification of nAChRs from the radioligand data described in the chapter 2 was difficult. However (-)-nicotine and some ligands such as epibatidine and α bungarotoxin were potent inhibitors of ligand binding, while the others displaced radioligand in insignificant quantities. Epibatidine and α-bungarotoxin competed with [^{3}H]-(-)-nicotine, indicating the possible existence of α7 subunits and α4/or β2 subunit containing nAChR on human peripheral blood lymphocytes. These tentative conclusions can be made because epibatidine is a potent agonist for α4β2 (with K*i* values ≈ 0.01-0.05 nM or 0.01-0.05 x 10^{-9} M) and α7 subunit containing nAChRs (with Ki ≈ 20.6 – 233 nM or 20.6-233 x 10^{-9} M) (Gerzanich *et al*., 1995; Gotti *et al*., 2000; Sharples and Wonnacott, 2001), and α-BTX, is a competitive antagonist which is very specific and sensitive to nAChR containing α 7 subunits (K*i* ≈ 0.35-3.5 nM or 0.35-3.5 x 10^{-9}M) (Sharples and Wonnacott, 2001). Thus, α bungarotoxin is diagnostic for the presence of this subunit although it will also label nAChR composed of alpha 8, (only found in chickens), and alpha 9 nAChR subunits, and the muscle nAChR (Anand *et al*., 1993; Bertrand *et al*., 1992; McGehee and Role, 1995; Gotti *et al*., 1997).

Epibatidine is also a useful ligand to distinguish between β2 and β4 containing nAChRs. The findings from Wang *et al.*, (1996) showed that the α3 nAChR in SH-SY5Y (human neuroblastoma cells) can be divided into 2 groups. One with β2 subunit combinations (α3β2, α3β2α5, α3β2β4, and α3β2β4α5) has a higher apparent affinity for epibatidine.

Another group of workers found α3 containing nAChRs without β2 subunits (α3β4, α3β4α5) with lower apparent affinity for epibatidine (Wang *et al*., 1996). Data from the competition binding studies, described in chapter 2 showed that epibatidine strongly displaced [^{3}H] - (-)-nicotine from human peripheral blood lymphocytes. This might be because of the presence of the β2 subunit containing nAChR in human peripheral blood lymphocytes.

Tritiated nicotine was first reported to bind α4β2 nAChR in brain tissue by Romano and Goldstein in 1980. Since then [^{3}H]-(-)- nicotine binding has been extensively characterised and the binding sites appears to be identical to those labelled with other agonists, namely [^{3}H]-acetylcholine, [^{3}H]-cytisine, [^{3}H]-methylcarbamycholine and [^{3}H]-ABT-418. Immunoprecipitation experiments indicate that these sites correspond to α4β2 (Flores *et al*., 1992; 1997).

[^{3}H]-(-)-nicotine binding is absent in β2 nAChR subunit knockout mice (Picciotto *et al*., 1995). All nAChRs bind nicotine, but only the α4β2 nAChR type appears to be sensitive enough for this binding to be measurable in ligand binding assays. It should be noted that the binding affinities of these agonists are lower than their EC50 values for activating α4β2 nAChR (1.0-10 μM or 1.0-10 x 10^{-6} M), and may reflect binding to the high affinity, desensitised state of the nAChR (Mark *et al*., 1996; Gotti *et al*., 1997). Competition binding assays with such agonists can provide information on the relative potencies of competing ligands. However, the results obtained can be misleading with

respect to receptor activation. Radioligand bind assays do not distinguish agonists from antagonists and full inhibitors of binding can be partial agonists with respect to nAChR function (Nordberg, 1995). Although all nAChRs, by definition, respond to nicotine, they differ with respect to the nicotine concentrations required for activation or desensitisation (Gotti *et al*., 1997).

