

The Assembly and Folding of Neuronal Nicotinic Acetylcholine Receptors

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List of abbreviations

5HT	5-hydroxytryptamine (Serotonin)
α -BTX	α -bungarotoxin
ACh	Acetylcholine
AChR	Acetylcholine receptor
AChE	Acetylcholine esterase
BiP	Immunoglobulin binding protein (GRP78)
<i>C. elegans.</i>	<i>Caenorhabditis elegans</i>
ChAT	Choline acetyltransferase
CIP	Calf intestinal phosphatase
CNS	Central nervous system
D α 3	Third nAChR α like subunit of <i>Drosophila</i>
D α 4	Fourth nAChR α like subunit of <i>Drosophila</i>
DDF	(p(<i>N,N</i>)-dimethyl-aminobenzenediazonium fluoroborate)
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FCS	Foetal calf serum
GABA	γ -aminobutyric acid
G-proteins	Guanine nucleotide regulatory proteins
GRD	GABA and glycine-like receptor of <i>Drosophila</i>
GRP78	Glucose-regulated protein 78 (BiP)
HEK	Human embryonic kidney cells
LCCH3	Ligand-gated chloride channel (<i>Drosophila</i> GABA receptor subunit)
mAbs	Monoclonal antibodies
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor

NMJ	Neuromuscular junction
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PNS	Peripheral nervous system
RDL	Resistance to dieldrin (<i>Drosophila</i> GABA _A receptor subunit)
RDL ^r	A mutated, dieldrin resistant form of RDL
SCG	Superior cervical ganglia
SDS	Sodium dodecyl sulphate
SH-SY5Y	Human peripheral neuroblastoma cell line
SOS	Special oocyte saline
TM (1-4)	Transmembrane domains 1 - 4
TRP	Transient receptor potential
TRPL	Transient receptor potential like

Abbreviations for *Drosophila* nAChR subunits (nomenclature suggested by FlyBase is given in parentheses):

ALS	α -like subunit	(nAcR α -96Aa)
ARD	β -like subunit (ACh receptor of <i>Drosophila</i>)	(nAcR β -64B)
SAD	Second α -like subunit <i>Drosophila</i>	(nAcR α -96Ab)
SBD	Second β -like subunit <i>Drosophila</i>	(nAcR β -96A)

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are pentameric hetero-oligomeric ion channels. To date five vertebrate muscle subunits (α , β , γ , δ and ϵ) and eleven vertebrate neuronal nAChR subunits ($\alpha 2$ - $\alpha 9$ and $\beta 2$ - $\beta 4$) have been cloned. In *Drosophila* only four nAChR subunits have been reported to date. These include two subunits (ALS and SAD) which show homology to the vertebrate α subunits and are assumed to be agonist binding subunits. The remaining two subunits (ARD and SBD) resemble the vertebrate non- α subunits in that they lack the two adjacent cysteine residues characteristic of the α subunits and are thought to have structural roles. A central aim of this research project was to investigate the folding, subunit assembly and functional properties of cloned nAChRs. Invertebrate (*Drosophila* and *C. elegans*) and vertebrate (rat) nAChR subunits were expressed in a variety of mammalian and invertebrate cell lines. In contrast to vertebrate nAChRs, no functional expression has been reported for any combination of the four *Drosophila* subunits expressed in *Xenopus* oocytes or cultured mammalian cells. Whereas the *Drosophila* SBD cDNA, reported previously, lacked part of the SBD coding sequence, here the construction and expression of a full-length SBD cDNA is reported. It seems plausible that the problems in expressing functional *Drosophila* nAChRs in *Xenopus* oocytes or in mammalian cell lines might be due to an inability of these expression systems to correctly assemble *Drosophila* nAChRs. Despite expression in what might be considered a more native cellular environment, functional nAChRs were not detected in a *Drosophila* cell line unless the *Drosophila* nAChR subunit cDNAs were co-expressed with vertebrate nAChR subunits. The results also indicate that the folding of *Drosophila* nAChR subunits is temperature-sensitive and strongly suggest that the inability of these subunits to generate functional channels in the absence of vertebrate subunits is due to a requirement for co-assembly with as yet unidentified *Drosophila* nAChR subunits. In the latter stages of this project a new (fifth) *Drosophila* nAChR subunit, D $\alpha 3$ (isolated by another group) was obtained and examined by heterologous expression. *Drosophila* cDNA libraries have been screened for novel nAChR subunits and a partial cDNA clone of a novel α subunit has been isolated and sequenced.

1: Introduction

1.1 Acetylcholine as a neurotransmitter

Acetylcholine (ACh) is an important neurotransmitter in both vertebrates and invertebrates which binds to two pharmacologically distinct families of receptor (Dale, 1914). These two families of ACh receptor are classified as "nicotinic" and "muscarinic" due to their selective activation by the agonists nicotine and muscarine.

ACh levels are regulated by the ACh enzymes choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), and a high affinity uptake system for choline. It has been shown by histochemical detection of esterase activity and immunohistochemical localisation of ChAT that both enzymes are widely expressed in the central nervous system (CNS) of vertebrates and in invertebrates such as the *Drosophila* (Buchner *et al.*, 1986) as is the uptake system for [³H]-choline, one of the products of ACh hydrolysis (Buchner and Rodrigues, 1983).

1.2 Acetylcholine receptors

The nicotinic and muscarinic receptors are members of distinct superfamilies of neurotransmitter receptors, the ligand-gated ion channel and guanine nucleotide regulatory protein (G-protein) linked superfamilies respectively. Before discussing the neuronal nicotinic acetylcholine receptors (nAChRs), the main subject of this research project, I shall briefly review the muscarinic receptors and the most extensively studied members of the ligand-gated ion channel family, the muscle type nAChRs.

1.2.1 Muscarinic acetylcholine receptors

The muscarinic acetylcholine receptor (mAChR) is a member of the G-protein coupled receptor superfamily which includes the α and β adrenergic receptors, the γ -aminobutyric acid (GABA) type B receptors (GABA_BR) and serotonin (5HT) receptors (eg. 5HT_{1B}, 5HT₂) receptors.

Each of these receptors consists of a single polypeptide with seven putative transmembrane domains and a long intracellular loop between the 5th and 6th transmembrane domains. The receptors modulate a variety of signal transduction pathways by interactions with G-proteins. Activation of the muscarinic receptors, as described below, can result in inhibition of adenylyl cyclase and the stimulation of phospholipase C, whilst other effects are indirect such as the increase in cAMP or the stimulation of arachidonic acid.

The muscarinic receptors have been classified pharmacologically into 3 subtypes: M1, M2 and M3 based mainly on their affinities for pirenzepine (Hammer *et al.*, 1980), 11-[[2-[(diethyl-amino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido-[2,3-b]-[1,4] benzodiazepine-6-one (AF-DX116) (Hammer *et al.*, 1986) and 4-diphenylacetoxy-*N*-methylpiperidine methbromide (4-DAMP) (Barlow *et al.*, 1976). These subtypes may be heterogeneous, however, their detailed pharmacological characterisation has been limited by the lack of sub-type specific antagonists. Since the development of molecular biological techniques, the cloning of five genes encoding different muscarinic receptor subtypes (m1 - m5) have been established (Kubo *et al.*, 1986; Bonner *et al.*, 1987). The muscarinic receptors each have a unique pattern of mRNA tissue distribution within the mammalian central nervous system (CNS), shown by *in situ* hybridisation, for example M3 is distributed predominantly in the forebrain and some thalamic nuclei whereas M1 is predominantly localised in the cerebral cortex, striatum and hippocampus (Buckley *et al.*, 1988; Vilaro *et al.*, 1991).

The M1, M3 and M5 muscarinic receptors preferentially couple to phosphoinositide hydrolysis via pertussis toxin-insensitive G-proteins (Bonner, 1988; Peralta *et al.*, 1988). Inhibition of adenylyl cyclase via pertussis toxin-sensitive G-proteins is the main coupling system of M2 and M4 (Peralta *et al.*, 1988). This is not always the case and promiscuous coupling to more than one second messenger system has been

reported for example m2 has been shown to couple with low efficiency to phosphoinositide hydrolysis (Peralta *et al.*, 1987).

Muscarinic receptors are also expressed in the mammalian peripheral nervous system (PNS) and have important roles such as inhibition of rate and force of heart beat and smooth muscle contraction. The roles of these receptors in the CNS are less well understood but they are implicated in the maintenance of short term memory (Hagan and Morris, 1988). The loss of M2 autoreceptors in the basal forebrain of Alzheimer's patients indicate that they may be involved in memory loss (Mash *et al.*, 1985).

1.3 Ligand-gated ion channels

The nAChRs are members of the superfamily of ligand-gated ion channels. The interaction of ACh with these receptors leads to the opening of an intrinsic ion channel and an influx of cations (predominantly Na⁺) into the cell.

The ligand-gated ion channel superfamily includes glycine receptors, GABA_A receptors, 5HT₃ receptors and the ionotropic glutamate receptors. The *Torpedo* electric organ nAChR was the first ligand-gated ion channel to be studied in detail and is the only receptor for which a high resolution three dimensional structure has been determined (Unwin, 1993; Unwin, 1995). By analogy to the *Torpedo* nAChR it is possible that all of the receptors in this superfamily are formed from five subunits arranged around a central membrane spanning pore. The three dimensional structure of the neuronal and muscle nAChR is likely to be broadly similar to that of the *Torpedo* electric organ nAChR (which is described in detail in the following section).

1.4 The *Torpedo* nAChR

Due to the rich source of cholinergic synapses, the electric organ of fish such as the marine ray *Torpedo* played an important role in the identification and purification of the nicotinic acetylcholine receptor (Changeux, 1975). The *Torpedo* electric organ is

composed of electrocytes, modified muscle cells, that have lost the ability to contract. Two 1 Kg electric organs from a large *Torpedo* can yield 0.2 g of pure receptor. After homogenisation, receptors purified by sucrose-gradient equilibrium centrifugation and alkaline treatment_x produce vesicles where cholinergic ligand binding and ion transport can be assayed. The detergent-solubilised form of the *Torpedo* nAChR was obtained by solubilisation of the membrane in Triton X-100 (Meunier *et al.*, 1972).

1.4.1 Subunit composition and receptor structure

The *Torpedo* nAChR is the most thoroughly characterised receptor in the superfamily of ligand-gated ion channels. The nAChR is an integral membrane glycoprotein of approximately 250 kD. It is a pentameric complex comprising four distinct subunits α , β , γ and δ with the apparent molecular weights of 40, 50, 60 and 65 kD, respectively (Raftery *et al.*, 1974; Weill *et al.*, 1974; Raftery *et al.*, 1975; Lindstrom *et al.*, 1979; Karlin, 1980). A group of neurotoxins isolated from the venoms of various snakes, including α -bungarotoxin (α -BTX) from the elapid snake (Lee, 1972), have been used extensively in the isolation, purification and characterisation of nAChRs. α -BTX is a highly potent antagonist of vertebrate nAChRs and can be radiolabelled without loss of binding and has been shown to bind irreversibly to the *Torpedo* nAChR with a K_d of approximately 10^{-11} M. Radiolabelled α -BTX has been used to label nAChRs on a sucrose gradient to show that the receptors migrate with a sedimentation coefficient of 9S. The *Torpedo* nAChR pentameric complexes form covalent links through cysteine disulphide bonds between the δ subunits to yield 500 kD AChR dimers which have a sedimentation coefficient of 13S (Raftery *et al.*, 1972). The 13S 'heavy form' can be converted to the 9S 'light form' by reducing agents such as dithiothreitol (Chang and Brock, 1977; Hamilton *et al.*, 1977; Sobel *et al.*, 1977). The properties of the 9S and 13S forms of the *Torpedo* nAChR have been found to be virtually identical (Anholt *et al.*, 1980; Montal, 1986). There is no evidence for dimers of vertebrate muscle or neuronal nAChRs or in invertebrate nAChRs.

1.4.2 Molecular cloning of *Torpedo* nAChR subunits

The nAChR subunits were cloned initially using mRNA isolated from *Torpedo* electric organ to prepare cDNA libraries. Synthetic oligonucleotides corresponding to determined amino acid sequences were used to screen cDNA libraries which led to the isolation of cloned DNAs and sequences for the α , β , γ and δ subunits (Noda *et al.*, 1982; Sumikawa *et al.*, 1982; Noda *et al.*, 1983b; Noda *et al.*, 1983c). Other groups were using similar approaches about the same time such as screening a *Torpedo* electric organ cDNA library using probes designed to *Torpedo* electric organ mRNA. This approach yielded cloned DNAs for the complete γ and α sequences (Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983) and partial sequences for the β and δ subunits (Hershey *et al.*, 1983). With the identification of sequence information, specific oligonucleotide probes were designed and partial clones used to probe the *Torpedo* electric organ cDNA libraries which resulted in the isolation of full-length α , β , γ and δ clones (Claudio *et al.*, 1983; Claudio *et al.*, 1987). The cDNA clones of the *Torpedo* nAChR have been used as probes to isolate nAChR subunits from other species which are described later.

1.4.3 Subunit topology

The four *Torpedo* nAChR subunits have similar hydropathy profiles, each consisting of four hydrophobic domains, thought to form the membrane spanning domains TM1 - TM4, interspersed by more hydrophilic regions. The longest hydrophilic domains are the relatively conserved amino terminal end of the subunit and the highly variable domain forming the cytoplasmic loop between TM3 and TM4. The amino terminal domain is where the ACh binding site is located (Devillers-Thiery *et al.*, 1993) and there is a short extracellular carboxy-terminal tail. The predicted membrane topologies of nAChR subunits, together with that of other ligand-gated ion channel subunits, are shown in Figure 1.1. The five subunits surround the central pore which has been shown to have an aperture of 6 - 7 Å by the channel being able to pass

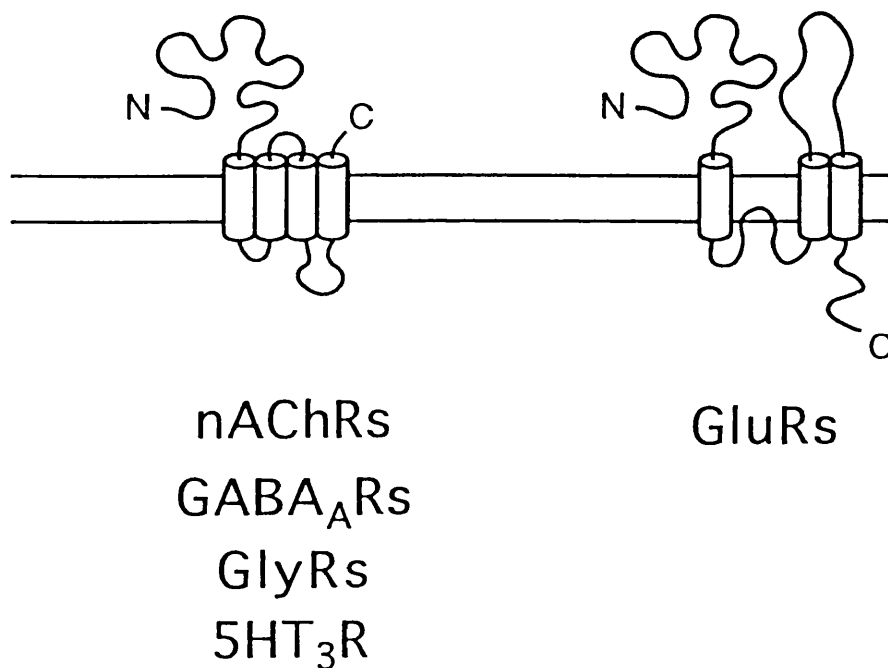


Fig. 1.1: The predicted membrane topologies of the nAChR subunits, together with that of other ligand-gated ion channel subunits are shown. The nAChR, GABA_AR, GlyR, 5HT₃R and GluR subunits contain an N-terminal signal sequence that is cleaved in the ER. The N-terminus of the mature receptor is located on the extracellular surface of the membrane as is the short C-terminal tail. Each subunit has four proposed membrane spanning domains (TM1-TM4).

The proposed topology of the glutamate receptor has three transmembrane domains with an extracellular N-terminus and intracellular C-terminus (Hollmann et al 1994., Bennett and Dingledine 1995).

neutral organic molecules as large as triethanolamine or glucosamine (Dwyer *et al.*, 1980; Dwyer and Farley, 1983).

The membrane spanning domains of the pentameric receptors are thought to be formed by twenty hydrophobic segments (four in each subunit), presumed until recently to form α -helices. This theory has been questioned by Akabas (Akabas *et al.*, 1992) and Unwin (Unwin, 1993) who suggested that not all the residues in the membrane spanning segments are in an α -helical conformation. Unwin determined the structure of the nAChR at 9Å resolution by microscopy of ordered arrays of receptors in the *Torpedo* electric organ membrane. From diffraction methods, images of four rods, interpreted to be α -helices, were observed in each subunit, however only one rod per subunit appeared to be in the membrane spanning domain. The speculation about the secondary and tertiary structural elements of the nAChR inferred an overall similar proposed structure, i.e. five subunits around a central pore. The three rods, or α -helices, located in the extracellular domains of each subunit are thought to form an ACh binding site. The fourth rod, a single kinked rod, observed in the membrane spanning domain, is thought to be a kinked α -helix formed from the TM2. No other secondary structures could be resolved and Unwin suggested that the three other membrane spanning domains, TM1, TM3 and TM4 may form β strands.

The complete amino acid sequences deduced from the cloned cDNA sequences have been used to predict the structure of the nAChR subunits, their molecular weights, potential glycosylation sites, predicted phosphorylation sites and the location of the ACh binding site.

