FUNCTIONAL ASPECTS OF NEURONAL NICOTINIC RECEPTOR DIVERSITY

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PhD Thesis

University College London
ABSTRACT

The agonist sensitivity of nicotinic acetylcholine receptors (nAChRs) in rat superior cervical ganglion (SCG) neurones was compared, under voltage-clamp, with that of cloned nicotinic receptor subunit combinations expressed in *Xenopus* oocytes. Agonist responses in SCG neurones indicated that cytisine was the most potent agonist and lobeline the least potent (Cytisine > DMPP > Nicotine > ACh > Carbachol > Lobeline). The α3β2 combination had a relatively high sensitivity to DMPP and low sensitivity to cytisine (DMPP > ACh > Lobeline > Carbachol > Nicotine > Cytisine). The α3β4 combination had a high sensitivity to cytisine and low sensitivity to DMPP (Cytisine > Nicotine ≈ ACh > DMPP > Carbachol > Lobeline). The results suggest that the nAChRs in the SCG have characteristics of both α3β4 and α3β2 subunit combinations.

Complete agonist concentration-response plots were determined for α3β2, α3β4, α4-1β2, α4-1β4 and α7 nAChR subunit combinations. In general, the relationships were poorly fitted by a single component Hill-Langmuir relationship. Improved fits were obtained with the sum of two components, suggesting that pairwise expression of neuronal nicotinic subunits in oocytes may produce more than one functionally distinct receptor.

This study also examined the effects of acute ethanol (EtOH) exposure on agonist responses of neuronal nAChRs expressed in oocytes. In some cells, α3β4 responses could be either potentiated or inhibited (25% to 237% of control response) by low EtOH concentrations (1-30mM). At high EtOH concentrations (100-300mM) robust potentiations were observed, (135% to 305%). The α3β2, α4-1β2 and α4-1β4 combinations were less sensitive to low EtOH concentrations, but respectively showed potentiations of up to 178%, 226% and 154% at high concentrations. α7 receptors were also relatively insensitive to low EtOH concentrations, but potentiation or inhibition could be seen at high concentrations (88% to 141%). This modulation may underlie some of the behavioural effects of ethanol. The α3β4 subunit combination may be especially sensitive to modulation by low EtOH concentrations. This remarkable sensitivity and plasticity may contribute to a process of mutual reinforcement in nicotine and alcohol addiction.
ACKNOWLEDGEMENTS

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Special thanks go to Donald Jenkinson for his assistance in transferring this work to Strathclyde and David Colquhoun for not giving up on me. Last, but not least, I would like to thank Alasdair Gibb and John Connolly for teaching me everything I know about neuroscience.
FULL PAPERS:


ABSTRACTS:


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<tbody>
<tr>
<td>Å</td>
<td>Ångströms</td>
</tr>
<tr>
<td>α-Bgt</td>
<td>alpha-bungarotoxin</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ANS</td>
<td>autonomic nervous system</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid</td>
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<td>°C</td>
<td>degrees Centigrade</td>
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<td>cyclic adenosine monophosphate</td>
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<td>centimetres</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>EtOH</td>
<td>ethanol</td>
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<td>FM</td>
<td>frequency modulation</td>
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<td>g&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>times the force of gravity</td>
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<td>GABA</td>
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<td>HEPEs</td>
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<td>IPN</td>
<td>interpeduncular nucleus</td>
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<td>K&lt;sub&gt;app&lt;/sub&gt;</td>
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<td>κ-βgt</td>
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<td>K_d</td>
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<td>LED</td>
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<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<tr>
<td>NBT</td>
<td>neuronal bungarotoxin / kappa-bungarotoxin</td>
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<td>n_H1</td>
<td>Hill slope / Hill coefficient</td>
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<td>picoamps</td>
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<td>phosphatydic acid</td>
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<tr>
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<td>protein kinase C</td>
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<td>peripheral nervous system</td>
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<td>ribonucleic acid</td>
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<td>rNTP</td>
<td>ribonucleotide triphosphate</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SCG</td>
<td>superior cervical ganglion</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SDS</td>
<td>sodium dodecylsulphate (a.k.a. lauryl sulphate)</td>
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<tr>
<td>S.E.</td>
<td>standard error</td>
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<td>SPL</td>
<td>lateral spiriform nucleus</td>
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<tr>
<td>TE</td>
<td>tris EDTA (see above &amp; below)</td>
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<tr>
<td>TMA</td>
<td>tetramethylammonium</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
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<td>U</td>
<td>units of enzyme activity</td>
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<td>ultraviolet</td>
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<tr>
<td>$V_H$</td>
<td>holding potential</td>
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<td>VIP</td>
<td>vasointestinal peptide</td>
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<tr>
<td>V</td>
<td>versus</td>
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<td>$Y_{max}$</td>
<td>maximum response</td>
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CHAPTER 1:
INTRODUCTION

1.1. GENERAL INTRODUCTION

The muscle nicotinic acetylcholine receptor is the most extensively studied and best understood of all ligand-gated ion channels discovered. In spite of this wealth of information, comparatively little is still known about the function of its neuronal counterparts, especially those of the central nervous system. One of the main gaps in our knowledge has been the elucidation of an actual physiological role for these CNS receptors. However, it is becoming increasingly clear that they have a role in the presynaptic modulation of release of a number of CNS neurotransmitters (for review see Wonnacott, 1997). The possible involvement of these receptors in the aetiology of various disease states is also only just coming to light. Similarly, there is at present considerable interest in neuronal nicotinic receptors as potential therapeutic targets in the treatment of a number of neurological disorders (see below; for review see Benowitz, 1996). Ultimately, however, the most enduring interest in these receptors is probably in the elucidation of the mechanisms underlying the induction and maintenance of nicotine addiction and smoking behaviour (for review see Dani & Heinemann, 1996) and the prevention of its associated health problems.

1.2. THE NICOTINIC RECEPTOR GENE FAMILY

Access to a rich source of receptor protein from the electric organs of the *Torpedo californicus* and *Electrophorus electricus*, and the availability of a high affinity ligand (α-bungarotoxin from the venom of *Bungarus multicinctus*), allowed the muscle-type nicotinic AChR to be the first ligand-gated ion channel protein to be purified and sequenced, and subsequently have its encoding genes cloned (Raftery et
It was found to be a 290-kD transmembrane glycoprotein consisting of four subunits sharing ~60% homology arranged in the stoichiometry 2α, β, γ, δ.

In at least some species, muscle AChRs exist in two developmentally regulated forms (for review see Schuetze & Role, 1987). Upon innervation, the γ subunit of the embryonic or foetal muscle nAChR is replaced by the homologous ε subunit in adult muscle (e.g. Mishina et al., 1986). The receptor switches from one composed of α(×2) βγδ subunits to one composed of α(×2)βε subunits (with corresponding changes in functional properties), although in one case (in adult extrinsic ocular muscle) both types appear to be present (Horton et al., 1993). More recently two distinct muscle α1 subunits have been cloned from Xenopus embryos: Both α1a and α1b, which exhibit 89% homology, encode a protein with a predicted signal peptide and a mature protein of 437 amino acids (Hartman & Claudio, 1990). Two α isoforms have also been found in humans that differ in that one of them contains an extra 22 amino acids encoded by a separate exon (Beeson et al., 1990). Additionally, two alternative splice variants of the rat muscle β subunit have been found (Goldman & Tanai, 1989). The function of these different muscle subunit isoforms is unknown.

While the primary structures of the electric organ and muscle nicotinic receptors were likely to be distinct from their neuronal counterparts it was predicted that they would be coded for by homologous genes (Patrick & Stallcup, 1977; Swanson et al., 1983). The cDNA clones for muscle nAChR subunits were, therefore, initially used as probes for reduced stringency screening of cDNA libraries obtained from a number of neuronal sources. This led to the identification from avian genomic and rat PC12 cell cDNA libraries of cDNAs encoding the chick α2 subunit and the rat α3 subunit (Nef et al., 1986; Boulter et al., 1986). Consequently, these two clones provided essential cDNA probes for the discovery of a multigene family encoding neuronal nicotinic receptors expressed in brain and peripheral ganglia. To date, a total of 8 neuronal α subunits and 3 neuronal β subunits have been identified (see McGehee & Role, 1995 for review). The amino acid sequences of different rodent nAChR α and
β subunits are 40 to 70% identical (Boulter et al., 1990). The α8 subtype has so far only been found in the chick, and the α9 subtype only in the rat (Schoepfer et al., 1990; Elgoyhen et al., 1994). The rat α4 subunit can exist in two splice variants: α4-1 and α4-2 (Goldman et al., 1987). α4-1 is the full length variant, whereas α4-2 differs firstly in that it starts at nucleotide 389 of clone α4-1. The second difference is in their 3' ends starting at nucleotide 1871 of α4-1. However, when expressed in functional combinations (see below) the two splice variants appear to be functionally indistinguishable (Connolly et al., 1992). The designation α refers to those subunits with a conserved pair of cysteines in the presumed 1st extracellular domain (see below), those without it being termed β subunits. To date, no neuronal nAChR subunits corresponding to the muscle γ, ε, and δ subunits have been identified. By convention, muscle AChRs are designated by the postscript 1 (e.g. α1, β1), and neuronal subunits by postscript numbers indicating the order in which the particular subunit was identified (e.g. chick α2, first; rat α9, most recent).

1.3. What Subunits Are Necessary For Obtaining Functional Neuronal Nicotinic Receptors?

Early studies in lipid bilayers revealed that the Torpedo α1, β1, γ & δ subunits together were both necessary and sufficient to form receptors functionally indistinguishable from those found in native tissues (e.g. Labarca et al., 1984). Similarly, there is close functional correlation between bovine α1, β1, γ & δ subunits coexpressed in Xenopus oocytes with those recorded from calf foetal muscle, and also between α1, β1, γ & ε subunit coexpression and adult calf muscle (Mishina et al., 1987). However, altering the ratios of injected mRNAs changes the functional characteristics of the receptors, and functional receptors can be formed with some (but not all) simpler combinations of subunits, as long as they contain the α1 subunit, including the pairs αγ and αδ (e.g. Liu & Brehm, 1993). An important point arises from this work. Since functional receptors can be formed with less than the full
complement of muscle subunits (e.g. $\alpha 1\beta 1\gamma$), it follows that just because a combination is functional in oocytes, it does not necessarily occur in nature.

In general, functional neuronal nicotinic receptors can be formed by the co-expression in oocytes of simple neuronal nicotinic $\alpha/\beta$ subunit pairs, as was first demonstrated for the rat $\alpha 2\beta 2$, $\alpha 3\beta 2$ and $\alpha 4\beta 2$ combinations (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988a), and subsequently with the $\beta 4$ subunit (Duvoisin et al., 1989). However, not all the subunits can form functional receptors in this manner (e.g. $\alpha 5$ and $\beta 3$ in all pairwise combinations tested to date, Deneris et al., 1989). Also, some subunits ($\alpha 7$, $\alpha 8$ & $\alpha 9$) can form functional receptors homomerically (i.e. without the addition of a $\beta$ subunit) (Séguëla et al., 1993; Gerzanich et al., 1994; Elgoyhen et al., 1994). Additionally, it has only been possible to demonstrate functional receptors with the $\alpha 6$ subunit when it has been co-expressed with a $\beta$ subunit from a different species (chick $\alpha 6$/human $\beta 4$, Gerzanich et al., 1997). These are the minimum subunit requirements for the formation of functional neuronal nicotinic receptors in *Xenopus* oocytes. This does not, however, exclude the possibility of the formation of functionally distinct receptors with more complex subunit combinations (see below for examples).

### 1.4. Nicotinic Receptor Structure

Most structural studies of ACh receptors have used receptors derived from the electric organs of the electric ray *Torpedo*. Unwin (1993) determined the three dimensional structure of the ACh receptor to a 9Å resolution by electron microscopy of two dimensionally ordered arrays of receptor in *Torpedo* electric tissue membrane fragments embedded in ice. After incubation at room temperature, a small fraction of the receptors form two-dimensional crystalline arrays that roll up into tubes. The resulting averaged images revealed a structure similar to previously proposed structures (e.g. Stroud et al., 1990)- a narrow-waisted cylinder of five subunits surrounding a central ion pore.
Hydrophobicity analysis of a 'typical' nAChR subunit (Stroud et al., 1990, review) reveals a long amino-terminal sequence region of ~200 amino acids rich in hydrophilic residues, which could form an extracellular domain. This is followed by four hydrophobic segments ~20 amino acids long (referred to as M1-M4) which could form membrane spanning regions (Guy, 1983; Finer-Moore & Stroud, 1984). The M2 segment is believed to be exposed to the central ion pore of the receptor, as mutations in this region alter the conductance properties of nAChRs expressed in *Xenopus* oocytes (e.g. Imoto et al., 1988; Leonard et al., 1988). Also, channel blockers label residues within this region (Hucho, 1986; Hucho et al., 1986; Giraudat et al., 1986, 1989). Labelling experiments with non-competitive blockers suggest that the M1 segment may also contribute to the channel pore (DiPaola et al., 1990; Akabas & Karlin, 1995). Between M3 and M4 there is a long sequence segment, of greatest sequence diversity between the different nAChR subunits, containing a region that suggests an amphipathic helix (MA) in the carboxy-terminal end (Finer-Moore & Stroud, 1984). The sequences of the four strongly hydophobic segments (M1-M4), and that of the MA region, are strongly conserved amongst different subunits and across species. Covalent labelling of all four segments with photoactive phospholipids and hydrophobic probes supports the contention that the M1-M4 regions are membrane spanning (Giraudat et al., 1985; Blanton & Wang, 1991; Blanton & Cohen, 1992).

Each subunit contains, in its N-terminal extracellular half, 2 disulfide-linked cysteine residues separated by 13 other residues, thus forming a 15 residue closed loop (Kao & Karlin, 1986). All α subunits also contain 2 cysteine residues at positions corresponding to α192 and α193 of the *Torpedo* receptor. All neuronal subunits containing this pair are designated α subunits, those without it being termed β-subunits. These 2 cysteine residues along with Tyr93 (and others) are thought to be involved in forming the agonist binding site of the receptor for ACh (Kao et al., 1984; Middleton & Cohen, 1991), and lie within 10 Å of a carboxylate group that is involved in binding the quarternary ammonium group in acetylcholine. Other residues may also
be involved in the binding site for other agonists such as nicotine (e.g., Tyr198, Cohen et al., 1991).

The predicted structure of neuronal nicotinic receptors is by analogy with the muscle and Torpedo receptors. Estimates of the molecular weight of neuronal nicotinic receptor macromolecules suggest that these receptors contain at least four subunits, but probably not more than five (Whiting & Lindstrom, 1986; Whiting et al., 1987). Conductance measurements of a mutated chick nicotinic α4β2 receptor expressed in Xenopus oocytes suggest that it contains only two α subunits (Cooper et al., 1991), a situation analogous to the muscle nAChR.

The chicken α4β2 neuronal nicotinic acetylcholine receptor expressed in Xenopus oocytes also appears to be a pentamer of two α4 acetylcholine-binding subunits and three β2 structural subunits (Anand et al., 1991). When the subunits are labelled with [35S]-methionine, 1.46 times more label is found in the α subunits than in the β subunits. Additional evidence for the presence of two agonist binding subunits (i.e. α subunits) comes from measurements of the Hill coefficients of agonist concentration response relationships for the various subunit combinations. These are routinely estimated at values between one and two (e.g. this study, Ch. 3) suggesting the involvement of a bi-molecular reaction of agonist with receptor in receptor activation.

1.5. ANATOMICAL DISTRIBUTION OF NEURONAL NICOTINIC RECEPTORS

a) Binding studies

Characterization of putative nicotinic receptors in rat brain using receptor binding techniques indicates that at least two major classes of binding sites exist. One is labelled by high-affinity binding of agonists such as [3H]L-nicotine, [3H]-ACh, [3H]-cytisine and [3H]-methylcarbachol (e.g. Abood & Grassi, 1986; Clarke et al., 1985;
Pabreza et al., 1991), and a second labelled with \( \alpha^{125}\text{I}}\)-bungarotoxin (Clarke et al., 1985; Marks et al., 1986)

Binding of \( \alpha\)-Bgt can be seen to be high in the cerebral cortex (especially layers I and IV), hypothalamus, hippocampus, inferior colliculus, and in a few brainstem nuclei (Clarke et al., 1985; Hunt & Schmidt, 1978a). The most heavily labelled cells in the hippocampus are the GABAergic interneurones in both the dentate gyrus and Ammon's horn (Freedman et al., 1993). L-\( ^3\text{H}\)-nicotine or \( ^3\text{H}\)-ACh binding is high in the interpeduncular nucleus, in several thalamic nuclei (including the medial habenula), and in the superior colliculus. Also, moderate binding is seen in the presubiculum, layers III and IV of the cerebral cortex, parts of the striatum, and the dorsal tegmental nuclei. Weaker binding is seen in the hypothalamus and the hippocampus.

b) In situ hybridization studies

The anatomical distribution of rat brain regions expressing the \( \alpha4 \) and \( \beta2 \) nAChR genes correlates fairly well with the distribution of high-affinity nicotine and ACh binding sites (Clarke et al., 1985; Wada et al., 1989). A reasonably good correspondence has also been noted between the distribution of the \( \alpha\)-Bgt-AChR gene \( \alpha7 \) and the distribution of high affinity binding sites for \( ^{125}\text{I}}\)-\( \alpha\)-Bgt (Clarke et al., 1985; Séguela et al., 1993).

In situ hybridization studies have mapped the distribution of transcripts of all known members of the neuronal nAChR gene family in the rat CNS. Each \( \alpha \) subunit's mRNA exhibits a unique pattern. \( \alpha2 \), \( \alpha5 \) and \( \alpha6 \) transcripts exhibit restricted but distinct patterns of distribution, whereas \( \alpha3 \), \( \alpha4 \), and \( \alpha7 \) mRNAs are more widely distributed throughout the brain (Wada et al., 1989; Séguela et al., 1993). High levels of \( \alpha7 \) transcripts have been localized by in situ hybridization in the olfactory areas, the hippocampus, the hypothalamus, the amygdala, and the cerebral cortex of the rat (Séguela et al., 1993).
Early studies showed that in many regions of the CNS, β2 was the only β subunit to be detected, and it appeared to be expressed in all areas where α mRNAs are expressed. The β4 subunit was believed to be restricted to the ventral two thirds of the medial habenula which gives a uniquely robust signal. However, later studies revealed that this subunit has a far more widespread distribution, including high levels in the presubiculum, parasubiculum, subiculum, dentate gyrus, cortical layer IV, IPN and the trigeminal motor nerve nucleus (Dineley-Miller & Patrick, 1992). The distribution of mRNAs for α2, α3, α4 and β2 genes in chicken brains is generally similar to that in rat for corresponding genes (Morris et al., 1990).

It should be noted that the levels of the mRNAs for some of these subunits appear to be dependent on the stage of development. Zoli et al. (1995) looked at α3, α4, β2 and β4 subunits in the rat PNS and CNS. In the majority of cases studied (caudal brain, spinal cord, dorsal root ganglia, trigeminal and geniculate ganglia) all four subunit mRNAs were initially (E11-13) detected but, during subsequent prenatal development, the level of some of these subunit mRNAs (α3 and β4 in the brain and spinal cord, α4 and β4 in the dorsal root ganglia, α4 in the visceral sensory ganglia, and α3, α4, and β4 in the somatic sensory ganglia) became undetectable. In the case of the cerebral cortex α3 and β2 mRNAs were initially (E12-13) expressed followed by a repression of the α3 subunit (E15) and the subsequent (E17-19) induction of the α4 subunit. Only some subunit mRNAs were initially (E13-15) expressed in the retina (α3-α4-β2β4), parasympathetic or sympathetic motor ganglia (α3-β2β4), and vestibulo-cochlear ganglia (α4-β2) and their level remained stable throughout prenatal and early postnatal development. Overall, in most central and peripheral structures the appearance of nAChR subunit mRNAs was precocious and temporally related to the timing of neuronal differentiation. In addition, in several structures the expression of the α3, α4 or β4 subunits appeared to be transient. This study also suggested that only a limited number of subunit combinations might be functional during development of the CNS and PNS.
For the most recently discovered subunit $\alpha_9$, in situ hybridization studies reveal a restricted pattern of gene expression that includes the outer hair cells of the rat cochlea, the pars tuberalis of the hypophysis, the nasal epithelium, and the skeletal muscle of the tongue, with no transcripts detected in the CNS (Elgoyhen et al., 1994).

Specific $\alpha_6$ mRNA labelling is restricted to a few nuclei throughout the brain; and is particularly high in several catecholaminergic nuclei: the locus coeruleus (A6), the ventral tegmental area (A10) and the substantia nigra (A9)] at levels significantly higher than those found for any other known nicotinic receptor subunit mRNA (La Novère et al., 1996). Labelling for $\alpha_6$ mRNA can also be detected at lower levels in the reticular thalamic nucleus, the supramammillary nucleus and the mesencephalic V nucleus, as well as some cells of the medial habenula (medioventral part) and of the interpeduncular nucleus (central and lateral parts). Some other areas (such as the substantia nigra, the ventral tegmental area and the locus coeruleus) previously thought to contain mainly $\alpha_3$ mRNA have been found to also contain high levels of $\alpha_6$ mRNA. There is also extensive colocalization of $\alpha_6$ and $\beta_3$ mRNA (La Novère et al., 1996). However, the $\alpha_6$ subunit does not appear to form functional receptors in combination with other subunits (although see above for the peculiar circumstances where it does). This raises the possibility that there may yet be additional neuronal non-alpha subunits to be cloned. They may have been missed in previous studies if they have a low sequence identity compared to the currently known subunits.

c) Rat Superior Cervical Ganglia

One of the great unanswered questions in this field of study is the exact subunit composition of native receptors. Of particular interest to the present work is the nature of the nAChR subunits present in the rat SCG. Several studies give information on which nicotinic subunit mRNAs are expressed in rat peripheral ganglia, although they do not all agree completely with one another. Rust et al. (1994) revealed the presence of mRNAs for $\alpha_3$, $\alpha_4$, $\beta_2$ and $\beta_4$ subunits in all peripheral ganglia tested (except the
adrenal medulla—see below). However, the distribution of α7 was tissue-dependent: α7 mRNAs were detected in the neurons of the superior cervical ganglion, adrenal medulla and ciliary ganglion, but were found only in a small fraction (1-3%) of the neurons of the sphenopalatine and otic ganglia. In the adrenal medulla only α3, α7 and β4 mRNAs were detected. α2, α5, α6 and β3 mRNAs were looked for in all preparations but not detected. In agreement with this study, Klimachewski et al. (1994), looked for just α4-1 and β2 mRNAs and found both of them to be present in rat SCG neurones. In contrast to these two studies, Mandelzys et al., (1994) looked for the same mRNAs (α2-α7 & β2-β4) in the rat SCG, but detected only α3, α5, α7, β2 and β4 subunits. This is supported by the study of Zoli et al. (1995) who looked for α3, α4, β2 and β4 mRNAs in the rat SCG, but found only α3, β2 and β4. Thus, for the rat SCG there are several studies supporting the presence of α3, α7, β2 and β4 subunits, but conflicting evidence concerning the presence of α4-1, and α5. Studies in rat PC12 cells (a sympathetic neuronal cell line derived from adrenal medulla tumor cells) have found α3, α5, β2 and β4 subunit mRNAs present, but not α2, α4 or β3 mRNAs (Boulter et al., 1990).

mRNA levels for α3 and α7 subunits in the rat SCG increase by more than threefold over the first 2 postnatal weeks, a period when most synapses are forming on the neurons (Mandelzys et al., 1995). However, there is no significant change in mRNA levels for α5, β2, or β4.

The presence of nAChRs subunit mRNAs in avian peripheral ganglia does not exactly correspond to those found in rat ganglia (see McGehee & Role, 1995 for review).

d) Antibody binding

When examining questions of gene expression, it is not only important to consider the amount of RNA transcribed, but also the amount of protein product. This is because a fast rate of protein degradation, or a poor efficiency of translation, can
mean that a strong expression of mRNA does not result in a high concentration of protein. Also, protein can be transported away from its site of production.

Several anti-nAChR antibodies, some of which are specific for the β2 subunit, bind to rat brain with a pattern similar to that seen for high affinity 3H-nicotine and 3H-ACh (Deutch et al., 1987; Swanson et al., 1987). More detailed subunit-specific studies for several subunits over the entire rat brain are not yet available. β4 immunoreactivity has been detected in most regions of the brain and is strongest in the striatum and cerebellum (Forsayeth & Kobrin, 1997), unlike in situ hybridization studies which find the highest signal in the medial habenula (e.g. Dineley-Miller & Patrick, 1994). The β3 subunit has been detected primarily in the striatum and cerebellum, and faintly in hippocampus (Forsayeth & Kobrin, 1997). Several studies have looked at α4 immunoreactivity in selected brain regions. α4-like immunoreactivity has been found through layers II to VI of the rat cerebral cortex (Nakayama et al., 1995), where it is most prominent in cell bodies and apical dendrites of pyramidal cells in layer V. α4-like immunoreactivity has also been found in many neuronal cell bodies and their fibers in the paraventricular nucleus (PVN) and in many axons and axon terminals in the median eminence (Okuda et al., 1993).

Immunohistochemical localization for the α7 subunit is well correlated with previous in situ localization of α7 transcripts and α-bungarotoxin autoradiographic binding studies, being expressed in most regions of the rat brain (Dominguez del Toro et al., 1994). Particularly strong immunoreactivity is observed in several sensory and motor nuclei of the brainstem as well as the red nucleus.

e) Cellular distribution

In rat SCG neurones α7-specific α-bungarotoxin binds with high affinity (K\textsubscript{i} ~ 6 \cdot 10^{-11} M) (Fumagalli et al., 1976), but there is conflicting information as to whether this binding is exclusively extrasynaptic or not (Smolen, 1983b; Fumagalli & DeRenzis, 1984). The binding of neuronal bungarotoxin (a toxin which binds with
strong affinity to the $\alpha3\beta2$ combination, e.g. Boulter et al., 1987; Wada et al., 1988; Luetje et al., 1990) is, however, concentrated at synaptic membranes on the proximal dendrites (Loring et al., 1988). Both mAb 35-AChR and $\alpha$-Bgt-AChRs are found in clusters that are widely distributed over the surface of embryonic chicken ciliary ganglion neurons. Some mAb 35-AChRs are located at synaptic sites, but the bulk of them are located extrasynaptically in well-defined patches measuring 1-4 $\mu$m in diameter. $\alpha$-Bgt-AChRs are found almost exclusively in these extrasynaptic sites, which thus contain both AChR types (Horsch & Sargent, 1995). Double-labelling immunofluorescence experiments with mAbs to AChRs and to synaptic vesicle antigens in chick SPL showed that most clusters of $\alpha5$-L1 and $\beta2$-L1 are extrasynaptic (Ullian & Sargent, 1995). These extrasynaptic receptors may therefore have a secondary role, and not be directly involved in synaptic transmission.

$\alpha4$ immunoreactivity has been detected in the postsynaptic membranes of neuronal cell bodies and apical dendrites of rat cortical neurones (Nakayama et al., 1995). Whereas postsynaptic nicotinic responses can be measured in a number of CNS preparations (e.g. Connolly et al., 1995), there is little evidence of a direct role in CNS synaptic transmission. However, there is now a wealth of neurochemical and electrophysiological information indicating that neuronal nAChRs are also located presynaptically (see below).

1.6. SUBUNIT COMPOSITION OF NATIVE NEURONAL NICOTINIC RECEPTORS

Immunoprecipitation studies reveal that chick ciliary ganglia express protein for at least three subunits: $\alpha3$, $\beta4$ and $\alpha5$ (Vernallis et al., 1993). Further studies in this cell type have revealed the presence of at least three distinct receptor subtypes (Conroy & Berg, 1995). Firstly, all of the synaptic-type acetylcholine receptors contain both $\alpha3$ and $\beta4$ subunits, and most, if not all, of the $\alpha5$ gene product present in fully assembled receptors is associated with these subunits. About 20% of the total receptors also contain the fourth gene product, $\beta2$. No $\beta2$ subunits are found coassembled with the
fifth acetylcholine receptor gene product expressed by the neurons, α7, which has been shown previously to comprise a class of abundant, nonsynaptic receptors on the cells. Studies in chick brain suggest various combinations of α3, α4, α5 and β2, possibly including α3α5β2, α4α5β2, or other triplet combinations (Whiting et al., 1991; Conroy et al., 1992).

Virtually all of the high affinity nicotinic receptors measured to date in the rat CNS appear to be composed of α4 and β2 subunits only. For example, only antisera generated against the α4 and β2 subunits are able to immunoprecipitate specifically receptors labelled by (3H)cytisine in rat brain (Flores et al., 1992), and a similar situation appears to be present in chick brain (Whiting et al., 1991). However, the demonstration of other subunit transcripts in a variety of central and peripheral nervous tissue suggests a greater degree of receptor heterogeneity. Recent studies using immunoprecipitation with subunit-specific antisera in rat trigeminal ganglion neurones have provided strong evidence that these cells at least possess the principal subtype α3β4, as well as the α4β2 subtype (Flores et al., 1996).

Other immunoprecipitation studies have established that β2, β3 and β4 subunits are co-assembled in the rat cerebellum, probably along with the α4 subunit (Forsayeth & Kobrin, 1997). It has been suggested in this study that the β3 subunit functions as a structural link between a relatively unstable α4β2 dimer and a relatively stable α4β4 dimer.

Monoclonal antibodies against the two α-Bgt AChR subunits have been used to demonstrate that at least two subtypes are present in embryonic day 18 chicken brain. The predominant brain subtype contains α7 subunits, while a minor subtype contains both α7 and α8 subunits, although both subtypes may also contain other subunits (Keyser et al., 1993; Gotti et al., 1994a). α8 homomers and α7α8 heteromers are also a major component in chick retina (Anand et al., 1993; Keyser et al., 1993; Gotti et al., 1994b). The α8 subunit has not, however, been found yet in rat brain.