Data obtained from competition studies, in the present study, show that (-)- nicotine, epibatidine and α bungarotoxin competed with [^{3}H]-(-)-nicotine while others do not significantly displace radioligand. Thus radioligand binding experiments from the present study suggested that the presence of α7 and possibly α4 or/and β2 containing nAChR subunits in human peripheral blood lymphocytes. This conclusion can be made because (-)- nicotine, and epibatidine are very potent agonists for α4β2 and epibatidine is also a potent agonist for α4 and α7 subunit containing nAChRs (Sharples and Wonnacott, 2001), while α bungarotoxin is a competitive antagonist which is very specific and sensitive for α7 subunit containing nAChR.

RT-PCR was then used to demonstrate the presence of mRNA for possible nAChR subunits in human peripheral blood lymphocytes. The data obtained show that human peripheral blood lymphocytes expressed mRNA for α4, α5, α7, β1 and β2 nAChRs subunits. The expression of mRNA for the α1, α2, α3, α6, α10, β3, and β4 subunits of nAChRs was not observed in the human peripheral blood lymphocytes from any of the samples. However, mRNA for the α5 nAChR subunit was detected in the lymphocytes

from each sample. The expression pattern of mRNAs for α4, α7, β1, and β2 subunits of nAChRs also varied between individuals.

The data obtained from the RT-PCR studies show the presence of α4, α5, α7, β1, and β2 mRNA nAChR subunits. However, of these only the α7 subunit is able to form a functional receptor in the absence of other receptor subunits (Couturier *et al*., 1990; Gerzanich *et al*., 1994). In contrast the α4 subunit can combine with a β2 subunit to form a functional receptor (Cooper *et al*., 1991; Luetje and Patrick, 1991; Flores *et al*., 1992; Sargent, 1993; Zhou *et al*., 2003). Therefore the results obtained from radioligand binding assays and RT-PCR showed that human peripheral blood lymphocytes might carry nAChR containing α7, α4β2 and/ or α4β2α5 subunits.

The α5 subunit does not form a functional nAChR by itself or any β subunit (Conroy *et al*., 1992; Wang *et al*., 1996). However α5 subunits can be incorporated into nAChR with three different subunits α3β2/4 and α4β2 subunits to form hetero-oligomers and produce changes in channel properties (Conroy *et al*., 1992; Conti-Tronconi *et al*., 1994; Galzi and Changuex, 1995; McGehee and Role, 1995; Ramirez-Latorre *et al*., 1996; Wang *et al*., 1996; Gerzanich *et al*., 1998). It is possible to conclude from the data presented in chapter 3 that human peripheral blood lymphocytes do contain mRNA for the α5 nAChR subunit, although the radioligand binding data described in chapter 2 could not identify whether or not the receptors present contained the α5 subunit. Furthermore, there seem to be no ligands available that are specific for α5 subunit

containing nAChR. Thus radioligand binding experiments are unable to address the question as to whether these subunits are present.

However, the presence of mRNA for the α5 subunit of nAChR in human peripheral blood lymphocytes, demonstrated in the present study, is consistent with other reports where the α5 subunit was expressed. This subunit has been identified in nAChR in human thymic stromal cells and thymocytes (Mihovilovic *et al*., 1997; Kuo *et al*., 2002). The presence of mRNA for the α5 nAChR subunit was also detected in human peripheral lymphocytes by others (Sato *et al*., 1999; Kuo *et al*., 2002). However, in the present study the mRNA for the α5 nAChR subunit was detected in human peripheral blood lymphocytes from all samples tested. This is in accordance with the findings of Sato *et al*., (1999) and those Kuo *et al*., (2002), who reported that the α5 mRNA subunit was detected from all samples tested. The widespread distribution of mRNA of α5 nAChR subunit suggests that the translation of the α5 mRNA subunit could be readily triggered in response to external influences and that this subunit could have a modulatory role in nAChR function. In this context the channel properties of neuronal nAChR expressed in oocytes in *in vitro* systems is modified by the inclusion of the α5 mRNA subunit (Ramirez-Lattore *et al*., 1996). Moreover, Orr-Urtreger *et al*., (2005) showed that mice deficient in the structural α5 nAChR subunit were more susceptible to experimentally induced inflammatory bowel disease than their wild type controls. The absence of the α5 nAChR subunit increased susceptibility to disease initiation and the presence of α5 nAChR subunit in the wild type animal appeared to enhance therapeutic sensitivity to