1.4.4 The location of the ACh binding site

The ACh binding site has been located on the α subunit of the *Torpedo* nAChR the binding properties of which are altered when a cysteine disulphide bond is reduced (Karlin, 1980; Changeux, 1981; Popot and Changeux, 1984). The α subunit has

seven cysteines at positions 128, 142, 192, 193, 222, 412, and 418. The last three are predicted to be located in the hydrophobic transmembrane domains and so are unlikely to form part of the binding site. The alkylating compound [³H]-maleimidobenzyltrimethylammonium (MBTA) has been used to affinity-alkylate the nAChR and has shown that Cys 192 and Cys 193 are labelled indicating they are part of the binding site (Kao *et al.*, 1984). It was subsequently shown that disulphide linkages are formed between Cys 192 and Cys 193 and between Cys 128 and Cys 142 (Kao and Karlin, 1986).

The ACh binding site has been investigated with several photoaffinity labelling probes such as DDF (p(*N,N*)-dimethyl-aminobenzenediazonium fluoroborate) and has identified three different regions of the N-terminal hydrophilic domain of the α subunit (Goeldner, 1980). [³H]-DDF has been used to label the *Torpedo* nAChR α subunits, which after cleavage by cyanogen bromide, produced three [³H]-DDF labelled peptides (Dennis *et al.*, 1988; Galzi *et al.*, 1990). Six residues in the *Torpedo* α subunit have been identified which are conserved in the α subunits of all species which have been examined. The six residues thought to be important in the agonist binding site on the α subunit are: Trp 86, Tyr 93 (both in loop A), Trp 149, Tyr 151 (both in loop B) and Tyr 190, Tyr 198, and the Cys doublet 192-193 (in loop C) (Kao *et al.*, 1984; Dennis *et al.*, 1988; Abramson *et al.*, 1989; Galzi *et al.*, 1990; Cohen *et al.*, 1991; Middleton and Cohen, 1991). Mutations of these residues cause a five-fold to a hundred-fold decrease in the apparent affinity of the receptor for ACh. The three [³H]-DDF-labelled peptides in the NH₂-terminal large hydrophilic domain suggested by their arrangement that several loops contribute to the ACh binding site. This has led to a three loop model of the ACh binding site (Dennis *et al.*, 1988; Galzi *et al.*, 1990) which is shown in Figure 1.2.

Photolabelling experiments with *d*-tubocurarine have identified γ Trp 55 and δ Trp 57 which may contribute to the ACh binding site (Blount and Merlie, 1988) in

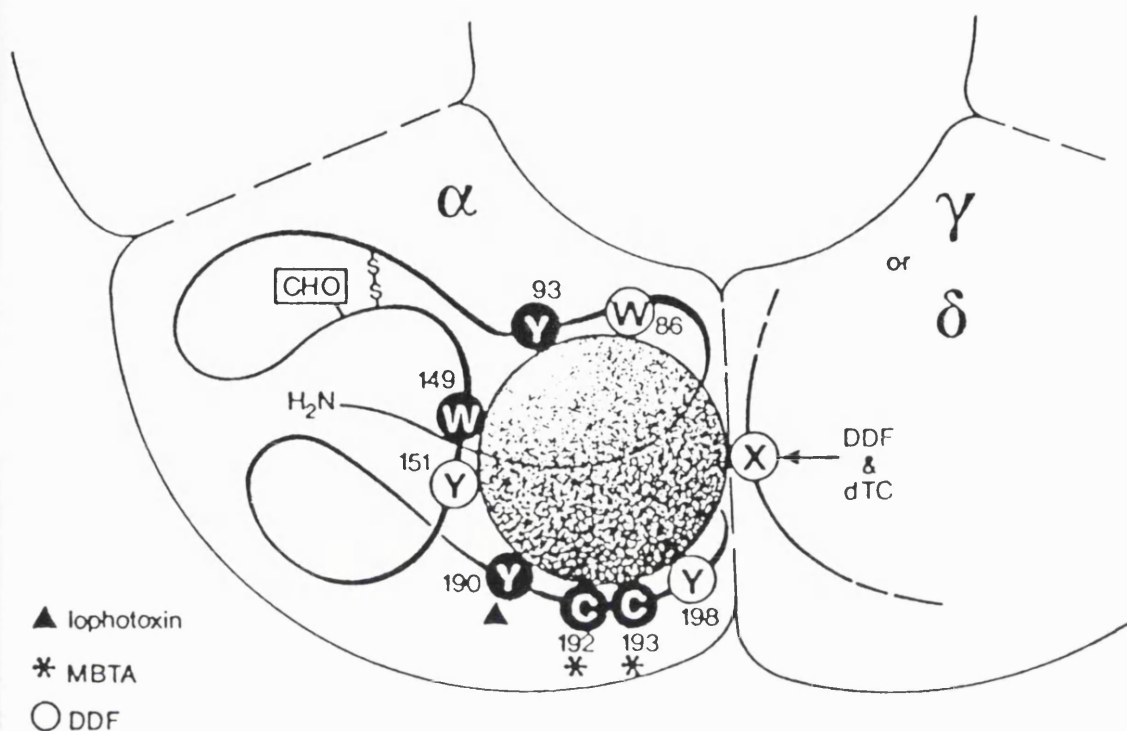


Fig. 1.2. Model of the agonist binding site of the nAChR from the *Torpedo* electric organ. Schematic model for the proposed folding of the amino-terminal extracellular segments of the α -chain in the AChR oligomer. The space occupied by the competitive antagonist DDF, in all possible orientations within its binding site, is represented by the sphere. The polypeptide chain is folded in such a way that the [^3H]-DDF labelled (O) amino acids (one letter code) are in contact with the sphere. Filled circles denote those residues clearly shown to be labelled and open circles those for which evidence indicative of labelling was obtained. The amino acids labelled by [^3H]-MBTA (*) and [^3H]-lophotoxin (▲) are also indicated. The numbers refer to the positions within the sequence of the α subunit of *Torpedo*. The X denotes as-yet unidentified amino acid(s) labelled by [^3H]-DDF on the γ subunit and by [^3H]-d-tubocurarine on the γ and δ subunits. The disulphide bond (S-S) linking α -Cys-128 and α -Cys 142 and the site of N-linked glycosylation at Asn 141 are indicated. The figure is from Galzi *et al* 1991.

conjunction with the residues located on the α subunits. The neuronal β nAChR subunits, like the α subunits, also have the conserved residues Trp 149 and Tyr 93 (Deneris *et al.*, 1988; Nef *et al.*, 1988; Deneris *et al.*, 1989). These are highly conserved amino acids which suggest they have an essential physiological role that is distinct from the muscle structural subunits which do not have these conserved residues.

1.4.5 The structure of the *Torpedo* nAChR ion channel

The lining of the pore of the ion channel is thought to be formed from the TM2 domains of the five subunits (Giraudat, 1986; Hucho, 1986; Giraudat, 1987) based on affinity labelling data. Mutational experiments have confirmed the functional role of TM2 and have shown that labelled amino acids belong to superimposed rings of homologous residues in the lumen of the channel referred to as the: Threonine ring, Serine ring, Equatorial Leucine ring, Valine ring and the Outer Leucine rings line the lumen of the ion channel (Imoto *et al.*, 1988; Akabas *et al.*, 1992). Mutations in the Serine ring, Equatorial Leucine ring, Valine ring and the Outer Leucine rings have been shown to alter ion channel gating (Revah *et al.*, 1991; Devillers-Thiery *et al.*, 1992). The proposed structure of the nAChR ion channel is shown in Figure 1.3.

The lining of the ion channel by the TM2 domains of the five subunits is supported by considerable data. The alignment of sequences has revealed superimposed rings of conserved amino acids. The inner ring of negatively charged amino acids is involved in monovalent and divalent ion permeability. The Serine ring has been linked to ion permeability. The Equatorial Leucine ring is thought to play a role in the gating of the ion channel. Mutations in the Serine ring, Intermediate ring and Outer Leucine rings have been shown to alter cation selectivity (Galzi *et al.*, 1992; Bertrand *et al.*, 1993; Devillers-Thiery *et al.*, 1993).

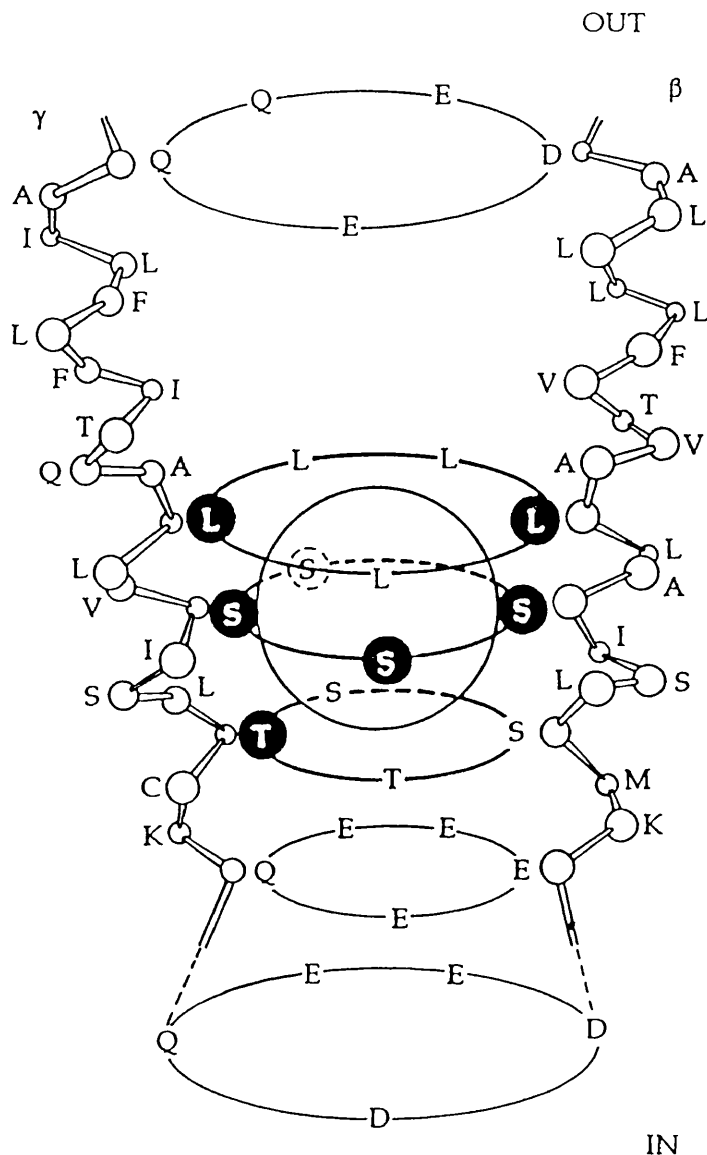


Fig. 1.3. Model of the high-affinity binding site for chlorpromazine within the AChR ion channel. The TM2 segments, arranged as transmembrane α helices, are quasi-symmetrically arranged around the central axis of the molecule and are tilted with respect to this axis. The figure displays the α carbons of the considered amino acids (single letter code).

The rings of homologous amino acids in the lumen of the channel from the outer surface to the cytoplasmic side are: (1) the "Outer Charged Ring", (2) the three rings of amino acids labelled by chlorpromazine, the "Leucine Ring", the "Serine Ring" and the "Threonine Ring" (filled circles), (3) The "Intermediate Ring" and the "Inner Charged Rings". The sphere represents the space occupied by a molecule of chlorpromazine in all possible orientations within its binding site. The figure is from Revah *et al.* 1990.

There is extensive sequence homology in the TM2 domain throughout the superfamily of ligand-gated ion channels, in particular the Inner ring of negatively charged amino acids, the Serine ring and the Equatorial Leucine ring (Devillers-Thiery *et al.*, 1993). Experiments where mutations were introduced in the TM2 domain, to change selected amino acids, have indicated that subtle changes in amino acid composition can lead to receptors that display different physiological and pharmacological properties. The data also show that correct subunit co-assembly is critical. The wrong stoichiometry or incorrect subunit co-assembly can have a profound effect on the ion channel lining which could in turn effect ion channel selectivity, gating, permeability or overall functional properties of the channel.

1.4.6 The subunit arrangement in *Torpedo* nAChR assembly

The structural arrangement of the individual subunits within the nAChR pentamer has been investigated by several experimental approaches. Using tagged α , β and δ subunits the single subunit between the two α subunits was inferred from electron microscopic images of receptor dimers to be the γ subunit and not the β or δ subunit (Karlin *et al.*, 1983; Karlin, 1991). This structural arrangement was supported by evidence that the two ACh binding sites are formed in the interfaces between α and γ , α and δ , whereas α and β do not form an ACh binding site (Kurosaki *et al.*, 1987; Blount and Merlie, 1989; Pedersen and Cohen, 1990; Sine and Claudio, 1991). In contrast electron diffraction studies suggested that the β subunit is located between the two α subunits (Kubalek *et al.*, 1987; Unwin, 1993).

α -BTX has been used to identify possible intermediates during the formation of the nAChR in conjunction with co-immunoprecipitation studies with antibodies to the β , γ and δ subunits. The [125 I]- α -BTX is co-precipitated with the $\alpha\delta$ and $\alpha\gamma$ combinations but not with the $\alpha\beta$ combination, indicating that the $\alpha\beta$ combination does not form an ACh binding site (Blount and Merlie, 1989; Blount *et al.*, 1990; Gu *et al.*, 1991; Saedi *et al.*, 1991). The proposed model for nAChR assembly derived from this

evidence suggests that the α subunit oligomerises with either δ or γ to form heterodimers $\alpha\delta$ and $\alpha\gamma$ which then associate with β and each other to form the pentameric complex. This model has been questioned recently and another model based on [^{35}S]-pulse chase labelling experiments in cultured cells expressing the *Torpedo* or mouse nAChR subunits has been proposed (Green and Claudio, 1993). These experiments demonstrated that $\alpha\beta\gamma$ and $\alpha\beta\gamma\delta$ are intermediates in the assembly of the $\alpha_2\beta\gamma\delta$ pentamer. This model suggests that the formation of the $\alpha\beta\gamma$ trimer is followed by the addition of the δ subunit and finally by the addition of the second α subunit to form the pentamer. It has been suggested that the use of different detergents for receptor solubilisation may account for discrepancies in the data that led to the two derived models (Green and Claudio, 1993; Green and Millar, 1995).

1.5 The vertebrate muscle nAChR

At the motor endplate, action potentials cause the release of a brief pulse of ACh into the synaptic cleft to a concentration of 0.1 - 1 mM for less than 1 msec (Katz and Miledi, 1977). ACh diffuses across the cleft and binds to postsynaptic nAChRs triggering opening of cation-selective channels through which Na^+ and K^+ flow passively to a threshold depolarisation when muscle contraction occurs. The concentration of ACh in the cleft returns to background levels by diffusion and degradation by ACh esterase (Kuffler and Yoshikami, 1975; Katz and Miledi, 1977).

The nAChR receptor at the neuromuscular junction (NMJ) of vertebrates was investigated using oligonucleotide probes based on the *Torpedo* nAChR subunits to isolate the subunits that form the muscle nAChR. Four nAChR subunits were cloned from calf muscle: $\alpha 1$ (Noda *et al.*, 1983a), $\beta 1$ (Tanabe *et al.*, 1984), γ (Takai *et al.*, 1984) and δ (Kubo *et al.*, 1985). A fifth subunit (the ϵ subunit) was isolated from calf muscle, (Takai *et al.*, 1985) which had the highest sequence homology to the *Torpedo* γ subunit and it was shown by expression studies that the calf ϵ subunit could replace the *Torpedo* γ subunit to form a functional receptor in combination with

Torpedo α , β and δ subunits. There is a developmental switch from foetal to adult tissue at the vertebrate NMJ from the γ subunit to the ϵ subunit (Mishina *et al.*, 1986; Karlin, 1991). The structure of the muscle nAChR at the NMJ is presumed to be pentameric in nature by analogy to the *Torpedo* nAChR (see Section 1.4). The stoichiometry of the four distinct subunits is $\alpha_2, \beta, \gamma, \delta$ in foetal tissue and $\alpha_2, \beta, \epsilon, \delta$ in the adult muscle where the ϵ subunit replaces the γ subunit.

1.6 Neuronal nAChRs of vertebrates

In addition to the five muscle nAChR subunits (α , β , γ , δ and ϵ), eleven "neuronal" nAChR subunits have been identified in the vertebrate central and peripheral nervous system (α_2 - α_9 and β_2 - β_4) (reviewed by Sargent, 1993, McGehee and Role, 1995, Green and Millar, 1995). All neuronal nAChR subunits contain two cysteines which are homologous with cysteines 128 and 142 of the muscle subunits. The classification of the nAChR subunits into α and β is based on whether they have adjacent cysteine residues at positions 192 and 193 (based on muscle α numbering) which are found in all α subunits from the electric organ and from muscle. The β_2 and β_4 have been reported to generate functional nAChRs when co-expressed with either the α_2 , α_3 or α_4 subunits, in contrast there has been no report of the β_3 subunit forming functional channels when co-expressed with any other single α subunit (Boulter *et al.*, 1987; Wada *et al.*, 1988; Deneris *et al.*, 1989). The α_7 , α_8 and α_9 subunits are atypical with respect to their ability to form functional homo-oligomeric channels in *Xenopus* oocytes which are sensitive to α -BTX (Couturier *et al.*, 1990a; Gerzanich *et al.*, 1993; Elgoyhen *et al.*, 1994).

The vertebrate neuronal nAChR subunits used in this study were cloned from rat. Table 1.1 summarises the rat nAChR subunits which have been cloned to date. In the following section the α and β subunits are described in more detail.

Table 1.1: Molecular cloning of rat neuronal nAChR subunit cDNAs.