The different possible subunit combinations that might occur in native neuronal nicotinic AChRs are summarized in Table 1.1.
<table>
<thead>
<tr>
<th>Region</th>
<th>Possible subunit combinations</th>
<th>Basis for prediction (References)</th>
<th>Selective agonists (e.g. other subtypes)</th>
<th>Relative Agonist Potency in oocytes (rat clones)</th>
<th>Specific antagonists</th>
<th>Oocyte Pharmacology References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>α₁β₂δ (foetal)</td>
<td>e.g. Witzmann et al. 1990</td>
<td>na</td>
<td>AN&gt;B&gt;A&gt;D&gt;C&gt;N&gt;L</td>
<td>α-Bgt</td>
<td>Compston et al. 1992</td>
</tr>
<tr>
<td></td>
<td>α₁β₂δ (adult)</td>
<td>e.g. Witzmann et al. 1990</td>
<td>na</td>
<td>na</td>
<td>α-Bgt</td>
<td>Cooper et al. 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Witzmann et al. 1990</td>
</tr>
<tr>
<td>Ganglion</td>
<td>α₃β₄</td>
<td>Flores et al. 1996</td>
<td>cytisine +</td>
<td>E&gt;C&gt;N&gt;A&gt;D&gt;C&gt;A&gt;L</td>
<td>NBT (co-applied)</td>
<td>Cowen et al. 1994</td>
</tr>
<tr>
<td></td>
<td>α₇</td>
<td>Enna et al. 1993; Zhang et al. 1994*</td>
<td>DMAC, anatoxin-A**</td>
<td>E&gt;DM&gt;N=D&gt;C&gt;A&gt;AB</td>
<td>α-Bgt, MLA?</td>
<td>Zhang et al. 1994*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DeFebré et al. 1995</td>
</tr>
<tr>
<td>Brain</td>
<td>α₄β₂</td>
<td>Flores et al. 1992</td>
<td>A-85380?</td>
<td>E&gt;N/A&gt;AN&gt;AB</td>
<td>NBT (pre-incubated)</td>
<td>Papke et al. 1993, 1996</td>
</tr>
<tr>
<td></td>
<td>α₈* (amacrine only)</td>
<td>Keyser et al. 1993; Gott et al. 1994a, 1994b</td>
<td>TMA?</td>
<td>C=N&gt;A&gt;D&gt;T*</td>
<td>α-Bgt, MLA?</td>
<td>DeFebré et al. 1995</td>
</tr>
<tr>
<td></td>
<td>α₃β₄</td>
<td>Flores et al. 1996; Clarke &amp; Reuben 1995; Fotavhat &amp; Kornh 1997</td>
<td>cytisine +</td>
<td>E&gt;C&gt;N&gt;A=AB=D&gt;CA&gt;L</td>
<td>NBT (co-applied)</td>
<td>Cowen et al. 1994</td>
</tr>
<tr>
<td>Cochlea</td>
<td>α₉</td>
<td>Elgavish et al. 1994</td>
<td>nicotine-insensitive</td>
<td>A&gt;D=O (w.p.a.)</td>
<td>α-Bgt</td>
<td>Elgavish et al. 1994</td>
</tr>
</tbody>
</table>

Table 1.1. Possible subunit composition of native neuronal nicotinic receptor subtypes compared to the well-established muscle-type receptor.

Predictions are based on pharmacological and electrophysiological properties of cloned receptors and immunoprecipitation studies, as well as in situ hybridization (see refs). All information relates to rat receptors, unless otherwise indicated. Relative potencies are estimated from the low concentration data from several sources (see refs).

well established for muscle receptor, w.p.a = weak partial agonist.* Chick only; predicted in chick; ** Assumption based on studies in hippocampal synaptosomes
+ Cytisine will be selective for β₄-containing α-Bgt-insensitive receptors

See pages 14-16 for abbreviations. See below for non-standard abbreviations.

A = ACh; AB = ABT-418; AN = Anatoxin-a; C = Cytisine; CA = Carbachol; DM = DMAC; D = DMPP; E = Epiptadine; L = L-lobeline; N = Nicotine; O = Oxotremorine-M; T = TMA
Nicotinic pharmacological profiles have been obtained in a number of different peripheral and central neurone preparations, but the studies obtained using specific receptor subunit combinations in oocytes do not appear to have thrown much light on the receptor subtypes present in these neurones. For example, rat sympathetic ganglia have an agonist profile similar (high potency for cytisine and nicotine), but not identical to (high potency for DMPP), that obtained with the α3β4 receptor in oocytes in parallel studies (Covemton et al., 1994- see Chapter 3). This lack of correspondence is not very surprising considering the variety of subunit mRNAs expressed in the rat SCG and the large amount of heterogeneity observed at the single channel level (e.g. in the SCG, Mathie et al., 1991: medial habenula, Connolly et al., 1995). Nicotinic agonist profiles have been obtained from neurones in the rat habenular and interpeduncular nucleus (Mulle et al., 1991). However, one major problem encountered when making comparisons between receptors expressed in oocytes and native receptors is the wide variety of experimental conditions used in different studies (e.g. divalent cation levels, concentration ranges, rates of drug application, and the age of preparation or length of cell culture, to name but a few: for review of heterogeneity of single channel studies see Papke, 1993).

In some preparations a number of pharmacologically distinct nicotinic responses have been obtained. In dissociated foetal rat hippocampal neurones ~83% of cells display a rapidly desensitising α-Bgt-sensitive current (termed type IA), ~5% of cells display a slowly desensitising dihydro-β-erythroidine sensitive current (type II), and ~2.5% of cells display a slowly desensitising methyllycaconitine sensitive current (type III). Type III responses tend to be more sensitive to classical neuronal nicotinic antagonists (e.g. mecamylamine) than type II responses. Additionally ~10% of currents display a hybrid current response (Type IB: Albuquerque et al., 1995). These authors suggest an α7-containing receptor is responsible for Type IA responses, an α4β2
receptor is responsible for Type II responses, and an $\alpha_3\beta_4$ receptor is responsible for Type III responses.

In most neuronal preparations such cell specific distinction does not occur and obviously a rapid perfusion system is required if one is to dissect hybrid currents into their components where rapidly desensitising components such as the $\alpha_3\beta_2$ and $\alpha_7$ type receptors may be involved. Recently such a component has been resolved in dissociated chick ciliary ganglia which can be blocked by $\alpha$-Bgt (Zhang et al., 1994), and also in a small percentage of cultured rat trigeminal ganglion neurones (Liu et al., 1993).

Some very distinctive species differences have recently been observed in mammalian cell preparations. Cultured cochleal outer hair cells which express the $\alpha_9$ receptor (see below) display different pharmacology in rat and guinea-pig preparations. The agonist DMPP is a weak partial agonist in the rat preparation, but a full agonist in the guinea-pig preparation (Chen et al., 1995).

1.8. AGONIST & ANTAGONIST PHARMACOLOGY OF CLONED RECEPTORS IN HETEROLOGOUS EXPRESSION SYSTEMS

a) Heteromeric receptors

Studies involving the co-expression of $\alpha/\beta$ subunit pairs in Xenopus oocytes reveal neuronal nicotinic receptor subtypes with very different pharmacological characteristics (for extensive review see McGehee & Role, 1995), and indicate that both the $\alpha$ and $\beta$ subunits can contribute to agonist sensitivity. For instance, expression of an $\alpha_2$, $\alpha_3$ or $\alpha_4$ receptor subunit along with either a $\beta_2$ or $\beta_4$ subunit results in the formation of functional receptors each with their own very distinctive agonist profile with agents such as nicotine, cytisine and DMPP (e.g. Luetje & Patrick, 1992, Connolly et al., 1992; Covernton et al., 1994, see Chapter 3). This could also be inferred from comparisons of some earlier papers (e.g. Boulter et al., 1987; Wada et
Thus, cytisine is a potent agonist on receptors containing the β4 subunit (e.g., 5× the potency of ACh for α3β4 receptors, See Chapter 3), but only a weak partial agonist on receptors containing the β2 subunit (e.g., 0.02× the potency of ACh for α3β2 receptors). The agonists nicotine and DMPP do not show such a clear cut difference between β2 and β4 containing receptors but do give very similar rank orders of potency for all β4 containing receptors (Luetje & Patrick, 1991). More recently there has been interest in the compound ABT-418 due to its cognition enhancing effects (see below), and also in the analgesic compound epibatidine. Both these agents are broad spectrum activators of neuronal nAChR subtypes, although ABT-418 does show some subtype selectivity in its potency relative to ACh (Papke et al., 1997). Epibatidine is the most potent agonist yet tested on all rat neuronal nAChR subtypes, with EC₅₀ values in the submicromolar range (Papke et al., 1997). It should be noted that several of these agonists can also act as antagonists on some subtypes due to their partial agonist activity (e.g. nicotine & cytisine on β2 containing receptors, Luetje et al., 1991; Papke & Heinemann, 1994).

The subunits α5 and β3 do not form functional receptors in any of the α/β paired combinations tested so far, but the α5 subunit has been shown to be incorporated into native receptors containing the α3 and β4 subunits (Vemallis et al., 1992). The agonist profiles of such 'triplet' receptors expressed in oocytes remain to be elucidated. However, expression of chick α4, α5 and β2 subunits in *Xenopus* oocytes reveals the presence of receptors functionally distinct from a simple α4/β2 pair (Ramirez-Latorre et al., 1995).

The rank order of potency for a range of agonists displayed by the human α3β2 and α3β4 nAChR subunit combinations (DMPP ≈ Cyt ≈ nicotine > ACh and DMPP > Cyt ≈ nicotine > ACh, respectively), differs from that reported for their rat homologs (Luetje & Patrick, 1991; Covernton et al., 1994; Chavez-Noriega et al., 1997).

Similarly, various antagonists have been shown to be selective for different receptor subtypes, although there is still an absence of compounds with enough selectivity to be of much use in distinguishing native receptors. The traditional
ganglion blocking drugs mecamylamine, trimetaphan, pentolinium and hexamethonium have a 9-22\times lower IC\textsubscript{50} on \(\beta_4\)-containing receptors than on \(\beta_2\)-containing receptors (Cachelin & Rust, 1995). The antagonist dihydro-\(\beta\)-erythroidine also shows subtype selectivity, but the amount of inhibition does not appear to be dependent on the presence of any particular subunit (Harvey \textit{et al.}, 1996). The antagonist d-tubocurarine (d-Tc) also has an interesting subtype specific pharmacology, causing inhibition of agonist-induced responses on \(\alpha_2\beta_2\) and \(\alpha_3\beta_2\) receptors but a mixed inhibition/potentiation of \(\alpha_2\beta_4\) and \(\alpha_3\beta_4\) receptors (with no agonist effects due to d-Tc alone, Cachelin & Rust, 1994).

The snake toxin neuronal bungarotoxin (NBT) shows subtype selectivity (Boulter \textit{et al.}, 1987; Wada \textit{et al.}, 1988; Duvoisin \textit{et al.}, 1989; Luetje \textit{et al.}, 1990; Papke \textit{et al.}, 1993), but the block appears to depend on the experimental protocol used: thus when co-applied with ACh, NBT causes a rapidly reversible block of \(\alpha_3\beta_4\) receptors, but requires a preincubation period to block receptors containing the \(\beta_2\) subunit and other receptors containing the \(\beta_4\) subunit (an effect which is only slowly reversible) (Papke \textit{et al.}, 1993). This effect may explain the inconsistent results obtained with this toxin in the past. It has been demonstrated that the first 131 amino acids of the subunit are sufficient to regulate the kinetics of NBT inhibition, and that this portion is also an important determinant of whether cytisine is a full or partial agonist in receptors formed with the \(\alpha_3\) subunit (Papke \textit{et al.}, 1993).

\textit{b) Homomeric receptors}

The subunits \(\alpha_7\), \(\alpha_8\) (chick only) and \(\alpha_9\) (rat only) differ from the other subunits not only in their ability to form receptors homomerically (although the \(\alpha_7\) and \(\alpha_8\) subunits can also form heteromers together, Gotti \textit{et al.}, 1994), but also in their distinctive pharmacology. All three subtypes again have their own distinctive agonist profiles and can be distinguished from heteromeric receptors by virtue of their block by \(\alpha\)-Bgt. Although strychnine block has been reputed to be a characteristic of these
three subtypes it has long been known that strychnine can cause a depressant effect in
the mammalian sympathetic ganglion and at the neuromuscular junction (Lanari &
Luco, 1939; Landau, 1967). Strychnine can also block α3β4 and α4-1β2 receptors
expressed in Xenopus oocytes (IC<sub>50</sub> ≈10μM, Patrick Coverton & Fiona Kempsill,
unpublished observations). The rat α7 receptor has an agonist profile distinct from that
of the heteromeric receptors with a rank order of potency Nic ≈ DMPP > Cyt > ACh at
low concentrations (Séguéla et al., 1992). The anabasine derivative 3-(4)-
dimethylaminocinnamylidine (DMAC) has recently been found to be a particularly
selective and potent agonist at rat α7 receptors (DeFiebre et al., 1995) with minimal
responses (1% of ACh maximal response) on α4β4, α4β2, α2β2 and α3β2 (α3β4 not
tested) receptor subunit combinations. Epibatidine appears to be the most potent
agonist yet tested for the rat α7 receptor (EC<sub>50</sub>=1μM, Papke et al., 1997), although the
agonist (+)-Anatoxin-a which is very potent on the chick α7 receptor (EC<sub>30</sub>=0.58μM,
Thomas et al., 1993; Amar et al., 1993) has not been tested on the rat receptor. The α7
subtype also reveals a species dependent pharmacological difference between human,
rat and chick clones in spite of a large sequence homology (94% for human/rat, 92%
for human/chick, Peng et al., 1994): the agonist DMPP acts as a potent full agonist on
human and rat α7 receptors (up to 60% of maximum in concentration range used), but
only a very weak partial agonist on chick α7 receptors (3.5% of maximum)(Peng et
al., 1994; Séguéla et al., 1993; Gerzanich et al., 1994). The agonist profile observed in
human α7 receptors differs from that reported for its rat homolog (Séguéla et al., 1993;
Chavez-Noriega et al., 1997).

The α8 receptor (so far only found in chick) has a pharmacological profile
distinct from that of the α7 subtype. In general α8 homomers are more sensitive to
agonists and less sensitive to antagonists than chick α7 homomers (Gerzanich et al.,
1994). Also, unlike the chick α7 receptor, DMPP is a full agonist on the α8 receptor.

The latest subtype cloned, α9 (found in rat cochlear hair cells and pituitary
gland, Elgoyhen et al., 1994), has several interesting characteristics. It was initially
reported that this receptor displayed an unusual mixed nicotinic-muscarinic
pharmacology due to its activation (as a weak partial agonist) by the muscarinic agonist oxotremorine-M, although it is not activated by several other classical muscarinic agonists (Elgoyhen et al., 1994). However, recent studies reveal that this agonist also evokes a nicotinic response in both muscle and sympathetic neurone preparations (Reinstetter et al., 1994; Xian et al., 1994). Oxotremorine-M can also activate α3β4 receptors expressed in Xenopus oocytes at high micromolar concentrations (Patrick Covemton, unpublished observations). In contrast with all other functional nicotinic receptors expressed in oocytes so far, α9 is not activated by nicotine (up to 1mM, Elgoyhen et al., 1994) and so the label 'nicotinic' becomes somewhat inappropriate. Also, in contrast with the α7 and α8 receptors, α9 is not activated by cytisine- but it is activated by DMPP (A weak partial agonist, as with chick α7).

c) Transfected cell lines

As well as using oocytes for expression systems, mammalian cell lines have also been used yielding similar results. Rat α3β4 receptors expressed in (HEK)-293 cells (a human kidney cell line) show a similar pharmacology (in spite of some confusing differences in potency terminology) to studies with this receptor expressed in oocytes with a rank order of potency Cyt > Nic > ACh > DMPP, and block by 100μM mecamyamine, hexamethonium and d-Tc (Wong et al., 1995). It has also been possible to express rat α7 receptors in human SH-SY5Y neuroblastoma cells where they display similar physiological and pharmacological profiles to receptors expressed in oocytes (Puchacz et al., 1994). However, recent industrial emphasis has been on expressing human receptors in cell lines rather than the rat receptors which are the prime interest in this thesis. The use of rat receptors enables comparisons with most of the classic behavioural, electrophysiological and anatomical studies.

1.9. NEURONAL NICOTINIC RECEPTOR MODULATION
a) Calcium

Nicotinic acetylcholine receptor (nAChR) responses of rat medial habenular neurons can be potentiated up to 3.5-fold by increasing the concentration of external Ca$^{2+}$ in the millimolar range (Mulle et al., 1992). This effect, independent of voltage, is probably due to the binding of Ca$^{2+}$ to an external site. External Ca$^{2+}$ decreases nAChR single-channel conductance at negative potentials, but it markedly enhances the frequency of opening of acetylcholine activated channels. The potentiating effect of Ca$^{2+}$ is mimicked by Ba$^{2+}$ and Sr$^{2+}$, but barely by Mg$^{2+}$. The potentiating effect of Ca$^{2+}$ has also been demonstrated for several neuronal nAChR subunit combinations ($\alpha2\beta2$, $\alpha3\beta2$, $\alpha3\beta4$ & $\alpha4\beta4$) expressed in *Xenopus* oocytes (Vemino et al., 1992). For instance, with the $\alpha3\beta4$ subtype the response with 1.8 mM Ca$^{2+}$ is almost double that recorded in 0.18 mM Ca$^{2+}$.

Mutations in several consensus Ca$^{2+}$ binding sequences from the N-terminal domain of the neuronal $\alpha7$ nicotinic acetylcholine receptor alter Ca$^{2+}$ potentiation of the chick $\alpha7$-V201-5HT$_3$ chimera (Galzi et al., 1996). Mutations E18Q or E44Q abolish calcium-enhanced agonist affinity but preserve the calcium increase of plateau current amplitudes and cooperativity. On the other hand, mutations of amino acids belonging to the 12 amino acid canonical domain ($\alpha7$ 161-172) alter all features of potentiation by enhancing (D163, S169), reducing (E161, S165, Y167) or abolishing (E172) calcium effects on ionic current amplitudes and agonist affinity.

b) Phosphorylation of nAChRs

Phosphorylation of ligand-gated ion channels may represent a major mechanism in the regulation of their function and play an important role in synaptic plasticity (Swope et al., 1992- review). Protein kinases generally increase desensitization of muscle and *Torpedo* nicotinic acetylcholine receptors (nAChRs).
There is evidence to show that peripheral ganglionic nAChRs can also be modulated by protein kinases, but to date little is known of any analogous modulation at central nicotinic AChRs. Activation of protein kinase C in chick lumbar sympathetic ganglia causes enhancement of the rate of desensitization of ganglionic nAChRs (Valenta et al., 1993). Substance P also enhances the rate of desensitization and slows recovery in ganglia (Role, 1984; Valenta et al., 1993), possibly by indirectly activating PKC. In contrast, protein kinase A activators enhance nicotinic currents in chick ciliary ganglia, (Margiotta et al., 1987). VIP activation of PKA in ciliary ganglion neurones also increases the magnitude of ACh-evoked currents via a cAMP-dependent mechanism (Gurantz et al., 1994), This effect is also seen in rat intracardiac neurones where it appears to be cAMP-independent ( Cuevas & Adams, 1996). Thus, neuronal nAChR activity can be both up and down-regulated by kinase-dependent mechanisms. This contrast with the effect of PKA on muscle receptors should not be surprising, since the cytoplasmic domains (where the potential phosphorylation sites are located) are the least conserved regions of the nicotinic AChRs. The α3 subunits in these peripheral receptors can be phosphorylated (Vijayaraghavan et al., 1990) and the degree of phosphorylation mimics the time course of the PKA enhancement of the ACh-activated current. Biochemical studies have also shown that nicotinic α4 subunits can be phosphorylated by PKA, (Nakayama et al., 1993). Habenular neurones in the rat brain also express the mRNAs for these subunits (Wada et al., 1989), and the nicotinic responses here have both pharmacological and electrophysiological similarities to ganglionic receptors (McGehee & Role, 1995 - review). It is, therefore, possible that analogous modulation might also occur in the CNS. Nicotinic AChR current responses recorded in rat medial habenular neurones increase with internal perfusion (Lester & Dani, in press). Chelation of internal Ca^{2+} with BAPTA increases the rate of increase in desensitization, and this effect is partially reversed by internal perfusion with Mg-ATP. This suggests the possible involvement of a Ca^{2+}-dependent protein kinase. The changes in desensitization run parallel to a rapid 'run-up' and subsequent 'run-down' of the nicotinic response. This effect has also been observed in rat peripheral ganglia (See
Chapter 3), PC12 cells (Ifune & Steinbach, 1993), bovine chromaffin cells (Nooney & Feltz, 1995), and also in rat medial habenular slices (Robin Lester, personal communication). In undialyzed medial habenula neurones (using the perforated-patch technique) nicotinic responses do not exhibit changes in desensitization or 'run-up/down' (Robin Lester, personal communication).

Recent studies also show that calcium flux through homomeric α7 receptors expressed in GH4C1 rat pituitary cells can be enhanced by activating PKA (Quik et al., 1997). This subtype is believed to form functional receptors throughout the brain.

c) Allosteric ligands

There are a number of non-competitive inhibitors of neuronal nicotinic receptors, such as the channel blocker hexamethonium (Gurney & Rang, 1984), the 11 amino acid peptide Substance P (Stafford et al., 1994), and several general anaesthetic agents (e.g. on the α7 receptor, Zhang et al., 1997). However, there are also a number of ligands that appear to potentiate neuronal nicotinic receptor function. For instance, the calcium-activated chloride channel blockers niflumic and flufenamic acid potentiate agonist responses of α3β4 receptors expressed in Xenopus oocytes, although they inhibit α3β2 receptors (Zwart et al., 1995). Also, anticholinesterase agents such as galanthamine and the neurotransmitter 5-HT can potentiate nicotinic responses in PC12 cells and hippocampal neurones (Storch et al., 1995; Schrattenholz et al., 1996), although at higher concentrations they cause inhibition. Furthermore, the present study reveals that ethanol can potentiate the agonist-induced responses of a range of neuronal nAChR subtypes (see Chapter 5).

1.10. Other Functional Characteristics Of Neuronal Nicotinic Receptors.

a) Single channel conductance & kinetics
Single channel studies in several neuronal preparations reveal extensive heterogeneity both within and between membrane patches (e.g., Mathie et al., 1991; Connolly et al., 1995, Moss & Role, 1993; Sivilotti et al., 1997). This is apparent for both single channel conductance, where multiple conductance states are observed, and open probability. This heterogeneity is also observed for receptor subtypes expressed in *Xenopus* oocytes (e.g. Charnet et al., 1992; Papke et al., 1992). An extensive review of the properties of single channels from various neuronal preparations and combinations expressed on oocytes (along with tables of the varying experimental conditions) is provided by Papke, 1993 (also see McGeehe & Role, 1995).

So far the main single channel conductances of all neuronal nAChR subunit combinations expressed in *Xenopus* oocytes do not appear to correspond to any of those encountered in any neuronal cell preparations. However, nAChRs in transfected cells appear to provide a more satisfactory (although not exact) match, suggesting that the oocyte system does not assemble these channels properly (Stetzer et al., 1996; Sivilotti et al., 1997).

**b) Rectification**

A characteristic of neuronal nicotinic macroscopic current responses is the strong inward rectification observed at positive potentials (with a non-conducting whole-cell current between approximately 0 and +50mV, e.g. in the medial habenula, Connolly et al., 1995). This phenomenon has been studied extensively in rat PC12 cells and rat sympathetic neurones (Ifune & Steinbach, 1991; Mathie et al., 1990), where it appears to be at least partly due to block by internal Mg\(^{2+}\).

The mechanisms underlying rectification have recently been investigated in rat α7 homomers expressed in oocytes (Forster & Bertrand, 1995), where it has been shown that a ring of negatively charged glutamate residues located at the inner mouth of the channel is essential for rectification, and that this rectification is at least
partially relieved by chelation of internal Mg$^{2+}$. In external barium, application of nicotine elicits an inwardly rectifying response with $\alpha 7$ receptors, whereas in calcium the response has a linear IV relation (Sands et al., 1993), although this could be due to involvement of Ca$^{2+}$-activated chloride currents.

c) Desensitisation rates

Several neuronal nicotinic receptor subtypes differ markedly in their activation/inactivation kinetics. The $\alpha 7$ and $\alpha 8$ (although not $\alpha 9$) homomeric receptors expressed in oocytes reveal very rapid rates of desensitisation (e.g. Gerzanich et al., 1994; Elgoyhen et al., 1994) making them easily distinguishable from most of the heteromeric receptor subtypes expressed so far, with the possible exception of $\alpha 3\beta 2$, although at low agonist concentrations the distinction may not be quite so obvious. However, there are further distinctions within the heteromeric receptor population with $\beta 2$-containing receptors usually showing faster rates of desensitization than $\beta 4$ receptors (Cachelin & Jaggi, 1991; Fenster et al., 1997).

As well as differences in the acute desensitization during a response, there are also subtype differences in the rates of recovery from desensitization (as measured with the agonist nicotine, Fenster et al., 1997). In general, receptors containing $\beta 4$ subunits (i.e. those that are less affected by acute desensitization) recover from desensitization more slowly than those containing $\beta 2$ subunits. Additionally, receptors containing $\alpha 4$ subunits recover more slowly than those containing $\alpha 3$ subunits.

c) Divalent cation permeability

All neuronal nicotinic receptors tested so far have a significantly greater calcium permeability than muscle receptors (Vemino et al., 1994), although Ca$^{2+}$ influx through nicotinic receptors at the neuromuscular junction is also sufficient to elicit Ca$^{2+}$-dependent processes (e.g. Decker & Dani, 1990). For example, the
Ca\textsuperscript{2+}:Na\textsuperscript{+} permeability ratio for muscle \(\alpha(\times2)\beta\gamma\delta\) receptors and neuronal \(\alpha3\beta4\) receptors is 0.2 and 1.1 respectively (Costa et al., 1994). Ca\textsuperscript{2+} influx through nAChRs in rat habenular neurones is sufficient to directly activate a Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} conductance (Mulle et al., 1993; Connolly et al., 1995). This characteristic therefore has implications for the involvement of these receptors in the modulation of activity in neuronal circuits (see below). This could take the form of regulation of Ca\textsuperscript{2+}-dependent neurotransmitter release via presynaptic neuronal nAChRs or in the regulation of other Ca\textsuperscript{2+} dependent processes (e.g. the Ca\textsuperscript{2+}-dependent translocation of protein kinase C seen in PC12 cells, Messing et al., 1989). One final possible consequence of this high calcium permeability is autoregulation of the receptor due to transient decreases in the modulation produced by external calcium (as suggested by Vernino et al., 1994).

1.11. THE PHYSIOLOGICAL SIGNIFICANCE OF NICOTINIC RECEPTORS IN THE BRAIN

Nicotinic receptors clearly have a role mediating fast synaptic transmission in autonomic ganglia (see Brown, 1979, 1980 for reviews). However, in spite of the apparent widespread distribution of nAChRs in the CNS, there is little evidence for any analogous role in the brain, although some involvement in synaptic transmission has been noted between neurones of the pedunculopontine tegmental nuclei and dopamine neurons in the substantia nigra pars compacta in rat brain slice preparations (Futami et al., 1995). There is also some evidence of nicotinic synaptic transmission in the rat hippocampal slice culture (D. Bertrand, personal communication). This has led to the suggestion that nAChRs might more commonly play a modulatory role, especially when their high Ca\textsuperscript{2+} permeability is brought into consideration (e.g. Mulle et al., 1992: for reviews see McGeehe & Role, 1995; Role & Berg, 1996). There are now several lines of evidence to support a role in the presynaptic regulation of neurotransmitter release. Firstly, nicotine and \(\alpha\)-Bgt binding sites can be detected
along the axons and within the terminal fields of both CNS and PNS neurones, suggesting a synaptic terminal location. (e.g. see Sargent, 1993 for review).

Additionally, studies of brain synaptosomes reveal several instances where nicotinic ligands affect neurotransmitter release. In superfused synaptosome preparations from striatum, nicotine is a potent agonist, acting with a sub-micromolar EC$_{50}$ to elicit [$^3$H]dopamine release (Clarke & Reuben, 1996; Grady et al., 1992; Whiteaker et al., 1995). This nicotine-evoked release was not blocked by $\alpha$-bungarotoxin but was effectively blocked by neuronal bungarotoxin, suggesting that $\alpha$7-type receptors were not involved (Grady et al., 1992). Clarke & Reuben also demonstrated in a parallel study that nicotine stimulates the release of noradrenaline in hippocampal synaptosomes, but was 40 times less effective, suggesting the involvement of different receptor subtypes. Wilkie et al., (1996) showed in the same preparation loaded with [$^3$H]choline that nicotine can also evoke a dose-dependent increase in [$^3$H]ACh release, although higher concentrations were less effective (perhaps through desensitization).

Nicotinic agonists have been demonstrated to decrease the amplitude of the afferent volley recorded in the rostral subnucleus of the IPN on stimulation of the fasciculus retroflexus. This action was insensitive to $\alpha$-bungarotoxin and to neuronal bungarotoxin, but was blocked by mecamylamine, hexamethonium, tubocurarine, and DHβE, with IC$_{50}$ values different from those reported for IPN postsynaptic nAChRs (Mulle et al., 1991). Whole-cell recordings performed on rat interpeduncular nucleus neurons using the thin-slice technique have shown that nicotine dramatically increases the frequency of postsynaptic GABAergic currents (Lena et al., 1993). Similarly, in relay neurons from slices of thalamic sensory nuclei, nAChR activation facilitates the release of the inhibitory neurotransmitter GABA, and micromolar concentrations of nicotinic agonists increase the frequency of miniature GABAergic synaptic currents and decrease the failure rate of evoked synaptic currents. These actions of nicotinic agonists are not observed in knock-out mice lacking the $\beta$2 nAChR subunit gene (see below, Lena & Changeux, 1997). Low concentrations of nicotine (170nM) have also
been shown to enhance synaptic transmission in co-cultures of chick medial habenular and IPN explants (McGehee et al., 1994). However, in this preparation the effects were shown to be sensitive to α-Bgt, suggesting the involvement of α7-type receptors, and this was supported with antisense deletion experiments.

Stimulation of presynaptic nicotinic receptors with submicromolar concentrations of nicotine has been shown to enhance glutamatergic synaptic transmission in both hippocampal cultures from postnatal rats and hippocampal slices from young rats. (Gray et al., 1996). Blockade of this effect by α-Bgt and methyllycaconitine indicated that this effect was via an α7-type nAChR. Using measurements of intracellular Ca²⁺ in single mossy-fibre presynaptic terminals, this study also showed that these nAChRs can mediate a Ca²⁺ influx that is sufficient to induce vesicular neurotransmitter release.