nicotine (Orr-Urtreger *et al*., 2005). It was reported that α4β2α5 nAChRs expressed in oocytes have about 125 times lower EC50 for ACh and twice the conductance of α4β2 nAChR (Ramirez-Latorre *et al*., 1996).

The results of experiments using the RT-PCR technique described in chapter 3 showed that mRNA encoding for β1 as well as β2 nAChR subunits are expressed in human peripheral blood lymphocytes. However, lymphocytes from only seven out of ten subjects expressed the β1 mRNA nAChR subunits. These results are in agreement with the findings of De Rosa *et al*., (2005), who found the β1 mRNA nAChR subunits in some, but not all, samples of rat and human lymphocytes examined. However, the findings of De Rosa *et al*., (2005) do not concur with those of Sato *et al*., (1999), who examined seven healthy donors for the presence of the β1 mRNA subunits in human peripheral mononuclear leukocytes (MNLs) and failed to find any mRNA for this subunit in cells from all seven of his subjects. The β1 subunit is known to only combine with α1,γ, δ, and ε subunits to form the pentameric muscle nAChRs. In the present study, no mRNA for the α1 nAChR subunit was detected from any of the samples from human peripheral blood lymphocytes studied. This finding is similar to data described by Sato *et al*., (1999) and by De Rosa *et al*., (2005) who reported that the α1 nAChR subunit was not observed in human peripheral blood lymphocytes. This observation is similar to that showing the presence of an isolated α6 subunit in the absence of complimentary subunits needed to form functional receptors (Sato *et al*., 1999). Although mRNA for the β1 nAChR subunit was found in human peripheral blood lymphocytes, the reason why this

mRNA was detected in some, but not all, samples remains unknown although it might reflect a diversity of expression of nAChR subunits among individual subjects and species. However, in the absence of the α1 nAChR subunit, any β1 subunits formed are unable to form a functional nAChR.

From the data obtained from RT-PCR experiments in the present study, it is clear that there is inter-subject variability in terms of nAChR mRNA subunit expression on human peripheral blood lymphocytes. This finding could explain some of the problems encountered in the radioligand binding studies described in chapter 2, although the two sets of experiments were done on cells from different donors and the receptor subunit expression profile is not known for the samples used in these experiments.

This intersubject variation in nAChR subunit expression can be seen from Western blot analysis as well as in the results obtained from radioligand binding studies in chapter 2 and RT-PCR in chapter 3. Proteins from human peripheral blood lymphocytes on a Coomassie brilliant blue stained SDS-PAGE gel were seen but the protein bands obtained varied in intensity from subject to subject. In addition, samples from some subjects produced a protein band that was very faint although the equal amounts of protein were loaded onto gel. A high degree of variability in the protein expression pattern was observed between individuals. This intersubject variation in nAChR subunit expression could relate to tobacco smoking history (Lebargy *et al*., 1996; Benhammou *et al*., 2000; Cormier *et al*., 2004) or to other environmental or genetic factors. Such effects have been reported from experiments with leukocytes and are discussed elsewhere in this

thesis. If the same were true of lymphocytes, a white cell subgroup, the presence of such cells may have contributed to some of the variation seen in terms of nAChR subunit expression obtained in the present study. Interestingly, recent work from De Rosa *et al*., (2005) suggested that such variation could be avoided by using human peripheral blood lymphocytes isolated from volunteers not exposed to tobacco smoke. However, in these cells, De Rosa *et al*., (2005) found that the expression pattern of mRNA for the α7 subunit and muscle type nAChR subunits still varied between individuals and within an individual studied at different times. Unfortunately, we were unable to obtain data on the smoking history of the donors whose blood we used.