Subunit	Probe	Mature peptide (No. amino acids)	Reference
$\alpha 2$	Chicken $\alpha 2$	55,500 (484)	(Wada et al., 1988)
$\alpha 3$	Mouse $\alpha 1$	54,800 (474)	(Boulter, 1986)
$\alpha 4$	rat $\alpha 3$	67,100 (600)	(Boulter, 1987)
$\alpha 5$	rat $\beta 3$	48,800 (424)	(Boulter, 1990)
$\alpha 6$	PCR *	53,300 (463)	(Lamar, 1990; Gerzanich, 1997)
$\alpha 7$	chicken $\alpha 7/\alpha 8$	54,200 (480)	(Seguela et al., 1993)
$\alpha 8$ **	-	-	-
$\alpha 9$	rat $\alpha 7$	(457) 52,000	(Elgoyhen et al., 1994)
$\beta 2$	rat $\alpha 3$	54,300 (475)	(Deneris et al., 1988)
$\beta 3$	rat $\alpha 3$	50,200 (434)	(Deneris et al., 1989)
$\beta 4$	rat $\beta 2$	53,300 (475)	(Duvoisin et al., 1989)

Table 1.1 summarises the neuronal nAChR subunits isolated to date from the rat. The hybridisation probe used to isolate the subunits and the mature peptide size (daltons) is also shown.

* Highly degenerate primers to conserved regions of nAChR subunits in the extracellular and membrane spanning domains were used to amplify rat genomic DNA. Precise details of the oligonucleotides were not reported.

** An $\alpha 8$ cDNA has been cloned from chick (Schoepfer *et al.* 1990), but an analogous subunit has not been identified in other species.

1.6.1 Structure of neuronal nAChRs

The neuronal nAChRs, like their muscle counterparts, are thought to be pentameric complexes with the five subunits arranged like barrel staves around a central pore. Whereas the subunit composition of the muscle nAChR has been established with reasonable confidence, the compositions of the subunits in the AChRs of the peripheral and central nervous system have yet to be elucidated with certainty.

Early studies on purified chicken and rat brains (Whiting and Lindstrom, 1986; Whiting and Lindstrom, 1987) revealed the predominant receptors to be composed of two subunit types, the ligand binding ($\alpha 4$) and the structural ($\beta 2$) subunits. When expressed in *Xenopus* oocytes the $\alpha 4/\beta 2$ receptor was shown, by quantification of [^{35}S]-methionine labelled receptors, to have a stoichiometry of two $\alpha 4$ and three $\beta 2$ subunits (Anand *et al.*, 1991). Site directed mutagenesis studies (creating subunits that confer different single channel properties) were used to assess subunit stoichiometry and suggested that the functional neuronal nAChRs are pentameric and comprised of two α and three β subunits (Cooper *et al.*, 1991).

Functional nAChRs are also formed from the co-assembly of more than two different types of subunit (Ramirez-Latorre *et al.*, 1996). Ganglionic postsynaptic nAChRs contain $\alpha 3$ subunits in combination with $\beta 4$ and $\alpha 5$ subunits and, sometimes, $\beta 2$ (Conroy *et al.*, 1992; Conroy and Berg, 1995). nAChRs of the chick ciliary ganglion have been shown to have the composition $\alpha 3/\beta 4/\alpha 5$ (80%) and $\alpha 3/\beta 2/\beta 4/\alpha 5$ (20%) (Conroy and Berg, 1995). Receptors comprising more than one different α and β subunit appear to be more widespread than receptors made up from one type of α and one type of β subunit (reviewed by Sargent, 1993; McGehee and Role, 1995).

More recent heterologous expression studies have shown that homomeric receptors can be formed from a single α subunit expressed alone ($\alpha 7$ - $\alpha 9$) (Couturier *et al.*, 1990a; Gerzanich *et al.*, 1993; Elgoyhen *et al.*, 1994). Functional homomeric $\alpha 7$, $\alpha 8$

or $\alpha 9$ nAChRs which are sensitive to α -BTX have been expressed in oocytes (Couturier *et al.*, 1990a; Elgoyhen *et al.*, 1994; Gotti *et al.*, 1994). The $\alpha 7$ and $\alpha 8$ subunits have also been shown to co-assemble as heteromers in some native chick AChRs (Schoepfer *et al.*, 1990; Keyser *et al.*, 1993). It is unknown whether other, as yet uncharacterised subunits, are associated with these subunits in native AChRs or if there are homomeric $\alpha 7$, $\alpha 8$ or $\alpha 9$ nAChRs *in vivo*. *In vitro*, the $\alpha 7$ nAChR subunit does not appear to co-assemble with the other known subunits ($\alpha 2$ - $\alpha 6$ and $\beta 2$ - $\beta 4$) to form hetero-oligomers that have functional properties distinct from those of $\alpha 7$ homo-oligomers (Couturier *et al.*, 1990a; Anand *et al.*, 1993).

There is evidence from heterologous expression studies that neuronal nAChR subunits can form functional homomeric ion channels and functional heteromeric channels, the latter of which can have more than two types of subunit. There is also evidence for nAChR *in vivo* with more than two types of subunit for example in the chick ciliary ganglia $\alpha 3/\alpha 5/\beta 4$ or $\alpha 3/\beta 2/\beta 4$ are the most probable subunit combinations (Papke, 1993; Vernallis *et al.*, 1993; Mandelzys *et al.*, 1995; McGehee and Role, 1995).

1.6.2 *In vivo* distribution of neuronal nAChRs

Vertebrate nAChRs are located at the NMJ, in brain and autonomic ganglia. In the CNS the receptors are localised in discrete regions. *In situ* hybridisation studies have shown that the individual nAChR subunits are expressed in discrete and sometimes overlapping nuclei (Wada *et al.*, 1989; Heinemann *et al.*, 1990; Morris *et al.*, 1990). In invertebrates, such as the *Drosophila*, nAChRs are located in the majority of sensory pathways and interneurons where ACh is the main excitatory transmitter (Pitman, 1971; Gerschenfeld, 1973; Restifo and White, 1990). In contrast to vertebrates, nAChRs of invertebrates are not found at the NMJ, where glutamate rather than ACh is the main transmitter at insect neuromuscular synapses (Gerschenfeld, 1973; Breer and Sattelle, 1987).

There have been reports that show that the subunit combinations $\alpha 4/\beta 2$ and $\alpha 3/\beta 4$ are located predominantly in the CNS and the autonomic ganglia of the peripheral nervous system (PNS), respectively (Whiting *et al.*, 1991). mRNAs for $\alpha 3$ and $\beta 4$ are the most abundant of the nAChR subunit mRNAs expressed in the rat superior cervical ganglia (SCG) followed by $\alpha 7$ and $\beta 2$ mRNAs. The evidence for $\alpha 5$ and $\alpha 4$ has been less clear cut (Klimaschewski *et al.*, 1994; Rust *et al.*, 1994; Mandelzys *et al.*, 1995; Zoli *et al.*, 1995). Of all the combinations expressed in *Xenopus* oocytes, $\alpha 3/\beta 4$ mimics the pharmacological properties of the nAChRs in rat SCG at whole cell level (Luetje and Patrick, 1991; Wong *et al.*, 1995). However, expression of the cloned rat $\alpha 3$ and $\beta 4$ cDNA in *Xenopus* oocytes generates channels whose properties do not resemble native channels in SCGs exactly (Sivilotti *et al.*, 1997). Interestingly it has been shown that when the $\alpha 3$ and $\beta 4$ subunits are expressed heterologously in a mouse fibroblast cell line, their channel properties mimic more closely those seen in native receptors (Lewis *et al.*, 1997). This has led investigators to suggest that the ion channel properties of neuronal nAChRs may be influenced by the host cell type (Lewis *et al.*, 1997).

Immunoprecipitation studies with the chick ciliary ganglia have shown co-precipitation of $\alpha 3$ and $\beta 4$ with $\alpha 5$ suggesting that at least three subunits form the chick ganglionic nAChR (Vernallis *et al.*, 1993). The most likely candidates for the "triplet" combinations in sympathetic ganglia are: $\alpha 3/\alpha 5/\beta 4$ (Vernallis *et al.*, 1993; McGehee and Role, 1995) or $\alpha 3/\beta 2/\beta 4$ (Papke, 1993; Mandelzys *et al.*, 1995).

The rat $\alpha 6$ nAChR subunit has been shown by *in situ* hybridisation to be restricted to a few nuclei throughout the brain such as the locus coeruleus and substantia nigra (Le Novère *et al.*, 1996). The same study found extensive co-localisation of $\alpha 6$ and $\beta 3$ indicating the possible existence of nAChRs containing both these subunits. $\alpha 6$ has been shown by immunohistochemical localisation to be present on dopaminergic

neurones in the mid brain suggesting a possible role for $\alpha 6$ in the modulation of dopaminergic transmission (Goldner *et al.*, 1997).

High levels of the rat $\alpha 7$ subunit transcript have been shown to be localised in the hippocampus, amygdala, hypothalamus, olfactory areas and the cerebral cortex by *in situ* hybridisation (Seguela *et al.*, 1993). The chick $\alpha 8$ subunit is the predominant receptor subtype found in the chick retina but has also been identified in chick brain in receptors containing both $\alpha 7$ and $\alpha 8$ subunits (Schoepfer *et al.*, 1990; Keyser *et al.*, 1993). The rat $\alpha 9$ nAChR subunit has been shown to have a restricted pattern of gene expression by *in situ* hybridisation. The $\alpha 9$ gene expression is located in the outer hair cells of the rat cochlea and may have a role in the cholinergic efferent innervation of the cochlea hair cells (Elgoyhen *et al.*, 1994).

1.6.3 Functional expression of cloned neuronal nAChR subunit combinations

Numerous studies report the successful expression in *Xenopus* oocytes of the vertebrate muscle nAChR subunits ($\alpha \beta \epsilon \delta$ and $\alpha \beta \epsilon \delta$) and of various combinations of neuronal nAChR subunits (for example $\alpha 3/\beta 4$ and $\alpha 4/\beta 2$). In addition, some vertebrate neuronal nAChR subunits ($\alpha 7$, $\alpha 8$ and $\alpha 9$) have been shown to form functional homo-oligomeric channels (Couturier *et al.*, 1990b; Elgoyhen *et al.*, 1994).

In the case of rat nAChRs, the injection of cDNAs or cRNAs encoding either the $\beta 2$ or $\beta 4$ subunits in pairwise combination with cDNAs or cRNAs encoding $\alpha 2$, $\alpha 3$ or $\alpha 4$ into *Xenopus* oocytes result in the formation of a nicotine-gated ion channel (Boulter *et al.*, 1987; Wada *et al.*, 1988). Injection of individual cDNAs or cRNAs does not lead to the formation of functional channels. The six combinations ($\alpha 2/\beta 2$, $\alpha 2/\beta 4$, $\alpha 3/\beta 2$, $\alpha 3/\beta 4$, $\alpha 4/\beta 2$ and $\alpha 4/\beta 4$) each have different pharmacological profiles which indicate that both the α and β subunits can influence the pharmacology of the receptor and probably contribute to the ligand binding site. The differences between pairwise combinations are usually subtle but sometimes these differences can be more

significant, for example, cytosine is the most potent agonist on all receptors containing the $\beta 4$ subunit and least on the receptors containing $\beta 2$ (Luetje and Patrick, 1991; Papke and Heinemann, 1993). Furthermore, neuronal bungarotoxin acts as an antagonist preferentially on receptors containing the $\beta 2$ subunit rather than the $\beta 4$ subunit (Duvoisin *et al.*, 1989; Luetje *et al.*, 1990; Wada *et al.*, 1990). Functional receptors are not formed when the α subunits $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ are heterologously expressed with the $\beta 3$ subunit (Deneris *et al.*, 1989; Boulter *et al.*, 1990).

The pharmacological differences are less subtle when the α ($\alpha 2$ – $\alpha 6$) subunits are varied and the β ($\beta 2$ – $\beta 4$) subunit is kept constant. However, in some cases dramatic differences have been observed, for example there is approximately a 100-fold difference in the concentration of nicotine required to activate the $\alpha 3/\beta 2$ compared to the $\alpha 2/\beta 2$ receptor (Luetje and Patrick, 1991).

nAChR subunits have been expressed in mammalian cell lines such as HEK-293 and BOSC 23 (Wong *et al.*, 1995; Gopalakrishnan *et al.*, 1996; Stetzer *et al.*, 1996; Ragozzino *et al.*, 1997). The combination $\alpha 4/\beta 2$ has been successfully expressed in HEK-293 cells and functional channels characterised using patch-clamping (Buisson *et al.*, 1996), binding assays and the $^{86}\text{rubidium}$ -efflux assay (Gopalakrishnan *et al.*, 1996). The rank order of potency of four nAChR ligands to activate the $\alpha 4/\beta 2$ receptors is nicotine>ACh>cytosine>ABT418. The putative ganglionic nAChR $\alpha 3/\beta 4$ subunit combination has also been expressed in HEK-293 cells and the functional channels analysed by single cell and whole cell patch-clamp studies (Wong *et al.*, 1995; Stetzer *et al.*, 1996) showed rank order of agonist potency: DMPP>cytosine=nicotine=ACh. More recently, a study using the human BOSC 23 cell line, investigated ACh-induced single channel currents with cells transiently transfected with the subunit cDNA combinations: $\alpha 3/\beta 4$ (rat and chick), $\alpha 3/\beta 2$,

$\alpha 4/\beta 2$, $\alpha 4/\beta 4$, $\alpha 7$ and $\alpha 8$ (chick) (Ragozzino *et al.*, 1997). Functional channels were observed for all combinations.

The $\alpha 5$ subunit appears to be unable to generate functional receptors when expressed in *Xenopus* oocytes in pairwise combination with $\beta 2$, $\beta 3$ or $\beta 4$ (Boulter *et al.*, 1987; Couturier *et al.*, 1990b) even though it appears to co-assemble with $\beta 2$ and $\beta 4$ subunits in neurones (Conroy *et al.*, 1992; Vernallis *et al.*, 1993; Conroy and Berg, 1995). A recent study, using receptors generated by heterologous expression in oocytes, has shown that the $\alpha 4/\beta 2$ receptor is pharmacologically distinct from the $\alpha 4/\beta 2/\alpha 5$ receptor (Ramirez-Latorre *et al.*, 1996). This suggests that the $\alpha 5$ subunit can co-assemble with $\alpha 4/\beta 2$ and contribute to the functional response. More recently $\alpha 5$ has been shown to co-assemble with $\alpha 3/\beta 2$ and $\alpha 3/\beta 4$ in *Xenopus* oocytes and the human neuroblastoma cell line SH-SY5Y (Wang *et al.*, 1996). The $\alpha 3/\beta 4/\alpha 5$ subunit combination produces channels that resemble those in the chick ciliary ganglia (Conroy *et al.*, 1992).

The $\alpha 5$ subunit has a cysteine pair homologous to the $\alpha 1$ cysteines 192 and 193 which are located in close proximity to the ACh binding site, hence the classification as an α subunit. The $\alpha 5$ subunit when aligned with the other nAChR α subunits lacks two of the amino acid residues that are conserved throughout the other α subunits, Tyr 93 and Tyr 190 (Kao *et al.*, 1984; Dennis *et al.*, 1988; Abramson *et al.*, 1989; Galzi *et al.*, 1990; Cohen *et al.*, 1991; Middleton and Cohen, 1991). Mutations of these residues has been shown to reduce the apparent affinity of the receptor for ACh (Karlin, 1993). These data suggest that the $\alpha 5$ subunit may have a different role to the other α subunits in that it may have more of a structural role rather than as a ligand binding subunit.

There has only been one report to date of the $\alpha 6$ subunit forming a functional channel (Gerzanich *et al.*, 1997). This study showed that when the chick $\alpha 6$ subunit was co-

expressed with the human $\beta 4$ subunit in *Xenopus* oocytes a functional nAChR was detected showing novel pharmacological properties and strong inward rectification. Pharmacological differences have not been observed with co-injection of the $\alpha 6$ or $\beta 3$ subunits in combination with any of the pairs of subunits mentioned above ($\alpha 2/\beta 2$, $\alpha 2/\beta 4$, $\alpha 3/\beta 2$, $\alpha 3/\beta 4$, $\alpha 4/\beta 2$ and $\alpha 4/\beta 4$). It has been shown that both the genes encoding the $\beta 3$ and $\alpha 6$ subunits are expressed in the rat CNS and PNS indicating that they may contribute to receptor function *in vivo*.

The $\alpha 7$, $\alpha 8$ and $\alpha 9$ nAChR subunits have all been shown to generate functional homo-oligomeric channels when expressed in *Xenopus* oocytes (Couturier *et al.*, 1990a; Gerzanich *et al.*, 1993; Elgoyhen *et al.*, 1994). $\alpha 7$ has been expressed in several cultured mammalian cell lines and functional responses have been observed in human SH-SY5Y neuroblastoma cells, HEK-293 cells and rat pituitary cells (GH₄C₁) (Puchacz *et al.*, 1994; Gopalakrishnan *et al.*, 1995; Quik *et al.*, 1996). In a recent study (Cooper and Millar, 1997) $\alpha 7$ was expressed in a variety of neuronal and non-neuronal cultured cell lines and the folding, assembly and subcellular localisation was found to be critically dependent on the nature of the host cell. When transfected with $\alpha 7$, rat pheochromocytoma (PC-12) cells and SH-SY5Y cells showed an increase in surface α -BTX binding, but this was not observed in seven other cell lines which were shown by immunoprecipitation to have high levels of expression of the $\alpha 7$ subunit suggesting that these cell lines do not have the ability to transport nAChRs from their site of synthesis, the ER, to the cell surface.