Finally, neuronal-bungarotoxin, but not α-bungarotoxin has been shown to decreases the evoked release of ³H-acetylcholine in rat cortical slices (Sugaya et al., 1990). Similarly, recordings of synaptic potentials made from pyramidal cells located in cortical layers II/III evoked by stimulation of superficial cortical layers, showed that iontophoretic applications of nicotinic agonists increased the amplitude of the monosynaptic excitatory postsynaptic potential mediated by non-N-methyl-D-aspartate glutamate receptors in 14% of cells (Vidal & Changeux, 1993). This effect was abolished by the selective nicotinic blocker, neuronal bungarotoxin (IC₅₀ = 0.6-0.7 μM) and by dihydro-β-erythroidine (IC₅₀ = 20-30 μM), whereas hexamethonium, mecamylamine, tubocurarine and α-bungarotoxin were ineffective. Thus, there is evidence for the involvement of multiple region-specific (and possibly species-specific) nicotinic receptor subtypes in the modulation of neurotransmitter release in the brain.

1.12. TRANSGENIC STUDIES
Studies of a transgenic strain of mice that contain a defective β2 subunit reveal that high-affinity binding sites for nicotine are absent from the brains of mice homozygous for this mutation (Picciotto et al., 1995). Electrophysiological recording from brain slices reveals that thalamic neurons from these mice do not respond to nicotine application (Picciotto et al., 1995). This study also demonstrated in behavioural tests that nicotine no longer augments the performance of β2(-/-) mice on passive avoidance, a test of associative memory. However, paradoxically, mutant mice are able to perform better than their non-mutant siblings on this task. Thus it might appear that either i) contrary to present views, β2-containing receptors are not physiologically important mediators of cognitive function, ii) other subunits become upregulated to compensate for the absent subunit, or iii) nicotine augmentation of passive avoidance is due to desensitization of β2-containing receptors, whose normal physiological function is to potentiate inhibitory circuits to provide a dynamic range of cognitive function.

1.13. NEURONAL NICOTINIC RECEPTORS IN DISEASE AETIOLOGY & TREATMENT

a) Nicotine Addiction

Nicotine concentrations in the brain during smoking are believed to reach high nanomolar to low micromolar levels (for review see Dani & Heinemann, 1996). Such levels can cause substantial receptor desensitization at central nAChRs while causing little receptor activation (e.g. Lester & Dani, 1995). Longer applications of these lower concentrations result in slower recovery from desensitization, and the time for recovery varies considerably among different receptor subtypes (Fenster et al., 1997). The α4-1β2 subtype appears to be the most sensitive to chronic desensitization and the α7 subtype the least sensitive (i.e. it recovers fastest). The study of Fenster et al. (1997) also suggests that the α4β2 subtype might be the only one at which significant activation occurs at smoking levels of nicotine.
There is direct evidence that nicotine acts upon the mesolimbic pathways. Nicotine stimulates the release of dopamine in the nucleus accumbens of freely moving rats and stimulates release from synaptosomes isolated from various areas including the nucleus accumbens (see above). Also, dopamine antagonists or lesions of the nucleus accumbens reduce nicotine self-administration in rats, although dopamine blockade appears to increase smoking behaviour in humans (for reviews see Stolerman & Shoai, 1991; Rose & Corrigall, 1997).

Chronic nicotine exposure results in an increase in the density of binding sites for putative nicotinic AChRs in the brains of rats, mice and humans (Benwell et al., 1988; Marks et al., 1983; Schwartz & Kellar, 1983), although it has little effect on the levels of mRNA expression (α2, α3, α4, α5, β2 subunits, Marks et al., 1992). It has been suggested that this change is due to nAChRs entering long-lasting inactivated states, due to desensitization from continuous low levels of nicotine (Lester & Dani, 1994; Peng et al., 1994). As a result, the number of nicotinic receptors appears to be regulated by a post-transcriptional mechanism that decreases receptor turnover (Peng et al., 1994). These changes would enable some cholinergic systems to move towards their initial levels of excitability, even as the number of nAChRs increases (Dani & Heinemann, 1996). However, not all cholinergic systems appear to return to such a state after chronic nicotine exposure. Nicotinic receptor function appears to be persistently upregulated in some systems and down-regulated in others after chronic nicotine-induced increases in receptor number (e.g. Marks et al., 1993; Peng et al., 1994; Gopalakrishnan et al., 1996).

b) Alzheimer's Disease

Nicotinic receptors are specifically and substantially reduced in number in Alzheimer's disease along with a loss of hippocampal cholinergic inputs (e.g. Rinne et al., 1991; Nordberg, 1994- review; Perry et al., 1995). Also, α4β2 receptors are diminished in the hippocampus of ageing mouse brains, (Kulmer et al., 1995). The
brains of patients with Alzheimer's disease show marked decreases in B(max) values for low-affinity $^{125}$I-kappa-bungarotoxin sites and both high- and low-affinity $^3$H-nicotine sites, whereas $^{125}$I-alpha-bungarotoxin sites are not significantly different in number from age-matched control brains (Sugaya et al., 1990). Activation of presynaptic nicotinic receptors enhances hippocampal synaptic transmission (Gray et al., 1996), although this effect is inhibited by $\alpha$-bungarotoxin. It is, therefore, possible that altered efficiency of hippocampal transmission could be a contributory factor in the cognitive deficits associated with this disease. Nicotine has been shown to enhance cognitive function in normal healthy people, and has therefore been proposed as a treatment for Alzheimer's. Experimental studies with brief exposure to intravenous or subcutaneous nicotine have shown some modest improvement in cognitive function in patients with Alzheimer's disease (Jones et al., 1992), and trials with new nicotinic ligands such as ABT-418 are underway.

Finally, decreased expression of $\alpha 4$ mRNA is correlated with increased expression of tau-protein in Alzheimer's patients. (Wevers et al., 1995).

c) Parkinson's Disease

Marked decreases of nicotinic binding sites and nAChR protein expression have been shown in Parkinson's disease patients with cognitive dysfunction (e.g. Perry et al., 1995). Some epidemiological studies suggest that smoking delays the onset of Parkinson's disease symptoms (e.g. Baron, 1986). Also nicotine can be neuroprotective to nigrostriatal neurones in lesioned rats (Fuxe et al., 1990). Activating presynaptic nicotinic receptors enhances dopamine release from nerve terminals in the striatum as well as in the mesolimbic systems of the brain, (see above, Balfour & Benwell, 1993, review: Soliakov & Wonnacott, 1996). Recently, several new nicotinic agonists have been developed which show promise of being able to alleviate some of the symptoms of Parkinson's Disease (e.g. SIB-1765F, Lloyd et al., 1995).
d) Schizophrenia

The observation that more than 95% of schizophrenics smoke as opposed to less than 50% of the general population has suggested that there may be some involvement of nicotinic AChRs in schizophrenia itself or in the side-effects (see below) of antipsychotic drugs (Lohr & Flynn, 1992; Connolly et al., 1992, Nisell et al., 1995; for review see Leonard et al., 1996). Smoking amongst schizophrenics can be pathological, and patients may smoke or chew tobacco to the point of nausea. Abnormalities of nicotinic acetylcholine receptor function have been found in schizophrenic patients and their 1st degree relatives, (Adler et al., 1993, Freedman et al., 1994, 1995). Over 95% of schizophrenics and 50% of their 1st-degree relatives are unable to effectively diminish or "gate" the auditory evoked potentials arising from repeated stimuli- they have what is termed a P50 gating deficit. The deficit, therefore, probably has a genetic component, and appears to be from the side of the family carrying the schizophrenia phenotype (Siegel et al., 1984; Waldo et al., 1991). Nicotine is able to temporarily reverse these deficits in auditory processing in both schizophrenic patients and their first degree relatives (Adler et al., 1992, 1993), whereas neuroleptics are ineffective (Freedman et al., 1983; Adler et al., 1990). However, other cholinergic agents and anticholinesterases had previously appeared to be ineffective in alleviating any symptoms of schizophrenia (Pfeifer & Jenny, 1957; Rosenthal & Bigelow, 1973; Berger et al., 1979). In a rat model of auditory gating, injection of α-bungarotoxin into the cerebral ventricles abolished the ability to gate, and this loss of function can be reversed by administration of nicotine. The P50 gating mechanism may involve nicotinic stimulation of GABAergic interneurones in the hippocampus in normal rats and humans (Miller & Freedman, 1995), and numbers of α7-type receptors are decreased in hippocampal brain tissue from schizophrenic patients (Freedman et al., 1995). These observations suggest that nicotinic α7-type receptors in this brain region may be involved in the P50 gating deficit in schizophrenics. Linkage analysis of this deficit has yielded positive lod scores to
markers mapping to a locus on chromosome 15, in the same region as the human α7 subunit (Freedman et al., 1997). Although there are sites on other chromosomes which may contain genetic determinants for some forms of schizophrenia (Peltonen, 1995, review), this work reinforces the idea that at least for some families, a deficit of functional α7 receptor may be one of the factors in the aetiology of schizophrenia. The high incidence of smoking among schizophrenics may be a reaction to counteract these deficits in nicotinic receptor function.

e) Tourette's Syndrome

Nicotine has been shown to potentiate the pharmacological effects of haloperidol in animal studies (Emerich et al., 1991). This provided the rationale for administering nicotine to patients with Tourette's syndrome who were being treated with haloperidol. In clinical trials transdermal nicotine has been reported to cause both acute and long-lasting reduction of motor tics in non-smoking Tourette's patients (Silver & Sanberg, 1993; Dursun et al., 1994). The treatment was effective both as a sole treatment or as an adjunct to haloperidol treatment, and in patients who are refractory to haloperidol treatment (Dursun et al., 1994; Dursun & Reveley, 1997). These treatments were effective for up to 4 weeks after a single 2 day treatment, and appeared to differentially affect different types of individually quantified tics (e.g. head-shakes, eye-blinks, vocal tics and facial grimace). This suggests a differential role of nicotinic receptors in the generation of different types of tics (Dursun & Reveley, 1997).

f) Neuronal nAChRs and other disease states

Epidemiological studies reveal that smokers have a lower incidence of ulcerative colitis (Calkins, 1989), and clinical trials have demonstrated that nicotine can produce short-term benefits (Pullan et al., 1994). However, the mechanisms
underlying this benefit are unclear. Conversely, the inflammatory bowel disorder Crohn's Disease is worsened by smoking (Calkins, 1989).

Other therapeutic applications of nicotinic agents currently under investigation include their use in obstructive sleep apnoea (Gothe et al., 1985).

g) Possible side-effects due to anti-psychotic treatment.

Neuronal nAChR subtypes can be blocked by commonly used antipsychotic and anti-depressive drugs at therapeutic concentrations (Connolly et al., 1992). Depression of cognitive function and tardive dyskinesia (i.e. Parkinson-like symptoms) are frequent and serious complications of antipsychotic treatment. As noted above, nicotinic agonists can enhance striatal dopamine and hippocampal glutamate release, and nAChRs are therefore likely to be involved in the neuronal circuits which mediate these cognitive and motor disturbances. Therefore, in addition to a direct involvement in some of the symptoms of schizophrenia (auditory gating deficit), there is also indirect evidence that nicotinic AChRs might be involved in the neuronal circuits which mediate some of the side-effects of anti-psychotic drugs.

Similarly, nicotine has also been shown to enhance vasopressin release (Seyler et al., 1986) and to reduce weight (Winders & Grunberg, 1990). If nicotinic AChRs are blocked by antipsychotic drugs, it could contribute to the symptoms of inappropriate vasopressin release syndrome and weight gain associated with antipsychotic medication (Tornatore et al., 1987). Also, a compromise of nicotinic circuits due to anti-psychotic treatment may contribute to the high levels of smoking among schizophrenics.

1.14. AIMS OF THIS STUDY

The anatomical and functional characteristics of nicotinic receptors in the brain reveal extensive diversity. For the investigation of neuronal nAChRs as potential
therapeutic targets it is necessary to know several things. These include: 1) What is the subunit composition of the functional nicotinic receptors present in a particular neuronal circuit? 2) What are the pharmacological properties and physiological roles (if any) of these receptors? 3) What mechanisms are available for the manipulation and regulation of the activity and density of these receptors? The aim of the present study was to use functional approaches to answer some aspects of these questions, with the view to increasing the understanding of this diverse group of receptors. In the long term this may prove valuable in the development of treatments for a number of neurological disorders in which neuronal nAChRs have recently been implicated.

1.15. SUMMARY OF EXPERIMENTS PERFORMED IN THIS THESIS

In order to answer some aspects of the questions raised above, the following experiments were performed:

1) Electrophysiological techniques were used to compare the pharmacological profile of the nicotinic responses in rat SCG cells with those of cloned nicotinic receptor subtypes expressed in *Xenopus* oocytes. This was in order to try to elucidate the subunit composition of the nicotinic receptors present in SCG cells. The nicotinic receptor subunit combinations used were based on the subunit mRNAs known to be expressed in SCG cells.

2) Complete agonist concentration-response relationships were determined for a range of neuronal nicotinic receptor subunit combinations expressed in *Xenopus* oocytes. This would enable the determination of equivalent points on the agonist concentration-response curves of the different subtypes. It is important to know the shape of such curves for studies of receptor modulation: the amount of change induced by a modulator can be different at different parts of the concentration-response curve. This information is also useful when trying to predict the responses of native nicotinic receptors of similar subunit composition. Such predictions can then be incorporated into detailed receptor models of clinical conditions such as nicotine addiction.
3) The effects of ethanol on the agonist responses of a range of neuronal nicotinic receptor subtypes expressed in *Xenopus* oocytes were investigated. These experiments utilized the results from (2) to allow comparisons of the different subtypes at equivalent agonist concentrations. We hoped to determine whether there was a direct action of ethanol on neuronal nicotinic acetylcholine receptors. If so, this could provide a molecular basis for the correlation between heavy consumption of tobacco and alcohol. If so, we then wished to discover whether some subtypes might be particularly sensitive to alcohol. By combining this knowledge with that of the anatomical distribution of similar receptors in the brain, we may be able to refine our models of addiction and the role of particular nicotinic receptors in the process.
CHAPTER 2:

GENERAL METHODS

The studies described in this thesis involved work on both native nicotinic receptors (University College London) and cloned receptor subtypes expressed in Xenopus oocytes (University of Strathclyde).

PART I - METHODS EMPLOYED AT UCL

2.1. PREPARATION OF SUPERIOR CERVICAL GANGLION NEURONES

a) The whole ganglion preparation

One of the problems encountered when using an acutely-dissociated enzyme-treated rat sympathetic ganglion cell preparation is the failure to consistently produce significant numbers of healthy cells. A variety of techniques were therefore investigated using various forms of slicing and enzyme treatment of the whole undissociated superior cervical ganglion. Attempts at slicing were unsuccessful, whereas enzyme treatment was found to be essential to obtaining significant numbers of patchable cells. This was due to the presence of a tough extracellular matrix, which prevented access to the cell surface if not digested away with enzyme treatment. The preparation found to be most successful was one based on that used by Mathie et al. (1990) using a similar (not identical) enzyme treatment, but without the subsequent mechanical dissociation of the cells.

Patch recordings from whole undissociated sympathetic ganglia have also been performed by Derkach et al. (1987, 1993) using a collagenase enzyme treatment, and by Gola et al. (1992) in rabbit ganglia using a continuous stream of saline (1.5-3 hr) after a short protease treatment.
In order to make recordings from receptors from mature SCG synapses it was decided to use rats between the ages of 4 to 6 weeks. Whereas Sprague-Dawley rats may not physically appear to be fully mature at this age, the sympathetic nervous system is fully developed with all synapses formed by the end of the first postnatal month (Smolen, 1983b).

b) Solutions for SCG neurones.

A modified Krebs’ solution (calcium-free) was used for preparation, enzyme treatment and recording from rat superior cervical ganglion neurones. Its composition was (in mM): NaCl 125, KCl 2.5, NaHCO₃ 26, NaH₂PO₄ 1.25, MgCl₂ 1, glucose 25. The pH was 7.4 when bubbled with 95% O₂ and 5% CO₂ (carbogen). The bath solution also contained 500nM atropine sulphate (to block endogenous muscarinic receptors) and 100nM tetrodotoxin (to prevent spontaneous firing of action potentials) (both from Sigma). The Krebs’ solution was bubbled continuously with 95% O₂ and 5% CO₂ before perfusion through the recording chamber.

c) Cell Preparation Protocol.

Whole superior cervical ganglia were obtained from 4-6 week old male or female Sprague-Dawley rats. Rats were killed by decapitation low in the neck. Once removed, whole ganglia were kept in ice-cold carbogenated modified Krebs’ solution and cleaned of connective tissue (‘desheathed’). The whole ganglia were then treated for 15 min in a Krebs’ solution containing 1 mg/ml trypsin (Sigma) followed by 45 min in a modified Krebs’ solution containing 1 mg/ml collagenase (type IA), 1 mg/ml trypsin inhibitor, and 0.1 mg/ml thermolysin (all Sigma). This was then followed by a 5 min treatment in a modified Krebs’ solution containing 150μM methanesulphonylfluoride (MSF, Aldrich). MSF is an irreversible cholinesterase inhibitor used to prevent any interference of endogenous acetylcholinesterase with
Figure 2.1. Patch clamp recordings in SCG neurones were made using an intact preparation. The picture above shows a photograph of the surface of an enzyme-treated undissociated rat superior cervical ganglion. The cells were viewed under infinity-corrected Nomarski differential interference contrast optics on the stage of an upright microscope (Zeiss Axioskop) using a 40× 0.75 water-immersion objective at a total magnification of 400×. The individual cells are approximately 15-30μm in diameter. Smaller satellite cells can be seen on the surface of the neurones. These were removed with a suction pipette prior to electrophysiological recording.
exogenously applied ACh. All enzyme treatments took place in 10 ml Krebs' solution at 37°C and were continuously bubbled with 95% O2 and 5% CO2.

The whole ganglia were then mounted in the recording chamber (volume=0.7ml) using a nylon-mesh grid (Edwards et al., 1989) to hold them in place. The recording chamber was continually perfused with carbogenated Krebs' solution. Healthy cells were identified as those having a smooth and shiny appearance, with the cell nucleus not visible. Most cells could be seen to possess several satellite cells on their surface (See for e.g. Figure 2.1. for photograph of the preparation). These could be removed using gentle suction and careful manipulation of a suitably sized cleaning pipette (~5 μm diameter) filled with Krebs' solution. Any other debris was removed from the vicinity using a larger pipette. Attempts to clean the cells without enzyme treatment (e.g. Edwards et al., 1989) were not successful.

2.2. ELECTROPHYSIOLOGICAL RECORDING FROM SYMPATHETIC NEURONES.

Macroscopic currents were recorded using the whole-cell patch clamp configuration with a pipette solution containing (in mM): CsF 135, CsCl 5, HEPES 10, EGTA 5, Mg-ATP 4, adjusted to pH 7.3 with NaOH. In some cells an ATP-regenerating system was included in the pipette solution (containing: 20mM phosphocreatine, 50U/ml creatine phosphokinase and 25U/ml protein kinase A catalytic subunit) in order to avoid possible loss of ATP due to spontaneous hydrolysis. Responses were indistinguishable from those recorded with the standard pipette solution.

Agonists were diluted in the SCG recording solution (see above). Stock solutions of acetylcholine chloride (ACh), (-)-nicotine hydrogen tartrate (Nic), (-)-lobeline hydrochloride (Lob), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), (-)-cytisine (Cyt) and carbachol (CCh) (all Sigma) were kept frozen, and thawed and diluted to the desired concentration each day before experimenting. With SCG
neurones the maximum agonist concentrations used for the low concentration potency ratios were as follows (in μM): Cyt, 3; DMPP, 5; Nic, 5; ACh, 10; CCh, 20; Lob, 30.

All recordings were made on the day of cell preparation and at room temperature (20-25°C). Patch clamp recordings were made using an Axopatch 1B amplifier (Axon Instruments). Patch electrodes were pulled on a two-stage electrode puller (Kopf Instruments) from thin-walled borosilicate glass (Clark Electromedical, GC150TF-7.5), coated with Sylgard® resin (Dow Corning 184), and fire polished on a Narashige (MF-83) microforge to a final tip resistance of 5-10 MΩ. Cells were viewed under infinity-corrected Nomarski differential interference contrast optics on the stage of an upright microscope (Zeiss Axioskop or Zeiss Jena) using a 40× 0.75 water-immersion objective with 1.6 mm working distance at a total magnification of 400×. Cells were voltage clamped at -60 mV.

Macroscopic currents were stored on FM tape (Racal Store 4, DC-5 kHz) after filtering at 10 kHz (-3 dB, 8-pole Bessel response), as well as being plotted directly onto a pen recorder after being filtered at 1 Hz (Barr & Stroud 8-pole Bessel). Filtering at this frequency did not affect the magnitude of the current responses. The noise levels of the responses on the chart recorder did not visually decrease until they were filtered at 3 Hz. Figures were obtained by digitizing the data stored on FM Tape at 200Hz via a CED1401 interface.

2.3. Drug Application Techniques For SCG Neurones

a) Stepper motor apparatus

A stepper motor apparatus with associated ‘linear actuator’ control circuit for fast solution exchanges was constructed by Mr David Osborne at the joint workshop of the Pharmacology and Physiology Departments at University College London, and set up for applying drugs described below.
Drugs were applied to a particular cell using a gravity-fed double-barrelled glass pipette (Clark Electromedical) pulled to ~100μm diameter for each barrel on the first stage of a Kopf Instruments two-stage puller. The double-barrelled pipettes did not break on this pull and were removed from the puller still intact. The pulled-out section could then be broken manually after brushing it lightly with a diamond-tipped pen. The broken cross section was then examined under a dissecting microscope in order to inspect the cleanness of the break. Pipettes with imperfect breaks were discarded so as to ensure laminar solution flow when connected to the perfusion apparatus.

b) Perfusion Apparatus.

Cells were exposed to control solution flowing from one barrel of the double-barrelled perfusion pipette and then to drug solution flowing from a second barrel by the lateral movement of the pipette across the surface of the ganglion using the stepper motor apparatus. Control solution continually flowed down one barrel, while the solution flowing down the second barrel could be switched between a number of different drug solution reservoirs using an 8-way tap (Ball-Hatchett). The flow rate could be optimised to give effective discrimination between the two solution streams, while causing minimal mechanical disruption of the ganglion surface, by altering the height of the drug and control solution reservoirs. The step distance was usually in the range of about 100μm, but could be adjusted to a minimum of 25μm if necessary. At this minimum step distance the solution changes could be completed within approximately 600μs (measured as the 10-90% response for an open recording pipette using a theta-perfusion pipette, i.e. with a flat interface between the two barrels)- see figure 2.2. However, theta perfusion pipettes were found to give unsatisfactory discrimination between the control and drug flows when recording with this preparation in the whole-cell configuration- therefore, circular double-barrelled perfusion pipettes were used in preference. Under these conditions no mixing between
Figure 2.2. The stepper motor driven perfusion apparatus enabled the use of rapid solution exchanges. The figure shows the minimum solution exchange time that could be obtained, using a theta (θ) pipette. The junction potential change when switching between low and high osmolarity salt solutions (20-200mM NaCl) was measured at an open glass recording pipette. The 10-90% rise time is approximately 600μs. Under the usual recording conditions using a double-barrelled flow pipette solution exchanges were complete in approximately 5ms. The double-barrelled flow pipette allowed better discrimination between the two solution streams than the θ pipette when applying solutions to whole cells.
the two flows was apparent, and solution exchanges were complete in less than 5ms. In
addition the whole bath was continually perfused with control solution, which was
gravity fed via a two-way Hamilton tap.

2.4. Preparation and Injection of Xenopus Oocytes.

The data for potency ratios at low concentrations for the α3β2 and α3β4
subunit combinations in Xenopus oocytes presented in Chapter 3 was obtained by Drs
Hiroshi Kojima and Lucia Sivilotti (Department of Pharmacology, University College
London) and a brief description of the experimental methods used by Dr Hiroshi
Kojima is given below.

a) Solutions for oocytes.

The solutions used for the preparation and injection of oocytes had the
following composition (in mM): nuclear injection buffer (NaCl 88, KCl 1, HEPES 15,
pH 7.0); sterile Barths' medium (NaCl 88, KCl 1, MgSO₄ 0.82, Ca(NO₃)₂ 0.33, CaCl₂
0.41, NaHCO₃ 2.4, Tris/HCl 15, penicillin 50 U/ml, streptomycin 50 U/ml, pH 7.4).
Recordings were made using a nominally calcium-free (to avoid interference by the
endogenous Ca²⁺-activated chloride conductance, see e.g. Miledi, 1982; Leonard &
Kelso, 1990) solution of the following composition (in mM): NaCl 115, MgCl₂ 1.8,
KCl 2.5, HEPES 10, and 500 mM atropine sulphate (Sigma) to block endogenous
muscarinic receptors; pH 7.2 with NaOH. Agonists (see above) were diluted in the
recording solution. With oocytes injected with α3β4 cDNA pairs the maximum
concentrations used did not usually exceed the following (in µM): Cyt. 1; Nic. 5; ACh.
6; DMPP. 1; CCh. 10; Lob. 20. Similarly for α3β2 cDNA pairs (in µM): DMPP. 3;
ACh. 3; Lob. 5; CCh. 50; Nic. 50; Cyt. 100.

b) Preparation of oocytes for injection and recording.
Cloned cDNAs suitable for direct nuclear injection were kindly supplied to us by Dr J. Patrick (Baylor College of Medicine, U.S.A.). The cDNAs were cloned into the pcDNA1neo vector and grown in *E. coli* strain MC1061/93 (as described in Luetje & Patrick, 1991). Plasmids were prepared by CsCl centrifugation and resuspended in nuclear injection buffer. Transcription is under control of a cytomegalovirus promoter in these vectors.

Mature *Xenopus laevis* were anaesthetized with 0.2% tricainemethanesulphonate (Sigma) and ovarian lobes were surgically removed (Methfessel *et al.*, 1986). The oocytes were manually dissected and stored in sterile Barth’s medium. One day after dissection, healthy oocytes at maturation stage V were used for injection. The aim was to inject into the nucleus the cDNAs that encode for the required subunit combination. A sharp bevelled pipette (tip diameter 5-10μm) was inserted into the animal pole of the oocyte, and about 10 nl of solution (cDNA concentration 0.1 mg/ml) was injected.

The follicle cell layer was removed two days after injection, to facilitate the electrophysiological measurements. This was done manually after treatment with 1mg/ml collagenase (type IA, Sigma) in Barth’s medium for 1 hr at 19°C, as described by Methfessel *et al.* (1986). Defolliculated oocytes were maintained at 19°C in Barth’s medium until used. Three days after injection the oocytes were tested for expression of nicotinic AChR by bath application of 10 μM cytisine and 20 μM DMPP for the α3β4 and α3β2 subunit combinations respectively. Oocytes giving responses of more than 200 nA (at -60 mV) were kept in Barth’s medium and used for subsequent experiments.

Oocytes were superfused continuously (at about 2-16 ml/min, chamber volume 300 μl) with the recording solution and agonists applied using a Hamilton valve to switch from control solution to agonist solution. The oocytes were exposed to agonist for up to 40 seconds. Current responses to agonist application were studied under 2-microelectrode voltage clamp (Axoclamp 2A, Axon Instruments, Burlingame, CA), at
a holding potential of -60 mV, and recorded on a chart recorder. Microelectrodes were filled with 3M KCl and had a resistance of 0.5-3.0 MΩ. Oocytes were washed for approximately 5 min between applications, which was sufficient time to allow recovery from desensitization.

PART II - METHODS EMPLOYED AT THE UNIVERSITY OF STRATHCLYDE

2.5. PREPARATION OF cDNA & cRNA

Rat subunit cDNAs under the control of the SV40 early promotor in the Flip vector (Bertrand et al., 1990) were kindly provided by Dr Robert Duvoisin (Dyson Vision Research Institute, Cornell University, New York). The other subunits were provided by Prof. S. Heinemann, Dr J. Boulter and Dr D. Johnson (Salk Institute, USA). The pGEM HE vector (Liman et al., 1992), into which the rat α7 subunit was subcloned, was originally provided by Dr Emily Liman (Harvard, USA). The following steps were carried out for each of the neuronal nicotinic receptor subunits α3, α4-1, α7, β2 and β4.

a) Growth of bacterial cultures

Transformed competent bacterial cells (E. coli strain DH5αF') containing the plasmid DNA for the relevant neuronal nAChR subunits and the ampicillin resistance gene were streaked onto agar plates (15g/l Bacto-agar) containing LB (Luria Bertani) Medium plus Ampicillin (Bacto-trypotne, 10g/l, Yeast Extract, 5g/l, NaCl, 5g/l, Ampicillin, 100μg/ml) and grown at 37°C until isolated colonies were observed. A single colony was picked, inoculated with 3 ml of LB medium + Ampicillin in Falcon 2059 tubes and grown for 12 hrs at 37°C on a shaking incubator. 1ml of this culture
media was then diluted 50% with glycerol and frozen to -80°C to make the glycerol stocks for long-term storage.

b) Plasmid preparation

First a platinum loop of the above glycerol stock was inoculated with 0.5 ml LB medium (with no ampicillin). This was grown for 1 hr at 37°C. 2.5 ml of LB medium (with 120 µg/ml ampicillin) was then added and the bacteria grown for 12 hr at 37°C on a shaking incubator. The bacteria were then streaked onto agar (plus ampicillin) plates and grown overnight at 37°C. A single colony was then inoculated in 3 ml LB medium (plus ampicillin) and grown again for 12 hr at 37°C on a shaking incubator. This was inoculated with 100 ml LB medium (plus ampicillin) in a 500 ml flask and incubated overnight (~14 hours) at 37°C on a shaking incubator. The cells were then harvested by centrifuging at x5000g for 20 min, pouring off the culture, and retaining the bacterial pellet.

Plasmid DNA was purified using the Qiagen plasmid purification procedure (Maxi protocol) based on the optimized alkaline lysis method of Birnboim & Doly (1979)- see Qiagen Plasmid Handbook. The bacterial pellet was first resuspended in 10ml of 50mM Tris-HCl/10mM EDTA (pH 8.0) with 100µg/ml RNase A, and the bacterial cells then lysed by adding 10ml of 200mM NaOH/1% SDS (sodium dodecylsulphate, a.k.a. lauryl sulphate) for 5 min at room temperature. The lysate was then neutralized by adding 10ml pre-chilled 3M potassium acetate (pH 5.5) and incubating on ice for 20 min. This solution was then centrifuged at 4°C for 30 min at ≥ 30,000×g. The supernatant was removed and re-centrifuged at 4°C for 15 min at ≥ 30,000×g and the next supernatant removed.