The inter-subject variability might reflect not only differences in smoking behaviour but also other differences such as the developmental profiles of the cells, or it could be genetically determined or environmental factors such as infection and physiological stress. It has been suggested that the changes in the number of nAChRs on lymphocytes is connected with the etiology of some neurological diseases such as Alzheimer's and Parkinson's diseases, myasthenia gravis, and/or immune process (Adem *et al*., 1986; Maslinski *et al*., 1992; Mihovilovic and Roses, 1993). A decrease in the number of nAChRs on lymphocytes has been observed in Alzheimer's disease (Adem *et al*., 1986). Moreover, the real number of nAChRs on lymphocytes may depend on their function (Strom *et al*., 1972), stage of maturation (Maslinski *et al*., 1987; 1992), and activation (Strom *et al*., 1972; 1974; Grabczewska *et al*., 1989; 1990). Moreover, the alterations of nAChRs on lymphocytes may be related to cell maturation (Mihovilovic and Roses, 1993; Rinner *et al*., 1994; Kuo *et al*., 2002). The changes described during development

and aging are specific to nAChRs and not only found on lymphocytes, although they are also found in the brain (Zoli *et al*., 1995; Gotti *et al*., 1997). Zoli and colleagues (1995) indicated that high affinity nicotine and alpha bungarotoxin binding sites are independently regulated during the development and aging of different brain areas in rat (Zoli *et al*., 1995). It is conceivable that the same results may apply to human brain and human peripheral blood lymphocytes, although this needs to be further investigated using appropriate techniques.

Because of this variability in experimental findings, the presence of nAChRs in lymphocytes remains controversial. However, all or some, of the factors reviewed above could explain some of the variability encountered in the radioligand binding experiments discussed in chapter 2 and the inter subject variation seen in RT-PCR and Western blot analysis in chapter 3 and 4.

If, at the outset, the intersubject variability had been known, we would not have started with radioligand binding. We would have started with RT-PCR work to detect the possible mRNA for the subunits of the nAChRs that might be present on human peripheral blood lymphocytes. The detection of any known mRNA subunits would have been following by radioligand binding assays using the relevant agonists and antagonists to study each of the mRNA subunits. Furthermore, we would have identified a group of subjects with known smoking history and used them throughout experiments so that we could have made the better comparisons and eliminated one area of doubt from our conclusion. However, this would have necessitated using blood from volunteers within

the institutions. We were unable to do this as the necessary medical support was not available to us.

The results obtained from radioligand binding studies, RT-PCR and Western blot analysis in the present study, therefore, provide evidence for the presence of one binding site specific for (-)-nicotine on human peripheral blood lymphocytes. The results from radioligand binding studies indicate that the one receptor on human peripheral blood lymphocytes, described in the present study, is cholinergic in nature and this binding site may comprise nAChRs with a subunit composition of α4β2, α4β2α5, and α7 subtypes as shown by RT-PCR studies and Western blot analysis. However, further experiments are necessary to determine whether one receptor, or combination of those listed in previous sentence, is responsible for the single binding site found in the radioligand binding studies.

Chapter 6 Future work

The results obtained from radioligand binding studies on human peripheral blood lymphocytes did not provide a definitive answer as to nature of the nAChR present because of low specific binding and intersubject variation. Not only was the specific binding obtained from radioligand binding in the present study low but also the specific binding obtained from the studies by others was also low (Adem *et al*., 1986; Maslinski, 1989; Grabczewska *et al*., 1990; Morgan *et al*., 2001). In order to avoid equivocal data, due to inter-subject variation, human peripheral lymphocytes samples should ideally have come from subjects with a known history and background (i.e. smoking history, condition of illness from any disease and ethnicity). Our data may indicate that blood group might be a factor in this variability but it would be preferable to have information relating to a number of possible variables from the donors. These idealised subjects could then have been used throughout the study to make easier and better comparisons of the data obtained using different techniques. Such an approach may not be possible because the radioligand binding studies require a large number of lymphocyte cells to perform one single binding assay. However, it may be possible to avoid inter-subject variation, to a large extent, by collecting the blood lymphocytes and using the information provided, on a sample-by-sample basis.