1.6.4 Presynaptic neuronal nAChRs

As well as postsynaptic neuronal nAChRs, there has been much attention to the function of presynaptic nAChRs and their ability to modulate the release of neurotransmitters (Cheeselet, 1984). There have been three particular areas studied in detail: the vertebrate nigrostriatal system, the hippocampus and the habenulo-interpenduncular pathway. These each possess cholinergic input and high densities of

[³H]-nicotine and [¹²⁵I]- α -BTX binding sites (Clarke *et al.*, 1985). There is very little evidence for nicotinic synaptic transmission in the vertebrate brain. This, together with the diversity of the receptors and their high permeability to calcium has lead to the idea that the main role of nAChRs in the brain could be purely or predominantly modulatory (McGehee and Role, 1995). There is evidence, however, that exogenously applied nicotinic agonists can act directly on cells to excite them and that nAChRs in the brain do have direct actions themselves and not only function as modulators of transmitter release (Clarke, 1993; Yang *et al.*, 1996). The occurrence of nAChRs on somatodendritic regions of neurones also suggests that these receptors do not have a purely presynaptic modulatory role (Clarke, 1993).

The release of several of the classical neurotransmitters, including noradrenaline, glutamate and GABA have been shown to be modulated by nicotine acting at the nerve terminal (McGehee *et al.*, 1995; Clarke and Reuben, 1996; Yang *et al.*, 1996). One of the most extensively studied systems is the action of presynaptic nAChRs regulating dopamine release using neurochemical techniques. Nicotine is a potent agonist acting on superfused synaptosome preparations from striatum to elicit dopamine release (Grady *et al.*, 1992; Clarke and Reuben, 1996). Cytisine is a partial agonist in this system on the rat striatum suggesting that the presynaptic receptors are composed of $\alpha 4$ and $\beta 2$ since this combination has the highest sensitivity to nicotine of any subunit combination expressed in *Xenopus* oocytes (Clarke and Reuben, 1996) whereas cytisine is ineffective at rat $\beta 2$ containing nAChRs expressed in oocytes (Papke and Heinemann, 1993). The partial agonist action of cytisine implies that $\beta 4$ may be part of the presynaptic receptor since it is most potent at receptors containing the $\beta 4$ subunit and least potent at receptors containing the $\beta 2$ subunit (Luetje and Patrick, 1991; Grady *et al.*, 1992; Papke and Heinemann, 1993; Clarke and Reuben, 1996). In the substantia nigra the predominant mRNAs present are $\alpha 4$, $\beta 2$, $\alpha 3$, $\alpha 5$ and $\beta 3$ but no $\beta 4$ expression has been observed (Deneris *et al.*, 1989; Wada *et al.*, 1989; Wada *et al.*, 1990). The situation is complex, the data indicate a presynaptic

role of $\alpha 4$ and $\beta 2$ but support more complex receptor compositions. The composition of the presynaptic receptors like their postsynaptic counterparts still has to be elucidated.

Clearly, the nAChRs are expressed in the CNS of vertebrates but their functions have yet to be fully elucidated. In contrast, in invertebrates, the nAChRs act as the major excitatory ligand-gated ion channels in the CNS which are reviewed in the following sections.

1.7 nAChRs of invertebrates

As in the vertebrate system, nAChRs are expressed abundantly in invertebrates (Sattelle, 1980; Sargent, 1993). In the insect nervous system, ACh appears to be the principal excitatory neurotransmitter (Pitman, 1971) and nAChRs are present in high density in insect neural membrane extracts (Sattelle, 1980; Breer and Sattelle, 1987). The majority of sensory pathways and interneurons use ACh as the excitatory transmitter, whereas at the NMJ, the transmitter is glutamate (Sattelle, 1980). The first demonstration of ACh receptors in the insect neurones was shown by locally applied ACh (Kerkut *et al.*, 1969). This has been investigated further in recent years using mainly the following species: the Cockroach (*Periplaneta americana*), the locusts (*Locusta migratoria* and *Schistocerca gregaria*), the flies (*Drosophila melanogaster* and *Musca domestica*) and the moth (*Manduca sexta*). The work presented in this thesis will focus predominantly on the cloned nAChRs from *Drosophila*. Expression studies with nAChR subunits cloned from the nematode *Caenorhabditis elegans* (*C.elegans*) are also presented.

1.7.1 The cholinergic system of *Drosophila melanogaster*

The *Drosophila* CNS has been shown to contain the essential components of the cholinergic system, the ACh synthesising enzyme ChAT, acetylcholinesterase and a high affinity uptake system for choline for the degradation and removal of ACh. Null

mutations of ChAT and AChE both result in recessive embryonic lethals which is in accordance with the presumed predominant role of ACh in the *Drosophila* CNS (Hall and Kankel, 1976; Greenspan, 1980). Immunohistochemical localisation of ChAT and AChE show wide expression throughout the CNS (Gundelfinger and Hess, 1992).

Both nicotinic and muscarinic receptors have been identified by binding studies with nicotinic and muscarinic antagonists such as α -BTX and quinuclidinyl benzylate. In contrast to the vertebrate system, the nicotinic binding sites are in large excess compared to the muscarinic sites (Dudai, 1979; Salvaterra and Foders, 1979). Four genes encoding *Drosophila* nAChR subunits have been identified (see below) which show considerable homology to the vertebrate nAChR genes. A *Drosophila* muscarinic receptor homologue (DM1) has also been identified (Onai *et al.*, 1989; Shapiro *et al.*, 1989).

1.7.2 nAChRs of *Drosophila*

Membrane fractions from *Drosophila* heads were shown to contain high affinity [¹²⁵I]- α -BTX binding sites with a dissociation constant (K_d) in the range of 0.1-2nM. Binding of [¹²⁵I]- α -BTX is displaced by unlabeled nicotinic ligands such as nicotine, ACh and *d*-tubocurarine but is insensitive to muscarinic ligands such as atropine (Dudai, 1979). More recently, saturation binding analysis has revealed two different classes of α -BTX binding sites from *Drosophila* head membrane preparations: K_d values of 0.1 nM (class 1) and 4 nM (class 2) (Schloss *et al.*, 1988). Solubilised α -BTX binding complexes were shown to have a molecular weight of 250,000 - 300,000 from sucrose density gradients (Schloss *et al.*, 1991). Preliminary reports revealed the existence of several polypeptides of 42, 57, 65 and 79 kDa, with the 42 kDa polypeptide as the major α -BTX binding site (Restifo and White, 1990). Another population of neuronal nicotinic receptors in *Drosophila* have been shown to be insensitive to α -BTX (Restifo and White, 1990). The variability of nicotinic ligands to bind to their receptors suggest that there is a heterogeneous population of

nAChRs. This diversity been supported by immunohistochemical data using monoclonal antibodies raised against the nAChR from the electroplax of the ray *Torpedo californica* (Chase *et al.*, 1987).

The molecular cloning of four nAChR subunits (ALS, ARD, SAD (or D α 2) and SBD) from *Drosophila* represents the first step towards understanding the molecular basis for this diversity. *Drosophila* nAChR cDNAs were identified by hybridisation with cDNA probes from vertebrate nAChRs and this is summarised in Table 1.2. The cDNA encoding the γ subunit from the electroplax of the fish *Torpedo californica* was used to isolate the cDNA and the gene for a putative nAChR subunit, the "AChR protein of *Drosophila*" (ARD) (Hermans-Borgmeyer *et al.*, 1986; Sawruk *et al.*, 1988; Wadsworth *et al.*, 1988). As in the classification for vertebrates, those subunits containing the two consecutive cysteine residues in positions analogous to 192 and 193 in the *Torpedo* α subunit are classified as ligand binding α subunits. An " α -like subunit" (ALS) gene and cDNA were identified by cross hybridisation with a fragment of the chick neuronal α 2 subunit gene (Bossy *et al.*, 1988). A "second α -like subunit of *Drosophila*" (SAD), which is also referred to as D α 2, and a "second β subunit of *Drosophila*" (SBD) were isolated by low stringency screening of *Drosophila* genomic and cDNA libraries with an ALS probe or with a mixture of oligonucleotides designed to a highly conserved region preceding the fourth hydrophobic region of all known nAChRs (Baumann *et al.*, 1990; Jonas *et al.*, 1990; Sawruk *et al.*, 1990a; Sawruk *et al.*, 1990b). The amino acid sequence alignment of these four *Drosophila* nAChR subunits is shown in Figure 1.4. There have also been preliminary reports of the isolation of a third α like subunit, "*Drosophila* α -like subunit 3" (D α 3 or ADR) (Gundelfinger and Hess, 1992). This will be discussed in Chapter seven of this thesis. Recently a partial expressed sequence tag sequence was deposited in the EMBL/Gen Bank nucleotide data bases and since this sequence is similar to, but not identical to, D α 3, it is referred to here as D α 4 and will be discussed in more detail in Chapter seven.

Table 1.2: Molecular cloning and nomenclature of *Drosophila* neuronal nAChR subunit cDNAs.

Subunit	Probe	Mature peptide (No. amino acids)	Reference
ALS	Chick $\alpha 2$	62500, (546)	(Bossy <i>et al.</i> 1988)
ARD	<i>Torpedo</i> γ subunit	57,652 (497)	(Hemans-Borgmeyer <i>et al.</i> 1986)
SAD	ALS	60,963 (535)	(Baumann <i>et al.</i> 1990) (Jonas <i>et al.</i> 1990)
SBD	ALS	57,270 (493)	(Sawruk <i>et al.</i> 1990)

Table 1.2 summarises the neuronal nAChR subunits isolated to date from *Drosophila*. The hybridisation probe used to isolate the subunits and the mature peptide size (daltons) are also shown.

Abbreviations for *Drosophila* nAChR subunits (nomenclature suggested by FlyBase is given in parentheses):

ALS	α -like subunit	(nAcR α -96Aa)
ARD	β -like subunit (ACh receptor of <i>Drosophila</i>)	(nAcR β -64B)
SAD	Second α -like subunit <i>Drosophila</i>	(nAcR α -96Ab)
SBD	Second β -like subunit <i>Drosophila</i>	(nAcR β -96A)

	1				50
ALSMGSVLFAAV	FIALHFATGG	L...ANPDAK
SBDAP	ISFEANPDTK
SAD	MAPGCCTTRP	RPIALLAHIW	RHCKPLCLLL	VLLLLCETV.	...QANPDAK
ARDMESSCKSWL	LCSILVLVAF	SLVSASEDEE
					*
	51				100
ALS	RLYDDLSSNY	NRLIRPVGNN	SDRLTVKMGL	RLSQLIDVNL	KNQIMTTNVW
SBD	RLYDDLSSNY	NRLIRPVVNN	TETLTVWLGL	KLSQLIEVNL	KNQVMTTNLW
SAD	RLYDDLSSNY	NRLIRPVSN	TDTVLVKLGL	RLSQLIDLNL	KDQILTTNVW
ARD	RLVRDLFRGY	NKLIRPVQNM	TQKVGVRFG	AFVQLINVNE	KNQVMKSNVW
	** *	* * * * *	* *	* * *	* *
	101				150
ALS	VEQEWNDYKL	KWNPDDYGGV	DTLHVPSEHI	WHPDIVLYNN	ADGNYEVTIM
SBD	VKQRWFDYKL	RWDPEEYGGV	EQLYVPSEHI	WVPDIVLYNN	WDGNYEVTLM
SAD	LEHEWQDHKF	KWDPSEYGGV	TELYVPSEHI	WLPDIVLYNN	ADGEYVVTM
ARD	LRLVWYDYQL	QWDEADYGGI	GVLRLPPDKV	WKPDI VLFNN	ADGNYEVRYK
	* *	* * * *	* *	* * * * *	* * *
	151				200
ALS	TKAILHHTGK	VVWKPPAIYK	SFCEIDVEYF	PFDEQTCFMK	FGSWTYDGYM
SBD	TKATLKYTGE	VFWEPPIYK	SSCEMNVEYF	PYDEQICFMK	FGSWTYNGAQ
SAD	TKAILHYTGK	VVWTPPAIFK	SSCEIDVRYF	PFDQQTCFMK	FGSWTYDGDQ
ARD	SNVLIYPTGE	VLWVPPAIYQ	SSCTIDVTYF	PFDQQTICIMK	FGSWTFNGDQ
	** *	* * * * *	* * *	* * * * *	* * * *
	201				250
ALS	VDLRHLKQTA	DSDN.IEVGI	DLQDYIISVE	WDIMRVPAVR	NEKFYSCCEE
SBD	VDLKHLDDQIP	GSNL.VQVGI	DLTEFYLSVE	WDILEVPATK	NEEYYPDTLE
SAD	IDLKHISQKN	DKDNKVEIGI	DLREYYPVSVE	WDILGVPAER	HEKYYPCCAE
ARD	VSLALYNNKNFV	DLSDYWKSGT	WDIIEVPAYL	NVYEGDSNHP
	*		** *	** *	
	251		TM1		300
ALS	PYLDIVFNLT	LRRKTLFYTV	NLIIPC VGIS	FLSVLVFYLP	SDSGEKISLC
SBD	PFSDITFKLT	MRRKTLFYTV	NLIVPCVALT	FLTVLVFYLP	SDSGEKVTLC
SAD	PYPDIFFNIT	LRRKTLFYTV	NLIIPC VGIS	YLSVLVFYLP	ADSGEKIALC
ARD	TETDITFYII	IRRKTLFYTV	NLILPTVLIS	FLCVLVFYLP	AEAGEKVTLG
	** *	* * * * *	** *	* * * * *	** *
	301	TM2		TM3	350
ALS	ISILLSLTVF	FLLLAEIIPP	TSLTVPLL GK	YLLFTMMLVT	LSVVVTIAVL
SBD	ISILVSLTVF	FLLLAEIIPP	TSLAVPLL GK	YLLFTMILVS	LSVWTTVCVL
SAD	ISILLSQTMF	FLLISEIIPS	TSLALPLL GK	YLLFTMLLVG	LSVVITIIIL
ARD	ISILLSLVVF	LLLVS KILPP	TSLVLPLIAK	YLLFTFIMNT	VSILVTVIII
	**** *	** *	** *	*****	* *
	351				400
ALS	NVNFRSPVTH	RMAPWVQRLF	IQILPKLLCI	ERPKKEEPEE	DQPPEVLTDV
SBD	NIHFRSPSTH	NMSRLVRKLF	LHFMPKLMMM	RRTQ.....
SAD	NIHYRKPSH	KMRPWIRSFF	IKRLPKLLLM	RVPKDLLRDL	AAN....KIN
ARD	NWNFRGPPTH	RMPMYIRSIF	LHYLPAFLFM	KRPR.....
	* * * *	* *	*		

	401				450
ALS	YHLPPDVDFK	VNYDSKRFSG	DYGIPALPAS	HRFDLAAAGG	ISAHCFAEPP
SBD	YTLP.....	DYDDSTPSNG	YTNEIDVRDS	ISD.....
SAD	YGLKFSKTKF	GQALMDEMOM	NSGGSSPDSL	RRMQGRVGAG	GCNGMHVTTA
ARDKTRL	RWMMEMPGMS	MPAHPHPSYG	SPAELPKHIS	AIGGKQSKME
	451				500
ALS	LPSSLPLPGA	DDDLFSP.SG	LNGDISPGCC	PAAAAAAAAD	LSPTFEKPYP
SBD	FPSE..FKDS	QDGAYDN.GM	QNSVDSDNVI	P.....RNLT
SAD	TNRFSGLVGA	LGGGLSTLSG	YNGLPSVLSG	LDDSLSDVAARKKYP
ARD	VMELSDLHHP	NCKINRKVNS	GCELGLGDGC	RRESESDSI	L.....LS
	501				550
ALS	REMEKTIEGS	RFIAQHVKNK	DKFESVEEDW	KYVAMVLDRM	FLWIFAIACV
SBD	PEVLQALRAV	RFIAQHIKDA	DKDNEIVEDW	KFVSMVLDRF	FLWLFTLSCV
SAD	FELEKAIHNV	MFIQHMQRQ	DEFNAEDQDW	GFVAMVMDRL	FLWLFMIASL
ARD	PEASKATEAV	EFIAEHLRNE	DLYIQTREDW	KYVAMVIDRL	QLYIFFIVTT
	*	** *	*	* ** *	* *
	<u>TM4</u>				600
ALS	VGTAIIILQA	PSLH...DQS	QPIDILYSKI	AKKKFELLKM	GSENTL....
SBD	FGTLAIICQS	PSLY...DTR	SPIDRQLSEI	PLRKNNFM.L	PPDIVRQVLT
SAD	VGTFVILGEA	PSLY...DDT	KAIDVQLSDV	AKQIYNLTEK	KN.....
ARD	AGTVGILMDA	PHIFEYVDQD	RIIEIYRGK.
	** *	*	*		

Fig. 1.4 Amino acid sequence alignment of the four *Drosophila* nAChR subunits ALS (Bossy *et al.* 1988), ARD (Hermans-Borgmeyer *et al.* 1986), SAD (Bauman *et al.* 1990, Sawruk *et al.* 1990) and SBD (Sawruk *et al.* 1990). Note, the SBD sequence is incomplete lacking the 5' coding region. Deduced amino acid sequences have been aligned by the GCG program Pile-up (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). Gaps (.) were introduced to maximise sequence identity. Asterisks show residues that are conserved in all four subunits. The putative transmembrane domains TM1, TM2, TM3 and TM4 are indicated by a line above the sequences.

The putative mature *Drosophila* nAChR subunit polypeptides consist of 493, (SBD), 535 (SAD), 497 (ARD) and 546 (ALS) amino acid residues and show 45 to 66% amino acid homology between the four subunits (see Table 1.3). The *Drosophila* nAChR subunits resemble the vertebrate subunits in their predicted membrane topologies and share between 33 - 50% sequence homology (Gundelfinger and Hess, 1992). The main regions of sequence homology are in the N-terminal extracellular domain and the M1, M2 and M3 regions whereas the putative cytoplasmic loop between M3 and M4 is the least homologous and is a fact exploited when raising monoclonal antibodies to the various subunits. ALS and SAD-like nicotinic acetylcholine receptor subunit genes are widely distributed in insects and have been shown by PCR in a variety of insect species representing varying degrees of evolutionary divergence (Sgard *et al.*, 1993). The observed nucleotide sequence identities ranged from 68% to 88%.