A Qiagen-tip 500 was equilibrated by gravity flow with 10ml QBT buffer (750mM NaCl: 50mM MOPS, pH 7.0; 15% ethanol; 0.1% Triton X-100), and this was followed by applying the supernatant mentioned above. The Qiagen-tip was then washed with 2 × 30ml QC buffer (1.0M NaCl: 50mM MOPS, pH 7.0; 15% ethanol).

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The eluents from all these stages were discarded. The DNA was then eluted from the Qiagen-tip with 15ml QF buffer (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% ethanol). The DNA could then be precipitated from the eluent by adding 10.5ml (0.7 volumes) isopropanol. This was then centrifuged at ≥15,000×g for 30 min at 4°C and the supernatant discarded leaving a glassy DNA pellet.

The DNA pellet was then washed twice to remove precipitated salt with 5ml pre-chilled 70% ethanol, centrifuging at ~12,000×g for 15 min with each wash, and rotating the tubes by 180° in the centrifuge between each wash to make sure the pellet travelled through the wash solution. The pellet was then air dried and resuspended in 250μl TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA).

The identity of the plasmids was confirmed by sequencing the 5' ends of the plasmid insert. This work was carried out by Roth Tate (Strathclyde Molecular Biology Lab) using an Applied Biosystems 273A automated DNA sequencer.

c) Linearization of plasmid DNA to obtain template DNA.

20μg of each subunit DNA was linearized in a total volume of 200μl of reaction mixture. This included: i) 20μg plasmid DNA (volume depending on concentrations obtained above), ii) 15μl of 10× reaction buffer (specific for each enzyme), ii) DEPC H2O (to give a final volume of 200μl), iv) restriction enzyme (volume adjusted depending on activity to give 10-15 Units activity per μg of plasmid. The restriction enzymes used were as follows: i) EcoRI (for α3 & α4-1 plasmid), ii) Nhel (α7), iii) HindIII (β2), iv) Xhol (β4).

The reaction mixture was incubated at 37°C for approximately 2 hours, and then put on ice. The template was then recovered using the following procedure. 2μl of 10% SDS was added (to give 0.1% in the mixture), followed by 20μl proteinase K (20mg ml stock, to give 200μg ml) to degrade RNAases and restriction enzymes. This was then incubated at 37°C for approximately 45 min.
The template mixture was then extracted twice with phenol-chloroform (1:1, pH 8.0) to remove all protein, centrifuging briefly and keeping the aqueous layer each time. It was then extracted once with chloroform/isoamyl alcohol (24:1). The remaining template/phenol-chloroform mixtures were then re-extracted with T.E. buffer, and the samples pooled.

The volume of the samples was then estimated and 0.25× this volume of 10M ammonium acetate was added. To the salt-DNA mixture, 2.5× this volume of cold absolute ethanol was then added. This mixture was then stored at -20°C for several hours to allow the DNA to precipitate, and then centrifuged at 15000×g at 4°C for 15-20 min. The supernatant was removed and the remaining pellet desalted by washing twice with 70% ethanol, centrifuging at 15,000×g at 4°C for ~20 min and discarding the supernatant each time. Finally, any remaining EtOH/ammonium acetate was driven off by centrifuging under vacuum, and the remaining DNA pellet resuspended in T.E. (to give ~1μg/μl stock, assuming 70% is re-extracted). Finally, the concentrations of the template DNA stocks were measured using the GeneQuant UV-spectrophotometer.

d) RNA transcription.

Capped RNA transcripts were synthesized from template DNA using mCAP™ mRNA Capping kits (Stratagene or Ambion). The following reagents were added in order to a 1.5ml microfuge tube at room temperature to give a final reaction volume of 50μl: i) 4μl of RNase-free DEPC-treated dH2O; ii) 10μl 5× Transcription Buffer; iii) 0.5μl RNase Block; iv) 2μl 0.7M DTT; v) 5μl mCAP analogue; vi) 3μl rNTPs; vii) 2μl (1μg) linearized template DNA; viii) 1.5μl 20U/μl RNA Polymerase (SP6 for α3, α4-1, β2; T3 for β4; T7 for α7). The mixture was briefly microfuged and incubated at 37°C for 1hr. Remaining template DNA was then removed by adding 2μl 10U/μl RNase-free DNase I, microfuging briefly, and incubating at 37°C for 10min.

Sometimes this mixture was directly injected, but usually the RNA was isolated from the reaction mixture using citrate-buffered (pH 4.5) phenol-chloroform
extraction, followed by ethanol-ammonium acetate precipitation. In all cases, estimates of RNA concentration and purity were made with re-precipitated RNA by measuring the optical density (O.D.) $260/280$ ratio using the GeneQuant UV-spectrophotometer.

2.6. Collection of *Xenopus Oocytes*

*a) Xenopus laevis anaesthesia.*

Female *Xenopus laevis* frogs were anaesthetised by placing them in a bucket containing 2g/l tricaine dissolved in 2-3 litres of tap water. The frogs were considered fully anaesthetised (usually after approx. 30 min) when they no longer presented the following behaviours:

i) **nose flare** (this is the final reflex seen before full anaesthesia and the first seen during recovery); ii) **swallow reflex**; iii) 'arm and finger movements'. The frogs usually remained anaesthetised for about one hour.

*b) Surgical removal of oocytes*

Once fully anaesthetised a small (approx. 1cm) vertical incision was made on either the left or right side of the frog’s abdomen. The incision was in two stages: (i) through the skin and then (ii) the underlying muscle layer. Part of the ovary could then be pulled out with forceps until about 3-4 lobes were exposed. These were then tied off at the base with 4-0 Ethilon monofilament nylon (Ethicon Ltd) and the tied off section cut away. The lobes of oocytes were transferred to a modified Ca$^{2+}$-free Barth’s saline solution of the following composition (in mM): NaCl, 88.0; KCl, 1.0; NaHCO$_3$, 2.4; HEPES, 15.0; MgSO$_4$·7H$_2$O, 0.82; Na$^+$Penicillin, 10μg/ml; Streptomycin Sulphate, 10μg/ml (pH 7.6 with NaOH). The knot was then pushed back into the abdomen and any excess single oocytes wiped away. The muscle layer was
then stitched together (usually 3 double-knotted stitches) using 4-0 Chromic gut (Ethicon Ltd) followed by stitching the skin in a similar manner. Any air bubbles were removed using a syringe needle, and Gentamycin Sulphate (0.1ml, 10mg/ml, Sigma) was injected into the abdomen through the incision.

c) *Xenopus laevis* recovery

The frog was allowed to recover by lying it on its back on damp paper towels in a transparent plastic container, and periodically moistening the skin by spraying with tap water. When it started to wake up it was transferred onto its belly. When fully awake (usually after about 2-3 hours) it was transferred back to a recovery tank.

Removal of oocytes was performed twice on all frogs, with a period of recovery after the first procedure until the wound had completely healed. After their second surgical procedure the frogs were sacrificed by destruction of the CNS and exsanguination while still under anaesthesia.

### 2.7. Preparation of *Xenopus* Oocytes

#### a) Selection of oocytes.

Healthy oocytes, Stages V-VI, were selected by their appearance. Stage V oocytes are the largest ones with a distinctive coloration- the animal pole is dark brown/black and the vegetal pole pale yellow/green. Stage VI oocytes are similar, but tend to have some dark pigment in the vegetal pole and a pale band around the equator. Healthy oocytes were those with clear definition between the two poles with an even coloration in each pole. They also had no tendency to burst during peeling. Any patches of discoloration, especially patches of white, in the animal pole were usually indicative of poor viability, and hence short life span for recording.
b) Collagenase treatment.

The lobes of oocytes were kept in sterile-filtered (0.2μm disposable syringe filters, Nalgene) Ca\(^{2+}\)-free Barths' saline (see above) and cut into small clumps under a dissecting microscope. Collagenase solution was made up in Ca\(^{2+}\)-free Barths' saline using ~1000U/ml collagenase (Type 1A, Sigma) and filtered (0.45μm & 0.2μm, Nalgene). Nominally Ca\(^{2+}\)-free solutions were used to prevent possible activation of any contaminating Ca\(^{2+}\)-dependent proteases.

The clumps of oocytes were transferred to 5cm Petri dishes containing ~10 ml collagenase solution and put on a rotating platform (Bellydancer) at room temperature for approx. 1 hour, or until the oocyte membranes were in a suitable condition for peeling. The oocytes were then rinsed 4 times with Ca\(^{2+}\)-free Barths' saline and transferred to Ca\(^{2+}\)-containing Barths' saline for peeling. Ca\(^{2+}\)-containing Barths' saline had the following composition (in mM): NaCl, 88.0; KCl, 1.0; NaHCO\(_3\), 2.4; HEPES, 15.0; Ca(NO\(_3\))\(_2\)•4H\(_2\)O, 0.30; CaCl\(_2\)•6H\(_2\)O, 0.41; MgSO\(_4\)•7H\(_2\)O, 0.82; Na\(^+\)Penicillin, 10μg/ml; Streptomycin Sulphate, 10μg/ml (pH 7.6 with NaOH).

c) Peeling of oocytes

Oocytes were peeled of their somatic cell layers with fine forceps under a dissecting microscope in Ca\(^{2+}\)-Barths' saline in 15cm Petri dishes. The oocytes were then ready for injection. This was usually done immediately after peeling, although occasionally oocytes were stored overnight at 4°C in Ca\(^{2+}\)-Barths' saline before injection.

2.8. Injection of DNA & RNA

Either 3-20ng (total for whichever subunit combination) of rat nicotinic AChR subunit RNAs, or 2-5ng of the cDNA insert in a Flip Cat (SV40) vector, in TE were
injected into defolliculated *Xenopus* oocytes. α:β subunit combinations were injected in a 1:1 ratio for DNA, and approximately 1:1.5 for RNA.

*a) Injection Pipettes.*

Pipettes for the injection of cDNA and RNA were pulled from 3.5" Drummond injection glass (#3-00-203-G/X, Drummond Scientific, U.S.A.) in two stages on a Flaming/Brown micropipette puller (Model p-87, Sutter Instrument Co., U.S.A.). Pipettes were pulled to an external tip diameter of approx. 3-5µm, and then broken back manually on the element of an Narashige (MF-83) microforge electrode polisher to a final tip diameter of ~10-15µm for nuclear injection of cDNA, or ~15-20µm for RNA injection. Only pipettes that broke cleanly with a bevelled tip were selected, and for RNA pipettes RNase-free conditions were used. The un-pulled ends of the injection pipettes were fire polished to prevent fragments of broken glass interfering with the seal on the injection apparatus and damaging the seal gaskets.

*b) Injection of cDNA.*

Injection pipettes were first loaded with Flourinert (FC-40, Sigma) non-compressible, water immiscible solvent using an automatic oocyte injector (Drummond Nanoject) mounted on a Narishige micromanipulator. Approx. 1-2µl of cDNA in injection buffer was deposited on a strip of Parafilm under a dissecting microscope and loaded into injection pipettes. Oocytes were arranged on a plastic mesh grid under Ca²⁺-Barths' saline with their animal poles at right angles to the axis of the incoming injection pipette. As the nucleus is not visible, it was assumed that it was located in a central position approximately one third of the way down from the animal pole in the vertical axis of the oocyte. The pipette was therefore inserted 'blind' into this approximate area and 20nl injected into each oocyte, giving around 5 seconds for the solution to leave the pipette. The pipette was then withdrawn and the
ooocyte given time to recover. Oocytes were then transferred to fresh Ca$^{2+}$-Barths' saline and stored in an incubator at 19.5°C.

c) Injection of RNA.

After removal from storage in DEPC-treated water at -80°C the RNA was stored on ice. Before injection it was heated to 65°C on a heating block (DB-2P Dri-Block, Techne Ltd) for 3 min, centrifuged (MSE Micro Centaur) at 1200 rpm for 5 min, then returned to ice. The purpose of the heating was to separate any RNA that had self-hybridised, and the centrifugation was to bring down any condensation that may have formed during the heating process to the RNA solution.

The RNA (2-3μl in TE buffer) was deposited onto a strip of Parafilm using RNase-free Gilson tips, and loaded into injection pipettes as described above. Oocytes were arranged under Ca$^{2+}$-Barths' saline on a nylon mesh grid in any orientation (preferably animal pole up, as oocytes appeared to seal up better afterwards when injected in this pole). The injection pipette was inserted until it just penetrated the membrane and ~50-70nl RNA solution injected into each oocyte. Oocytes were then left to recover and stored as above.


Electrophysiological recording usually took place 2-6 days after oocytes were injected with cDNA or RNA, although occasionally it was possible to obtain good quality recordings up to 14 days after injection. Two-electrode voltage-clamp recordings were made using a Geneclamp 500 voltage and patch clamp amplifier (Axon Instruments). Recording electrodes were pulled from thin-walled borosilicate glass (Clark Electromedical GC150TF-7.5) in two stages on a Flaming/Brown micropipette puller (Model p-87, Sutter Instrument Co., U.S.A.). Voltage electrodes were filled with 3M KCl and current electrodes filled with 0.25M CsCl, 0.25M CsF
and 100mM EGTA (pH 7.2 with CsOH). Recording electrodes were usually pulled to the resistance required for the voltage electrode (2-5 MΩ), and a suitable resistance for the current electrode (0.5-2 MΩ) was usually obtained by breaking the pipette back on the bottom of the recording chamber.

Oocytes were placed in a groove at the bottom of a narrow Perspex chamber (designed by Dr John Connolly) with a volume of 0.5ml, and after voltage pipette impalation were continuously perfused with a modified Ringer solution of the following composition (in mM): NaCl, 115.0; KCl, 2.5; HEPES, 10.0; BaCl₂, 1.8 (pH 7.2 with NaOH), and 1μM atropine sulphate. After current pipette impalation, clamp gain and stability were monitored on a digital storage oscilloscope and adjusted for optimum clamp speed and capacity (Geneclamp 500 ‘Gain’ and ‘Stability’ controls). In this bath oocytes could be perfused at up to 30ml/min if required. Oocytes were usually voltage clamped at -60mV and typically had holding currents in the region of -50nA. Responses were recorded on heat-sensitive paper using a Gould TA240 EasyGraf™ chart recorder. Where possible (e.g. when the desensitization rate was not too fast) the magnitude of the current responses was noted directly from the Geneclamp LED current/voltage meters- otherwise the responses were measured by hand from the chart-recorder traces.
CHAPTER 3:

COMPARISON OF NEURONAL NICOTINIC RECEPTORS IN RAT SYMPATHETIC NEURONES WITH SUBUNIT PAIRS EXPRESSED IN XENOPUS OOCYTES.

3.1. INTRODUCTION

Several subunit types for neuronal nicotinic acetylcholine receptors (neuronal AChRs) have now been cloned (e.g. Boulter et al., 1986; for review see Deneris et al., 1991, McGehee & Role, 1995) with a total of eight $\alpha$ and three $\beta$ subunits so far, $\alpha_8$ having as yet been found only in the chick (Schoepfer et al., 1990) and $\alpha_9$ only in the rat (Elgoyhen et al., 1995). The term $\alpha$ refers to those subunits containing a pair of cysteine residues (analogous to Cys 192-193 of the Torpedo $\alpha$) in the agonist binding region of the receptor, those without it being termed $\beta$. The mRNAs encoding these subunits have distinct patterns of expression in different parts of the nervous system (e.g. Wada et al., 1989; Séguela et al., 1993; Dineley-Miller & Patrick, 1992; Elgoyhen et al., 1994), but it is not known to what extent this correlates with the functional diversity among native receptors.

The subunit structure of the Torpedo nicotinic acetylcholine receptor is now well understood and the muscle receptor is probably the same. It is a pentamer of subunits termed $\alpha$, $\beta$, $\gamma$ and $\delta$ arranged around a central ion pore in the stoichiometry 2:1:1:1 (for review see Karlin, 1993) with the $\gamma$ subunit (in muscle) switching to the $\varepsilon$ subunit during development (Mishina et al., 1986). The structure of native neuronal AChRs is, however, still largely unknown. Studies in oocytes reveal that, unlike the muscle nicotinic AChR, expression of $\alpha/\beta$ subunit pairs is usually sufficient to form functional receptors (Boulter et al., 1987; Duvoisin et al., 1989), although in native receptors the subunit composition may be more complex (e.g. Listerud et al., 1991; Conroy et al., 1992; Vernallis et al., 1993; Conroy & Berg, 1995). However, not all neuronal subunits appear to be functional when expressed in this manner (e.g. $\alpha_5$ and

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β3 in all combinations tested so far) (Boulter et al., 1990) and some form functional receptors homomerically (α7, α8, α9) (Couturier et al., 1990; Séguéla et al., 1993; Gerzanich et al., 1994; Elgoyen et al., 1995). It is believed that, like the muscle receptor, nicotinic receptors on neurones are also pentamers containing at least two α subunits (Anand et al., 1991; Cooper et al., 1991).

Rat superior cervical ganglion (SCG) neurones express messenger RNAs for the α3, α7, β2 and β4 subunits of the nicotinic acetylcholine receptor, with conflicting information concerning the presence of α4-1 and α5 mRNAs (Klimaschewski et al., 1994; Mandelzys et al., 1994; Rust et al., 1994; Zoli et al., 1995; see Ch. I ), but it is not known which of these are involved in forming the native functional receptors. The receptors in these neurones have been extensively studied and show a number of functional as well as structural differences from those found in muscle (e.g. Paton & Zamis, 1951; for reviews see e.g. Colquhoun et al., 1987; McGeehee & Role, 1995).

One way to characterize receptor subtypes is by measuring the relative potencies of a series of agonists; for example cytisine is a potent agonist, relative to acetylcholine, for α3β4 receptors, but very weak for α3β2 receptors (Luetje & Patrick, 1991, and present work). In order to cast light on the nature of the native receptors in rat sympathetic neurones, it was decided to make parallel measurements, under comparable conditions, of agonist potency ratios on (a) neurones of the rat superior cervical ganglion, and (b) rat nicotinic AChRs expressed in *Xenopus laevis* oocytes using α3β2 or α3β4 subunit pairs. These two pairs would appear to be a good choice for comparison with the rat SCG as the α3 and β4 mRNAs have the highest level of expression in this tissue (Mandelzys et al., 1994; Rust et al., 1994; etc.). Also, at the time that this study was being undertaken these were the only three subunit mRNAs to have been detected in the rat SCG (Mandelzys et al., 1991).

An additional benefit of these studies is to attempt to validate the usefulness of nicotinic receptors expressed in oocytes as pharmacological models for the investigation of action of therapeutic and addictive drugs.
3.2. Specific Methods

a) Drug application protocol

Drugs were applied approximately every 3 mins for a duration of approximately 40 s using a stepper-motor-driven perfusion system (see General Methods, Chapter 2). This was sufficient time to allow complete recovery from desensitization. The amplitudes of the whole cell currents were measured from the pen recorder traces. The agonist-induced inward current amplitude increased with time in the majority of recordings, and never decreased. The extent of any whole-cell 'run-up' was calculated for each recording from response versus time plots for a drug standard (usually 10μM ACh) which constituted every third drug application. The extent of the 'run-up' at any time during the recording could thus be estimated and current amplitudes corrected by linear interpolation. Due to this procedure, the actual magnitude of some of the whole-cell responses may differ from the corrected amplitudes used for final analysis (e.g. for the fitted data in figures 2c & d).

For oocyte drug application protocols see Ch. 2, General Methods, Part I.

b) Analysis of Potency Ratios

Potency ratios were first estimated in individual cells. Then the data from all the separate results was pooled to give an average potency ratio. Initial attempts to normalize the responses from different cells via the response to a standard dose of agonist were not very effective.

Extensive desensitization at high agonist concentration makes full dose-response curves misleading, so the aim was to find the low-concentration limit of the relative potency of pairs of agonists. Low concentrations of agonist that gave responses well below the maximum were therefore used. All the curves from a single cell were fitted simultaneously by weighted least-squares with Hill equations that were
constrained to be parallel (i.e. they all had the same Hill slope). The parameters that were estimated during fitting were: $K_{app}$ (for one of the curves, the ‘standard’), $n_{II}$ (the common Hill coefficient), and potency ratios for each of the other curves, relative to the ‘standard’. The maximum response was fixed at a value much larger than any observed response, and the fit was weighted by assuming that the coefficient of variation (CV) was the same for each response (in all cases CV=0.2). This procedure amounts to fitting curves that are linear, constrained to be parallel, and have constant errors when plotted on double-logarithmic co-ordinates (see for example figure 3.6.). The adequacy of the parallel simultaneous fit was judged by eye, and by performing a variance ratio test on the decrease in the sum of squared deviations when each curve was fitted separately with its own $n_{II}$ value. Potency ratios so estimated were averaged and converted to a common standard (ACh). Errors for the ratios were estimated by Fieller’s theorem (e.g. Colquhoun, 1971).

### 3.3. Results

#### 3.2.1. Agonist-Induced Current Responses

**a) SCG neurones**

Figure 3.1.c shows typical inward current responses of a single SCG neurone to the nicotinic agonists ACh, DMPP and cytisine. The average peak response to 10μM ACh in SCG neurones clamped at -60 mV was 113 ± 24 pA (n=12). This value applies to the first application of 10μM ACh as subsequent applications tended to result in larger current responses due to the effects of ‘run-up’ (see below). 10μM was the highest concentration of ACh used on SCG neurones in these experiments and was usually the concentration at which a component of desensitization started to appear with this agonist. No significant desensitization was observed with any of the other agonists up to the highest concentrations used, even though the magnitude of the
(a). Oocyte: α3-β2

![Image of currents for α3-β2 oocytes]

(b). Oocyte: α3-β4

![Image of currents for α3-β4 oocytes]

(c). SCG neurone

![Image of currents for SCG neurone]

Figure 3.1. Examples of whole-cell responses in oocytes and rat SCG neurones. Inward current responses to bath applications of the nicotinic agonists ACh, DMPP and cytisine in: (a) a single oocyte with an α3-β2 cDNA pair (DMPP is clearly the most potent agonist), and (b) a single oocyte injected with an α3-β4 cDNA pair (cytisine is clearly the most potent agonist). Both oocytes were voltage clamped at -60 mV, and the traces were obtained directly from the chart recorder. (c) Inward current responses to stepped flow-pipe applications of the same agonists in a single neurone from an undissociated rat SCG: cytisine is clearly the most potent agonist. The neurone was voltage clamped at -60 mV. Traces were replayed from FM tape, filtered at 3 Hz, and sampled at 200 Hz.
Figures 3.2. Examples of whole-cell responses from rat SCG neurones used to construct low concentration-response plots. Inward current responses to stepped flow-pipe applications of several low concentrations of the nicotinic agonists ACh and cytisine. Recordings are from a single neurone in an undissociated rat SCG. The neurone was voltage clamped at -60 mV. Responses from individual cells such as these were used to plot the log-log concentration response plots (e.g. Figure 3.6). Values for potency ratios relative to ACh could then be calculated for the responses from each cell and the data pooled and meaned. Cytisine responses were generally slower to reach peak activation than ACh responses.
Figures 3.3. Examples of whole-cell responses from rat SCG neurones used to construct low concentration-response plots. Inward current responses to stepped flow-pipe applications of several low concentrations of the nicotinic agonists ACh and nicotine. Recordings are from a single neurone in an undissociated rat SCG. The neurone was voltage clamped at -60 mV. Responses from individual cells such as these were used to plot the log-log concentration response plots (e.g. Figure 3.4). Values for potency ratios relative to ACh could then be calculated for the responses from each cell and the data pooled and averaged. Nicotine responses were usually the slowest to reach peak levels of activation, whereas ACh responses were generally the fastest.
response may have been larger than that of 10μM ACh. The time to reach peak response was shortest for ACh (<1 s at 10μM) and longest for nicotine (up to 20 s at 5 μM) with solution exchanges complete in around 5 ms.

Figures 3.2 and 3.3 show the data from two individual SCG neurones. The examples here show one cell to which ACh and cytisine were applied, and another with ACh and nicotine. In each case several low concentrations of the two different agonists were applied in random order with a standard 10μM ACh constituting every third drug application in order to monitor the stability of the recording. Responses from individual cells such as these were used to plot the log-log concentration response plots (e.g. Figure 3.6). Values for potency ratios relative to ACh could then be calculated for the responses from each cell and the data pooled and meaned.

b) Nicotinic current responses in SCG neurones exhibit 'run-up'.

The agonist-induced current responses recorded in SCG neurones had a tendency to increase in magnitude with time for any given concentration of agonist. This phenomenon has also been observed for nicotinic responses in rat medial habenula neurones (Lester & Dani, 1995). This effect necessitated the use of a repeated standard during the course of a recording, so that responses could be corrected for "run-up" by linear interpolation. Figs 3.4 shows some examples of 10μM acetylcholine responses from a single SCG neurone exhibiting this phenomenon. Note that the increase in peak response was accompanied by an increase in the rate of desensitization, and hence a decrease in the residual current after equal durations of agonist application. An extreme example of this run-up was recorded in a single neurone with the agonists cytisine and DMPP and is presented as a response vs time plot in figure 3.5. Thus, this effect appears to be independent of the agonist used. The time course in this example appears to be linear, but in most cells this was not the case. However, the concomitant increase in desensitization was never as apparent with agonists other than ACh. This might be expected, as these agonists do not exhibit
Figure 3.4. Nicotinic agonist-induced responses in SCG neurones exhibit 'run-up' and time-dependent increases in desensitization. The responses recorded in SCG neurones had a tendency to increase in magnitude with time for any given concentration of agonist. This ran parallel to an increase in the rate of desensitization. In this example agonists were applied 28.5 minutes after establishing the whole-cell recording (to allow the baseline to stabilize). After 76.5 minutes the peak response to 10μM ACh had increased from 250 pA to 285 pA (an increase of 14%). The time constant (τ) for the rate of desensitization has decreased from 19.7 s to 15.0 s. However, this phenomenon was not observed in all cells, although the magnitude of the effect was much greater in other cells (e.g. Figure 3.5.).
Figure 3.5. 'Run-up' can be extensive in some neurones. An extreme example of 'run-up' was recorded in a single neurone with the agonists cytisine and DMPP and is presented here as a response vs time plot. This effect appears to be independent of the agonist used, although in this example they do run up at slightly different rates. The DMPP response increases at approximately 5.7% per minute, whereas the increase in cytisine response is 4.7% per minute. The time course in this example appears to be linear, but in most cells this was not the case. However, the concomitant increase in desensitization was never as apparent with these agonists, as they tended not to exhibit much acute desensitization at low concentrations.
much of a component of desensitization at the low concentrations used for these experiments.

This phenomenon has now been described in some detail in rat dissociated medial habenular neurones (Lester & Dani, 1995; Lester & Dani, in press), and PC12 cells (Ifune & Steinbach, 1993), and has also been noted in bovine chromaffin cells (Nooney & Feltz, 1995). In medial habenular neurones 'run-up' is specific to nAChRs, associated with a loss of Ca\textsuperscript{2+} sensitivity (i.e. the degree of potentiation it causes) and may be regulated by internal ATP (Lester & Dani, in press).

c) Oocytes injected with cDNA pairs

Fig.3.1a. shows typical inward current responses of a single oocyte injected with the \( \alpha3\beta2 \) cDNA combination to the nicotinic agonists ACh, DMPP and cytisine. The average peak response to 1\( \mu \)M DMPP in \( \alpha3\beta2 \)-injected oocytes clamped at -60 mV was 24.6 ± 3.4 nA (n=9). Fig.3.1b. shows typical responses of a single oocyte injected with an \( \alpha3\beta4 \) cDNA pair to the same three agonists. The average peak response to 1\( \mu \)M cytisine in \( \alpha3\beta4 \)-injected oocytes clamped at -60 mV was 255.6 ± 91.7 nA (n=8). These mean current responses may appear smaller than those usually quoted for neuronal nicotinic AChRs expressed in oocytes, but the absence of Ca\textsuperscript{2+} from the extracellular medium is likely to result in smaller responses (see below for reasons). For responses in oocytes injected with the \( \alpha3\beta2 \) combination a desensitizing component was often observed, though at the low concentrations used it tended not to be very marked. Such a component was not observed in \( \alpha3\beta4 \)-injected oocytes. This effect has previously been reported by Cachelin & Jaggi (1991), where it appears to be a more consistent observation, but it should be noted that they used a much larger concentration range (into the mM range) and the presence of extracellular Ca\textsuperscript{2+} may contribute to this component by activating chloride currents (see below, and Miledi, 1982; Leonard & Kelso, 1990).
3.3.2. DOSE-RESPONSE CURVES

a) SCG neurones

Figs 3.6, 3.7 & 3.8 show three typical sets of data obtained from three separate SCG neurones. For the cell in Fig.3.6, the rank order of potency is: Cyt > DMPP > ACh, and the calculated potency ratios (relative to ACh) are: Cyt = 6.66, DMPP = 2.66. For the cell in Fig.3.7, the rank order of potency is: ACh > CCh > Lob, and the calculated potency ratios (relative to ACh) are: CCh = 0.32, Lob = 0.068. It was usually only possible to obtain single points for the agonist lobeline. This drug was slow to wash off and inhibited subsequent agonist responses. It was, therefore applied last to the cell. Figure 3.8. shows the fits for a cell to which the agonists ACh and nicotine were applied. The agonist nicotine can be seen to be more potent than ACh with a potency ratio of 1.82. In these three examples the separate fits for each agonist (i.e. where the dose-response curves are not constrained to be parallel) can be seen as dashed lines.

It is apparent from Fig.3.6. that DMPP gives a steeper Hill slope estimate than the other two agonists - in this particular case $n_{H1}$ for ACh = 1.84, $n_{H1}$ for Cyt = 1.83, $n_{H1}$ for DMPP = 2.69. It was a consistent observation that DMPP gave a steeper Hill slope estimate than any other nicotinic agonists applied to the same SCG neurone, but no corresponding difference was observed in oocytes injected with either the $\alpha3\beta2$ or cDNA pairs.