The experiments should also follow the following sequence; first, RT- PCR experiments would be used to demonstrate the presence of mRNA subunits for nAChR that may be present on human peripheral blood lymphocytes. This would be followed by radioligand binding assays using the relevant agonists and antagonists specific for each of mRNA subunits of nAChR, which were detected from the RT-PCR work. Then the expression of protein in human peripheral blood lymphocytes would be confirmed by Western blot analysis.

Immunofluorescence antibody technique is another method, which could be used to confirm the expression of nAChRs on immune cells by using a specific monoclonal antibody for each nAChR subunit to reveal the presence of the nAChR subunit on all peripheral blood lymphocytes or on a subset of lymphocytes.

There is increasing evidence that nAChRs are widely expressed on immune cells such as monocytes, neutrophils, and lymphocytes. The expression of these nAChRs on the surface of immune cells may be indicative of a link between the nervous and the immune systems. However, little is known about the functional role of this extra-neuronal cholinergic system. Different nAChRs subunits have been detected in human lymphocyte cells but their functional roles are still unclear. Therefore, the functional roles of nAChRs on human lymphocytes should be determined.

The results obtained in the present study provide the possible nAChR subunit composition on human peripheral blood lymphocytes. The patch clamp technique could

be used to investigate the functional role of the nAChRs on individual human peripheral blood lymphocytes. In this technique, a small heat polished glass pipette is used. The pipette is pushed against the cell membrane to form a seal across the tip of the pipette. The seal can be formed tightly enough to permit current flow. The flow of current originates from a small patch of membrane into the pipette and then into current measurement circuitry. When the membrane underneath the patch is disrupted, the change in cell current can be monitored in the whole cell.

The use of this technique would clarify whether there are any inward cation channels on human peripheral lymphocytes that are activated by nicotine. Moreover, this technique could be used to characterise the ion channel further to give an idea of ligand selectivity, which also might help to identify the nAChR subunit composition through the use of subunit selective agonists or antagonists.

It would be of interest to determine if the nAChR expression is restricted to a specific subset of lymphocytes. Different subsets of human blood peripheral lymphocytes might express different subtypes of nicotinic acetylcholine receptors, and also their roles might be changed as cells mature. In this regard, Richman and Arnason (1979) suggested that expression of nAChR in T suppressor cells might result in a proliferation or an activation of these cells and the subsequent suppression of proliferation in the overall lymphocyte population. Shok *et al*., (2007) also suggested that murine mature newly generated B-lymphocytes of the bone marrow expressed α4(α5)β2 nAChR, while the number of α7(α5)β4 receptors increased with B cell maturation in the spleen. The subsets of

lymphocytes in a sample can be separated via one of the two methods, either using fluorescence activated cell sorter (FACS analysis) (Flow cytometry) or magnetic bead separation.

Finally, in order to determine the functions of nicotinic acetylcholine receptors in human peripheral blood lymphocytes, the model of α4, β2 and/or α7 knockout mice could be used to determine the functions of these receptor subtypes. By using cells from mice deficient in a particular nAChR subunit the consequence of this genetic deletion can be determined and thus the function of the nAChR determined. However, there is no guarantee that any function of nAChR in the immune system of mice is the same as that in man.