1.7.3 Distribution of the *Drosophila* nAChR subunits

If the isolated genes and cDNAs of the four *Drosophila* subunits: ALS, ARD, SAD and SBD encode functional nAChR subunits then it would be expected that their expression would be confined specifically to the nervous system. This has been confirmed by *in situ* hybridisation studies in late *Drosophila* embryos where the transcripts for the four analysed nAChR genes were shown to be expressed throughout the CNS but no expression was detected outside the CNS (Wadsworth *et al.*, 1988; Sawruk *et al.*, 1990a; Sawruk *et al.*, 1990b). There is a highly regulated expression of nAChR genes during development (Sawruk *et al.*, 1990b) and levels are particularly high in late embryos and in late pupae i.e. when there are periods of synapse formation and terminal differentiation in the CNS (Hermans-Borgmeyer *et al.*, 1986; Wadsworth *et al.*, 1988; Jonas *et al.*, 1990; Sawruk *et al.*, 1990a). During the larval stage the levels of the ALS transcript remain high whereas the levels of ARD, SBD and SAD all decrease (Bossy *et al.*, 1988).

Table 1.3: Percent amino acid identity between *Drosophila* nAChR subunits.

	SAD	SBD	ARD	Chick $\alpha 2$
ALS	66	61	47	54
SAD		60	45	51
SBD			45	50
ARD				52

The percentage amino acid identity was calculated by multiple sequence alignments of the nAChR proteins. The regions between TM3 and TM4 were omitted from the calculations of similarity as these are the most highly variable parts of the proteins. The chick $\alpha 2$ subunit was included as it shows the highest sequence similarity to *Drosophila* subunits of all the vertebrate nAChR subunits (Gundelfinger and Hess, 1992).

Abbreviations for *Drosophila* nAChR subunits (nomenclature suggested by FlyBase is given in parentheses):

ALS	α -like subunit	(nAcR α -96Aa)
ARD	β -like subunit (ACh receptor of <i>Drosophila</i>)	(nAcR β -64B)
SAD	Second α -like subunit <i>Drosophila</i>	(nAcR α -96Ab)
SBD	Second β -like subunit <i>Drosophila</i>	(nAcR β -96A)

1.7.4 Structural arrangement of *Drosophila* nAChRs

In some of the first studies investigating the nAChRs of *Drosophila* it was shown that α -BTX blocks the action of insect nAChRs and autoradiography has shown that specific [125 I]- α -BTX binding is distributed almost evenly over the neuropile of the *Drosophila* CNS (Dudai, 1978; Rudloff, 1978; Sattelle, 1986). These data suggest that the nicotinic cholinergic system of *Drosophila* may be relatively simple, possibly comprising only one nAChR subtype. The discovery that the locust α L-1 nAChR subunit can form functional homomeric channels when expressed in *Xenopus* oocytes (Marshall *et al.*, 1990) supported the possibility that there may be homo-oligomeric *Drosophila* nAChRs *in vivo*.

The association, dissociation and equilibrium binding of [125 I]- α -BTX to *Drosophila* head membrane extracts reveals a heterogeneity of toxin binding receptors with at least two different affinities suggesting a more complicated system (Schloss *et al.*, 1988). The cloning of four *Drosophila* nAChR subunits including the proposed structural subunits ARD and SBD whose mRNAs are abundant in the CNS, strongly indicates that, as in the vertebrate system, hetero-oligomeric nAChRs are present in the CNS of insects.

There is evidence, (from immunoprecipitation studies) to suggest that the *Drosophila* ARD subunit is part of a high-affinity α -BTX-binding nAChR (Schloss *et al.*, 1988) and that the ALS and ARD subunits co-assemble into an α -BTX-binding complex (Schloss *et al.*, 1991). Antisera to the cytoplasmic loop between TM3 and TM4 of ALS have been shown to immunoprecipitate one class of the α -BTX-binding sites and has been used to show co-assembly of ALS and ARD. This is supported by the co-expression of ALS and ARD in *Xenopus* oocytes which generates nicotine-elicited currents which are not observed in oocytes expressing either subunit alone (Schloss *et al.*, 1991). However, it still hasn't been possible to prove that ALS and ARD form functional nAChRs *in vivo* consisting of only two types of subunit or if other subunits

are also required. Despite the evidence for hetero-oligomeric channels, homomeric channels may also exist in the CNS of *Drosophila*.

1.8 nAChRs of the nematode *C. elegans*

The nematode *C. elegans* is a useful model system in which to study nAChRs for several reasons. It is a free living nematode with a short life cycle. The *C. elegans* nervous system has been completely mapped and shown to consist of only 302 neurones (White *et al.*, 1986). The sequencing of the entire genome is underway making the *C. elegans* genetic map the most complete of any multicellular organism (Wilson *et al.*, 1994; Hodgkin *et al.*, 1995).

Nematodes have been shown to possess nAChRs (Johnson and Stretton, 1980; Fleming *et al.*, 1993; Squire *et al.*, 1995; Treinin and Chalfie, 1995; Ballivet *et al.*, 1996) and much of the work done has been focused on gene products sensitive to levamisole. Levamisole is an agonist that is more potent than acetylcholine at the nematode muscle nAChRs (Lewis *et al.*, 1980). Levamisole is an antihelmintic agent which is used to treat adult and larval nematode infections in domestic animals. Other antihelmintic agents include pyrantel and morantel which are also thought to exert their effect by their potent action as agonists at the ACh receptors causing muscle contraction and paralysis.

1.8.1 nAChR subunits of the nematode *C. elegans*

Four nAChRs had been cloned, ACR-2, LEV-1, UNC-29 and UNC-38 (Squire *et al.*, 1995; Fleming *et al.*, 1997) when this current project was started. These four nematode nAChR subunits show conservation of many mammalian and other invertebrate nAChR sequence features, such as the four transmembrane domains and cysteine residues at positions corresponding to positions 128 and 142 of the *Torpedo* α subunit, which implies a common evolutionary origin for these proteins (Squire *et al.*, 1995).

ACR-2 was isolated by screening a λ library of *C. elegans* genomic DNA with a probe designed on the *Drosophila* α gene (Squire *et al.*, 1995). The isolated cDNA encodes a putative structural nAChR subunit which is most similar to the *Drosophila* subunit ARD (49% amino acid identity). ACR-2 lacks the two vicinal cysteine residues at positions corresponding to 192 and 193 of the *Torpedo* α subunit and so is classified as a non- α subunit. UNC-38 has the pair of cysteines at positions 192 and 193 and is classified as an α subunit (Fleming *et al.*, 1997). UNC-38 has 58% identity to the rat α 2 subunit and 49% homology to ALS. LEV-1 is classified as a non- α subunit (Fleming *et al.*, 1997). It shares 66% amino acid identity with UNC-29. UNC-29, another structural subunit has been isolated and shows similarity to other nAChR β subunits ARD (50%) and rat β 2 (39%) (Fleming *et al.*, 1997).

Since the start of the *C. elegans* genome sequencing project, over 20 potential nAChR sequences have been identified. These include the *deg-3* (u662) gene which encodes a nAChR α subunit, which shows similarities (52% amino acid identity) to the rat and chicken α 7 subunit in the region of the putative second transmembrane domain (Treinin and Chalfie, 1995). Ce21, an α subunit shows most similarity to the vertebrate α 7 subunit, 46.8% amino acid identity and the ability to form homomeric channels in oocytes (Ballivet *et al.*, 1996). Ce21 was kindly provided midway through the project and some data on this clone are shown in the Results (Chapter 7). Ce13 and Ce21 were isolated by screening a *C. elegans* library with a probe encoding conserved domains of the avian α 5 nAChR. Ce13 closely resembles the *Drosophila* ARD subunit (50.3% amino acid identity) (Ballivet *et al.*, 1996). More recently another *C. elegans* nAChR subunit, ACR-3, has been published (Baylis *et al.*, 1997).

The work in this project focuses on the four subunits cloned prior to the start, namely ACR-2, LEV-1, UNC-29 and UNC-38, however, some experiments on the α subunit Ce21 are also described.

1.8.2 Functional co-expression of nAChR subunits of the nematode *C. elegans*

It is only recently that the cloning and functional expression of several *C. elegans* subunits has been published. When expressed alone in *Xenopus* oocytes neither ACR-2 or UNC-38 generate levamisole-gated channels. However, when the two subunits are co-expressed a functional channel is formed which is activated by the agonist levamisole and antagonised by mecamylamine (Squire *et al.*, 1995).

Ce21, the $\alpha 7$ like subunit, forms a functional homomeric nAChR when expressed in *Xenopus* oocytes (Ballivet *et al.*, 1996). The Ce21 receptor is not activated by the antihelmintic agent levamisole, however, both Ce21 and chick $\alpha 7$, when expressed alone in oocytes, form channels that are activated by ACh and this response is antagonised by levamisole (Ballivet *et al.*, 1996). Unlike the $\alpha 7$ receptor the ACh response on the Ce21 channel is not antagonised by α -BTX or methylcaconitine. A recent paper (Fleming *et al.*, 1997), has shown that functional nAChRs are formed when UNC-38 is co-expressed with LEV-1 and UNC-29 in *Xenopus* oocytes. The channels respond to 100 μ M levamisole and are blocked by the nicotinic antagonist mecamylamine but not by α -BTX.

1.9 Expression systems

The *Xenopus* oocyte has provided a powerful means of assessing the functional properties of putative ligand gated ion channel subunits (Sigel, 1990). Injection of nAChR subunit cDNA or mRNA transcribed from cDNA clones (cRNA) in which the coding sequence is placed downstream of a promoter such as the cytomegalovirus (CMV) or simian virus (SV40), frequently results in the formation of functional ligand-gated ion channels on the oocyte surface. Oocytes do not express an endogenous functional nicotinic receptor but do express a calcium gated chloride channel (Miledi and Parker, 1984) that can confound analysis since some nAChRs have significant calcium permeability which can activate these chloride channels (Vernino *et al.*, 1992; Elgoyhen *et al.*, 1994). There is evidence for very low levels of

a nAChR α subunit mRNA in oocytes (Hartman and Claudio, 1990). It is, therefore, possible that injected subunits may co-assemble with this endogenous subunit (Buller and White, 1990). The existence of an endogenous nAChR subunit may detract from the use of the oocyte as an expression system for nAChRs and another system in which there are no endogenous subunits may be preferable. This is one of the main reasons for trying other expression systems such as the *Drosophila* S2 cell line in this study.

The possibility has been suggested that receptors expressed in the amphibian oocyte may exhibit properties which differ from those exhibited by the same subunit combinations expressed in their native cellular environment. A recent study of recombinant nAChRs (Sivilotti *et al.*, 1997) expressed in *Xenopus* oocytes investigated combinations of rat nAChR subunits including $\alpha 3/\beta 4$ and $\alpha 4/\beta 4$. The heterologously expressed receptors do not appear to resemble the native sympathetic ganglion receptors in either single-channel behaviour or agonist potency. The authors suggested that this could be due to further subunits remaining to be cloned or because nAChRs expressed in oocytes generally differ from native ganglion receptors in their single channel conductance. Oocytes may not be able to assemble neuronal nAChR subunits efficiently into channels with the correct composition or stoichiometry due to different post translational modifying systems compared to the native mammalian neurones. Recent data obtained from the expression of $\alpha 3/\beta 4$ in a cultured mammalian cell line showed that channel properties varied considerably from those seen when this subunit combination was expressed in oocytes (Lewis *et al.*, 1997). The results indicated that the properties of ion channels such as single channel conductance may be influenced by the choice of the heterologous expression system.

One of the aims of this project was to examine the properties of nAChR subunit combinations expressed in expression systems other than the *Xenopus* oocyte. The mammalian HEK-293 cell line is a widely used expression system used successfully

for many types of receptor including some of the nAChR subunits for example the vertebrate $\alpha 3/\beta 4$ subunit combination (Wong *et al.*, 1995; Stetzer *et al.*, 1996) and the $\alpha 4/\beta 2$ combination (Buisson *et al.*, 1996; Gopalakrishnan, 1996). It is likely, however, that an invertebrate cell line may provide a more native cellular environment for the expression of invertebrate ion channels.

One of the most extensively used invertebrate expression systems uses the high levels of heterologous gene expression that can be achieved with recombinant baculovirus expression vectors. For example, the transient baculovirus expression system in cultured *Spodoptera fugiperda* cells which have been used to express many receptor types such as GABA receptors (Miller, 1988; Lee, 1993). This system is limited to transient gene expression. Following viral infection of cells, foreign gene expression is usually controlled by promoters that are active in the final stages of the viral life cycle and so the host cells are only viable for a short period.

Expression systems which enable stable transfection of cell lines have advantages over transient systems such as not needing to inject, transfect or virally infect cells prior to each experiment. Also large numbers of cells can be more easily generated from stably transfected cell lines. *Drosophila* S2 cell line has been used successfully to establish stably transfected cell lines for the functional expression of several ion channels and receptors (Millar *et al.*, 1994; Millar *et al.*, 1995; Han *et al.*, 1996) and was chosen as a host cell for investigating the expression of nAChR subunits in this study.

1.10 Assembly of proteins

The assembly of ion channels is a complex series of events that are critical for the correct functioning of the channel. The subunit stoichiometry and their relative positions to each other have to be precise for the channel to function correctly. The subunits have to be partitioned correctly into transmembrane, extracellular and

cytoplasmic regions and to have accurate interactions with neighbouring subunits. There is evidence for a "quality control mechanism" whereby misfolded or incorrectly assembled proteins are prevented from being expressed on the cell surface (reviewed by Green and Millar, 1995).

Multi-subunit ion channels undergo many post-translational processes to establish correct functional expression. After the subunit proteins are synthesised in the endoplasmic reticulum (ER) they undergo several post-translational modifications. These include proteolytic cleavage, glycosylation, phosphorylation, fatty acylation and disulphide bond formation which occur in the ER and Golgi apparatus (reviewed by Green and Millar, 1995). The ER is responsible for the biosynthesis and maturation of proteins and glycoproteins for secretion and transport to their cellular destination such as the plasma membrane. Some of these events are described in the following sections.

1.10.1 Protein folding

Accurate subunit folding and oligomerisation must be achieved in order to generate functional ion channels. Misfolded proteins are sometimes retained in the ER where they are often rapidly degraded (Corless *et al.*, 1987; Schlesinger and Schlesinger, 1987; Hurlley *et al.*, 1989; Hurlley and Helenius, 1989). Correctly folded proteins are exported to their appropriate subcellular locations.

Translated cellular proteins have been shown to fold spontaneously. In their natural environment proteins fold in a specific manner via a series of defined intermediates. The first step is a native secondary structure which is converted to a mature protein via slower reaction steps such as proline isomerisation and disulphide bond formation (Bergman and Kuehl, 1979; Freedman, 1987). These reactions are mediated by chaperone proteins which are resident in the ER that aid and are essential for folding, oligomerisation and assembly of proteins from molten globular proteins to correctly

folded oligomerised proteins (Gething and Sambrook, 1992; Bergeron *et al.*, 1994). The chaperones are thought to prevent inappropriate intramolecular and intermolecular interactions during protein maturation. The ER is rich in chaperone proteins which are present in a large excess to the newly synthesised proteins (Koch, 1987; Marquardt *et al.*, 1993).

The lumen of the ER forms a highly specialised environment for the folding membrane and soluble proteins. The redox environment and ionic composition is carefully maintained and there is regulation of the levels of the chaperone proteins and folding enzymes. The concentration of Ca^{2+} in the ER is in the milimolar range and protein maturation, including disulphide bond formation, is dependent on the concentration of calcium (Lodish and Kong, 1990; Suzuki *et al.*, 1991). Proteins generated in the ER are often rich in cysteines and often form numerous disulphide bonds (Gilbert *et al.*, 1990) which are important for the final stability of the protein and for progress through the folding pathway. The main buffer inside the ER is probably glutathione which helps to maintain optimal conditions for spontaneous disulphide bond formation (Gilbert *et al.*, 1990). The addition of reducing agents such as dithiothreitol (DTT) alters the redox conditions in the ER and alters protein folding and maturation but does not affect protein synthesis. The result of DTT addition is an accumulation of proteins that cannot be transported from the ER and a rapid expansion of the ER takes place which is reversible on removal of DTT (Gilbert *et al.*, 1990; Helenius *et al.*, 1992). Disulphide bonds are an essential feature of nAChRs, they have been shown to be involved in the formation of *Torpedo* nAChR dimers (Raftery *et al.*, 1972; Chang and Brock, 1977; Hamilton *et al.*, 1977; Sobel *et al.*, 1977). A recent paper investigating the effect of DTT on the muscle α subunit has shown that there is irreversible misfolding of DTT-arrested subunits which does not affect the ability of the chaperone calnexin to bind to or dissociate from the subunit (Gelman and Prives, 1996). The data suggest that calnexin is involved in

preventing misfolding in the initial events of the subunit assembly and folding but not an essential participant in the late stages of α subunit maturation.

The ER membrane contains a specific ATP translocator which transports ATP from the cytosol into the lumen of the ER (Clairmont *et al.*, 1992). ATP is essential for the function of some of the ER proteins including the chaperone protein GRP78 (BiP). When cells are depleted of ATP the folding process is inhibited (Helenius *et al.*, 1992).