In some cells the concentration ranges of some of the agonists were extended to include higher concentrations. The pooled data for the agonists ACh, DMPP and Cytisine are presented as concentration-response relationships in Figure 3.9. Again these plots reveal the steeper Hill slope obtained with the agonist DMPP ($n_{H1}= 2.50$), whereas the ACh and cytisine plots are almost parallel ($n_{H1}= 1.71$ and 1.79 respectively). The maxima on these curves were not very well defined as the original aim of this study was to use low concentrations. However, the EC$_{50}$ estimates based on
Figure 3.6. Example of fitted dose-response curves for sets of whole-cell responses from a single SCG neurones. Data sets containing the whole-cell responses from each individual cell were fitted separately to form a set of parallel dose-response curves (see Methods) from which the relative potency ratios were obtained. In this example the agonists ACh (the standard for all cells), DMPP and cytisine were used. The rank order of potency is: Cyt > DMPP > ACh, and the calculated potency ratios (relative to ACh) are: Cyt = 6.66, DMPP = 2.66. Separate fits for each agonist (where the dose-response curves are not constrained to be parallel) are shown as dashed lines. DMPP gives a steeper Hill slope estimate than the other two agonists- in this particular case $n_H$ for ACh = 1.84, $n_H$ for Cyt = 1.83 and $n_H$ for DMPP = 2.69.
Figure 3.7. Example of fitted dose-response curves for sets of whole-cell responses from a single SCG neurones. Data sets containing the whole-cell responses from each individual cell were fitted separately to form a set of parallel dose-response curves (see Methods) from which the relative potency ratios were obtained. In this example the agonists ACh (the standard for all cells), carbachol and lobeline were used. The rank order of potency is: ACh > CCh > Lob, and the calculated potency ratios (relative to ACh) are: CCh = 0.32 and Lob = 0.068. It was usually only possible to obtain single points for the agonist lobeline. This drug was very difficult to wash off, and inhibited subsequent agonist responses. It was, therefore applied last to the cell. Separate fits for each agonist (where the dose-response curves are not constrained to be parallel) are shown as dashed lines.
Figure 3.8. Example of fitted dose-response curves for sets of whole-cell responses from a single SCG neurone. Data sets containing the whole-cell responses from each individual cell were fitted separately to form a set of parallel dose-response curves (see Methods) from which the relative potency ratios were obtained. In this example the agonists ACh (the standard for all cells), and nicotine were used. The data was obtained from the responses shown in Figure 3.3. Nicotine is more potent than ACh with a potency ratio of 1.82. Separate fits for each agonist (where the dose-response curves are not constrained to be parallel) are shown as dashed lines.
Figure 3.9. In some cells the concentration ranges of some of the agonists were extended to include higher concentrations. The pooled data for the agonists ACh, DMPP and Cytisine are presented as concentration-response relationships in (a) and its log-log transform in (b). Again these plots reveal the steeper Hill slope obtained with the agonist DMPP ($n_H = 2.50$), whereas the ACh and cytisine plots are almost parallel ($n_H = 1.71$ and 1.79 respectively). The maxima on these curves were not well defined: the original aim of this study was to use low concentrations. However, the EC$_{50}$ estimates based on these crude curves were as follows: EC$_{50}$(ACh) = 48.3 μM; EC$_{50}$(DMPP) = 12.0 μM; EC$_{50}$(Cytisine) = 6.3 μM. Error bars represent the actual values of S.E.M. (n=3-12, except 2μM ACh & 30μM DMPP, n=2). Fits were weighted to $1/variance$ obtained from the 'smoothed' standard deviations.
Figure 3.10. Example of fitted dose-response curves for sets of agonist responses from single oocytes. Data sets containing the agonist responses from each individual 2-electrode voltage clamped cells were fitted separately to form a set of parallel dose-response curves (see Methods) from which the relative potency ratios were obtained for comparison with SCG neurones. (a) shows data from a single oocyte injected with an α3β2 cDNA pair using the agonists ACh, DMPP, Nicotine, Carbachol and Cytisine. In this example the rank order of potency is DMPP > ACh > Nic > CCh > Cyt, and the calculated potency ratios (relative to ACh) are: DMPP = 1.54, Nic = 0.025, CCh = 0.024, Cyt = 0.005. (b) shows data from a single oocyte injected with an α3β4 cDNA pair using the agonists ACh, cytisine, nicotine and DMPP. In this example the rank order of potency is: Cyt > ACh >> Nic > DMPP, and the calculated potency ratios (relative to ACh) are: Cyt = 5.12, Nic = 1.01, DMPP = 0.45.
these crude curves were as follows: \( EC_{50}(ACh) = 48.3 \text{ M} \); \( EC_{50}(DMPP) = 12.0 \text{ M} \); \( EC_{50}(Cytisine) = 6.3 \text{ M} \). Interestingly, the value of 48.3 \( \mu \text{M} \) obtained for ACh is similar to that obtained with this agonist for the higher affinity component with the \( \alpha3\beta4 \) subunit combination (i.e. 39.0 \( \mu \text{M} \), see Ch. IV).

b) Oocytes injected with cDNA pairs

Fig.3.10a shows a typical set of data using nicotinic agonist responses from a single oocyte injected with an \( \alpha3\beta2 \) cDNA pair. The data has been fitted with the Hill equation as described in the methods to obtain a set of dose-response curves constrained to be parallel. For the particular agonists applied to this oocyte a clear rank order of potency can be seen - DMPP > ACh > Nic >> CCh > Cyt. In this example the calculated potency ratios (relative to ACh) are: DMPP = 1.54, Nic = 0.025, CCh = 0.024, Cyt = 0.005. Fig.3.10b. shows a similar set of data from a single oocyte injected with an \( \alpha3\beta4 \) cDNA pair. In this example the rank order of potency is: Cyt > ACh >> Nic > DMPP. The calculated potency ratios (relative to ACh) are: Cyt = 5.12, Nic = 1.01, DMPP = 0.45.

3.3.3. Potency Ratios

Table 3.1. shows the final mean potency ratios calculated for both the native and the cloned receptors. It is clear from this table that in rat SCG neurones cytisine is the most potent of the agonists tested here, being almost five times as potent as ACh. DMPP and nicotine are also potent agonists when compared to ACh. For SCG neurones the complete rank order of potency is Cyt > DMPP > Nic > ACh > CCh > Lob. For oocytes injected with \( \alpha3\beta4 \) cDNA pairs the rank order of potency is Cyt > Nic > ACh > DMPP > CCh > Lob. Similarly for oocytes injected with \( \alpha3\beta2 \) cDNA pairs the rank order of potency is DMPP > ACh > Lob > CCh > Nic > Cyt. There is clearly a marked difference between the rank orders of potency obtained with these
Table 3.1. Potency ratios for nicotinic agonists in oocytes and rat SCG neurones. The relative potency ratios calculated individually from each oocyte and neurone were pooled and expressed as the mean potency ratio relative to ACh (± Standard Error).

<table>
<thead>
<tr>
<th></th>
<th>SCG neurone (n=3-4)</th>
<th>Oocyte α3β4 (n=4-7)</th>
<th>Oocyte α3β2 (n=7-21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytisine</td>
<td>4.76 ± 0.70</td>
<td>5.06 ± 0.76</td>
<td>0.020 ± 0.012</td>
</tr>
<tr>
<td>DMPP</td>
<td>2.50 ± 0.24</td>
<td>0.43 ± 0.08</td>
<td>1.62 ± 0.49</td>
</tr>
<tr>
<td>Nicotine</td>
<td>1.98 ± 0.13</td>
<td>1.10 ± 0.31</td>
<td>0.038 ± 0.012</td>
</tr>
<tr>
<td>Carbachol</td>
<td>0.361 ± 0.022</td>
<td>0.25 ± 0.04</td>
<td>0.068 ± 0.028</td>
</tr>
<tr>
<td>Lobeline</td>
<td>0.066 ± 0.014</td>
<td>0.18 ± 0.04</td>
<td>0.207 ± 0.081</td>
</tr>
<tr>
<td></td>
<td>SCG neurone</td>
<td>Oocyte α3β4</td>
<td>Oocyte α3β2</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ACh</td>
<td>1.62 ± 0.06 (12)</td>
<td>1.70 ± 0.08 (6)</td>
<td>1.13 ± 0.07 (5)</td>
</tr>
<tr>
<td>Cytisine</td>
<td>1.75 ± 0.08 (3)</td>
<td>1.97 ± 0.21 (4)</td>
<td>0.95 (1)</td>
</tr>
<tr>
<td>DMPP</td>
<td>2.65 ± 0.04 (3)</td>
<td>1.33 ± 0.16 (4)</td>
<td>1.25 ± 0.09 (21)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>1.55 ± 0.12 (4)</td>
<td>1.48 ± 0.09 (4)</td>
<td>0.82 ± 0.13 (5)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>1.85 ± 0.08 (3)</td>
<td>1.66 ± 0.20 (4)</td>
<td>0.87 ± 0.14 (5)</td>
</tr>
<tr>
<td>Lobeline</td>
<td>NA</td>
<td>1.37 ± 0.20 (5)</td>
<td>1.59 ± 0.28 (7)</td>
</tr>
</tbody>
</table>

**Table 3.2.** Hill slope estimates for dose-response curves from oocytes and rat SCG neurones. Mean Hill slope ± Standard Error (the number of cells used to calculate each mean is given in parentheses). The number of cells is lower for the very weak agonists as it was not always possible to test enough concentrations to get a good estimate. In SCG neurones a steeper Hill slope estimate was consistently obtained with DMPP.
two cDNA pairs, the most striking being cytisine which is the most potent agonist with α3β4 cDNA pairs, but the weakest agonist with α3β2 cDNA pairs.

Thus the rank order of the potency ratios in SCG neurones does not exactly match those obtained with either of the cDNA pairs, but it does match that of the α3β4 cDNA pair with only one agonist in a different position (i.e. DMPP is more potent than ACh with SCG neurones, but less potent with the α3β4 cDNA pair). Closer inspection of the actual values of the relative potency ratios shows that the values for cytisine are almost identical for SCG neurones and oocytes injected with α3β4 cDNA pairs (i.e. 4.76 ± 0.70 & 5.06 ± 0.76 respectively, but only 0.020 ± 0.012 with the α3β2 pair). Similarly the value for nicotine with the SCG neurones is close to that obtained with the α3β4 cDNA pair (i.e. 1.98 ± 0.13 & 1.10 ± 0.31 respectively, but only 0.038 ± 0.012 with the α3β2 pair). This is also the case with carbachol (0.361 ± 0.022 & 0.25 ± 0.04 respectively, but only 0.068 ± 0.028 with the α3β2 pair). However, the value for DMPP in SCG neurones is similar to that in oocytes injected with α3β2 cDNA pairs (i.e. 2.50 ± 0.24 & 1.62 ± 0.49 respectively, but only 0.43 ± 0.08 with the α3β4 pair). Lobeline gives similar values for both α3β4 and α3β2 cDNA pairs (i.e. 0.18 ± 0.04 & 0.207 ± 0.081 respectively), but has a lower potency in SCG neurones (i.e. 0.066 ± 0.014). However, it was usually not possible to apply more than one concentration of lobeline to an SCG neurone, as it was very slow to wash off and inhibited subsequent agonist applications, precluding further use of the recording.

3.3.4. Hill slope estimates

The steeper Hill slope estimates encountered with DMPP in the SCG neurones (see Table 3.2.) do cast some doubt on the accuracy of the DMPP potency ratio value when calculated using parallel fits. However, this would probably not be sufficient to account for such a large discrepancy, if in fact we were looking at just a single receptor subtype with a simple subunit combination in the rat. Studies using voltage recording in chick cultured skeletal muscle fibres showed that DMPP had a much
steeper concentration response relationship than all the other nicotinic agonists tested (which all gave similar slopes) (Harvey & Dryden, 1974), but no other precedents for this behaviour can be found. The reasons for the steeper Hill slope in rat ganglia obtained with DMPP are unclear, as factors such as desensitization or receptor heterogeneity will tend to decrease the Hill slope. Specific DMPP evoked neurotransmitter release via a presynaptic receptor is unlikely to be involved due to the presence of TTX and the absence of external Ca\textsuperscript{2+}. However, there is evidence for TTX-insensitive nicotinic agonist-induced neurotransmitter release (Marshall et al., 1996; Marshall et al., 1997), and there is the possibility depolarization-induced activation of voltage-sensitive Ca\textsuperscript{2+} channels (e.g. Soliakov & Wonnacott, 1996), although in these external Ca\textsuperscript{2+}-free conditions they would have to be on internal stores.

A Hill slope estimate greater than two might suggest that DMPP is activating a population of receptors with three agonist binding sites. If this was the case then we would have a heterogeneous receptor population which would tend to decrease the Hill slope. However, we are only looking at the very bottom of the concentration-response relationship, so if DMPP had a significantly different affinity for the other receptor(s) then an overall decrease in Hill slope might not be observed.

Our Hill slope estimates in oocytes (see Table 3.2) are somewhat lower than those reported by other authors - i.e. for α3β2, \( n_{II}(ACh) = 1.13, n_{II}(DMPP) = 1.25 \) and for \( n_{II}(ACh) = 1.70, n_{II}(DMPP) = 1.33 \). For example, Cachelin & Jaggi, (1991) report for rat α3β2, \( n_{II}(ACh) = 1.4, n_{II}(DMPP) = 1.7 \) and for rat, \( n_{II}(ACh) = 1.8, n_{II}(DMPP) = 1.8 \) (see also Gross et al., 1991, chick; Connolly et al., 1992). The lower the agonist concentrations, the better the estimate of the Hill slope due to minimal desensitization, and any factor that enhances the response of the oocyte to a nicotinic agonist will allow the use of lower concentrations. Steeper Hill slope estimates could therefore be due to higher levels of receptor expression or due to the presence of Ca\textsuperscript{2+} in the extracellular medium, as used by most authors (including those listed above). Extracellular Ca\textsuperscript{2+} increases whole-cell agonist responses in neuronal nicotinic AChRs.
(with 1.8 mM Ca$^{2+}$ almost doubling the response recorded in 0.18 mM Ca$^{2+}$; Vernino et al., 1992), and in outside-out patches it increases the frequency of channel opening (Mulle et al., 1992b) although this is associated with a decrease in the single channel conductance at negative potentials.

Another problem encountered when using extracellular Ca$^{2+}$ is that neuronal nicotinic AChRs have a significant Ca$^{2+}$ permeability (Vemino et al., 1992; Mulle et al., 1992a). Ca$^{2+}$ influx will activate the $I_{Cl(Ca)}$ (calcium-activated chloride conductance) (Miledi, 1982; Leonard & Kelso, 1990) in the oocyte (which is inward under the these recording conditions) and therefore amplify the response to nicotinic agonists resulting in an increased Hill slope. The use of Ca$^{2+}$-activated Cl$^{-}$ channel blockers such as flufenamic acid and niflumic acid is inadvisable, as at the concentrations usually used for this purpose these agents have direct effects on nicotinic channels, causing an inhibition of $\alpha3\beta2$ and potentiation of $\alpha3\beta4$ agonist-induced current responses recorded in Xenopus oocytes (Zwart et al., 1995). Recent studies have also demonstrated the presence of a Ca$^{2+}$-activated Cl$^{-}$ current in both rat and mouse sympathetic ganglion cells (Sanchez-Vives et al., 1994; De Castro et al., 1997). However, in rat ganglia this only appears several days after axotomy (Sanchez-Vives et al., 1994). Similarly, Ca$^{2+}$ influx through nAChRs in rat habenula neurones is sufficient to directly activate a Ca$^{2+}$-dependent Cl$^{-}$ conductance (Mulle et al., 1993). We have used nominally Ca$^{2+}$-free extracellular media in order to allow a direct comparison of oocytes with the SCG neurones.

3.4. DISCUSSION

The results of this study reveal that the nicotinic receptors in rat SCG neurones possess an agonist profile that is similar to that of the $\alpha3\beta4$ receptor subunit combination (see especially the high potencies of cytisine and, to a lesser extent, nicotine). However, the relatively high potency of DMPP in the SCG neurones, which is shown to be diagnostic of an $\alpha3\beta2$ combination in oocytes, shows that the potency
ratios are not wholly consistent with a simple $\alpha_3\beta_4$ combination, but possess characteristics of both the $\alpha_3\beta_4$ and $\alpha_3\beta_2$ subunit combinations.

*a) Comparisons with other studies*

The oocyte data presented here is largely in agreement with that published previously with fewer agonists under different conditions by other authors (e.g. Luetje & Patrick, 1991; Cachelin & Jaggi, 1991), although no comparable values for potency ratios are available from these publications. However, studies using the $\alpha_3\beta_2$ combination by Connolly *et al* (1992) give a relative nicotine potency approximately 10 times greater than in our experiments, although the presence of external calcium meant that lower agonist concentrations could be used. No previous published agonist profiles are available for rat SCG neurones or other peripheral preparations, (now there are) but it should be noted that other peripheral preparations such as the PC12 cell line, chick sympathetic ganglia and chick ciliary ganglia may have a different pattern of mRNA expression to that present in rat peripheral neurones: these three preparations contain mRNA for the $\alpha_5$ subunit which may be absent in the rat ANS (Boulter *et al.*, 1990; Listerud *et al.*, 1991; Corriveau & Berg, 1993; Rust *et al.*, 1994; Mandelzys *et al.*, 1994). Subsequent to this study, DeKoninck *et al.* (1995) obtained agonist profiles (although no comparable potency ratios) consistent with the data presented here although the study took place using neonatal rats and low concentrations were not used. The agonist profile in rat SCG neurones does not appear to match any observed in central neurones such as the rat habenular and interpeduncular nucleus (Mulle *et al.*, 1991) or those observed for other rat subunits expressed in oocytes such as $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_4-1\beta_2$, $\alpha_4-2\beta_2$, $\alpha_4-1\beta_4$ (Luetje & Patrick, 1991; Connolly *et al.*, 1992, Sivilotti *et al.*, 1997), the homomeric $\alpha_7$ receptor (Séguéla *et al.*, 1993), or the homomeric $\alpha_9$ receptor (Elgovhen *et al.*, 1995), although in all these studies external calcium was present. Similarly, they do not exactly match
any of the pharmacological profiles obtained with rat subunit cDNAs transfected into mammalian cell lines (e.g. α3β4, Wong et al., 1995; α7, Puchacz et al., 1994).

b) Receptor heterogeneity

Evidence for nicotinic receptor heterogeneity at the single channel level in dissociated rat SCG neurones has previously been reported from this lab (Mathie et al., 1991) revealing a large variability in both conductance and kinetic measurements both within and between patches, so it is not entirely unexpected that the whole-cell SCG agonist profile does not resemble that of a simple single α/β pair. Similar heterogeneity is also observed in the rat medial habenular (Connolly et al., 1995) where current responses have similar pharmacological and electrophysiological characteristics to ganglionic receptors. Also, like the rat SCG, the medial habenula contains high levels of α3 and β4 mRNA, e.g. Wada et al., 1989; Dineley-Miller & Patrick, 1993).

Single channel studies in avian sympathetic neurones (Moss & Role, 1993) reveal nicotinic AChR subtype segregation on a cell-by-cell basis. No such situation was apparent when looking at the relative potencies of the agonist-induced whole-cell responses in the present work, but it should be noted that all recordings were made from cells on the surface of the ganglia and there is the possibility of segregation from cells located deeper in the ganglion. However, considerable heterogeneity can also be observed for both the kinetic and conductance measurements of simple nicotinic subunit combinations expressed in *Xenopus* oocytes (e.g. α4-1β2, Charnet et al., 1992; α1β1γδ, Gibb et al., 1990; also see Ch.4).

c) Would single-channel studies be useful?

It is clear from the results presented in this paper that an agonist profile alone at the macroscopic level is not sufficient to give a satisfactory answer to the question
of which subunits make up the functional nicotinic AChR in rat SCG neurones, although this may not necessarily be the case in other cell types. Studies by Papke et al. (1991) reveal differences in conductance, open times and most notably burst kinetics when comparing rat α3β2 and α3β4 subunit pairs, but it is difficult to compare studies such as these with those conducted in sympathetic neurones due to the wide variety of recording conditions used (see Papke, 1993 for review). A detailed analysis at the single channel level using parallel studies would be useful, but is complicated by the marked run-down of neuronal nicotinic channels that is encountered in outside-out patches and to a lesser extent in inside-out patches. Recent studies of single channel conductance in rat SCG neurones could not find a match when made in parallel with α3β4, α4β4, α3α5β4 and α3β2β4 subunit combinations expressed in Xenopus oocytes (Sivilotti et al., 1997). This study gave main conductances of 22, 29, 24.9 and 22.1 pS respectively compared to 34.8 pS from adult and 36.6 pS from 1 day old rats, all made with 1mM Ca\(^{2+}\) in the external media. However, a single-channel study of the α3β4 subunit combination transfected into HEK-293 cells gave a value of 29 pS in 2mM external Ca\(^{2+}\) (Stetzer et al., 1997). As the presence of external calcium will tend to decrease the single channel conductance then this value appears to be a closer estimate. Thus, as suggested by Sivilotti et al., 1997, it is possible that Xenopus oocytes do not assemble neuronal nicotinic receptor subunits into functional receptors in the same way as neurones, or indeed in the same way as when transfected into mammalian cell lines. Recently expression of the cloned neuronal nicotinic α7 subunit in several cultured mammalian cell lines has revealed that the folding, assembly, and subcellular localization of this protein are critically dependent upon the nature of the host cell (Cooper & Millar, 1997).

It should be noted that most patch recordings are made from the cell soma, whereas most synaptic contacts in rat SCG neurones are found on the proximal dendritic processes (as obtained with α- and neuronal bungarotoxin labelling; Loring et al., 1988), the nicotinic AChR density at postsynaptic membranes being approximately one hundred times greater than that found extrasynaptically. Many of
these dendritic processes may be lost when SCG neurones are dissociated and the nature of the nicotinic AChRs present on new dendritic growth induced during the culturing process is uncertain. Measurements of receptor turnover in chick sympathetic ganglia in culture suggest that new receptors are formed within 24 hours (Listerud et al., 1991). The use of fresh undissociated SCG neurones in this study will remove any such uncertainty that may be encountered when recording whole-cell currents from dissociated and cultured neurones. Similarly, we have used 4-6 week old rats as the adult number of synapses is not attained until the end of the first postnatal month (Smolen & Raisman, 1980; Smolen, 1983a).

d) Possibilities for the actual subunit composition

If the native functional nicotinic AChRs do not consist of a simple α3β2 or α3β4 subunit pair, then what could the real subunit composition be? The data presented here would be consistent with a situation in which two populations with both these subunit compositions existed, but the high potencies obtained with cytisine and nicotine would suggest that an α3β4 population was dominant, whereas the high potency obtained with DMPP would suggest that an α3β2 population was dominant. Certainly there is evidence from immunoprecipitation studies in rat trigeminal ganglia that receptors consisting of a simple α3β4 pair do exist in peripheral neurones (Flores et al., 1996). However, immunoprecipitation studies in chick ciliary ganglia reveal a population of receptors composed of three different subunits, α3, α5 and β4 (Vernallis et al. 1993; Conroy & Berg, 1995). Wang et al. (1996) studied the co-expression of various combinations of cloned human α3, α2, α4, and α5 subunits in Xenopus oocytes and found expression on the surface membrane for combinations of α3β2, α3β4, α3β2α5, and α3β4α5 subunits, indicating that certain triplets of subunits will coassemble. Other studies suggest the incorporation of α7 into native receptors containing the α3 subunit (Listerud et al., 1991). It is therefore not unlikely that a similar situation might exist in the rat SCG, with receptors containing more than one α
and/or β subunit. With mRNAs encoding a total of three α and two β subunits present in the rat SCG there is scope for a large variety of different combinations. When chick α4, α5 and β2 subunits are coexpressed in *Xenopus* oocytes, a functionally heterogeneous receptor population is formed containing receptors distinct from those formed with a simple α/β pair (Ramirrez-Latorre et al., 1995), and similar functionally distinct receptors are obtained with rat and human subunit triplets (Sivilotti et al., 1997; Wang et al., 1996). There is also the possibility of as yet undiscovered subunits being involved in the functional receptors present rat ganglia, as well as other cell types. This appears to be the case in chick ciliary ganglia where a novel subpopulation of α-Bgt-sensitive nicotinic receptors that do not involve alphas 7, 8 or 9 is present (Pugh et al., 1995).

e) Is there a contribution from the α7 subtype?

There has been much interest in the α-bungarotoxin-sensitive homomeric α7 receptor (Couturier et al., 1990; Séguéla et al., 1993) due to its high calcium permeability and, therefore, its possible role in activating calcium dependent cellular mechanisms. Presynaptic α-bungarotoxin-sensitive nicotinic receptors are also believed to be involved in controlling synaptic transmission in the CNS (e.g. Gray et al., 1996). α-bungarotoxin binds with high affinity ($K_i \approx 6 \times 10^{-11} \text{M}$) to rat SCG neurones (Fumagalli et al., 1976), but there is conflicting information as to whether this binding is exclusively extrasynaptic or not (Smolen, 1983; Fumagalli & DeRenzis, 1984). However, α-bungarotoxin has no effect on synaptic transmission (e.g. Brown & Fumagalli, 1977) and has been demonstrated not to block the effects of exogenously applied nicotinic agonists in the rat SCG (Trouslard et al., 1993; Mandelzys et al., 1995), although such an effect has now been demonstrated in the embryonic chick ciliary ganglion (Zhang et al., 1994), and also in a small percentage of cultured rat trigeminal ganglion neurones (Liu et al., 1993). There is, therefore, no evidence at present to suggest the presence of functional homomeric α7 receptors in rat SCG.
neurones, although this does not exclude the possibility of incorporation of the α7 subunit into receptors with multiple subunits as suggested for chick sympathetic ganglia (e.g. Listerud et al., 1991). We have not undertaken any parallel study with the rat α7 receptor, but Séguéla et al. (1993) have obtained a rank order of potency with Nic > Cyt > DMPP > ACh in terms of approximate EC₅₀, and Nic ≈ DMPP > Cyt > ACh at low concentrations.

f) Other approaches

Apart from single channel analysis, what other methods could be employed in following up this study? Firstly there is the use of selective antagonists or neurotoxins (e.g. α-bungarotoxin - see above) to remove components of the whole-cell or single channel currents. In oocytes neuronal bungarotoxin (NBT) has been shown to block a number of α/β subunit pairs depending on the manner in which it is applied (Papke et al., 1993): e.g. α3β2 receptors show a long-lasting inhibition after pre-incubation with NBT, whereas α3β4 ,α4-1β2 and α4-1β4 receptors (and several other combinations including the muscle α1β1γδ) are at least partly blocked by co-application of NBT with the agonist. Co-application with NBT has been demonstrated to completely block whole-cell responses to ACh in rat sympathetic neurones (Mathie et al., 1988), this result resembles most closely that reported for α3β4 receptors (if the receptors actually contain only two subunit types). Re-examination of the block of nicotinic AChRs by neuronal bungarotoxin in the rat SCG might be useful in the light of the oocyte data now available, but this toxin is extremely difficult to acquire and produces variable results. Subsequent to this study DeKoninck et al. (1995) demonstrated in the rat SCG that NBT blocks nicotinic currents rapidly, but the kinetics for NBT removal were dependent on the duration of the application: brief applications were quickly reversible, whereas prolonged applications took orders of magnitude longer to reverse. These observations, like the potency ratios quoted in this study, reveal characteristics of both the α3β2 and α3β4 subtypes.
Some studies have reported that antagonists such as hexamethonium, mecamylamine, trimetaphan (all ganglion blockers) inhibit α3β4 receptors better than α3β2 receptors expressed in oocytes (e.g. Cachelin & Rust 1995), and that dihydro-β-erythroidine has the reverse selectivity (Harvey & Luetje, 1996). However, in general these compounds do not exhibit sufficient selectivity to be of any considerable value in assessing functional subtype subunit composition. For some of these compounds (e.g. hexamethonium) the position is complicated further by their activity as channel blockers (e.g. Gurney & Rang, 1984) which gives them a partial non-competitive nature. The IC_{50} values obtained for these compounds on various nicotinic AChR subunit combinations may, therefore, be critically dependent on the concentrations of agonist used. This was demonstrated by Cachelin & Rust (1994b) where the IC_{50} value for hexamethonium was increased three-fold by increasing the ACh concentration from 20μM to 30μM with the α3β4 subtype and from 2μM to 30μM for the α3β2 subtype.

So far work has concentrated on α/β pairs and recent studies involving triplet receptors expressed in oocytes has thrown little light on native subunit composition (e.g. Sivilotti et al., 1997). Examining the pharmacology and electrophysiology of receptors containing three or more subunits (based on the mRNAs present in a particular neurone cell type) is quite a daunting task considering the possible combinations that could feasibly exist. However, such studies may be made easier by immunoprecipitation studies with monoclonal antibodies (such as those by Vernallis et al., 1993 and Flores et al., 1996) to determine which subunits are actually coassembled in the native receptors, although the extent to which this is linked to actual functional receptors is unclear. Other biochemical methods such as single cell PCR may also be useful to see if there is in fact cell-by-cell segregation when looking at the pattern of mRNA expression.
In conclusion, this study demonstrates that agonist potency ratios may provide a quite subtle means of distinguishing receptor subunit composition in native cells by comparison with results from receptors expressed in oocytes. In the case of receptors in rat SCG neurones, the results are consistent with that of an $\alpha_3\beta_4$ subunit combination, except for the potency of DMPP which is approximately 6-fold more potent in the SCG than expected from the $\alpha_3\beta_4$ combination in oocytes. This single result therefore highlights the need for other corroborative functional and biochemical studies to be undertaken before a firm conclusion can be reached regarding the subunit composition of nicotinic ACh receptors in the rat SCG.

None-the-less, cloned nicotinic receptors expressed in *Xenopus* oocytes do appear to have all the general pharmacological properties found in native receptors. They are, therefore, useful systems for the initial study of agents which may modulate nicotinic receptor function in health and disease.
The data presented in the next chapter indicate that it may be necessary to advise caution when interpreting comparisons of agonist potency ratios for native cells and receptors expressed in *Xenopus* oocytes. As already mentioned, there are now several lines of evidence to show that oocytes do not assemble neuronal nicotinic receptors with identical functional properties to those expressed in transfected cells, and native receptors are matched more closely with their predicted subtypes expressed in these transfected cells. (e.g. Sivilotti *et al.*, 1997; Lewis *et al.*, 1997). The possibility that multiple receptor subtypes might occur in oocytes injected with simple α/β subunit pairs leads to the question of which receptor subpopulation we are looking at the low concentrations used in this study. It is possible that different agonists will have very different relative potencies on different subpopulations. Also, if the oocyte can produce multiple simple α/β pairs of different subunit stoichiometries or with different modes of post-translational modification, then it might not be surprising if a functional match could not be found with a native neurone, even if all the correct subunits were present.