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Appendix I

1. Reagent preparation for Western blotting

1.1 x2 Sample Buffer

		Final Concentrations
Glycerol 1 ml		20%
Stacking gel buffer	1.25 ml	0.125 M Tris.HCl pH 6.8
10% SDS	2 ml	4%
2- mercaptoethanol	or Dithiothreitol 0.1ml	2%
(Volume 5 ml)		

- Gloves must be worn; the glycerol was measured out using pipette.
- Stacking gel buffer and SDS were added and mixed
- In a fume cupboard, 2-mecaptoethanol or dithiothreitol was added and mixed well, a tiny amount of bromophenol blue powder was then added and mixed to dissolve
- distilled water was added to make up a final volume of 5 ml
- Making an aliquot and then kept at -20 ºC

1.2 10% Sodium Dodecyl Sulphate

SDS 30g

Volume 300 ml

- In fume cupboard SDS was weighed and distilled water added (wearing gloves and mask)
- Heat on magnetic stirrer to dissolve. Kept at room temperature.

1.3 Polymerising reagents

- Pre-weigh ammonium persulphate in 0.0075g in small bottle and store with desiccant in the dark at room temperature (Wearing gloves).
- Store TEMED as for ammonium persulphate

1.4 Resolving gel buffer

Tris 45.5 g (0.75M)

Volume 500 ml

- Dissolve Tris in approximately 300 ml distilled water
- The pH was adjusted to 8.8 (Wearing gloves)
- Distilled water was added to make up a final volume of 500 ml and store at 4º C

1.5 Stacking gel buffer

Tris 15.12 g (0.5M)

Volume 250 ml

- Dissolve Tris in Approximately 150 ml of distilled water
- The pH was adjusted to 6.8
- Distilled water was then added to make a final volume of 250 ml and store at 4ºC.

1.6 Reservoir buffer (RB)

			Final Concentrations	**10xRB**
Tris (MW 121)	1.2 g	7.5 g	(0.025 M)	15g
Glycerine (MW 75)	5.76 g	36g	(0.192 M)	72g
10% SDS	4 ml	25 ml	(0.1%)	(Add before use)
Volume	400 ml	2500 ml		500 ml

- Tris and glycine were dissolved in approximately 300 ml or 2400 ml of distilled water
- 10 %SDS was then added (Wearing gloves)
- Distilled water was then added to make up a final volume

1.7 Western blotting buffer

		Final concentrations
Tris (MW121)	9.0 g	0.025M
Glycine (MW 75)	43.2 g	0.192 M
Methanol	600 ml	20%

Volume 3000 ml

- Dissolve Tris and glycine in approximately 2000 ml of distilled water, and the pH was checked. This should be near 8.3
- 600 ml of methanol was added (in the fume cupboard and wearing gloves)
- Distilled water was added to make a final volume of 3000 ml; the solution was then kept at room temperature.

1.8 Coomassie blue staining solution

		Final concentrations
- Coomassie brilliant blue	1g	0.1%
- Methanol	500 ml	50%
- Glacial acetic acid	100 ml	10%
- Volume	1000 ml	

1.9 Destaining/fixing solution

			Final concentrations
Methanol	900 ml	450 ml	30%
Glacial acetic acid	300 ml	150 ml	10%
Volume	3000 ml	1500 ml	

- In fume cupboard, methanol was measured out into a 5 litres volumetric flask, gloves must be worn.
- In fume cupboard, acetic acid was measured out and added.

- Distilled water was then added to make up a final volume. The solution was then kept at room temperature in dark bottle.

2. Reagent preparation for RT-PCR

2.1. 6 x mini gel stop mix		**Final concentration**
- Sucrose	10 g	20%
- Ficoll 400	5 g	10%
- EDTA	0.19 g	10 mM
- Bromphenol blue	0.125 g	0.25%

(Volume 50 ml), Made up in 1 x TAE buffer

2.2 TAE (Tris acetic acid-EDTA buffer)		**Final concentration**
- Tris (base)	4.84 g	0.04 M
- EDTA	0.372 g	0.001 M
- Volume	1000 ml, the pH was adjusted with glacial acetic acid	

2.3 TE buffer		**Final concentration**
- 1 M Tris-HCl pH 7.4	10 ml	0.1 M
- 0.5 M EDTA pH 8.0	2 ml	0. 001 M

Appendix II

1. Protein Assay

All protein samples determined the concentration of protein using the method based on the Bradford (1976), as described below.