1.10.2 Chaperones and folding enzymes in the ER

The ER contains many molecular chaperone proteins and folding factors which are essential for the successful navigation of polypeptides through the maturation pathway. The majority of proteins resident in the ER are related to folding and assembly (Koch, 1987; Marquardt *et al.*, 1993). The most abundant chaperones are BiP, GRP94, calnexin and calreticulin (Haas and Wabl, 1983; Helenius *et al.*, 1992; Ou *et al.*, 1993; Peterson *et al.*, 1995). BiP is involved in the folding, retention and oligomeric assembly (Haas and Wabl, 1983) and is a strong candidate for a chaperone that is involved in the retention of denatured proteins (Pelham, 1986). Calnexin is a 64 kD transmembrane protein of the ER which associates with newly synthesised glycoproteins and is involved in their folding and the retention of misfolded proteins (Ou *et al.*, 1993; Bergeron *et al.*, 1994; Hammond and Helenius, 1994). The association of calnexin with substrates depends on the presence of monoglucosylated N-linked oligosaccharides which result from the activities of glucosidase I and II (Kornfield and Kornfield, 1985; Tatu and Helenius, 1997). Calreticulin is a 46 kD soluble protein located in the ER which interacts with an overlapping but not identical group of proteins to calnexin and is oligosaccharide dependent like calnexin (Peterson *et al.*, 1995). It has an N-terminal signal sequence and a C-terminal KDEL (single letter amino acid code) retrieval sequence for its retention in the ER (Michalak *et al.*, 1992). Calreticulin is a ubiquitous protein found across species and tissues and is

thought to have a fundamental role in the cell including being a calcium sequestering protein. There is still much to learn about chaperone proteins and their roles and functions as a matrix for the binding of early folding and assembly intermediates.

There are other factors in the ER involved in the folding and assembly process. These include the folding enzyme protein disulphide isomerase which is involved in the formation and reshuffling of disulphide bonds during folding (Freedman, 1989) and its action depends on the redox conditions. The oligosaccharide-transferase enzyme adds N-linked core sugar moieties to polypeptides which are necessary for the folding of many proteins in the ER (Paulson, 1989). The addition of such moieties are thought to be involved in: directing peptide segments to the surface of the immature protein for the correct positioning of the regions, to inhibit chaperone binding, and to make the protein intermediates more soluble so reducing irreversible-aggregation (Marquardt and Helenius, 1992). Another important process carried out in the ER is the removal of the signal sequence and may have implications for folding. The signal sequence is hydrophobic and would remain associated with the membrane which would be restrictive for the mobility of secretory proteins and their mobility through the ER.

1.10.3 The involvement of chaperone proteins in nAChR assembly

The early events of the muscle nAChR subunit $\alpha 1$ folding have been investigated using conformational epitopes for monoclonal antibodies at the ligand binding site. The nAChRs are assembled intracellularly and exported through the Golgi complex to the cell surface. The $\alpha 1$ subunit does not acquire the ability to bind α -BTX until 15-30 min after synthesis, shown by pulse-chase metabolic labelling (Merlie and Lindstrom, 1983) and during this period the AChR subunits have been shown to undergo several post-translational modifications such as disulphide bond formation and N-linked core glycosylation. Calnexin has been shown to form transient complexes with individual subunits of hetero-oligomeric membrane proteins

including the muscle $\alpha 1$ subunit (Gelman *et al.*, 1995) in the ER of cultured muscle cells and transfected COS cells. The interaction is thought to be an early post-translational event with a half life of around 20 min which is involved in the folding of the $\alpha 1$ subunit. Assembly, with the δ and γ subunits, is believed to occur after calnexin has dissociated from the $\alpha 1$ subunit. Calnexin has been shown to be involved in oligomerisation of other membrane proteins. Studies with brefeldin A (BFA), which causes disruption of the Golgi complex, suggest that assembly of the nAChR receptor is complete before leaving the ER (Klausner *et al.*, 1992). For the muscle nAChR $\alpha 1$ subunit the calnexin interaction complex is thought to be formed soon after biosynthesis, during folding, and calnexin is not thought to be involved in assembly.

The chaperone protein BiP has been shown to interact with AChR subunits in the muscle cell line BC3H1, transfected fibroblasts and transfected COS cells (Blount and Merlie, 1991; Forsayeth *et al.*, 1992). BiP forms transient complexes with unassembled α subunits but not conformationally mature or assembled AChR α subunits. The time course of association with BiP is different to that of calnexin and is observed to increase during pulse chase experiments up to 6 h. BiP forms complexes with misfolded subunits and those subunits unable to assemble whereas calnexin is thought to facilitate subunit folding. One possibility is that misfolded proteins are complexed with BiP and eventually get degraded but the involvement of BiP may not be that clearcut. It has been reported that unassembled α subunits are almost completely degraded within 50 min, whereas the association of the α subunit with BiP has been shown to increase up to 6 h (Gu *et al.*, 1989; Forsayeth *et al.*, 1992). The time course of association and the stability of the α subunits with BiP does not infer that BiP plays a role facilitating the degradation. BiP may be involved in a slower degradation process or possibly the complex is actually an intermediate in the folding and oligomerisation of the nAChR.

From the data generated so far on the chaperone interactions with the muscle nAChR subunits, calnexin seems to dissociate from the α subunit before subunit assembly which probably involves further, as yet, unidentified ER components for the assembly and export of the AChR from the ER. BiP may be involved in the removal and degradation of immature and misfolded subunits. There is much to learn about these processes in relation to the muscle and the neuronal nicotinic nAChRs for which there is even less known. The complicated assembly pathway with its defined steps and specific factors may explain some of the problems experienced when trying to express neuronal nAChRs in cell lines. The choice of host cell must have the correct ER environment for subunit assembly and any missing factor or subtle difference in pH or redox conditions for example may affect the folding and assembly of nAChRs.

1.10.4 Degradation of misfolded proteins

Quality control of protein folding and assembly is performed in the ER of the cell and attempts to stop misfolded proteins, incompletely assembled receptors, or abnormal products being transported to their final cellular destination where their imperfect structure could have a deleterious effect on the cell. The ER retains these structures where they are degraded before they can be passed to the Golgi and exported to their final subcellular location (Lippincott-Schwartz *et al.*, 1988). Degradation of some misfolded and unassembled products is blocked by inhibitors of lysosomal degradation suggesting that it takes place in lysosomes or autophagic vacuoles (Davis and Hunter, 1987) and there is evidence for degradation by an autophagosomal route (Masaki *et al.*, 1987). The time and rate of protein maturation varies from minutes to several hours and depends on rate of folding and rate of oligomer assembly. This can vary in different cells due to physiological differences and oligomerisation depends on concentration levels of the individual proteins that make up the oligomer and this in turn depends on expression levels. There must be a fine balance between having enough unassembled subunits to oligomerise to form mature proteins and degradation of unassembled proteins.

Misfolded proteins are often retained in the ER as aggregates, frequently found to be associated with BiP, these complexes are not transported to the cell surface (Sitia *et al.*, 1990). The identity of the enzymes involved in degradation are still not known, some degradation is blocked by inhibitors of lysosomal degradation which suggests that degradation takes place in lysosomes or autophagosomal vacuoles (Davis and Hunter, 1987) and some degradation occurs via the autophagosomal route (Masaki *et al.*, 1987).

The control of transport of proteins from the ER and the retention of incompletely assembled proteins and of resident proteins such as the chaperones involve several mechanisms. Many of the resident ER proteins have C-terminal KDEL or HDEL sequences which serve as retrieval signals (Pelham, 1991), these and other retention determinants may interact with receptors in the ER and the Golgi. Unassembled subunits and assembly intermediates may be retained by structural properties such as hydrophobicity or flexibility of exposed peptide groups. These are unlike misfolded proteins which form stable aggregates and associate with BiP. Unassembled subunits and assembly intermediates may be retained by weak interactions with a protein scaffold in the ER which may restrict diffusion to retain the proteins in the ER and allow enough freedom for subunit mixing for oligomerisation (Pfeffer and Rothman, 1987). Finally, misfolded proteins seem to be retained by aggregate formation, often in association with BiP. Aggregation occurs soon after synthesis and may be due to poor solubility of the incorrectly folded proteins. The association with BiP in the aggregates may be important for retention since BiP has a KDEL sequence which is how it is retained in the ER (Hurtley, 1989).

1.10.5 Glycosylation of nAChRs

The subunits of ion channels are synthesised in the ER and then are transferred to various intracellular locations via the Golgi body. Tunicamycin is an inhibitor of protein glycosylation and has been used to study *in vivo* glycosylation of ion channels.

Tunicamycin can be added directly to cell growth medium to inhibit glycosylation of asparagine residues and changes in apparent molecular weight determined by polyacrylamide gel electrophoresis. Endoglycosidases H and F have been used to study carbohydrate side chains added to proteins in the ER, sensitive to both endoglycosidases, and more complex carbohydrates modified in the Golgi apparatus are insensitive to endoglycosidase H. This has been a useful technique to study the subcellular location of assembled and unassembled nAChR subunits (Ross *et al.*, 1991). Site-directed mutagenesis has also been used to study the importance of glycosylation in assembly, cell surface expression and function of nAChRs (Mishina *et al.*, 1985; Blount and Merlie, 1990; Gehle and Sumikawa, 1991).

A common structural feature that may be involved in post-translational modification has been identified in muscle nAChR subunits (Noda *et al.*, 1983d), neuronal nAChRs (Boulter, 1986; Goldman, 1987) and GABA_A receptors (Schofield *et al.*, 1987) located at asparagine 141 in the nAChR α subunit there is a conserved N-linked glycosylation site. Site-directed mutagenesis of this site produces muscle α subunits that are not glycosylated and that do not achieve a mature conformation (Blount and Merlie, 1990). When expressed in oocytes, the mutated subunits do retain the ability to assemble with the δ subunit. Complexes containing the mutated α subunit do not bind α -BTX and are rapidly degraded (Blount and Merlie, 1990). The results suggest that incorrectly assembled receptor complexes are rapidly degraded and preclude surface expression of non-functional nAChRs in oocytes.

1.10.6 Phosphorylation of nAChRs

Protein phosphorylation can modulate the functional properties of many cellular proteins. Phosphorylation has been studied by expressing cloned ion channels in mammalian cell lines by metabolic labelling with [³²P]orthophosphate and immunoprecipitation (Miles *et al.*, 1989). The sites of phosphorylation have been determined by site-directed mutagenesis, for example, serine or threonine are altered

to alanine residues, tyrosine to phenylalanine residues. The functional effects of such mutations can be determined by comparing wild type and mutated receptors.

Protein phosphorylation has been implicated in modulating synaptic transmission and regulating the function of ion channels such as the nAChRs (Huganir and Greengard, 1987). The *Torpedo* nAChR is phosphorylated *in vitro* by three different protein kinases. Protein kinase C phosphorylates the α and δ subunits (Huganir *et al.*, 1984; Safran *et al.*, 1987), cyclic AMP dependent protein kinase phosphorylates the γ and δ subunits (Huganir and Greengard, 1983) and an endogenous protein tyrosine kinase phosphorylates the β , γ and δ subunits (Huganir *et al.*, 1984). Phosphorylation of the *Torpedo* nAChR increases the rate of desensitisation of the receptor (Huganir *et al.*, 1986).

Protein phosphorylation of the *Torpedo* nAChR is also thought to be involved in the assembly of the receptor. *Torpedo* nAChR subunits expressed in mouse fibroblasts were incubated with forskolin, which increases the levels of cAMP, which in turn increases receptor expression 2-3 fold by stimulating subunit assembly. The increased assembly and increased cell surface expression was shown to be due to phosphorylation of the γ subunit but not the δ subunit (Green *et al.*, 1991). This was later shown not to be due to direct phosphorylation (Jayawickreme *et al.*, 1994). The phosphorylation of the γ subunit is thought to increase the association with the α subunit (Claudio, 1989). The fact that unassembled subunits are phosphorylated indicates that phosphorylation occurs in the ER, possibly by protein kinase A. The kinase(s) that act in the ER may have a modulatory role in the signal transduction whereby changes in cAMP levels could regulate protein assembly and protein release from the ER. An increase in cAMP levels has been shown to cause an increase in surface expression of neuronal nAChRs such as the $\alpha 7$ receptor in SH-SY5Y cell (Cooper and Millar, 1997).

The nature of the host cell is thought to be important for the expression of nAChRs. There are many processes involved in protein folding, assembly and trafficking to the final subcellular destination such as glycosylation, phosphorylation and the presence of chaperone proteins. These are critical events that lead to the formation of functional nAChRs and disruption or absence of any of these can result in failure to express a functional receptor. Once a suitable host cell has been established and functional receptors can be expressed then nAChRs can be investigated *in vitro* to study the action of nicotinic ligands at these receptors and to screen potentially useful therapeutic compounds.

1.11 Potential therapeutic relevance of drugs acting at nAChRs

Nicotine acts at nAChRs found in the brain, autonomic ganglia and the vertebrate neuromuscular junction. Nicotine is believed to be the addictive constituent in tobacco. In vertebrates, the sites in the brain with the greatest number of nicotine binding sites are in the: cortex, thalamus and interpenduncular nucleus as well as substantial binding in the amygdala, septum, brain stem motor nuclei and locus ceruleus (Clark *et al.*, 1985). The mesolimbic system has been implicated in nicotine addiction (Corrigall *et al.*, 1994). Nicotine replacement therapy has been available for approximately 15 years in the form of gum and transdermal patches with a nasal spray and inhaler under development and this therapy is used widely to aid smoking cessation (Levin *et al.*, 1994).

There is evidence that nicotine enhances some form of memory, one mechanism by which this occurs could be by increasing the strength of synaptic communication between neurones in the hippocampus (a centre for learning and memory) which has rich cholinergic innervation and dense nAChR expression (Gray *et al.*, 1996). In Alzheimer's dementia, there is a reduction in nAChR number and degeneration of the cholinergic inputs. The reported negative correlation between smoking and Alzheimer's disease and other dementia's such as Parkinson's disease (Levin, 1992;

Whitehouse, 1993; Court and Perry, 1994) and the ability of nicotine to enhance certain forms of memory suggests a role for nicotinic agents in the treatment of some of the deficits due to this condition. The diversity of nAChRs potentially allows the development of selective compounds which have fewer side effects than current treatments. Experimental studies with intravenous or subcutaneous exposures to nicotine or nicotinic analogues such as ABT 418, have shown modest improvements in cognitive function with some Alzheimers patients (Jones *et al.*, 1992; Anderson *et al.*, 1995). A recent report (Gray *et al.*, 1996), has shown that hippocampal synaptic transmission is enhanced by low levels of nicotine.

The mechanism by which cigarette smoking may have a protective effect against Parkinson's disease is unknown. It may involve nicotine facilitating dopamine release from neurone in the nigrostriatal region which is the area depleted of dopamine containing neurones in Parkinson's disease. Clinical trials of nicotine in Parkinson's disease are ongoing (Grady *et al.*, 1992; Fagerstrom, 1994; Clemens, 1995).

Tourette's syndrome is characterised by repetitive motor and vocal tics. Haloperidol, a dopamine antagonist, is the usual treatment for this syndrome, but more recently the use of nicotine has been investigated which may potentiate the action of Haloperidol. Clinical trials are underway (Silver and Sanberg, 1993).

The neuronal nAChRs have been associated with schizophrenia. A recent paper (Freedman, 1997), has linked the $\alpha 7$ - nicotinic receptor gene with the inheritance of a pathophysiological aspect of schizophrenia. High doses of nicotine was shown to transiently normalise the epileptic abnormality in patients and their relatives. This was blocked by α -BTX but not by mecamylamine the latter of which is known to block all known nicotinic receptors except the $\alpha 7$ receptor.

A major potential use of drugs that act on the nAChRs are insecticides. Nitromethylenes such as imidocloprid are an important class of insecticides which have a broad spectrum of insecticidal activity and relatively low mammalian toxicity. Nitromethylenes have been shown to act at invertebrate nAChRs (Marshall *et al.*, 1990; Buckingham *et al.*, 1995).

The antihelmintic agents morantel, pyrantel and levamisole have been shown to have an action at the ACh receptors of the nematode *Ascaris suum* (Harrow and Gratton, 1985). They are thought to bring about their therapeutic effect by acting at ACh receptors to increase conductance and depolarisation of the muscle bag membrane resulting in maintained muscle contraction.

The therapeutic applications of nicotine and its analogues are potentially widespread and a critical aspect of these potential areas depends heavily on our understanding of the underlying mechanisms which include the structure and distribution of the neuronal nAChRs and their interactions with other neurotransmitter receptors. The diversity of the receptor subtypes offers a wealth of possibilities for novel drug design for the treatment of dementia's and other disorders.

1.12 Aims of the project

The aims of this project were to investigate the assembly and expression of the vertebrate and invertebrate neuronal nAChRs. Of particular interest was the question of whether the inability of cloned *Drosophila* nAChR subunits to form functional nAChRs in *Xenopus* oocytes could be attributed to an inability of this expression system to correctly assemble *Drosophila* nAChRs. In this study this question was addressed by expressing the *Drosophila* nAChR subunits in what may be a more native cellular environment, the *Drosophila* S2 cell line.

Another aim of the project was to isolate a full length *Drosophila* nAChR subunit SBD (previously isolated as an incomplete cDNA clone) and to co-express the full-length SBD subunit cDNA with other cloned *Drosophila* nAChR subunits.

A further aim was to search for novel nAChR subunits by screening *Drosophila* cDNA libraries. Novel *Drosophila* and *C.elegans* nAChR subunits identified during this project were also examined by heterologous expression.

2: Materials and Methods

2.1 Cell lines and cell culture

Drosophila S2 cells (Schneider, 1972) were provided by Dr Thomas Bunch, University of Arizona. *Drosophila* S2 cells were grown in Sheilds and Sang M3 medium (Sigma) containing 12.5% heat inactivated foetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco) at 25°C.

Human embryonic kidney cells (HEK-293) were obtained from the European Collection of Cell Cultures. HEK-293 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco), 10% heat inactivated FCS in 5% CO₂ at 37°C. For growth at lower temperatures, HEK-293 cells were grown in sodium bicarbonate-free DMEM containing 25 mM HEPES and 10% FCS.