We do not know which (if any) of the different receptors being formed in the oocyte might also occur in neurones. The concentration-response plots constructed for the SCG neurones unfortunately did not have enough data points or a large enough concentration range to observe any heterogeneity analogous to that which appears to occur with the α3β4 and α4-1β2 subunit combinations (see Chapter 4). Interpretation of such data would, however, be complicated by the presence of at least five subunits in these neurones. In fact, it is surprising that complete nicotinic receptor agonist concentration-response data from other studies in neurones do not appear to display more complex relationships considering the large numbers of different mRNAs present.
3.5. APPENDIX:

BASIS OF LOW CONCENTRATION ASSUMPTION FOR APPROXIMATE HILL PLOTS.

If we consider a simple case for the binding of \( n \) molecules of agonist \( A \) to a receptor \( R \), where \( k_{+1} \) is the association rate constant, and \( k_{-1} \) is the dissociation rate constant:

\[
nA + R \xrightleftharpoons{\frac{k_{+1}}{k_{-1}}} A_n R
\]

At equilibrium, according to the law of mass action, the rate of the forward reaction equals the rate of the reverse reaction:

\[
k_{+1} [A]^n [R] = k_{-1} [A]_{n} R
\]

If we divide both sides of this expression by \([R]_T\) (i.e. the total number of receptors), then:

\[
k_{+1} [A]^n p_R = k_{-1} p_{AR}
\]

where \( p_R = [R]/[R]_T \) and \( p_{AR} = [A_n R]/[R]_T \), i.e. the proportion of unoccupied and occupied receptors respectively.

The equilibrium dissociation constant, \( K_A = k_{-1}/k_{+1} \) ([A]=\( K_A \) when \( p_{AR}=0.5 \), i.e. half the receptors are occupied), therefore:

\[
[A]^n p_R = K_A p_{AR}
\]
In this simple model the receptor can be either occupied or unoccupied, therefore:

\[ p_R + p_{AR} = 1 \]

Substituting gives:

\[ \frac{K_A}{[A]''} p_{AR} + p_{AR} = 1 \]

Rearranging gives:

\[ p_{AR} = \frac{[A]''}{K_A + [A]''} \]

This is the Hill-Langmuir equation. By rearranging and taking logs the following expression is obtained:

\[ \log \frac{p_{AR}}{1 - p_{AR}} = n \log[A] - \log K_A \]

Thus a plot of \( \log(p_{AR}/1-p_{AR}) \) vs \( \log[A] \) should give a straight line of slope \( n \). This is termed a Hill Plot, and the slope \( n \) (or \( n_H \)) is the Hill coefficient or Hill slope.

However, when measuring concentration-response relationships the relationship between occupancy and response is not necessarily one of direct proportionality, even when measuring the response directly, as in the case of ion channels.
In general, concentration response curves do follow the general form of the *Hill-Langmuir* equation, and can be fitted 'empirically' with the expression below:

\[
y = y_{\text{max}} \frac{[A]^{n_\text{H}}}{[A]_{50}^{n_\text{H}} + [A]^{n_\text{H}}}
\]

where \([A]_{50}\) is the concentration at which the response is half maximal (although it does not necessarily refer to an equilibrium constant). This is usually termed the *Hill equation*, and is a generalization of the Hill-Langmuir equation. It makes no assumptions as to a model of receptor activation or to any relationship between receptor occupancy and response. Rearranging and taking logs gives:

\[
\log\left(\frac{y}{y_{\text{max}} - y}\right) = n_\text{H} \log[A] - n_\text{H} \log[A]_{50}
\]

Usually \(y_{\text{max}}\) cannot be measured accurately due to the effects of desensitization. However, at low concentrations, i.e. where \(y \ll y_{\text{max}}\):

\[
\log\left(\frac{y}{y_{\text{max}} - y}\right) \approx \log\left(\frac{y}{y_{\text{max}}}\right) = \log y - \log y_{\text{max}}
\]

Therefore,

\[
\log y = n_\text{H} \log[A] - (n_\text{H} \log[A]_{50} - \log y_{\text{max}})
\]

Thus, at low concentrations a plot of \(\log y\) vs \(\log[A]\) will be of a linear form with a slope of \(n_\text{H}\). In the low concentration experiments used here \(y_{\text{max}}\) was not
known, and was therefore fixed at a value well outside the range of the actual responses for the fitting process. Estimates could then be made for the value of \(n_{11}\).

If the above expression is fitted to the responses obtained with low concentrations for each of several agonists, and the slope \((n_{11})\) is constrained to be equal for all the fits (i.e. the best average slope is obtained), then a set of relative potencies (potency ratios) can be obtained (see Colquhoun, 1973). As the fits are constrained to be parallel, then the horizontal distance between the fitted lines will be a constant. This horizontal distance is equal to the log of the potency ratio (as we are using log-log coordinates). The potency ratio is, therefore, the antilog of the horizontal distance between the fitted lines.
CHAPTER 4:

DETERMINATION OF AGONIST CONCENTRATION-RESPONSE RELATIONSHIPS FOR NEURONAL NICOTINIC RECEPTOR SUBTYPES EXPRESSED IN XENOPUS OOCYTES

4.1. INTRODUCTION

In the previous chapter the pharmacological properties of native nicotinic receptors in rat SCG neurones were compared with those expressed in *Xenopus* oocytes from combinations of cloned subunit cDNAs. The aims of that study were two-fold. The first was to try to gain evidence for the presence of particular subunits in native receptors. It seemed that the α3β4 combination most closely resembled the properties of the SCG neurones. The second aim was to see how useful oocytes might be as model systems for investigating the action of therapeutic or addictive drugs. As mentioned earlier, there has been considerable interest in neuronal nicotinic receptors as therapeutic targets, and in their role in tobacco addiction. The results in Chapter 3 show that it is possible to produce receptors in oocytes which are pharmacologically very similar to native neuronal nicotinic receptors. Therefore, the work in Chapter 3 does help to establish that oocytes are a good primary system for the investigation of the action of centrally acting therapeutic and addictive drugs.

One of the first steps in the analysis of the interaction of a therapeutic or addictive drug with a biological system is the determination of the relationship between concentration and response. This gives us some idea of the concentration range over which the agonist may activate the system *in vivo*. Macroscopic measurements of responses over the entire concentration range in *Xenopus* oocytes are of considerable value for comparison with cells from various native *'in vitro'* preparations. They are very useful experiments for comparing simple receptor subtypes in conditions free from other contaminating nicotinic ligand-gated ion channels. Although the exact subunit composition of native receptors may not be
reproduced, general pharmacological properties are well reproduced in oocytes. They have also proved extremely useful for structure-function studies within the receptor (e.g. the M2 domain, Konno et al., 1991; α7/5-HT3 chimera, Eisele et al., 1993).

Estimates of effective concentrations of agents at neuronal nicotinic receptors is presently of considerable interest to the pharmaceutical industry. These receptors are being investigated as potential targets for therapeutic agents (e.g. Benowitz, 1996). There is also considerable interest in the elucidation of the nicotinic receptor subtypes and mechanisms involved in the induction and maintenance of nicotine dependence.

Those interests are also central to this present study. Therefore, in keeping with our overall aim of trying to understand how particular nicotinic receptor subtypes might participate in disease states, we decided to investigate the action of alcohol on the agonist responses of several subunit combinations expressed in *Xenopus* oocytes. One complication of such studies is that the extent of the effects of some modulators can be critically dependent upon the agonist concentration used relative to the maximal concentration. This can be seen for the potentiation of GABA$_A$ receptor responses by general anaesthetics (Lin et al., 1992). Therefore, it is important to compare different subtypes at equivalent positions on their concentration-response curves. Also, an effect may not be seen if we are at a saturating concentration of agonist. We wanted to obtain approximate equivalent concentrations of agonist for the different subunit combinations at the lower end of their concentration-response relationship (e.g. $<EC_{25}$). This would ensure minimal desensitization between agonist applications and better recovery.

### 4.2. Specific Methods

**a) Drug Application Protocol & Data Analysis**

At low agonist concentrations it was possible to apply a range of drug concentrations to the same oocyte. However, at higher concentrations of agonist there
was slow recovery of the peak current response. To minimize this problem, a ‘single-shot’ protocol was adopted. In this protocol, a reproducible control response was first obtained using a non-desensitizing standard concentration of agonist. This was followed by a single application of a high concentration of agonist (see fig. 4.1.). Only one high concentration of agonist was applied to each oocyte, and several oocytes were used for each concentration, until complete concentration-response relationships were obtained. All drug applications were made at 5 minute intervals. Standard agonist concentrations used for each subunit combination were as follows: \( \alpha_3 \beta_2, 10\mu M \text{ACh} \); \( \alpha_3 \beta_4, 10\mu M \text{ACh} \); \( \alpha_4 \beta_2, 1\mu M \text{ACh} \); \( \alpha_4 \lambda_2, 1\mu M \text{ACh} \); \( \alpha_7, 10\mu M \text{nicotine} \). All responses of a particular combination were normalized to the standard concentration.

Agonist concentration-response relationships were fitted by the method of weighted least squares with the Hill equation (See Appendix, Ch. 3) using the ‘Cvfit’ program (D. Colquhoun, University College London, U.K.). The mean data was fitted with either one or two components (assuming either one or two independent sites), and was given either equal weight or weights of \( 1/\text{variance} \) (obtained from the ‘smoothed’ standard deviations). The parameters that were estimated (one for each component) during fitting were \( K_{\text{app}} \) (apparent macroscopic equilibrium constant, or \( EC_{50} \)), \( n_H \) (Hill coefficient) and \( Y_{\text{max}} \) (maximum response). The minimum was fixed at zero. Errors for the parameters were expressed as the approximate standard deviation. Relative improvements between one and two site models were assessed by manually calculating the \( F \)-statistic from the ratios of the minimum sums of squares of deviations (see equation below, e.g. Kenakin, 1993).

\[
F = \frac{(SS_1 - SS_2)/(d.f._1 - d.f._2)}{SS_1/d.f._2}
\]

\( SS_1 \) = sum of squares for 1-component fit

\( SS_2 \) = sum of squares for 2-component fit
d.f.₁=degrees of freedom for 1-component fit (i.e. number of points fitted – number of parameters estimated)
d.f.₂=degrees of freedom for 2-component fit

b) Weighting of data

It is usual practice when fitting a single component Hill equation to a set of data to give the data points equal weights. The objective of such fits is to obtain an EC₅₀ value (i.e. the concentration at which the response is half maximal), with little interest in whether the data is fitted well by this equation. If all that is required is a half-maximal concentration, then this approach is sufficient. However, giving the points equal weights is an artificial situation especially when the ‘true’ values for the weights are available from the actual values of 1/variance. It is visually apparent from some of the plots fitted here that they contain more than one component (see for instance the kinks in the log-log plots in fig.4.2, and below). It can also be seen from the log-log plots in fig.4.2 and fig.4.3 that giving equal weights to the points results in a very poor fit to the lower concentration points to the extent that they do not appear to influence the fit at all. These points are invariably the most accurate points that can be measured due to the minimal affects of desensitization, although as you approach the resolution (i.e.± 1 nA) of the recording they become less accurate.

A case can therefore be made for attempting to fit the data with two components using weights of 1/variance. It can be seen below from the resulting fits in fig.4.2 and fig.4.3 that this procedure tended to result in the most satisfactory fits of the data. It should be noted, however, that the maxima obtained in these experiments are probably inaccurate due to the effects of desensitization that are bound to occur in a system such as the oocyte. The agonist concentration will reach a steady-state at different times at different points around the oocyte, due to the large cell size.

4.3. Results
Full agonist concentration-response relationships to acetylcholine were obtained for all the heteromeric subunit combinations tested for sensitivity to ethanol (see Chapter 5). This was done so that the different combinations could be tested at approximately equivalent positions on their respective concentration-response curves. A full agonist concentration-response relationship to nicotine was obtained for the homomeric α7 receptor. Figure 4.1 shows two examples of the high concentration ‘one-shot’ responses used to construct the curves.

a) α4-1β2 subunit combination

Figure 4.2a shows the concentration-response relationship for ACh on the α4-1 β2 subunit combination. The dashed line indicates a one-site equal weight fit of the Hill equation to the data giving an EC_{50} of 70.9 ± 14.6 μM. However, as can be seen from the log-log plot of the same fit in Figure 4.2b, the curve is a very poor fit at the lower concentrations, which should be regarded as the most accurate data points due to lower levels of desensitization. A more satisfactory fit to these low concentration points could be obtained by using the ‘true’ weights of 1/variance. However, under these conditions the higher concentration points were poorly fitted with the plateau well short of the observed maximum, resulting in a distorted (i.e. decreased) estimate of the EC_{50} value. A better fit to the entire α4-1β2 concentration range was obtained by fitting the sum of two components with the data weighted to 1/variance. This gave EC_{50} estimates of 0.30 ± 0.08 μM and 58.3 ± 21.1 μM for the high and low affinity components respectively. Further justification for fitting the data to two components can be seen from the distinct central plateau followed by a further increase in slope that is observed, especially on the log-log relationship. Such an increase in slope would not be seen with a simple Hill relationship. A decrease in Hill slope can indicate the influence of acute desensitization or the presence of a heterogeneous population of receptors activated by the same agonist. The subsequent increase in slope makes the
Figure 4.1. Typical examples of high concentration 'one-shot' responses used to construct full agonist concentration-response relationships. (a) Consistent inward current responses to the standard concentration of 1μM ACh for the α4-1β2 combination followed by a single high concentration of 300μM ACh. The bar above each response indicates the duration of agonist application. (b) Consistent inward current responses to the standard concentration of 10μM ACh for the α3β2 combination followed by a single high concentration of 1mM ACh. The responses to the higher agonist concentrations are shown on a smaller scale. As shown here α3β2 responses tended to desensitize more rapidly than α4-1β2 responses.
desensitization case alone unlikely, although there is almost certainly a significant amount of acute desensitization occurring during the course of a single response to high concentrations of agonist. Calculation of the $F$ statistic suggests that the two component model gave a significantly better fit than one component using both equal weighting ($p<0.01$) and weights of $1/\text{variance}$ ($p<0.01$). The 3mM ACh concentration point was not included in the fitting process. At this concentration the $\alpha4-\beta2$ combination may be sensitive to open channel block. Similar effects at mM concentrations of agonist have been seen for nicotinic responses in muscle (e.g. Ogden & Colquhoun, 1985) and BC3H1 cells (Sine & Steinbach, 1987).

b) $\alpha3\beta4$ subunit combination

Figures 4.3a. & 4.3b. show the concentration-response relationship for ACh on the $\alpha3\beta4$ subunit combination. The need to avoid using excessively high ACh concentrations (>10mM) which could affect the osmolarity of the external recording solution resulted in a poorly defined maximum plateau. However, like the $\alpha4-\beta2$ combination, the most satisfactory fit was obtained by fitting the data to the sum of two components with weights of $1/\text{variance}$. This resulted in estimates for the EC$_{50}$ of each component of $39.0 \pm 4.81 \mu$M and $2919 \pm 3466 \mu$M. However, the poorly defined maximum results in large errors for the lower affinity component giving its EC$_{50}$ estimate poor accuracy. The dashed line shows the single component fit with equal weights giving an EC$_{50}$ estimate of $1840 \pm 2319 \mu$M, again with very large errors due to the poorly defined maximum. The two component model gave a significantly better fit than one component using both equal weighting ($p<0.01$) and weights of $1/\text{variance}$ ($p<0.05$). The concentration range used here went up to higher concentrations than those attempted in previous oocyte studies with this combination (Cachelin & Rust, 1994; Cohen et al., 1995; Harvey & Luetje, 1996). This may account for the low affinity component previously being undetected.
Figure 4.2. Acetylcholine concentration-response relationship for the α4-1β2 subunit combination expressed in *Xenopus* oocytes. The responses are agonist-induced inward current responses for 2-electrode voltage-clamped oocytes (V_H=-60mV). The standard concentration to which all responses were normalized was 1μM ACh. Dashed lines indicate fits for a single Hill component using equal weights. Solid lines indicate fits for the sum of two Hill components using weights of 1/variance obtained from the 'smoothed' Standard Deviations of the responses at each agonist concentration. Error bars represent the actual Standard Error of the Mean. The concentration-response relationship is displayed in (a) log-linear format, and (b) as a log-log transformation of the plot in (a). The log-log plot illustrates the inflection in the curve produced by the second component, and the improvement of fit obtained at the low concentrations by weighting to 1/variance. The 3mM point was not included in the fit, as described in the results section. Each point represents the data from 4-5 oocytes.
Figure 4.3. Acetylcholine concentration-response relationship for the \( \alpha3\beta4 \) subunit combination expressed in *Xenopus* oocytes. The responses are agonist-induced inward current responses for 2-electrode voltage-clamped oocytes (\( V_H = -60 \text{mV} \)). The standard concentration to which all responses were normalized was 10\( \mu \text{M ACh} \). Dashed lines indicate fits for a single Hill component using equal weights. Solid lines indicate fits for the sum of two Hill components using weights of 1/variance obtained from the 'smoothed' Standard Deviations of the responses at each agonist concentration. Error bars represent the actual Standard Error of the Mean. The concentration-response relationship is displayed in (a) log-linear format, and (b) as a log-log transformation of the plot in (a). The log-log plot illustrates the inflection in the curve produced by the second component, and the improvement of fit obtained at the low concentrations by weighting to 1/variance. Each point represents the data from 4-7 oocytes.
Figure 4.4. Agonist concentration-response plots for: (a) α4-1β4 subunit combination; standard concentration was 1μM ACh; 4-6 oocytes were used for each concentration point, except for the 30nM point where n=3; (b) α3β2 subunit combination; standard concentration was 10μM ACh; 4-7 oocytes were used for each concentration point; (c) α7 homomeric receptor; standard concentration was 10μM nicotine; 4 oocytes were used for each concentration point. Dashed lines indicate fits for a single Hill component using equal weights. Solid lines indicate fits for the sum of two Hill components using weights of 1/variance obtained from the 'smoothed' Standard Deviations of the responses at each agonist concentration. Error bars represent the actual Standard Error of the Mean.
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<th>Subtype</th>
<th>EC$_{50}$ ($\mu$M)</th>
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<tr>
<td>$\alpha_4-\beta_2$</td>
<td>70.9 ± 14.6</td>
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<td>$\alpha_4-\beta_4$</td>
<td>28.0 ± 6.95</td>
<td>0.85 ± 0.14</td>
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<td>$\alpha_3\beta_4$</td>
<td>1840 ± 2319</td>
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<td>$\alpha_3\beta_2$</td>
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<tr>
<td>$\alpha_7^*$</td>
<td>63.9 ± 10.3</td>
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<tr>
<th>Subtype</th>
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<tr>
<td>$\alpha_4-\beta_2$</td>
<td>0.30 ± 0.08</td>
<td>58.3 ± 21.1</td>
<td>1.35 ± 0.12</td>
<td>0.96 ± 0.15</td>
<td>9.2 ± 1.9</td>
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<tr>
<td>$\alpha_4-\beta_4$</td>
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<td>64.0 ± 18.1</td>
<td>1.52 ± 0.04</td>
<td>2.35 ± 1.18</td>
<td>42.7 ± 7.5</td>
<td>57.3 ± 12.5</td>
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<td>$\alpha_3\beta_4$</td>
<td>39.0 ± 4.81</td>
<td>2919 ± 3466</td>
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<td>1.37 ± 1.16</td>
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<td>$\alpha_3\beta_2$</td>
<td>6.89 ± 2.02</td>
<td>170 ± 120</td>
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<td>$\alpha_7^*$</td>
<td>13.5 ± 1.13</td>
<td>124 ± 37.1</td>
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<td>1.54 ± 0.09</td>
<td>69.3 ± 6.6</td>
<td>30.7 ± 5.6</td>
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**Table 4.1.** Comparison of concentration-response characteristics obtained when fitting (a) 1-component (equal weight) or (b) 2-component (1/variance weight) models. Hill coefficients ($n_H$), EC$_{50}$ and relative Y$_{max}$ values are given for the various components. All values are given ± the approximated standard deviation. *$\alpha_7$ values are for nicotine, whereas all others are for ACh.
c) $\alpha4-\beta4$, $\alpha3\beta2$ & $\alpha7$ subunit combinations

Figures 4.4a., 4.4b. and 4.4c. show the concentration-response relationships for ACh on the $\alpha4-\beta4$ and $\alpha3\beta2$ subunit combinations and for nicotine on the $\alpha7$ homomeric receptor. These three receptor combinations also appeared to follow the general trend of more satisfactory fitting with the two component model (see Table 4.1 for $EC_{50}$ values). However, the improvement in fit was not significant with either weighting method for the $\alpha4-\beta4$ combination ($p>0.05$) and was only significant when using weights of 1/variance with the $\alpha3\beta2$ ($p<0.05$) and $\alpha7$ combinations ($p<0.01$).

Assuming that the data follow a Hill relationship it is clear that, in general, all the subunit combinations have a more satisfactory fit with a two component model. This is especially evident at the low agonist concentrations, but is not significant with all the subtypes. For all the subunit combinations tested, a comparison of the $EC_{50}$ and Hill slope values obtained when fitting with either one or two components is given in Table 4.1.

4.4. DISCUSSION

a) Agonist concentration-response relationships

The data in this chapter describes the agonist concentration-response relationships of several neuronal nicotinic receptor subtypes, some of which may occur in nature (e.g. $\alpha3\beta4$, Flores et al., 1996; $\alpha4\beta2$, Whiting et al., 1991). It is evident from the agonist concentration-response curves that some of the heteromeric receptor subtypes cannot be fitted in a satisfactory manner by a single Langmuir hyperbola, and that the best fit is obtained with the sum of two Langmuir components. $EC_{50}$ values are quoted by several authors using a single hyperbolic component (e.g. Cachelin & Rust, 1994; Cohen et al., 1995; Harvey & Luetje, 1996), although it would appear that
biphasic fits were not attempted. It is possible that in these studies a second component could be missed if it was assumed that a plateau had been reached and the concentration range had not been extended (as appears to be the case in these studies. Similarly, the true magnitude of low concentration points could have been missed due to problems with recovery from desensitization. Alternatively, these low points might simply not have been attempted if the concentration-response relationship appeared to be approaching a minimum, but in reality had a long 'foot'. However, a precedent can be found for the α4-1β2 receptor in the Papke & Heinemann (1994) study, where a distinct biphasic relationship was obtained with the weak partial agonist cytisine, although the data was not fitted. This high affinity component has also been noticed (although not considered) by other labs with the agonist nicotine (Robin Lester, personal communication; Fenster et al., 1997; Laura Chavez-Noriega, personal communication). Interestingly, in the study of Fenster et al. (1997), this component only appeared to be observed in the presence of external Ca²⁺, whereas in our study external Ca²⁺ was always absent. However, they also demonstrated that the α4-1β2 subtype was more desensitized by the continued presence of 300nM nicotine when external Ca²⁺ was not present. In our study there may be less desensitization due to our agonist application protocol, and the high affinity component may have been even more prominent in the presence of Ca²⁺.

b) Comparison of EC₅₀ estimates with previous studies

Some of the EC₅₀ values estimated in this study do not appear to be consistent with those quoted in other studies. For example, values for the rat α3β4 subtype expressed in Xenopus oocytes, all in Ca²⁺-free media, and fitted with a single component include 63μM (Zwart et al., 1995), 77μM (Cachelin & Rust, 1994), and 219μM (Cohen et al., 1995). Compare this to the value of 1840μM ACh obtained for the single-component fit in this study, albeit with a very large estimate of the standard error. However, some of these other values are relatively close to the value of 39μM
obtained for the higher affinity component in this study, and could be higher than this value due to the inclusion of part of the lower affinity component, as the maximum concentration used did not exceed 3mM ACh. In the study of Cachelin & Rust (1995), the EC50 values for β4 containing receptors were decreased approximately 2 fold by the presence of external Ca2+ (1mM), resulting in a value of 52μM ACh for α3β4, although Fenster et al. (1997) found no significant differences with the agonist nicotine on a range of subunit combinations. Other values obtained for the α3β4 combination in the presence of 1.8mM Ca2+ include 210μM and 207μM (Harvey & Luetje, 1996; Papke et al., 1997). These are similar to the value of 202μM (using 2mM external Ca2+) obtained with the rat α3β4 combination expressed in transfected HEK-293 cells (Wong et al., 1995), suggesting that agonist EC50 estimates are independent of the expression system used. Thus, it would appear that the EC50 values from different studies can vary considerably and also do not appear to correlate with the presence of external Ca2+ as suggested by Cachelin & Rust (1994). This variability could possibly be accounted for by poorly defined maxima at a point where a low affinity component starts to become evident, but has been missed.

Similar to the α3β4 combination, the EC50 value for ACh obtained here with the rat α4-1β2 combination with a single component does not appear to correlate relatively well with those quoted by other authors. For instance, these other studies include EC50 estimates of 2.5μM, 197μM and 270μM (Papke & Heinemann, 1994; Harvey et al., 1996; Papke et al., 1997), all carried out in 1.8mM Ca2+. Compare these values to the single component value of 71μM obtained in this study. Overestimates in other studies could be due to poorly defined maxima causing the curve fitting process to essentially find its own maximum. The value obtained in this study may also be a slight overestimate due to the decision not to include the 3mM point in the fit. Underestimates in other studies could be due to reaching a 'false' maximum (as suggested above for the α3β4 subtype), especially if the receptor expression is high and external Ca2+ is included resulting in large currents.
The single-component EC$_{50}$ estimates obtained with the other subunit combinations in this study do appear to correlate with those obtained in other studies. Estimates for the $\alpha 3\beta 2$ combination include 11$\mu$M and 24$\mu$M in Ca$^{2+}$-free media (Cohen et al., 1995; Cachelin & Rust, 1994) and 30$\mu$M, 44$\mu$M and 71$\mu$M with 1-1.8mM external Ca$^{2+}$ (Papke et al., 1997; Cahelin & Rust, 1994; Harvey & Luetje, 1996), which compare well with the 35$\mu$M obtained here. Harvey et al. (1996) obtained an ACh EC$_{50}$ of 55$\mu$M for the $\alpha 4\beta 4$ combination (in 1.8mM Ca$^{2+}$) which again compares well with the value of 28$\mu$M obtained here.

Nicotine EC$_{50}$ values available in the literature for the rat $\alpha 7$ homomer expressed in oocytes include 234$\mu$M in Ca$^{2+}$-free solutions and 90$\mu$M with Ca$^{2+}$ (Fenster et al., 1997; with $n_{II} = 1.10$ for both) compared with the value of 64$\mu$M obtained here with an identical Hill slope. The unfitted concentration-response plot of Séguéla et al. (1993) gives a nicotine EC$_{50}$ estimate of approximately 30$\mu$M (in 1.8mM external Ca$^{2+}$).

It is interesting to note that the two subtypes ($\alpha 4$-1$\beta 2$ and $\alpha 3$-$\beta 4$), whose single component ACh EC$_{50}$ values generally do not appear to be consistent with other studies are the ones whose concentration-response relationships have the most convincing biphasic shape.

c) What are the consequences of the biphasic relationships obtained in this study?

There are several consequences of the two-component relationships obtained here. Firstly, it may be necessary to reappraise EC$_{50}$ values quoted in earlier studies, especially where the maximum or minimum is not well defined, or where contamination by Ca$^{2+}$-activated Cl$^{-}$ currents and the effects of cumulative desensitization may interfere with interpretation. Secondly, it is possible to postulate several mechanisms to account for this phenomenon. It is likely that due to the effects of desensitization (especially in a large, slowly perfusing system like the oocyte) the calculated Hill slopes might be underestimates of their 'true' values. A Hill slope value
greater than 2 (for instance 2.35 for $\alpha_4$-$\beta_4$) might imply the presence of a receptor with 3 agonist binding sites, possibly with bi-liganded activations occurring at lower concentrations. Hill slope estimates greater than two have been noted for the rat SCG with the agonist DMPP (See Chapter 3, and Covernton et al., 1994). There is also the possibility of the formation of more than one distinct receptor subtype with different subunit stoichiometries of the same subunits occurring in the oocyte. This situation is suggested by the multiple single-channel conductance states obtained with simple $\alpha/\beta$ pairs injected into Xenopus oocytes (e.g. Papke et al., 1989; Papke & Heinemann, 1991; Charnet et al., 1992; Sivilotti et al., 1997). The study of Papke et al. (1989) gives strong evidence to support the possibility of multiple stoichiometries: driving the $\alpha_2:\beta_2$ ratio of injected RNA from 1:1 to 1:9 results in the loss of the primary single-channel conductance state. It could also be that receptors of the same stoichiometry are able to function in two independent modes, perhaps requiring post-translational modification to alter their behaviour. This was suggested for the behaviour of muscle $\alpha_1\beta_1\gamma\delta$ subunits expressed in Xenopus oocytes which exhibit distinct patch to patch heterogeneity in the $P_{\text{open}}$ of clusters at high ACh concentrations, and in shut time distributions and burst $P_{\text{open}}$ at low ACh concentrations (Gibb et al., 1990). There is no reason to suppose that any of these types of heterogeneity might not occur in vivo.

A further possibility to explain the biphasic nature of the concentration-response curve could be the presence of as yet unknown subunits native to the Xenopus oocyte that could contribute to the functional receptors being expressed.

The high affinity component (ACh $EC_{50}=0.3\mu M$) obtained with the $\alpha_4$-$\beta_2$ combination is of special interest. In this concentration range, ACh is approximately equipotent with nicotine on this subtype (Connolly et al., 1992). This subtype is also the most sensitive to nicotine of all the rat subtypes tested so far (Connolly et al., 1992; Vibat et al., 1995, Fenster et al., 1997), with $EC_{50}$ estimates ranging from 0.3$\mu M$ to 15$\mu M$. Studies on the desensitization characteristics of different neuronal nAChRs suggest that, at the levels of nicotine found in the brain during smoking (high nanomolar, Benowitz et al., 1989), the $\alpha_4$-$\beta_2$ subtype is the only one that will be
activated without being completely desensitized by the continued presence of the drug (Fenster et al., 1997), although other untested subtypes may exist in the brain. This leaves a window between 10nM and 1μM nicotine where desensitization increases, but receptor activation can still occur (Fenster et al., 1997). It has been suggested (e.g. Fenster et al., 1997) that the mixed receptor activation and desensitization that can occur in this portion of the concentration-response relationship may account for the up- and down-regulation of receptor function that has been observed in different studies (e.g. Marks et al., 1993; Peng et al., 1994; Gopalakrishnan et al., 1996) after chronic nicotine-induced increases in receptor number. If this is the case then it is possible that the α4-1β2 high affinity component revealed in this study will be critically involved in these processes, and hence in the development and maintenance of tobacco addiction. However, studies in rat hippocampal slices show that nanomolar concentrations of nicotine can enhance glutamatergic synaptic transmission via an α-bungarotoxin-sensitive α7-type receptor (Gray et al., 1996). Hence other receptor subtypes may also be involved in mediating the effects of smoking levels of nicotine.