1. Standard protein preparation

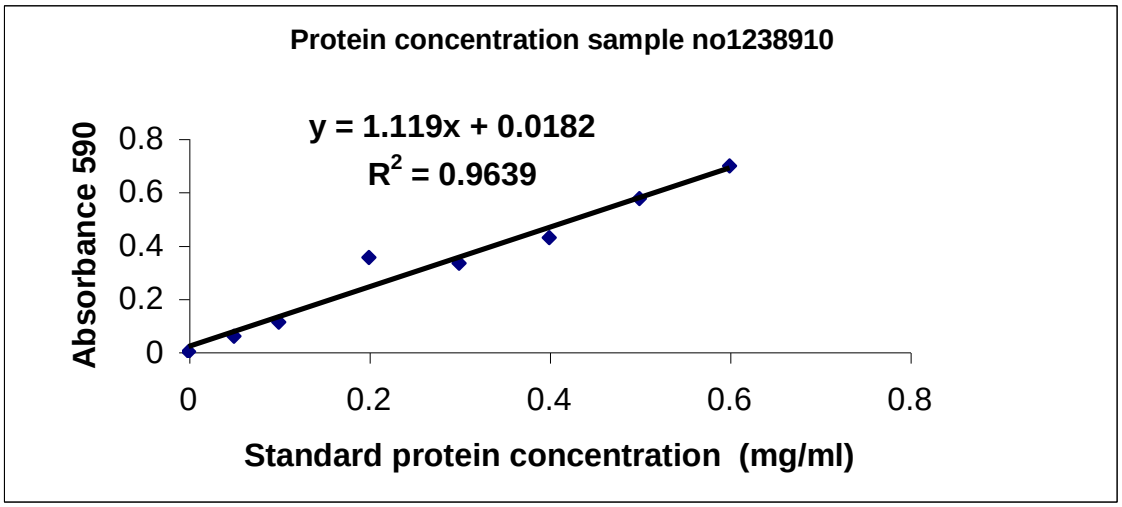
   The stock solution (0.6 mg/ml) was prepared into seven concentrations thus:

Final concentration (mg/ml)	**Stock protein solution (µl)**	**Distilled Water (µl)**
0.05	**10**	**110**
0.1	**20**	**100**
0.2	**40**	**80**
0.3	**60**	**60**
0.4	**80**	**40**
0.5	**100**	**20**
0.6	**120**	**0**

2. 10 µl of standard and sample protein were transferred to a 96 well plate. Distilled water was used as a control. The sample used should be neat, 1:5, 1:10).

3. Make a dilution 1:5 of Bradford reagents (1 part of Bradford + 4 parts of distilled water).
4. The absorbance was read at 590 nm by an ELISA plate reader
5. A protein standard curve was plotted and used for protein concentration measurement.

0.05 mg/ml	0.1 mg/ml	0.2 mg/ml	0.3 mg/ml	0.4 mg/ml	0.5 mg/ml	0.6 mg/ml	Sample1	Sample2	Sample3	Distilled water
1	2	3	4	5	6	7	X	Y	Z	D
1	2	3	4	5	6	7	X	Y	Z	D
1	2	3	4	5	6	7	X	Y	Z	D
1	2	3	4	5	6	7	X	Y	Z	D
1	2	3	4	5	6	7	X	Y	Z	D
1	2	3	4		6	7	X	Y	Z	D
1	2	3	4	5	6	7	X	Y	Z	D
Mean 1	Mean 2	Mean 3	Mean 4	Mean 5	Mean 6	Mean 7	Mean x	Mean y	Mean Z	Mean D



2. Atypical protein standard curve for protein assay using Bradford's method