2.2 Plasmids and cDNA libraries

The *Drosophila* expression vector pRmHa3 was provided by Dr Thomas Bunch, University of Arizona. This plasmid is a modified form of the expression vector pRmHa1 (Bunch *et al.*, 1988) in which the multiple cloning site has additional unique restriction sites (*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI and *Sal*I) (Bunch, 1989). The multiple cloning site of pRmHa3 was modified to contain the additional unique restriction sites (*Not*I, *Bcl*II and *Xba*I) by ligation of the synthetic oligonucleotides, 5'-CCGGGCGGCCGCTGATCATCTAGAC-3' and 5'-TCGAGTCTAGATGATCAGCGGCCGC- 3', into the *Xma*I and *Sal*I sites of pRmHa3 to create the plasmid pRmHa4.

The hygromycin selection plasmid, pCOHygro (van der Straten *et al.*, 1989), was provided by Dr. Martin Rosenberg, SmithKline Beecham Pharmaceuticals, Philadelphia.

The rat nAChR subunit cDNAs $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 3$ and $\beta 4$ in the cloning vector pcDNA1/neo were obtained from Dr Jim Patrick, Baylor College of Medicine, Houston.

The *Drosophila* nAChR subunit cDNAs ALS, SAD, ARD and SBD (Hermans-Borgmeyer *et al.*, 1986; Bossy *et al.*, 1988; Sawruk *et al.*, 1990a; Sawruk *et al.*, 1990b) were obtained in the expression vector pBluescript from Dr Heinrich Betz, Max-Planck Institut fur Hirnforschung, Frankfurt, Germany. A novel *Drosophila* nAChR subunit, the third α like subunit cDNA (ADR or D $\alpha 3$) (unpublished) was kindly provided by Dr Bertram Schmitt, Max-Planck Institute fur Hirnforschung, Frankfurt, Germany, in the expression vector pBluescript.

The *C. elegans* nAChR subunit cDNAs LEV-1, ACR-2 and UNC-29 (in the cloning vector pBluescript-KS) and UNC-38 (in pBluescript-SK) were supplied by Dr David Sattelle, The Babraham Institute, Cambridge (Squire *et al.*, 1995; Fleming *et al.*, 1997). The *C. elegans* nAChR subunit cDNA Ce21 (in the cloning vectors pMT3 and pFLIP-21) was kindly provided by Dr Marc Ballivet, University of Geneva (Ballivet *et al.*, 1996).

The cloning vector pcDNA3 was obtained from Invitrogen. The expression vector pGEMHE was a gift from Dr Emily Liman, Mass General Hospital, Boston. The pGEMHE vector was engineered from the vector pGEM 3Z (Liman, 1992).

The *Drosophila* cDNA library in pNB40 (Brown and Kafatos, 1988) was provided by Dr. Nicholas Brown (Harvard University). The *Drosophila* adult 5' Stretch Plus cDNA library was obtained from Clontech. The *Drosophila* Embryo cDNA library in the vector λ EXlox was obtained from Novagen. Two other cDNA libraries in Lambda Zap II (Stratagene) derived from mRNA: "*Drosophila* head library #1" (1.3+

Kb cDNA) and "*Drosophila* head library #2" (0.6 - 1.3 Kb cDNA) were obtained from Dr. Ron Davis, Baylor College of Medicine, Houston.

2.3 Antibodies

Monoclonal antibodies mAb299 and mAb290 (specific for the rat $\alpha 4$ and $\beta 2$ subunits, respectively) (Whiting and Lindstrom, 1988) were provided by Dr Jon Lindstrom, University of Philadelphia. mAb270 (raised against the chick $\beta 2$ subunit but which also recognises the rat $\beta 2$ subunit) (Whiting *et al.*, 1987) was purified from the hybridoma (HB189) which was obtained from the American Type Culture Collection. mAbFLAG-M2 (Hopp *et al.*, 1988) was obtained from IBI. Two monoclonal antibodies to the *Drosophila* ALS and SAD nAChR subunits, mAb D4 and mAb C3 respectively, were kindly supplied to us by Dr. Eckart Gundelfinger, University of Hamburg, Germany (Schuster *et al.* 1993, Jonas *et al.* 1994).

2.4 Production of competent *E. coli*

E. coli were made competent for plasmid transformation. *E. coli* strain (HB101 or XL1 Blue) were streaked from an agar stab (Invitrogen) onto SOB agar plates and incubated at 37°C overnight. Cells were then transferred from the SOB plate to 500 ml of sterile SOB liquid media and incubated at 37°C in a shaking incubator at 200 RPM until the OD₅₅₀ was between 0.50 - 0.55. Cells were then transferred to sterile 250 ml centrifuge bottles, cooled on ice for 30 min, and then centrifuged at 2500 RPM for 15 min. Cell pellets were resuspended in 20 ml ice-cold RF1 buffer (100 mM RbCl, 50 mM MnCl₂. 4H₂O, 30 mM Potassium acetate, 10 mM CaCl₂.2H₂O, 15% (w/v) glycerol, pH adjusted to 5.8 with 0.2 M acetic acid) and stored on ice for either 2 h (HB101) or 20 min (XL1 Blue). The RF1 buffer was filtered sterilised prior to addition of the cells by passing the buffer through a 0.22 μ m filter Stericup (Milipore). Cells were centrifuged at 2500 RPM for 9 min and then pellets resuspended with 3.5 ml ice-cold filter sterilised RF2 buffer (10 mM RbCl, 10 mM MOPS, 75 mM CaCl₂.2H₂O, 15% (w/v) glycerol, pH adjusted to 6.8 with 0.2 M

NaOH) and incubated on ice for 15 min. Aliquots, typically 50 - 100 µl, were frozen rapidly in an ethanol/ice bath and then stored at -70°C.

2.5 Bacterial transformation

All bacterial transformations were performed using frozen stocks of competent *E. coli* strain HB101 or XL1 Blue prepared as described in the previous section. Plasmid DNA (20 ng), which contained a gene conferring resistance to ampicillin, was added to a 50 µl aliquot of competent cells and incubated on ice for 30 min. Tubes were then transferred to a water bath equilibrated to 42°C and incubated for 90 seconds. The tubes were immediately transferred onto ice and after 2 min 500 µl SOC (2% bactotryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added and the cells placed in a shaking incubator at 37°C for 60 min. An aliquot (50 µl - 150 µl) of each sample was spread onto an LB-agar plate, supplemented with 50 µg/ml ampicillin, and grown overnight at 37°C.

2.6 Small-scale preparation of plasmid DNA

A single bacterial colony was transferred to 2 ml of LB medium containing 40 µg/ml ampicillin and incubated at 37°C overnight with vigorous shaking. 1.5 ml of the cell culture was centrifuged in a microfuge tube at 12,000g for 30 seconds. The supernatant was aspirated and the bacterial pellet was then lysed using the protocol originally described by (Birnboim and Doly, 1979). The bacterial pellet was resuspended in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0)). 200 µl of Solution II (0.2 M NaOH and 1% SDS) was added and the tube inverted five times followed by addition of 150 µl Solution III (60 ml potassium acetate (5M), 11.5 ml glacial acetic acid and 28.5 ml H₂O) and the cell lysate stored on ice for 5 min. The resulting Solution III is 3M with respect to potassium and 5M with respect to acetate. The lysate was centrifuged at 12,000g for 5 min and the supernatant transferred to a clean tube. An equal volume of

phenol:chloroform was added, vortexed and then centrifuged at 12,000g for 2 min. The supernatant was transferred to a fresh tube. The double-stranded DNA was precipitated with two volumes of 96% ethanol, vortexed and then centrifuged after 5 min at 12,000g for 5 min. The supernatant was aspirated and the precipitate washed with 70% ethanol. The DNA precipitate was then resuspended in 20 µL TE buffer containing RNAase (20 µg/ml) and stored at -20°C.

2.7 Small and large scale plasmid purification

For procedures requiring better quality plasmid DNA, (e.g. DNA sequencing or transfection of cultured cell lines), small scale (~20 µg) plasmid preparations were performed with either the Wizard DNA clean up System (Promega) or a QIAprep-spin Plasmid Purification Procedure (QIAGEN). For the QIAprep-spin column kit the plasmids were prepared from bacterial cultures (HB 101 or XL1-Blue) grown in the presence of an appropriate antibiotic (usually ampicillin, 40 µg/ml). 5 ml cultures of *E. coli* were grown and 2 ml were pelleted in 2 ml microfuge tubes, the supernatant aspirated and then a further 2 ml of culture added and the centrifugation repeated. The bacterial pellet was resuspended in 250 µl buffer P1 and 250 µl buffer P2 was added and incubated for 5 min at room temperature. 350 µl of chilled buffer N3 was added and mixed by gentle inversion and after incubation on ice for 5 min the solution was centrifuged for 10 min at 10,000g. The supernatant was transferred to a QIAprep-spin column in a 2 ml microcentrifuge tube and centrifuged for 1 min at 10,000g. 0.5 ml buffer PB was added to the column and the centrifugation repeated. 0.75 ml buffer PE was then added and the centrifugation repeated. Excess buffer PE was removed and the centrifugation repeated to remove remaining buffer. The QIAprep-spin column was placed in a clean microfuge tube and the DNA eluted by adding 50 µl 10 mM Tris/HCl (pH 8.5) or TE (pH 8.5) and centrifugation for 1 min. DNA yields between 5-20 µg were obtained and were stored at -20°C.

The Wizard DNA Clean Up Resin was for the purification of DNA fragments by removing heat stable restriction enzymes, alkaline phosphatase, kinases, polymerases, exonucleases and endonucleases, DNA fragments smaller than 175 bp and was used routinely in these studies to purify DNA from low melting point agarose gels. The DNA fragments were typically in a volume of 50 - 500 μ l (DNA bands excised from agarose gels were heated to 65°C to melt the agarose). 1 ml of Wizard™ DNA clean up resin was added to the DNA sample. The DNA/resin mix was pipetted into the syringe barrel attached to the minicolumn and the solution passed through the column. 2 ml of 80% isopropanol was added to the syringe barrel and passed through the column. The column was then centrifuged at 10,000g in a microcentrifuge for 2 min to remove residual isopropanol. The column was transferred to a clean microfuge tube and 50 μ l of prewarmed (65 - 70°C) water was added to the column and after 1 min the column was centrifuged for 30 sec to elute the bound DNA. The eluted DNA was stored at -20°C.

For larger scale production of plasmid DNA QIAGEN Maxi prep protocols were followed. 250 ml of a bacterial culture from a single colony were grown in LB containing the appropriate selective antibiotic for 12 - 16 hours with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000g for 10 min at 4°C. The pellet was resuspended in 10 ml buffer P1 (50 mM Tris.Cl (pH 8.0), 10 mM EDTA and 100 μ g/ml RNase A). 10 ml buffer P2 (200 mM NaOH and 1% SDS) was added and incubated at room temperature for 5 min. 10 ml of chilled buffer P3 (3.0 potassium acetate pH 5.5) was then added and incubated on ice for 20 min. The solution was centrifuged at 20,000g for 30 min at 4°C and the supernatant transferred to a QIAGEN-tip 500 which had been equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS (pH 7.5), 15% isopropanol and 0.15% Triton X-100). The supernatant was passed through the column by gravity and then washed twice with 30 ml wash buffer QC (1.0 M NaCl, 50 mM MOPS (pH 7.0) and 15% isopropanol). The DNA was eluted with 15 ml buffer QF (1.25 M NaCl, 50 mM Tris, Tris.Cl (pH 8.5))

and 15% isopropanol). The DNA was precipitated by addition of 10.5 ml isopropanol (room temp) and centrifuged at 15,000g for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and then resuspended in 1 ml TE (pH 8.0) or 10 mM Tris.Cl (pH 8.5) or water. DNA yields in the range 100 - 500 µg were obtained.

2.8 Determination of nucleic acid concentration

DNA or RNA (typically 5 - 10 µl) was diluted to a volume of 1 ml with TE (Tris-HCl 10 mM (pH 7.6), EDTA 0.1 mM) and its absorbance at 260 nm and 280 nm measured against a TE blank in a 1 ml quartz cuvette in a spectrophotometer. For DNA solutions if the $A_{260}:A_{280}$ ratio was less than 1.8:1, the sample were phenol extracted in order to remove protein contamination. The concentration of the nucleic acid was calculated as follows:

DNA concentration (µg/ml) = $A_{260} \times 50 \times \text{dilution factor}$.

RNA concentration (µg/ml) = $A_{260} \times 40 \times \text{dilution factor}$.

2.9 Restriction enzyme digestion of DNA

Restriction digests were carried out using enzymes from Promega, New England Biolabs or Boehringer Mannheim using buffers supplied by the manufacturers. Reactions were carried out typically in a total volume between 30 and 80 µl and at 37°C unless recommended otherwise. When cleaving DNA with more than one enzyme then this was carried out simultaneously when both enzymes cut efficiently in the same buffer. For enzymes with different buffer requirements then the digestion with one enzyme was carried out e.g. enzyme that works best in a buffer of lower ionic strength. Where possible, the first enzyme was heat inactivated by incubating the solution at 65°C for 10 min. The buffer was then modified e.g. by addition of NaCl to provide optimal conditions for the second enzyme and the digestion with the second enzyme was carried out. Restriction enzymes were heat inactivated at 65°C for 10 min at the end of the incubation period. When restriction enzymes were known

to be resistant to heat inactivation, they were removed by phenol / chloroform extraction.

2.10 DNA Ligations

Ligation of DNA fragments was carried out at 14°C using T4 DNA ligase (New England Biolabs or Boehringer Mannheim) for 5 - 20 hours (Weiss, 1968) in ligation buffer (New England Biolabs or Boehringer Mannheim). After incubation, the ligase was heat inactivated for 10 min at 65°C.

2.11 Agarose gel electrophoresis of DNA

A 0.8% (w/v) agarose gel was prepared by dissolving 0.8g agarose (Sigma) into 100 ml of TAE (40 mM Tris-acetate, 1 mM EDTA). The solution was allowed to cool to ~55°C and 10 µg/ml ethidium bromide was added prior to pouring into the gel mold. 2 µl gel loading buffer (bromophenol blue 0.25%, w/v, xylene cyanol FF 0.25%, Ficoll 15%) was added to 20 µl DNA sample. The samples were loaded on the gel with molecular weight markers of bacteriophage Lambda DNA (Gibco) that had been digested with the restriction enzyme *Hind* III heated to 70°C for 2 min before loaded onto gel. Gel electrophoresis was performed for 1 h at 110 amps (constant current). DNA was visualised on an ultra violet transilluminator.

2.12 Purification of DNA fragments from low melting point agarose gels

DNA gel electrophoresis was carried out on a 0.8% (w/v) low melting point agarose gel as described above. Gel electrophoresis was for 60 - 90 min at 50 Amps (constant). Ethidium bromide stained DNA bands were visualised on an ultra violet transilluminator and the desired bands excised with a scalpel blade. Exposure to the ultra violet illumination was kept to a minimum to prevent damage to the DNA. The excised bands were then purified using the Wizard DNA Clean up Kits (Promega) which is described in Section 2.7. The DNA was eluted for the columns in 50µl water and stored at -20°C until required.

2.13 DNA sequencing

DNA samples were prepared using either the QIAGEN plasmid Maxi Kit or the QIAprep Spin Plasmid Kit (Section 2.7). 1.3µg of double stranded DNA was used per sequencing reaction. Oligonucleotide primers (typically of 24 bases in length) were synthesised on the Applied Biosystems 392 DNA/RNA synthesiser and a concentration of 3.2 pmoles of primer was used in each sequencing reaction. The DNA and primers were mixed with the ready made DyeDeoxy Terminator Cycle sequencing promix (Applied Biosystems) containing the four ABI dye labelled dideoxynucleotides: G, A, T and C dye deoxy terminators and the thermally stable enzyme Ampli Taq DNA polymerase. The reagents were then covered with 40µl mineral oil and placed in a Hybaid Omni Gene thermal cycler. The conditions for the cycling reactions were as follows:

- Rapid thermal ramp to 96°C (1°C per second).
- 96°C for 30 seconds.
- Rapid thermal ramp to 50°C.
- 50°C for 15 seconds.
- Rapid thermal ramp to 60°C.
- 60°C for 4 minutes.
- Then repeat for a total of 25 cycles.
- Finally rapid thermal ramp to 30°C and hold.

The reactions were then purified to remove excess dyedeoxy terminators from the completed sequencing reactions by phenol extraction. At the end of the thermal cycling 80 µl of H₂O was added to the reaction mixture with 100 µl water-saturated phenol. The reagents were vortexed and centrifuged and the terminators were extracted into the phenol. The extraction was repeated using another 100 µl of phenol. After the sample was vortexed and centrifuged for 1 minute the aqueous upper layer was transferred to a clean tube. The extension products were precipitated

with 300 µl 100% ethanol and 15 µl of 2M sodium acetate, pH 4.5. The mixture was centrifuged for 15 min, 13,000 g at room temp. The pellet was washed with 70% ethanol and dried and stored at -20°C.

Gel plates were washed and air dried for approximately 30 minutes. An acrylamide gel mix was prepared (60 ml total volume) with a final acrylamide concentration of 4.75%. 30 g urea, 7.2 ml 40% 19:1 acrylamide (Amresco), 25 ml milliQ water and 1g Amberlite MB-1 were stirred for approximately 30 min until all the urea had dissolved. 6 ml 10X TBE (ABI recipe) was drawn through a Nalgene 0.2 micron filter followed by the rest of the gel mix. The mixture was then degassed for 5 min. 33 µl Temed (Kodak) was then added and the mixture swirled followed by addition of 300 µl 10% ammonium persulphate (Sigma). The mixture was swirled again and the gel was then poured and allowed to set for 2-6 h.

Loading buffer consisted of 1 part 50 mM EDTA to 5 parts deionised formamide. The samples were resuspended in 4 µl loading buffer, heated to 90°C for two minutes, then loaded on the gel. The gel was run for 14 h at 2500V, 40mA, and 32W. The sequences obtained were then checked.