**d) Do the neuronal nicotinic receptors expressed in Xenopus oocytes accurately reflect those encountered 'in vivo'?**

Although the ‘one-shot’ protocol used in this study goes some way to eliminating desensitization problems, the oocyte expression system is far from ideal for making comparisons with native neurones. This situation is further complicated by the presence of run-up or run-down depending on the individual oocyte recording. Sivilotti et al. (1997) have suggested that at the single channel level the oocyte expression system may not accurately reflect the situation in neurones, whereas transfected cells can sometimes provide a more satisfactory comparison (e.g. Stetzer et al., 1996). The receptors may exist as part of a larger signalling complex along with various kinases and anchoring proteins (e.g. between the NMDA receptor and protein tyrosine kinase Src. Yu et al., 1997). These components may be absent from certain
expression systems, and may have a significant influence on receptor function. However, it is just as likely that there may still be new subunits to be cloned, and that these will provide a match with functional studies in neuronal preparations. This is suggested by the presence of a novel sub-population of α-bungarotoxin-sensitive nicotinic responses that do not involve α7, α8 or α9 subunits, that is present in chick ciliary ganglia (Pugh et al., 1995).

On the other hand, the observations obtained when recording from neurones (or transfected mammalian cell lines) may not accurately reflect what would be observed in a completely intact undialyzed neurone ‘in vivo’. In this case, recording from a large cell, such as an oocyte, where the dialysis is far less complete, may reflect the ‘true’ case more faithfully. For example, as noted in Chapter 3, both the peak current amplitude and the desensitization rate of nicotinic responses in rat ganglion neurones tend to change with time during the course of a whole-cell recording. This phenomenon is also observed in rat medial habenula neurones where it has been investigated more thoroughly (Lester & Dani, 1995; Lester & Dani, in press) At the single channel level there are several examples of the properties of receptors changing during the course of a single recording. This can be seen in neuronal nicotinic receptors where severe ‘run-down’ can be observed in inside- and outside-out patch recordings. It has also been observed that the properties of a receptor can change depending on the configuration of the patch recording. Covarrubias & Steinbach. (1990) observed that the excision of membrane patches from clonal BC3H1 cells to the inside-out configuration reduces both the mean open time and burst duration of skeletal nicotinic acetylcholine receptors.

e) Conclusions of this study

The overlapping expression patterns of several types of α and β subunit mRNAs and proteins seen in numerous brain regions and specific cell types (e.g. Wada et al., 1989; Vernallis et al., 1993) suggests a propensity for extensive and possibly
very specific neuronal nicotinic receptor diversity. This diversity encompasses the possible expression of any number of a variety of subunit combinations with different functional properties from simple homomeric and $\alpha/\beta$ paired receptors to triplet receptors (e.g. $\alpha_4\alpha_5\beta_2$, Ramirez-Latorre et al., 1996) and possibly more. Although other explanations are possible, the two component concentration-response relationships described here are further evidence (additional to the heterogeneity encountered in single-channel studies) that this functional diversity may be enhanced by occurring within a single simple $\alpha/\beta$ subunit combination, resulting in the formation of more than one functionally distinct receptor subtype. This also raises the possibility of regulation of receptor function (either long or short term) by shifting from one receptor mode to the other.

However, the two site analysis demonstrates two additional points. Firstly, the previously published EC$_{50}$ values, a mainstay of the pharmaceutical industry, may need to be re-evaluated. Secondly, some of the subunit combinations (e.g. $\alpha_4\beta_2$ & $\alpha_3\beta_4$) may be more sensitive to low concentrations of agonists such as nicotine and acetylcholine than was previously suspected based on published EC$_{50}$ estimates.
Other explanations for the apparent biphasic concentration-response relationships.

The assumption has been made that the apparent poor fit of the data by a single Langmuir hyperbola might indicate the presence of more than one receptor subtype. However, there are several factors which might effect the shape of the concentration-response relationship. Any effects of desensitization and/or open channel block by the agonist will tend to distort the shape of the relationship at higher agonist concentrations. This may result in an asymmetrical relationship with an shallower or steeper (depending on the relative contributions and the concentration range over which the effects progress) development of the apparent saturation of the response at high concentrations. A single (and symmetrical) hyperbola fitted to such data may, therefore, appear to lie above or below (as encountered with the α4-1β2 combination in this study) the low concentration points depending on the way the above factors contribute to the distortion.

Cooperative models of bimolecular ligand-receptor interactions predict that at low and high agonist concentrations the concentration-response relationship will deviate from a simple hyperbola (with lower gradients at the extremes) (e.g. Takeuchi & Takeuchi, 1967). The two-site fits described in the present study assume that putative single or multiple receptor populations can be described by simple hyperbolic components. It may not be surprising, therefore, that the ‘wrong’ equation does not provide an adequate fit to the data.

None of the above factors, however, can account for a decrease and subsequent increase in the gradient of the relationship, as appears to be the case here for the α4-1β2 and α3β4 receptor subtypes. This has also been noted in other studies on the α4-
1β2 subtype (e.g. with the weak partial agonist cytisine, and also with nicotine, Papke et al., 1994; Robin Lester, personal communication). The accuracy of the fits in the present study is, however, limited by the number of data points used for the fitting process. A larger number of concentration points would provide a more reliable conclusion. Although adding parameters will generally provide a better fit to the data it would then also be worth adding more degrees of freedom (i.e. a third component) to see if further significant improvements of fit can be obtained with a larger data set.
5.1 INTRODUCTION

Having established the agonist concentration-response characteristics of different subunit combinations, it was then possible to examine the actions of alcohol on neuronal nicotinic receptors.

There is a particularly strong correlation between the excessive consumption of both alcohol and nicotine, (Kozlowski et al., 1993). This correlation is also seen in animal studies of ethanol-nicotine interactions at both behavioural (Dar et al., 1993; Blomqvist et al., 1996) and genetic levels (De Fiebre & Collins; 1993 Blomqvist et al., 1996). Nicotinic receptors are also thought to be involved in ethanol-induced dopamine release in the limbic forebrain (Blomqvist et al., 1993). The question therefore arises as to whether there might be direct actions of alcohol on neuronal nicotinic acetylcholine receptor subtypes which underlie the synergism between alcohol and nicotine addiction.

Alcohol is known to potentiate the activity of muscle nicotinic acetylcholine receptors (AChRs) (Arcava et al., 1991), and is also able to modulate the activity of a variety of other neuronal receptors such as glycine, 5HT\textsubscript{3}, GABA\textsubscript{\text{A}}, AMPA/kainate, NMDA and ATP (Samson & Harris, 1992, review; Li et al., 1993). Also, acutely applied alcohol has recently been shown to inhibit the chick \text{\textalpha}7 neuronal nicotinic receptor in a non-competitive mechanism involving the amino-terminal domain of the receptor (Yu et al., 1996). In other receptors, some aspects of its mechanism of action have been elucidated. For example the ethanol-induced enhancement of the activity of recombinant GABA\textsubscript{\text{A}} receptors requires the presence of the \gamma2L subunit (Wafford et al., 1991), and is thought to be partly mediated by protein kinase C, (Wafford & Whiting, 1992; Lin et al., 1994). This effect may also occur in hippocampal neurons in...
the brain, (Weiner et al., 1994). Protein kinase C also mediates one of the pathways through which alcohol inhibits the kainate responses of glutamate receptors, (Dildy-Mayfield and Harris, 1995), although there is also evidence of an additional PKC-independent mechanism. However despite years of intensive effort, there is still considerable debate about how alcohol exerts its effects on receptors. For example, perturbation of the fluidity of annular lipids or a discrete hydrophobic pocket within the receptor are also mechanisms that have been proposed, (Franks & Lieb, 1994, review). Ethanol can also alter the activity of Phospholipase D (Pai et al., 1988). Recent studies also reveal direct effects of ethanol on GABA\textsubscript{A} and glycine receptors, where two specific amino-acid residues in transmembrane domains 2 and 3 are critical for allosteric modulation (Mihic et al., 1997). It therefore seems probable that alcohol has more than one mechanism through which it exerts its effects.

It was therefore decided to examine the effects of alcohol on the agonist responses of several neuronal nicotinic AChR subtypes functionally expressed in Xenopus oocytes. Studies were carried out over the entire physiological concentration range (1-300mM). Table 5.1. gives an indication of physiologically relevant ethanol concentrations and the observed physiological and behavioural characteristics associated with them (adapted from Dietrich, R. A. & Palmer, J. D., 1994; Guinness Book of Records, 1995). These associated behaviours were summarized more concisely by Gaddum (see Table 5.2.). Minimal physiological effects occur at a blood-ethanol concentration of around 5mM, whereas the highest recorded blood-ethanol (with survival) is 328mM (reported for a 24-year-old female, Guinness Book of Records, 1991). The current drink driving limit in the United Kingdom is 17.4mM, and ranges from 17.4-32.6mM, depending on the State, in the U.S.A. (see Bowman & Rand, 1980, Ch.42 for review).

After ingestion, ethanol is rapidly and evenly distributed in all the tissue fluid. The metabolism of ethanol in the body saturates at very low levels, and little is lost through first-pass metabolism. Consequently it is metabolised at only approximately 8g (or 1 Unit) per hour, although this can vary between the sexes and across different
Table 5.1. Physiologically-Encountered Ethanol Concentrations In Humans

<table>
<thead>
<tr>
<th>Plasma $[\text{EtOH}]$ mg/100ml</th>
<th>Equivalent $[\text{EtOH}]$ (mM)</th>
<th>Total EtOH Males (ml)</th>
<th>Total EtOH Females (ml)</th>
<th>Physiological/Behavioural Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-50</td>
<td>0-10.85</td>
<td>0-29.75</td>
<td>0-24.06</td>
<td>Loss of inhibitions, excitement, incoordination, impaired judgement, slurred speech, body sway.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0-3 Units)</td>
<td>(2.5 Units)</td>
<td></td>
</tr>
<tr>
<td>50-100</td>
<td>10.85-21.70</td>
<td>29.75-59.50</td>
<td>24.06-48.13</td>
<td>Impaired reaction time, further impaired judgement, impaired driving ability, ataxia.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3-6 Units)</td>
<td>(2.5-5 Units)</td>
<td></td>
</tr>
<tr>
<td>100-200</td>
<td>21.70-43.40</td>
<td>59.50-119.0</td>
<td>48.13-96.25</td>
<td>Staggering gait, inability to operate a motor vehicle.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6-12 Units)</td>
<td>(5-9.5 Units)</td>
<td></td>
</tr>
<tr>
<td>200-300</td>
<td>43.40-65.10</td>
<td>119.0-178.5</td>
<td>96.25-144.4</td>
<td>Respiratory depression, danger of death in presence of other CNS depressant, blackouts.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12-18 Units)</td>
<td>(9.5-14.5 Units)</td>
<td></td>
</tr>
<tr>
<td>&gt;300</td>
<td>&gt;65.10</td>
<td>&gt;178.5</td>
<td>&gt;144.4</td>
<td>Unconsciousness, severe respiratory and cardiovascular depression, death.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&gt;18 Units)</td>
<td>(&gt;14.5 Units)</td>
<td></td>
</tr>
<tr>
<td>1510</td>
<td>328</td>
<td>899.4</td>
<td>727.5</td>
<td>Highest known blood concentration with survival (24-year-old female, Guinness Book of Records, 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(80 Units)</td>
<td>(73 Units)</td>
<td></td>
</tr>
</tbody>
</table>

Calculations are for a 70kg (11 stone) person, assuming 68% total body water for males & 55% for females. 1 Unit= 8g = 10mls EtOH: this is equivalent to 0.5 Pints 3.5% EtOH or 25mls 40% EtOH (i.e. 1 measure spirits). Drink-Drive limit = 80mg/100mls plasma concentration = 17.4mM = 47.7mls total EtOH (=5 Units) for males 38.6mls for females (=4 Units). Adapted from Dietrich, R. A. & Palmer, J. D., Ch. 31, p425, Human Pharmacology: Molecular to Clinical, 2nd Ed., Brody, T. M. et al [eds], 1994, Moseby-Year Book Inc.


Table 5.2. Gaddum Classification of Ethanol Intoxication

<table>
<thead>
<tr>
<th>Classification</th>
<th>Blood Ethanol Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Dizzy &amp; Delightful</strong></td>
<td>1 mg/ml 21.7 mM</td>
</tr>
<tr>
<td><strong>2. Drunk &amp; Disorderly</strong></td>
<td>2 mg/ml 43.5 mM</td>
</tr>
<tr>
<td><strong>3. Dead Drunk</strong></td>
<td>3 mg/ml 65.2 mM</td>
</tr>
<tr>
<td><strong>4. Danger of Death</strong></td>
<td>4 mg/ml 87.0 mM</td>
</tr>
<tr>
<td><strong>Lethal Concentration</strong></td>
<td>5-8 mg/ml 108.7-173.9 mM</td>
</tr>
</tbody>
</table>

races due to the presence of different phenotypes for alcohol dehydrogenase (see Bowman & Rand, 1980, Ch.42 for review).

5.2. SPECIFIC METHODS

Agonist Ethanol co-application protocols

Initially, reproducible current responses were obtained to the standard agonist concentration. All drug applications were made at 5 minute intervals. For all the subtypes tested, agonist responses in Ba2+ often exhibited a gradual ‘run-up’ or ‘run-down’ depending on the oocyte. Oocytes which could not reach a reproducible control response (i.e. within 10% of the previous response) were not used for analysis. After a reproducible control agonist response was obtained, the same concentration of agonist was co-applied with alcohol. Following this a recovery control response was obtained. Data was not used from cells where the immediate recovery response was not within 20% of the previous control value.

Agonist concentrations routinely used were in the range 1-10μM (ACh) for the heteromeric receptor subtypes and 10-30μM (nicotine) for the homomeric α7 receptor. If very high concentrations of agonist were used with short application intervals, the test recovery responses could be badly affected by desensitization. If the interval between applications was made long enough for recovery from high agonist concentrations, then run-up/down of the response could also jeopardize estimation of the degree of modulation. Therefore, in order to obtain the most reliable estimation of the degree of modulation of the agonist response by ethanol, we used low micromolar concentrations of agonist combined with short time intervals between drug applications.

As discussed below, there was evidence of the development of tolerance of the agonist response to modulation by ethanol. Therefore, only the 'first-exposure' co-
applications of agonist and ethanol were used to construct concentration-response relationships.

5.3. RESULTS

The concentration-response relationships shown in Chapter 3 reveal that it is problematical to find accurate equivalent levels of receptor activation for comparison of ethanol's effects between the different nicotinic receptor subtypes. It was therefore decided to make comparisons at 1-10μM ACh for the heteromeric receptors. This is approximately equivalent to a level ≤EC_{25} for all the subtypes if their concentration-response curves were fitted with only one component, and is a low enough concentration to allow adequate recovery from desensitization between agonist applications. For the homomeric α7 receptor, nicotine (10-30μM) was used in preference to ACh due to the relatively low potency of the latter agonist. This was also equivalent to a value ≤EC_{25}. Some results from early experiments on the α3β4 combination using 10μM nicotine have also been included in the ethanol concentration-% control plots, and these are identified by different symbols.

5.3.1. ACTIONS OF ETHANOL ON OOCYTES EXPRESSING THE α3β4 RECEPTOR SUBTYPE

a) Modulatory actions of ethanol at low concentrations on responses of the α3β4 subunit combination

Low concentrations of ethanol can both potentiate and inhibit the response of the α3β4 combination to nicotinic agonists. Figure 5.1. shows examples of inward current responses from 4 different oocytes revealing both potentiation and inhibition of α3β4 responses at remarkably low concentrations of EtOH (1-3mM). In the top trace 1mM EtOH potentiates the response to 3μM ACh to 136% of the control value. In the
Figure 5.1. Low concentrations of ethanol can both potentiate and inhibit the response of the α3β4 combination to nicotinic agonists. Inward current responses from 4 separate oocytes showing both potentiation and inhibition of α3β4 responses at low concentrations of EtOH (1-3mM). In the top trace 1mM EtOH potentiates the response to 3μM ACh to 136% of the control value. In the second trace 1mM EtOH inhibits the response to 10μM ACh to 85% of the control value. In the third trace 3mM EtOH potentiates the response to 3μM ACh to 148% of control. In the bottom trace 3mM EtOH inhibits the response to 1μM ACh to 68% of control. No discernible currents were observed when EtOH was applied alone at a concentration of less than 100mM.
second trace 1mM EtOH inhibits the response to 10µM ACh to 85% of control. In the third trace 3mM EtOH potentiates the response to 3µM ACh to 148 % of control. In the bottom trace 3mM EtOH inhibits the response to 1µM ACh to 68% of control.

Despite the problem of tolerance (see fig. 5.5.) these effects were qualitatively reproducible within a given oocyte. For example, in one oocyte three consecutive applications of 1mM ethanol + 10µM acetylcholine were made (separated by 10µM ACh control responses). The average of the responses in the presence of ethanol was 148 ± 2.08 nA (± S.E.). This was significantly greater (p<0.005, Students' t-test) than the average of the control current responses (103.3 ± 1.5 nA). When the same concentrations of ethanol and acetylcholine were applied to a different oocyte consistent inhibitions were seen. In this case the three responses with ethanol averaged only 346 ± 2.6 nA, significantly lower (p<0.05) than the control response of 397.5 ± 10.8 nA.

However, these modulatory effects were only observed in some oocytes- a fact that could cause them to be overlooked. With 1mM EtOH, 3 out of 8 oocytes showed effects which exceeded a 15% potentiation or inhibition of the control response, and for 3mM EtOH there were only 2 out of 9 oocytes. To obtain an average of the agonist responses in the presence of ethanol from all the oocytes tested, the data from potentiated, inhibited and unaffected cells must be combined together. Unsurprisingly, this population mean is not significantly different from the average control result. However, as shown above, this population result conceals the fact that agonist responses within individual oocytes can be significantly altered by low concentrations of ethanol. The mean inward current (± S.E.) obtained for α3β4 with 3µM ACh was 388.3 ± 40.8nA (n=16), and no discernible currents were observed when EtOH was applied alone at concentrations equal to or less than 30mM (n=22).

Figure 5.2. shows inward current responses from 2 different oocytes revealing the extremes of potentiation and inhibition of α3β4 responses that can sometimes be observed at the same ethanol concentration (in this case 30mM ethanol). In the top trace 30mM EtOH potentiates the response to 1µM ACh to 237% of the control value.
Figure 5.2. In different oocytes expressing the α3β4 combination large potentiations or inhibitions can be observed at the same ethanol concentration. In the top trace 30mM EtOH potentiates the response to 1μM ACh to 237% of the control value. In the bottom trace, in a different oocyte, 30mM EtOH inhibits the response to 10μM Nicotine to 25% of control. These effects are not dependent on the type of nicotinic agonist used.
In the bottom trace 30mM EtOH inhibits the response to 10μM nicotine to 25% of control. Thus, ethanol can modulate the responses of α3β4 receptors to either ACh or nicotine. Therefore, the effects we have observed are independent of the agonist used.

**b) Effects of high ethanol concentrations on α3β4 receptors**

At the upper end of the range, (100-300mM EtOH), robust potentiation of the agonist-induced current was observed on every occasion. An example of this is shown in Figure 5.3, where 300mM EtOH potentiates the response to 1μM ACh to 305% of the control value. At ethanol concentrations less than 100mM, ethanol alone had no effect on membrane current in the absence of agonist. However, in Figure 5.3, 300mM EtOH alone causes a very small outward current. Such currents were often observed at EtOH concentrations of 100 and 300mM, and were also seen in uninjected oocytes. The mean first exposure value in all oocytes of current produced by 300mM ethanol alone was 5.4 ± 0.9 nA (n=7). This outward current would tend to fractionally diminish the measured inward current generated by application of agonist plus ethanol to the oocyte. Since the largest current ever observed for alcohol alone was less than 3.5% of the smallest current response to agonist + alcohol for α3β4, the agonist + alcohol values were not adjusted for a possible contribution to the current by ethanol alone. Similarly, the agonist + alcohol responses of the other combinations were not adjusted. Figure 5.3 also shows a repeated co-application with 300mM EtOH on the same oocyte showing a similar level of potentiation.

**c) Both potentiations and inhibitions can occur in the same cell**

Figure 5.4 shows an example of α3β4 receptor agonist induced responses exhibiting both inhibition and potentiation by different concentrations of EtOH in the same oocyte. The traces run sequentially from top to bottom. At 3mM EtOH the response to 1μM ACh is inhibited to 68% of the control value (first exposure to
Figure 5.3. A continuous trace showing the reproducibility of robust potentiations produced by the co-application of high concentrations of ethanol to the α3β4 combination. In the upper trace the response to 1μM acetylcholine is potentiated to 305% of the control value by 300mM ethanol. However, in the middle trace 300mM ethanol alone only results in a small outward current. The lower trace shows a repeated co-application on this cell. This again resulted in potentiation to 305%.
Figure 5.4. Example of α3β4 receptor agonist-induced responses exhibiting both inhibition and potentiation by different concentrations of EtOH in the same oocyte. The traces run sequentially from top to bottom. At 3mM EtOH the response to 1μM ACh is inhibited to 68% of the control value. At 30mM EtOH the response to 1μM ACh is inhibited to 67% of the control value. However, at 300mM EtOH the response to 1μM ACh is potentiated to 172% of the control value.
EtOH). At 30mM EtOH the response to 1μM ACh is inhibited to 67% of the control value. However, at 300mM EtOH the response to 1μM ACh is potentiated to 172% of the control value.

d) Development of tolerance

Tolerance was defined as a decrease in the degree of ethanol modulation of an unchanging control response following repeated applications of ethanol. An example of this is shown in Figure 5.5.. The first two α3β4 responses are the first and second exposure of the oocyte to agonist + ethanol (1mM in this case) and are potentiated to a similar level (145% of control). The third trace shows a 1mM EtOH co-application later on in the same recording after several higher EtOH concentrations (up to 100mM) had been applied. The potentiating effect of the 1mM EtOH had been abolished. This tolerance was not observed in every cell, but it invalidated a protocol of carrying out agonist concentration-response curves in the presence of ethanol. Nor was it valid at a given agonist concentration, to compare effects seen with the first exposure to ethanol with subsequent effects in individual oocytes. To overcome these problems, we adopted the 'first exposure' approach (see methods) when seeking to compare the actions of ethanol on different receptor subtypes.

e) Effects of entire clinically relevant range of ethanol concentrations on α3β4 responses.

Figure 5.6. shows a scatter plot (♦ symbols) of all the '1st-exposure' responses of α3β4 receptors in the presence of EtOH. The magnitude of the responses is expressed as a % of the control response. The control response was defined as the average of the responses to agonist alone immediately before and after the agonist co-application with ethanol. Superimposed on this scatter plot are the points representing the means of the responses in the presence of a particular concentration of ethanol.
Figure 5.5. Repeated exposure can lead to the development of tolerance to the effects of low concentrations of ethanol in some cells. In this example the α3β4 responses are all from the same oocyte and run sequentially from top to bottom. In the top trace the 1st co-application of 10μM ACh and 1mM EtOH to this cell reveals a potentiation to 145% of the control agonist response. This was followed by a 2nd co-application (middle trace) in which approximately the same level of potentiation is seen. Following these two co-applications several higher concentrations of EtOH (up to 100mM) were applied. Although the magnitude of the control response has remained stable throughout, the lower trace shows that, after these high EtOH concentrations, the potentiating effect of 1mM EtOH is no longer evident. Thus, tolerance to the potentiating effect of EtOH has occurred.
Figure 5.6. A scatter plot (• symbols) of all the 'first-exposure' responses of α3β4 receptors in the presence of EtOH. The magnitude of the responses is expressed as a % of the control response. The control response was defined as the average of the responses to agonist alone immediately before and after the agonist co-application with ethanol. Superimposed on this scatter plot (○ symbols) are the points representing the means of the responses in the presence of a particular concentration of ethanol. Each data point represents a single oocyte, and each concentration represents responses from at least 3 different batches of oocytes. Note the large scatter of data points at the low EtOH concentrations (<100mM) reflecting the reproducible potentiations and inhibitions seen in some oocytes, as well as the less affected responses found in others. The overall mean is therefore close to 100%. The control value (100%) is indicated by a dotted line on the plot. At EtOH concentrations of 100mM and above only large potentiations were observed. At 100mM EtOH the mean averaged response to ACh was increased to 146.2±4.15% (range=135.7-161.0%) of the control value (n=5, p<0.0005), and at 300mM EtOH the mean averaged response to ACh was increased to 249.8±16.5% (range=206.5-305.1%) of the control value (n=5, p<0.001). Responses with 10μM nicotine/30mM EtOH are indicated by the ○ symbols (n=4).
Each data point represents a single oocyte, and each concentration represents responses from at least 3 different batches of oocytes. Note the large scatter of data points at the low EtOH concentrations (<100mM) reflecting the reproducible potentiations and inhibitions seen in some oocytes, as well as the less affected responses found in others. The overall mean is therefore close to 100%; see Table 5.3. The variability of modulation by low concentrations of ethanol was not dependent on the batch of oocytes or preparation of RNA from which the responses were obtained, but seemed to be associated with individual oocyte variation within a batch. However, at EtOH concentrations of 100mM and above only large potentiations were observed. At 100mM EtOH the mean averaged response to ACh was increased to 146 ± 4.15% (range=136-161%) of the control value (n=5, p<0.0005), and at 300mM EtOH the mean averaged response to ACh was increased to 250 ± 16.5% (range=207-305%) of the control value (n=5, p<0.001). Responses with 10µM nicotine/30mM EtOH are indicated by the ◦ symbols (n=4).

5.3.2. Ethanol Effects On Other Heteromeric Neuronal Nicotinic Receptors

The other three heteromeric subunit combinations (α3β2, α4-1β2 & α4-1β4) all appeared to behave in a similar manner in their modulation by ethanol. The typical characteristics were i) no apparent effect of ethanol at low concentrations (1-30mM); ii) Potentiation of the agonist-induced current at high ethanol concentrations (>30mM). The degree of potentiation was not identical between these three combinations, and was significantly less than that observed with the α3β4 combination. Each combination is described individually in more detail below.

a) Actions of ethanol on α4-1β4 receptors
The mean inward current obtained for $\alpha 4$-$1\beta 4$ with $1\mu M$ ACh was $1227 \pm 155$ nA ($n=17$). Figure 5.7b. shows the effects of ethanol on $\alpha 4$-$1\beta 4$ responses. It displays a scatter plot (♦ symbols) with superimposed mean data plot (O symbols) showing the effect of all '1st-exposure' agonist-EtOH co-applications relative to control (100%) for the $\alpha 4$-$1\beta 4$ subunit combination. Note also that the average potentiations at EtOH concentrations of 100mM and above are significantly ($p<0.05$) lower than those obtained with the $\alpha 3\beta 4$ subunit combination, (see Table 5.4.). Each data point represents an individual oocyte (data from a total of 40 oocytes were used for this plot).

Figure 5.8a. shows typical inward current responses obtained from a Xenopus oocyte injected with the $\alpha 4$-$1\beta 4$ subunit combination, showing potentiation of the response to $10\mu M$ ACh when co-applied with $300mM$ EtOH. The potentiation in this example is only $143\%$ of the control value, which is close to the mean value of $143 \pm 2.94\%$ ($n=6$).

b) $\alpha 4$-$1\beta 2$ receptors

Figure 5.7c shows the effects of clinically relevant concentrations of ethanol on $\alpha 4$-$1\beta 2$ responses. The mean inward current obtained for $\alpha 4$-$1\beta 2$ with $10\mu M$ ACh was $350 \pm 50.0nA$ ($n=27$). Like the combination $\alpha 4$-$1\beta 4$ there appears to be little effect of EtOH at concentrations less than 100mM, and potentiations were observed at this concentration and above. Agonist-induced current responses for the $\alpha 4$-$1\beta 2$ subtype were increased to $113 \pm 3.71\%$ ($p<0.05$, Students' t-test, $n=5$, range: 108-128\%) of the control value by 100mM EtOH, and increased to $189 \pm 14.1\%$ ($p<0.005$, $n=5$, range: 143-226\%) by 300mM EtOH. The potentiation at 300mM EtOH is significantly lower than that of the $\alpha 3\beta 4$ combination ($p<0.05$), but also significantly higher than that of the $\alpha 4$-$1\beta 4$ combination ($p<0.05$). Each data point represents an individual oocyte (a total of 34 oocytes for this plot).
Figure 5.7. (a-e) Scatter plots of individual cell responses to 'first-exposure' agonist-ethanol co-applications for the various subunit combinations. • symbols are for acetylcholine and ○ symbols are for nicotine. Each data point represents a single oocyte, and each concentration represents responses from at least 3 different batches of oocytes. At each ethanol concentration point the minimum number of oocytes was 6 for concentrations less than 100mM, and a minimum of 5 observations for concentrations of 100mM and 300mM. Superimposed on this data are symbols (○) indicating the mean response to agonist-ethanol co-applications at a particular concentration. The data points are expressed as a % of the control agonist alone responses. The control value (100%) is indicated by a dotted line on the plot. Note that in these experiments, apart from the α3β4 combination, EtOH had relatively little effect on the agonist responses when applied at concentrations less than 30mM. The largest poteniations at EtOH concentrations greater than 30mM are also seen with the α3β4 subunit combination.
Figure 5.8. Examples of modulation by ethanol of agonist responses of α4-1β4 and α7 subunit combinations. (a) Typical inward current responses obtained from a *Xenopus* oocyte injected with the α4-1β4 subunit combination showing potentiation of the response to 10μM ACh when co-applied with 300mM EtOH. The potentiation in this example is only 142% of the control value, which is close to the mean value of 143.3% (n=6). This is significantly less than the mean value of 249.8% for the α3β4 response (n=5). As with all the effects of ethanol, there is complete and rapid recovery of the control response. In the lower trace, a subsequent application of 300mM ethanol alone shows only a small outward current. (b) Example of an α7 response showing potentiation by 300mM EtOH to 131% of the
Table 5.3. Effects of low concentrations of ethanol (EtOH) on the response of the nicotinic α3β4 subunit combination to acetylcholine. The Mean values ± Standard Error (S.E.) of the agonist test response in the presence of different concentrations of ethanol are expressed as a % of the control response in the absence of ethanol. n = the number of oocytes in which first-exposure responses were studied for each ethanol concentration. A Students t-test of the mean (Mini-Tab) did not reveal a significant difference between the mean response in the presence of ethanol and the control response (100%). However the range of the test responses in the presence of alcohol demonstrates that there is considerable modulation by low concentrations of ethanol. NS= not significant.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>[EtOH] (mM)</th>
<th>Mean ± S.E.</th>
<th>n</th>
<th>Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3β4</td>
<td>1</td>
<td>107 ± 7.69</td>
<td>8</td>
<td>84.8, 145</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>103 ± 6.84</td>
<td>9</td>
<td>68.4, 148</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>101 ± 0.35</td>
<td>7</td>
<td>100, 103</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>30*</td>
<td>106 ± 19.4</td>
<td>9</td>
<td>24.8, 237</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Includes 10 μM nicotine data (n=4)
Table 5.4. Effects of high concentrations of ethanol (EtOH) on the responses of all the nicotinic subunit combinations to agonist. The Mean values (± Standard Error, S.E.) of the agonist response in the presence of 100 and 300 mM ethanol are expressed as a % of the control response in the absence of ethanol. n = the number of oocytes in which first-exposure responses were studied. In nearly all cases robust poteniatations are seen which are significantly greater than the control responses (Students t-test of the mean, Mini-Tab). NS= not significant.
c) \( \alpha 3 \beta 2 \) receptors

Figure 5.7d shows the effects of ethanol on \( \alpha 3 \beta 2 \) responses. The mean inward current obtained for \( \alpha 3 \beta 2 \) with 10\( \mu \)M ACh was 91.1 ± 8.7 nA (n=35). Like the combination \( \alpha 3 \beta 2 \) there appears to be little effect of EtOH at concentrations less than 100mM, and potentiations are observed at this concentration and above. However, a single inhibition (to 87% of control) was observed at 100mM EtOH. Agonist-induced current responses for the \( \alpha 3 \beta 2 \) subtype were increased to 116 ± 6.3% (p=0.05, Students' t-test, n=6, range: 86.7-126%) of the control value by 100mM EtOH, and increased to 161 ± 7.6% (p<0.002, n=5, range: 137-178%) by 300mM EtOH. Each data point represents an individual oocyte (a total of 35 oocytes for this plot).

5.3.3. Ethanol Effects On Homomeric \( \alpha 7 \) Receptors

Like the \( \alpha 3 \beta 2 \), \( \alpha 4-1\beta 2 \) & \( \alpha 4-1\beta 4 \) combinations, the \( \alpha 7 \) homomer was not strongly modulated at low concentrations of ethanol (1-30mM) under these conditions. (Figure 5.7e). At concentrations above 30mM the effects were more varied than the heteromeric combinations with some oocytes exhibiting potentiations, some small inhibitions and others apparently not affected. With 100mM EtOH the mean response was 116 ± 7.31% (n=6, range: 98.9-142%), and with 300mM EtOH the mean response was 108 ± 6.7% (n=6, range: 88.4-131%). An example of potentiation with 300mM EtOH is shown in Figure 5.8b. The mean inward current obtained for \( \alpha 4-1\beta 2 \) with 10\( \mu \)M nicotine was 189 ± 22.4 nA (n=36).

The effects of high concentrations of ethanol on all the subunit combinations are summarised in Table 5.4.

5.3.4. Rare Effects Of Ethanol On Nicotinic Receptors
As discussed above, to avoid problems with tolerance, we adopted a 'first-exposure' protocol. However, during early studies with repeated ethanol exposure some rare effects were observed for the α3β4 combination. In one case there was a cumulative inhibition from 3-100 mM EtOH (See Figure 5.9.). In another, robust potentiations were observed at 3-30 mM EtOH but the extent was inversely proportional to the concentration. In a third example a robust inhibition was observed with 30 mM EtOH, but with repeated applications, the control response exhibited marked 'run-down' and the EtOH switched to potentiation relative to control. Also, as is apparent from Figure 5.6d, we have a first exposure example of inhibition of α3β2 at 100 mM ethanol. In a preliminary experiment (John Connolly, personal communication) α4-1β4 responses were inhibited by ethanol in a single oocyte. These rare events suggest that there can be occasional oocytes in which exceptional behaviour occurs.

However using the more consistent, first exposure, Ca²⁺ free conditions employed here, four different phenomena become apparent: (i) variable potentiation at lower ethanol concentrations, (ii) variable inhibition at lower ethanol concentrations, (iii) the development of tolerance, and (iv) a consistent potentiation at the highest ethanol concentrations.

5.5. DISCUSSION

a) Modulation of neuronal nicotinic ACtRs by ethanol.

The data presented here suggests that different neuronal nicotinic receptor subtypes can differ in their characteristics of modulation by physiologically encountered concentrations of ethanol. These differences do not appear to be dependant upon the presence of any particular single α or β subunit, but do appear to involve a synergistic effect between α and β subunits. The effects of ethanol on the different subtypes appear to fall into three categories divided as follows: i) α3β4-type
Figure 5.9. In one case the α3β4 subtype exhibited cumulative inhibition from 3-100mM EtOH. This figure shows one of the rare effects observed with the α3β4 subunit combination. The responses do not run sequentially, but could be reproduced in duplicate. In the top trace 3mM EtOH inhibits the response to 10μM Nicotine to 89% of control. In the middle trace 10mM EtOH inhibits the response to 10μM Nicotine to 71% of control. In the bottom trace 30mM EtOH inhibits the response to 10μM Nicotine to 24% of control. Further inhibition was also observed with 100mM EtOH, but a recovery response could not be obtained due to breakdown of the recording.
effects characterised by the presence of both potentiations and inhibitions at low ethanol concentrations, and robust potentiations at high ethanol concentrations; ii) generally $\alpha_3\beta_2$, $\alpha_4-1\beta_2$ and $\alpha_4-1\beta_4$ receptors are less sensitive at low concentrations, but show potentiations at high EtOH concentrations; iii) homomeric $\alpha_7$ receptors are relatively insensitive at low EtOH concentrations, but show both potentiations and inhibitions at high EtOH concentrations. However, this characterization may be somewhat arbitrary. The low concentration effects observed with $\alpha_3\beta_4$ are rare events. Therefore, it is possible that, under different conditions such as in the presence of Ca$^{2+}$ (e.g. Dildy-Mayfield & Harris, 1995), the other subtypes may show greater sensitivity than they have in this particular set of experiments. It is clear, however, that the amount of potentiation observed at the high concentrations varies greatly between the different subtypes, with the most robust potentiations (up to 305% of control) occurring with the $\alpha_3\beta_4$ combination.

In contrast to the variability of the effects of low ethanol concentrations, potentiation of responses at high ethanol concentrations is nearly always seen. However Fig. 5.6a-e and Table 5.4 demonstrates that the average magnitude of the potentiation produced by 300 mM ethanol is different for different combinations. The order of decreasing sensitivity to ethanol is $\alpha_3\beta_4 > \alpha_4-1\beta_2 > \alpha_3\beta_2 > \alpha_4-1\beta_4 > \alpha_7$. The responsiveness of the $\alpha_3\beta_4$ combination is considerably greater than that of any other. This cannot be attributed to the presence of an independent site on the $\beta_4$ subunit, since the $\alpha_4\beta_4$ combination shows the least tendency to potentiation of any of the heteromeric combinations. Nor can it be attributed solely to the presence of the $\alpha_3$ subunit, as the $\alpha_3\beta_2$ combination shows less potentiation than the $\alpha_4\beta_2$ combination. It would seem that both $\alpha_3$ and $\beta_4$ subunits are required. Perhaps both subunits contribute juxtaposed domains which together form a binding site for alcohol. In other combinations, the domains may be less well aligned and the synergism would not occur.

Studies on the responses of nicotinic AChRs in ganglia, which also express $\alpha_3$ and $\beta_4$ subunits, show that the activity of the receptors can be both upregulated and
downregulated, (Valenta et al., 1993; Gurantz et al., 1994). We also see this with α3β4 receptors expressed from cDNAs in oocytes. The variability of the degree of modulation of α3β4 responses by low concentrations of ethanol has several interesting implications. If ethanol worked simply by acting on a hydrophobic pocket in the protein or on the annular lipid to disrupt protein structure, then we would perhaps expect more consistency in the degree and direction of current modulation. The inconsistency at low concentrations tends to argue against a simple change in membrane fluidity as the sole mechanism, and studies on the Torpedo receptor in lipid vesicles suggest that the lipid-protein interface, at least deep in the bilayer, is quite insensitive to the presence of ethanol up to 0.9M (Abadji et al., 1994). One possibility is that the rapid potentiation and inhibition of nicotinic responses at low concentrations of ethanol may be mediated by intracellular signalling pathways. This may involve intimate association of the receptor with a regulatory protein such as occurs between the NMDA receptor and protein tyrosine kinase Src (Yu et al., 1997).

The α3 and β4 subunits are not just restricted to ganglia, but are also found throughout the brain (Dinely-Miller & Patrick, 1994). α3 and β4 subunits also occur together in receptors in the mammalian nervous system (Flores et al., 1996). In oocytes, this combination is characterized by a long open time, (Papke & Heinemann, 1991) and like all neuronal nicotinic receptors, is more permeable to Ca\(^{2+}\) than muscle nAChRs (Mulle et al., 1992, Vernino et al., 1992, 1994). Nicotinic single channel activity with analogous characteristics has been observed in the medial habenula (Mulle et al., 1992, Connolly et al., 1995) where α3 and β4 subunits are also expressed, (Duvoisin et al., 1989) and so it seems probable that the observations described here will have relevance to the actions of ethanol on native nicotinic AChRs containing α3 and β4 subunits in brain tissue. Both these subunits are also expressed in PC12 cells (Boulter et al., 1990), where Nagata et al., (1996) have recently shown that low concentrations of ethanol (30μM to 10mM) can produce variable effects of potentiation and inhibition of nicotinic responses.
h) Comparisons with other studies

The effects of ethanol on the rat α7 homomeric receptor presented here appear to contradict those obtained for the chick α7 in another study. In the work of Yu et al. (1996) ethanol caused a dose-dependent inhibition of the nicotine-induced current response (IC50 = 33mM), whereas we observed mixed inhibition/potentiation at concentrations above 30mM. The reasons for this difference are unclear, as the agonist concentrations used were similar (10μM vs 10-30μM nicotine) and acute ethanol applications were used (although not using a ‘one-shot’ protocol). The chick α7 receptor is more sensitive to nicotine than the rat α7 receptor. The nicotine EC50 = 13μM for the chick receptor in the study of Yu et al., (1996) and 64μM for the rat receptor in this study (see Ch.4). These values are consistent with those obtained in other studies (e.g. Bertrand et al., 1992; Amar et al., 1993; Gerzanich et al., 1994, Corringer et al., 1995; Séguéla et al., 1992). We are, therefore, lower on the dose-response curve than with the chick α7 receptor at equivalent nicotine concentrations. This, however, does not appear to account for the differences in ethanol modulation. In this study there was no difference in behaviour in the few cells where higher nicotine concentrations were used. For instance, in one cell the response of rat α7 to 100μM nicotine was only reduced by 5% in the presence of 100mM EtOH. A second response in the same cell to 300μM nicotine was reduced by only 2.5%. In a second cell the response to 300μM nicotine was not altered in the presence of 300 mM ethanol (data not shown). It would therefore seem possible that there is a species difference between the rat and chick α7 receptors in their pattern of ethanol modulation. Several pharmacological differences between these two receptors have previously been described- for instance the agonist DMPP is a potent near-full agonist on the rat α7 receptor, but a very weak partial agonist on the chick α7 receptor (Séguéla et al., 1993: Bertrand et al., 1992), even though there is considerable (90%) amino acid sequence homology between them (Couturier et al., 1990; Séguéla et al., 1993). Species differences for the effects of alcohols have been noted for the 5-HT3 receptor.
which is a closely related member of the ligand-gated ion channel superfamily. 5-HT₃ receptors of guinea-pig nodose ganglion neurones are inhibited by trichloroethanol, whereas in a similar preparation from the rabbit the responses are inhibited (Gill et al., 1994). However, another study appears to contradict that of Yu et al., (1996), suggesting that the chick α7 receptor has a similar pattern of modulation by EtOH to the rat receptor under investigation in the present study (Susan Shepherd, University of Dundee, Personal Communication). De Fiebre et al. (1995) had suggested that inhibition by EtOH may also occur with the rat α7 receptor, although this now appears not to be the case due to defective recording equipment (Personal Communication).

c) What are the implications of these results?

The argument that the observations described here may have importance beyond the oocyte is reinforced by the fact that mecamylamine, a nicotinic antagonist, can antagonize ethanol-induced dopamine release in the rat nucleus accumbens (Blomqvist et al., 1993). Therefore, it seems possible that alcohol enhancement of nicotinic receptor activity in the mesolimbic pathway may contribute to the mutual reinforcement of drinking and smoking behaviour. Similarly, it is possible that the modulation of neuronal nicotinic receptor subtypes may contribute to the induction of alcohol dependence due to chronic high alcohol exposure. The concentration of ethanol at which the potentiating effect starts to occur in all the heteromeric receptors tested here (and sometimes with the α7 receptor) is equivalent to that regularly experienced by heavy drinkers (i.e. >30mM, which is equivalent to approximately 10 Units: 1 Unit=8g=10ml EtOH).

In rat substantia nigra reticulata and ventral palladium, Criswell et al., (1993) examined the nicotine induced changes in neuronal firing rate. In 7 of 9 cells which were excited by nicotine, the increase in firing rate was enhanced by alcohol. However, Frölich et al., (1994) noted that alcohol could inhibit the excitatory effects of nicotine, kainate and NMDA on neuronal firing rates in rat locus coeruleus, where α
3β4 is also expressed. Furthermore, EtOH-induced depressions of neuron firing can be enhanced by nicotine and antagonized by mecamylamine (Freund & Palmer, 1997). From these results it is apparent that the effects of ethanol on nicotinic receptors are not uniform in the brain, but may be subtype specific, or depend upon the intracellular signalling status of the cell under investigation. These apparently contradictory actions of ethanol support the variable observations at low ethanol concentrations with the α3 β4 subunit combination, and possibly also for the high concentration effects on the α7 receptor.

Criswell and colleagues also noted that the degree of inhibition of the NMDA response declined with prolonged incubation with ethanol. We have observed similar evidence of an initial strong sensitivity to low concentrations of ethanol, which fades with repeated exposure. This raises the possibility that if an individual rapidly consumes even a small amount of alcohol, then there could be a window of time before tolerance sets in, during which the person's performance is strongly affected.

Another implication of these results is that in the brain, the control of the activity of nicotinic AChRs may be highly dynamic and specific for each subunit combination. The diversity of the MIII-MIV cytoplasmic domains may determine some of this plasticity, enabling each receptor subtype to rapidly respond to a particular set of intracellular signalling influences. Unfortunately, dialysis of neurons during patch clamp may remove or disrupt some of these signalling pathways.

Much of our thinking about nicotinic receptors has been moulded by analysis of *Torpedo* and muscle type receptors, whose properties are comparatively static and which lie at the endpoint of a neuro-effector pathway. Here we have evidence that the control of the activity of neuronal receptors is highly dynamic, and that mechanisms may be present to both up and down-regulate agonist-induced receptor activity. They may be specialized for tasks in intermediate neuronal circuits involving integration of intracellular signalling activity and flexibility of response.
c) Phosphorylation sites may mediate part of the sensitivity of ligand-gated ion channels to alcohol

The ethanol-induced enhancement of the activity of recombinant (Wafford & Whiting, 1992, Lin et al., 1994) and brain (Weiner et al., 1994) GABA<sub>A</sub> receptors is thought to be partly mediated by protein kinase C (PKC), although there is also evidence for direct allosteric effects of ethanol on the receptor (Mihic et al., 1997). In contrast, Krishek et al. (1994) found that activation of PKC by phorbol esters results in reduction in GABA-activated currents carried by recombinant and native receptors. This reduction was diminished if the consensus PKC phosphorylation sites were mutated out. However there was an initial enhancement of the response of recombinant receptors expressed in oocytes. Protein kinase C (under conditions of elevated Ca<sup>2+</sup>) also mediates one of the pathways through which alcohol inhibits the kainate responses of glutamate receptors (Dildy-Mayfield & Harris, 1995), although there is also evidence of an additional PKC-independent mechanism. The actions of alcohol on neuronal nicotinic AChRs have not yet been linked to receptor phosphorylation, but there is firm evidence that phosphorylation of Torpedo and ganglionic nicotinic AChRs affects receptor function (see below; for review see Swope et al., 1992).

In addition to the effects described above, there is evidence for direct effects of ethanol on the activity of PKC. PKC contains a hydrophobic binding site for alcohols and anaesthetics (Slater et al., 1993), and ethanol has been shown to inhibit diacylglycerol-stimulated PKC activity at physiologically relevant concentrations (Slater et al., 1997). There is also evidence for indirect effects of ethanol on PKC activity. The presence of ethanol during the hydrolysis of phosphatidyl choline by phospholipase D results in the formation of the metabolically stable phosphatidylethanol instead of phosphatidic acid (PA) (Pai et al., 1988). This reduces the amount of PA that would normally be converted to diacylglycerol, an activator of protein kinase C.
e) Phosphorylation and neuronal nicotinic AChRs

Phosphorylation affects the function of many ligand-gated ion channels, (Swope et al., 1992; Browning & Rogers, 1994, reviews). Phosphorylation of muscle and Torpedo AChRs by protein kinase A (PKA), PKC and tyrosine kinase generally increases receptor desensitization (Huganir et al., 1986; Albuquerque et al., 1986; Middleton et al., 1986, 1988; Mulle et al., 1988; Ross et al., 1988; Eusebi et al., 1985; Hopfield et al., 1988; Mileo et al., 1995) although tyrosine kinases are also important in receptor aggregation (e.g. Gillespie et al., 1996; Qu et al., 1996). However, among members of the nicotinic receptor gene family, there is great diversity among the cytoplasmic domains. Hence it is not possible to assume that the effects of phosphorylation on any two subunits will be the same.

Much less work has been done on neuronal nicotinic AChRs, but activating protein kinase C in chick lumbar sympathetic ganglia causes enhancement of the rate of desensitization of ganglionic nicotinic AChRs, (Downing & Role 1987, Valenta et al., 1993). Substance P also enhances the rate of desensitization in ganglia, (Stallcup & Patrick, 1980; Clapham & Neher, 1983; Role, 1984; Simmons et al., 1990) possibly by indirectly activating PKC. In contrast, protein kinase A activators enhance nicotinic currents in chick ciliary ganglia, (Margiotta et al., 1987; Gurantz et al., 1994). Perhaps this contrast with the effect of PKA on muscle receptors should not be surprising, since the cytoplasmic domains are the least conserved regions of the nicotinic AChRs. The mechanism of PKA enhancement is not clear, but was proposed to involve the conversion of non-functional receptors to functional ones. The α3 subunits in these receptors can be phosphorylated (Vijayaraghavan et al., 1990) and the degree of phosphorylation mimics the time course of the PKA enhancement of the acetylcholine activated current. Since α3 subunits are present in the α-bungarotoxin insensitive receptor in chick ciliary ganglia, the above studies raised the question of whether
phosphorylation at PKA sites is an absolute requirement for function of nicotinic AChRs containing the α3 subunit.

Biochemical studies have shown that α4 subunits can also be phosphorylated by PKA, (Nakayama et al., 1993). Recent studies also show that calcium flux through α7 receptors expressed in GH4C1 rat pituitary cells can be enhanced by activating PKA (Quik et al., 1997). In summary, there is evidence that the activity of neuronal AChRs can be modulated by kinases.

f) Preliminary results for the effects of phosphorylation on neuronal nAChRs.

Preliminary data was obtained in this study using a mutant α3 subunit (supplied by Dr Robert Duvoisin, Cornell University) with the consensus phosphorylation site for PKA removed (4 serines at positions 412-416 changed to alanines). In combination with the β4 subunit a functional receptor was formed when expressed in oocytes (see figure 5.10). The modulation of this receptor by high ethanol concentrations appeared to be unchanged compared to the wild-type. At 300mM EtOH the mean averaged response to ACh (1-10μM) was increased to 269 ± 22.2% (n=9, range=160-357%) of the control value. Insufficient numbers of cells were investigated to observe modulation that might be present at low EtOH concentrations: in 4 oocytes 3mM EtOH produced effects ranging from 99.0-103% of the control response (3μM ACh).

A preliminary experiment using the PKC inhibitor G67874 appeared to result in potentiation of ACh-activated α3β4 responses (see Figure 5.11.). However, reproducible results could not be obtained and accuracy was compromised by the length of incubation required with this compound.
Figure 5.10. Preliminary results suggest that inhibiting protein kinase C can enhance responses of the nicotinic α3β4 receptor. 2-electrode voltage clamp recordings were made from *Xenopus* oocytes expressing the α3β4 receptor subtype. The figure shows inward current responses separated by 20 minute intervals running sequentially from left to right. The first two responses (left) show consistent control responses to 10μM ACh. Immediately following these the oocyte was incubated with the selective protein kinase C inhibitor Gö7874 (10nM) for 20 minutes, as indicated by the hollow bar. The subsequent (3rd) response to 10μM ACh has been enhanced to 111% of the control response (100%). The fourth response shows that after a 20 minute wash in control solution the response to 10μM ACh has returned to the control level. The fifth response shows that co-applying 10μM ACh and 10nM Gö7874 without preincubation does not enhance the response (i.e. there is no direct modulatory effect of Gö7874 on the receptor).
Figure 5.11. Preliminary results suggest that removal of concensus PKA phosphorylation sites does not affect ethanol modulation. A mutant α3 subunit with the concensus phosphorylation site for PKA removed (4 serines at positions 412-416 changed to alanines) was supplied by Dr Robert Duvoisin (Cornell University). In combination with the β4 subunit a functional receptor was formed when expressed in oocytes. The modulation of this receptor by high concentrations of ethanol appeared to be unchanged compared to the wild-type. In this example 100mM EtOH potentiates the response to 156% of control. Although no effects were observed at low EtOH concentrations, insufficient numbers of cells were investigated to make conclusive comparison with the wild type receptor.
In the light of the present results, it is tempting to suggest that the direct effects of alcohol on neuronal nicotinic receptors are responsible for the intense need to smoke that some people feel when drinking alcohol. It will be informative to determine whether it is the inhibitory effect or the potentiating effect which is important, and again which subtypes are involved. The answers to these questions will help define the mechanisms of reward in drug dependence. They may also help in the design of more effective strategies for smoking cessation, for if the effects described here occur in the brain, then there may be a case for advising those hoping to give up smoking that they should avoid heavy drinking at all costs!
CHAPTER 6:
CONCLUSIONS & FUTURE DIRECTIONS

a) Conclusions

This study has utilized a functional approach to investigate the pharmacological diversity of native and cloned neuronal nicotinic acetylcholine receptors. Having characterized some aspects of this diversity, we have then investigated how different subtypes of nicotinic AChRs might be involved in nicotine and alcohol addiction. This has involved patch-clamp experiments in rat SCG neurones and 2-electrode voltage clamp experiments in *Xenopus* oocytes.

The first part of the study attempted to predict the subunit composition of native receptors by pharmacological comparison with cloned receptors. In this study, the factors which hamper adequate comparisons between neurones and oocytes, such as desensitization and external calcium, were minimized. Although this was not entirely successful, it demonstrated that agonist potency ratios may provide a subtle means of distinguishing receptor subunit composition in native cells. The results did, however, reveal that the nicotinic receptors in rat SCG neurones have pharmacological properties similar, but not identical, to those obtained with $\alpha_3\beta_4$ receptors expressed in *Xenopus* oocytes. Expression of cloned receptors in oocytes, therefore, represents a good primary model for pharmacological studies. This study also demonstrated that, in common with some other neuronal cell types, the nicotinic responses in SCG neurones exhibit time-dependent changes in activity during the course of a recording.

The second part of the study involved constructing full agonist concentration response relationships for several subunit combinations. Although this has also been attempted by other authors, this study has revealed for the first time that, at the macroscopic level, simple neuronal nicotinic $\alpha/\beta$ pairs expressed in *Xenopus* oocytes can produce a functionally heterogeneous receptor population. It is evident from
numerous studies, including this one, that neuronal nicotinic receptor diversity can be
provided by the large number of functionally distinct receptors possible with different
subunit combinations. This study provides evidence that further neuronal nAChR
diversity can occur within a single simple α/β subunit combination by the formation of
more than one functionally distinct receptor subtype. It also indicates the need for
cautions in interpreting agonist concentration response data obtained for neuronal
nAChRs. Such measurements are a mainstay of the pharmaceutical industry in the
first-line screening of agents with potential therapeutic value.

The high-affinity component obtained with the α4-1β2 combination is of
particular interest. The α4-1β2 receptor is believed to be one of the most common
brain receptors, and if so, then it is likely to be strongly activated by the concentrations
of nicotine achieved by smoking cigarettes.

Effects of ethanol on nicotinic receptors were suggested by the positive
correlation between smoking and drinking behaviour in humans. The present study has
demonstrated for the first time that the activity of a number of defined mammalian
neuronal nAChR subtypes can be modulated by ethanol. This may have implications in
the development of ethanol addiction as well as the synergism of ethanol consumption
with nicotine addiction, especially if presynaptic nicotinic receptors in the brain are
subject to this modulation. Further functional diversity has been revealed in the
patterns of ethanol modulation exhibited by each subtype. In particular the α3β4
subtype appears to be particularly sensitive and variable in its modulation by ethanol.
The results suggest that in the brain the control of the activity of nicotinic AChRs
could be highly dynamic and specific for each subunit combination.

It would be tempting to suggest that the different patterns of modulation
exhibited by ethanol on nicotinic receptors could be diagnostic for a particular subtype
present in neurones, especially α3β4-type receptors. However, this approach would be
complicated by the variable behaviour obtained with this subtype, as well as the
probability of multiple receptor subtypes in a particular cell type. At present there is a
shortage of suitable selective nicotinic agents for studying nicotinic receptors.
especially those that are insensitive to α-bungarotoxin. Nicotinic receptors have been implicated in the aetiology of, and as potential therapeutic targets for, a number of serious neurological disorders. The development of suitable diagnostic methods for the elucidation of the nature and role of the functional nicotinic receptors present in particular neuronal circuits will be of considerable value in the future development of potential nicotinic therapeutic agents.

However, a major problem is to determine which particular nicotinic receptor subtype is associated with the neural circuits mediating particular disease states. In the present work two approaches have been used to try and answer this central question. The first has been to use pharmacological profiling to identify the subunit composition of native receptors and document their unique pharmacological properties. This will help to identify their presence in particular neuronal circuits in the brain.

The second approach has been to use drugs which mediate the disease states of addiction, and to determine which receptors are most sensitive to them. We decided to look at the interaction of alcohol with nicotinic receptors, based on the positive correlation between nicotine and alcohol consumption. Here we show that the α3β4 subtype is the most sensitive to alcohol.

Combining these approaches can help towards the development of subtype-specific drugs which can help combat the whole range of disease states in which nicotinic receptors are involved.

**h) Future Directions**

In the present study full concentration-response curves were obtained for ACh on several heteromeric subunit combinations. To make this more relevant to the addiction process this study could be extended to include the drug nicotine. We could then obtain values for the parameters of the biphasic relationships with this drug, especially for the α3β4 and α4-1β2 subtypes.
In the present study we have also demonstrated that a range of neuronal nAChR subtypes expressed in *Xenopus* oocytes can be modulated by ethanol. It would be interesting to see if such behaviour can also be reproduced in native receptors (e.g. using brain slice preparations). Of particular interest would be the question of whether cells which possibly contain α3β4-type receptors (e.g. in the medial habenula) show the special sensitivity to ethanol revealed in this study.

We obtained some preliminary data on the effects of mutations in the cytoplasmic domain on receptor function, particularly with respect to ethanol modulation. Additional mutations of the putative phosphorylation sites in this region would have been useful, not only for the possible elucidation of the mechanisms of ethanol modulation, but also in investigating their effects on normal receptor function. These studies could be supplemented by experiments with specific protein kinase inhibitors and/or catalytic subunits, as was briefly touched upon at the end of this study. This could also be extended to include native receptors. At present little is known about the effects of phosphorylation on neuronal nicotinic receptor function.

The determination of which subunits make up the functional nicotinic (or indeed any other) receptor in a particular native cell is not a simple task. A good place to start is to look at which mRNAs are present in that cell type using in situ hybridization studies. This, however, does not necessarily tell you with what efficiency individual subunit proteins are being expressed. Co-immunoprecipitation studies are of more value in that they will answer this question and also tell you which subunits are co-assembling to form complete receptors. This, however, does not necessarily tell you which assembled receptors are the functional ones, especially if there are multiple subtypes. Such studies will, however, give a good indication of the nature of the functional receptors, and are a solid starting point. The ultimate proof lies in the direct comparison of the functional properties of native receptors with receptors of known composition. However, it is becoming apparent that certain receptor expression systems (e.g. *Xenopus* oocytes) may not assemble some receptor proteins in the same manner as native cells. The requirements to answer this fundamental question are.
therefore, two-fold. Firstly, there is the need for adequate co-immunoprecipitation data to act as a predictor of the actual subunits in the assembled receptors in a particular cell type. Secondly, there is the need for reliable receptor expression systems that will faithfully reproduce the receptor under investigation. This second factor is, however, problematical in that, in order to confirm faithful receptor assembly, functional comparison with native receptors is required. Also, a particular expression system may faithfully reproduce one type of receptor, but not another. The perfect expression system would be the particular native cell under investigation with the native subunits removed. This is because receptors are being increasingly viewed as part of a larger complex of specifically associated kinases and binding proteins, and these factors will be very specific for the cell-type in which the native receptor is expressed. Unfortunately, in the absence of such 'perfect' expression systems we have to make a compromise, as in the use of *Xenopus* oocytes.

An additional factor is that in cases where multiple receptor subtypes are present in a cell type, suitably selective antagonists are required to remove components and define a single receptor subtype. The absence of such ligands for neuronal nicotinic receptors has severely hampered the progress of their investigation. The large number of non-selective drugs currently available are useful for the crude determination of the involvement of nicotinic receptors in brain physiology and disease. However, the stimulus for developing useful subtype-selective therapeutic agents ultimately lies in predicting which specific nicotinic receptor subtypes are involved in the aetiology of, or are potential therapeutic targets for, a particular disease state.
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**Other References:**


**Added References:**