2.14 Protein Assay

Protein concentration (e.g. of membrane preparations used for radioligand binding) were determined using the Bio-Rad Protein assay kit. The principal of the kit is based on the Bradford method of protein determination (Bradford, 1976).

2.15 Cloning of a full-length *Drosophila* nAChR SBD cDNA

The 5' end of the *Drosophila* nAChR SBD subunit mRNA was isolated from a *Drosophila* cDNA library (in plasmid pNB40) by the polymerase chain reaction (PCR). PCR primers were designed to the cDNA library plasmid (5'-ATATTGTCGTTAGAACGCGGCTAC-3') and the previously reported SBD cDNA

sequence (Sawruk *et al.*, 1990b) (5'-GCTGAGACAATTTTCAGACCTAACC-3'). The resultant PCR product was initially subcloned into plasmid pCRII (Invitrogen). After sequencing to confirm the identity of the PCR product the 5' SBD cDNA fragment was excised from the vector pCRII and subcloned into the cloning vector pBluescript. The resultant full-length SBD cDNA clone was then subcloned into the plasmid expression vectors pRmHa3 and pcDNA3.

2.16 Subcloning *Drosophila* nAChR subunit cDNAs into pRmHa3

The *Drosophila* SAD nAChR subunit cDNA was excised from pBluescript-SK by digestion with *Kpn*I and *Sma*I. This was then blunt ended with T4 DNA polymerase to remove 3' overhangs and to fill in 5' overhangs. SAD was subcloned into the *Sma*I site of the *Drosophila* expression vector pRmHa3 which had been treated with calf intestinal phosphatase (CIP) to remove the 5' phosphate groups from the plasmid to reduce self ligation and resulted in the construct pRmHa3-SAD.

The *Drosophila* ALS nAChR subunit cDNA was excised from pBluescript-KS with *Kpn*I and *Not*I digestion and subcloned directionally into *Kpn*I and *Not* I digested pRmHa4 to give plasmid pRmHa4-ALS. Sequencing revealed two ATG start codons upstream of the main starting codon neither of which had optimal consensus sequences .

The *Drosophila* ARD nAChR subunit cDNA was excised from pBluescript-KS with *Kpn*I and *Bam*HI and subcloned directionally into pRmHa3. Sequencing revealed ATG start codons upstream of the main starting codon and these were removed with further subcloning. The ARD cDNA was excised from pRmHa3 with *Bst*XI and blunt ended with T4 DNA polymerase and subcloned into the CIP treated *Sma*I site of the *Drosophila* expression vector pRmHa3 to give plasmid pRmHa3-ARD. Sequencing revealed that only the main starting codon remained with a good consensus sequence (A--ATGG).

The *Drosophila* SBD nAChR subunit cDNA was excised from pBluescript-SK with *EcoRI* and subcloned into the CIP treated *EcoRI* site of pRmHa3 to give plasmid pRmHa3-SBD. The main start codon did not have an optimal consensus sequence and sequencing revealed further start codons upstream. The original SBD cDNA lacked part of the coding sequence and details of the construction and expression of a full-length SBD cDNA are described later.

2.17 Subcloning *Drosophila* nAChR subunit cDNAs into pcDNA3

The *Drosophila* ALS subunit cDNA was excised from pRmHa4 by a double digest with *KpnI* and *XbaI* and subcloned "directionally" into *KpnI* and *XbaI* cut pcDNA3.

The *Drosophila* ARD subunit cDNA was subcloned "directionally" into pcDNA3 from the cloning vector pRmHa3 using the restriction enzymes *BamHI* and *KpnI*.

The *Drosophila* SAD subunit cDNA was excised from the pBluescript vector with the enzymes *KpnI* and *NotI* and subcloned "directionally" into *KpnI* and *NotI* cut pcDNA3.

The full-length *Drosophila* SBD subunit cDNA was subcloned "directionally" from the pBluescript cloning vector into pcDNA3 using the restriction endonucleases *XbaI* and *XhoI*.

2.18 Subcloning *Drosophila* nAChR subunit cDNAs into pGEMHE

The pGEMHE vector was derived from the pGEM 3Z vector (Promega) (Liman, 1992) but contains 5' and 3' untranslated regions of a *Xenopus* β -globin gene separated by a small polylinker with 5 restriction sites (3' -*HindIII*, *XbaI*, *EcoRI*, *BamHI* and *SmaI* -5'). The vector contains 3 unique restriction sites (*NheI*, *PstI* and *SphI*) which are suitable for linearisation of the plasmid prior to *in vitro* transcription.

This plasmid has been found to be well suited to the *in vitro* synthesis of mRNA for injection into *Xenopus* oocytes.

The pGEMHE construct was modified in this study by cutting with *NheI* and *PstI* and inserting a polylinker (5' -CTAGATAAGAATGCGGCCGCTAAACTAT- 3' and 5' -ATAGTTTAGCGGCCGCATTCTTAT- 3') introducing the unique *NotI* site resulting in the modified vector pGEMHE-2.

The *Drosophila* ALS subunit cDNA was excised from pRmHa4 by the restriction enzymes *EcoRI* and subcloned into *EcoRI* cut pGEMHE. The *Drosophila* ARD subunit cDNA was excised from the expression vector pRmHa3 by digestion with *HindIII* and the single strand overhangs removed with T4 DNA polymerase. The construct was then digested with *EcoRI*. The *Drosophila* ARD cDNA subunit was subcloned "directionally" into *SmaI* and *EcoRI* cut pGEMHE-2. The SAD nAChR subunit cDNA was excised from the expression vector pcDNA3 by digestion with *HindIII* and the single strand overhangs removed with T4 DNA polymerase. The construct was then digested with *XbaI*. The *Drosophila* SAD cDNA subunit was then subcloned "directionally" into *SmaI* and *XbaI* cut pGEMHE.

2.19 Introduction of an epitope-tag into the *Drosophila* nAChR subunits

Each of the *Drosophila* nAChR subunit cDNAs were "tagged" with an eight amino acid "FLAG-epitope" in the region between the third and fourth transmembrane spanning domains of the encoded proteins (Hopp *et al.*, 1988). The FLAG-epitope is a foreign epitope-tag introduced into the individual subunits to which a mAb was available for immunoprecipitation studies. This was necessary due to the lack of specific antibodies to the *Drosophila* nAChR subunits. A specific antibody to the FLAG-epitope was available and had been used successfully for immunoprecipitation experiments.

The pRmHa4-ALS construct was cut at a unique site between the third and fourth transmembrane spanning domains with the restriction enzyme *NcoI*. Two oligonucleotides containing the FLAG-epitope were annealed slowly from 65°C (5' - CATGGACTACAAGGACGACGATGACAAGGG - 3' and 5' - CATGCCCTTGT-CATCGTCGTCCTTGTAGTC - 3') and then ligated into the *NcoI* site. Following ligations and small scale production of plasmid DNA the constructs that had been successfully FLAG-tagged were isolated by PCR using the forward FLAG primer (5' - CATGGACTACAAGGACGACGATGACAAGGG - 3') and a reverse primer to the pRmHa4 vector (5'-AGGAGAAGAATGTGAGTGTGCATC-3'). The FLAG-tagged ALS construct was excised from pRmHa4 with *KpnI* and *XbaI* and subcloned "directionally" into pcDNA3.

The pRmHa3-ARD construct was digested with the unique restriction enzyme *NcoI* which cuts within the intracellular loop between Tm3 and Tm4. Two oligonucleotides were synthesised (5' -CATGGACTACAAGGACGACGATGACAAGGG - 3' and 5' - CATGCCCTTGTCATCGTCGTCCTTGTAGTC - 3') which when annealed contain the FLAG construct and with overhangs at each end that could be ligated directly into *NcoI* cut pRmHa3-ARD. Following small scale production of plasmid DNA the constructs were screened by PCR using the forward FLAG primer (5' -CATGGACTACAAGGACGACGATGACAAGGG - 3') and a reverse primer to the vector pRmHa3 (5' - AGGAGAAGAATGTGAGTGTGCATC -3'), to establish which constructs contained the FLAG-epitope.

The pBluescript-SAD plasmid was digested with the unique cutting enzyme *SphI* between the third and fourth membrane spanning regions. Two oligonucleotides, (5' - GACTACAAGGACGACGATGACAAGGGCATG - 3' and 5' - CCCTTGTCATCGT-CGTCCTTGTAGTCCATG - 3'), containing the FLAG-epitope and overlapping ends, were annealed and then ligated directly into the *SphI* site. pBluescript-SAD constructs were sequenced to establish which constructs had been FLAG-tagged. The

FLAG-tagged SAD cDNA was excised from pBluescript by digestion with *KpnI* and treated with T4 DNA polymerase to remove the single strand overhang and then digestion with *SmaI*. The FLAG-tagged SAD nAChR cDNA was subcloned into CIP treated *SmaI* cut pRmHa3 and its orientation checked by sequencing. The FLAG-tagged SAD construct was excised from pBluescript by the restriction enzymes *KpnI* and *XbaI* and subcloned directionally into pcDNA3.

The full-length pBluescript-SBD construct was digested with *SphI*, which cuts at a unique site in the intracellular loop between the third and fourth transmembrane domains. The oligonucleotides (5'-GACTACAAGGACGACGATGACAA-GGGCATG - 3' and 5' - CCCTTGTCATCGTCGTCCTTGTCATG - 3'), were annealed and ligated directly into the *SphI* site. The FLAG-tagged constructs were identified by sequencing. The FLAG-tagged SBD cDNA was excised from pBluescript by digestion with *SacI* and subcloned into CIP treated *SacI* cut pRmHa3. Correct orientation was checked by sequencing the full-length pRmHa3-SBD constructs.

2.20 Subcloning rat neuronal nAChR subunit cDNAs into pRmHa3

The rat neuronal $\alpha 3$, $\alpha 5$ and $\beta 4$ nAChR subunit cDNAs were excised from pcDNAI/Neo by digestion with *NotI* and *EcoRI* and subcloned "directionally" into pRmHa4 to give plasmids pRmHa4- $\alpha 3$, pRmHa4- $\alpha 5$ and pRmHa4- $\beta 4$. The rat neuronal nAChR $\beta 3$ cDNA was excised from pcDNAI/Neo by digestion with *NotI* and *SacI* and subcloned "directionally" into pRmHa4 to give plasmid pRmHa4- $\beta 3$. The rat neuronal nAChR $\alpha 4$ and $\beta 2$ subunit cDNAs were excised from pcDNAI/Neo by Dr Neil Millar and subcloned into the *Drosophila* expression vector pRmHa3 to give plasmids pRmHa3- $\alpha 4$ and pRmHa3- $\beta 2$ respectively.

2.21 Subcloning rat neuronal nAChR subunit cDNAs into pGEMHE

The rat neuronal nAChR $\alpha 3$, $\alpha 4$ and $\beta 4$ subunit cDNAs in pcDNAI/Neo were cut with *HindIII*, the single strand overhangs were removed by treatment with T4 DNA polymerase, and then the cDNA excised with *XbaI*. The subunit cDNAs were then subcloned "directionally" into *SmaI* and *XbaI* cut pGEMHE. The rat neuronal nAChR $\beta 2$ subunit cDNA was excised from pcDNAI/Neo by digestion with *BamHI* and *XbaI* and subcloned "directionally" into pGEMHE.

2.22 Subcloning *C. elegans* nAChR subunit cDNAs into pRmHa3

The *C. elegans* UNC38 nAChR subunit cDNA was excised from pBluescript-SK by digestion with *SacI* and *Sal I* and cloned "directionally" into pRmHa3 to give plasmid pRmHa3-UNC38.

The *C. elegans* UNC29 nAChR subunit cDNA was excised from pBluescript-KS by digestion with *HindIII* and *BamHI*, the single strand overhangs were removed by treatment with T4 DNA polymerase, and the excised subunit cDNA was then subcloned into the CIP-treated *SmaI* site of the *Drosophila* expression vector pRmHa3 to give plasmid pRmHa3-UNC29.

The *C. elegans* LEVI nAChR subunit cDNA was excised from pBluescript-KS by digestion with *KpnI* and *SacI* and cloned "directionally" into pRmHa3. Sequencing revealed several ATG start codons 5' to the main start codon and these were removed with further subcloning. The LEVI cDNA was excised from pRmHa3 by digestion with the restriction enzymes *Hind III* and *XbaI*, the single strand overhangs were removed by treatment with T4 DNA polymerase, and the subunit cDNA was then subcloned into the CIP-treated *SmaI* site of the *Drosophila* expression vector pRmHa3 to give plasmid pRmHa3-LEVI. One start codon with an optimal consensus sequence (A--ATGA) was revealed by sequencing.

The *C. elegans* ACR2 nAChR subunit cDNA was excised from pBluescript-KS by digestion with *Eco*RI and subcloned into the CIP-treated *Eco*RI site of pRmHa3. Sequencing revealed a long sequence of continuous adenines at the 5' end of the ACR2 and this was excised with further subcloning. The ACR2 cDNA was excised from pRmHa3 by digestion with *Hga*I. A double stranded oligonucleotide was ligated to the 5' end of ACR2 containing the *Eco*RI site and nucleotides to improve the consensus sequence of the start codon. The resulting ACR2 with the 5' oligonucleotide was then digested with *Eco*RI and subcloned into the CIP-treated *Eco*RI site of pRmHa3 to give plasmid pRmHa3-ACR2. Sequencing revealed one start codon with an optimal consensus sequence and no sequence of continuous adenines at the 5' end of the ACR2.

During the course of this project a fifth *C. elegans* nAChR subunit was isolated (Ballivet *et al.*, 1996) and this Ce21 subunit, an α like subunit, was kindly supplied to us in the cloning vectors pMT3 and pFLIP. Ce21 was excised from the pFLIP cloning vector by digestion with *Bam*HI and *Hind*III, the single strand overhangs were removed by treatment with T4 DNA polymerase, and the Ce21 subunit cDNA was then subcloned into CIP-treated *Sma*I cut pRmHa3.

2.23 Subcloning the *Drosophila* GABA receptor β subunit LCCH3 into pRmHa3

The *Drosophila* GABA receptor subunit cDNA LCCH3 (Ligand-gated chloride channel) (Henderson *et al.*, 1993) was excised from the cloning vector pSP64 by digestion with *Eco*RI and *Bam*HI and subcloned into the *Eco*RI and *Bam*HI sites of pRmHa3 to create the plasmid pRmHa3-LCCH3.

2.24 Subcloning *Drosophila* TRPL subunit into pRmHa3

The *Drosophila* TRPL (transient receptor potential like) subunit (Phillips *et al.*, 1992) was excised from pBluescript with *Eco*RI and subcloned into *Eco*RI cut CIP-treated pRmHa3 to create the plasmid pRmHa3-TRPL.

2.25 Subcloning locust α L-1 cDNA into pcDNA3

The locust α L-1 nAChR subunit cDNA (Marshall *et al.*, 1990) was excised from pBluescript with *Eco*RI and subcloned into CIP-treated *Eco*RI cut pcDNA3 to create the plasmid pRmHa3- α L-1.

2.26 Cloning of PCR products via single adenine overhang

The TA Cloning Kit using the pCR2.1 vector (Invitrogen) which contains a single thymine overhang was used to provide a quick, one step cloning strategy for the direct insertion of a PCR fragment. The ligation reactions and transformation protocols were as manufacturers specifications.

2.27 *Drosophila* cDNA library screening

Several *Drosophila* cDNA libraries (Section 2.2) were screened by PCR using various primers in an attempt to identify novel *Drosophila* nicotinic receptor subunits. Primers were designed to regions of the four cloned *Drosophila* nAChR receptor subunits that were conserved between *Drosophila* subunits and with nAChR subunits from other species. The PCR conditions were, 95°C for 30 seconds, then an annealing temperature of either 50, 55 or 60°C for 30 seconds, followed by extension at 72°C using Pfu DNA polymerase (Stratagene). Different annealing temperatures were used to optimise the PCR conditions to minimise non-specific PCR products.

The first strategy used degenerate oligonucleotides to the conserved TM3 and TM4 regions of the four *Drosophila* subunits ALS, ARD, SAD and SBD. The forward primer 5' - CTGCTGGGAAA(A/G)TATCT(A/G/T)CT(C/G)TT(C/T)AC- 3' and the reverse primer 5'- A(G/T)CCAGAGAAACAAGCGATCCA(A/G/T)(C/G/T)ACCAT- 3' were used for PCR to screen cDNA libraries. The PCR products from the cDNA libraries were compared to PCR products from reactions using the individual subunit cDNA subunits as the template. The PCR products which appeared to be of different size to the four PCR products from the subunits ALS, ARD, SAD and SBD were

subcloned into the TA cloning vector and sequenced. PCR products that were of similar size to the four control products were also cloned into the TA cloning vector and sequenced.

When sequenced the PCR products from screening the *Drosophila* cDNA libraries did not show any potential new *Drosophila* nAChR subunit. However, the technique itself was shown to work because all four of the previously identified *Drosophila* nAChR subunits were identified using these primers to screen the cDNA libraries. Many of the PCR products were non-specific with the same primer at the 5' and 3' end of the product, usually the forward primer. In order to try to reduce the number of non-specific products another strategy was used.

The second strategy used the reverse degenerate primer (5'-A(G/T)CCAGAGAAACAAGCGATCCA(A/G/T)(C/G/T)ACCAT- 3') with forward primers designed to the TM3 region of the individual cloned subunits: ALS 5'-CTGCTGGGAAAGTATCTGCTCTTCAC- 3', ARD 5'-CTGATCGCCAAA-TATTTGCTGTTCAC- 3', SAD 5'-CTACTGGGAAAATATCTACTGTTCAC- 3', and SBD 5'-CTGCTGGGCAAGTACCTTCTCTTTAC- 3'. PCR conditions were the same as for strategy one. PCR products from screening the cDNA libraries that differed in size to the PCR products obtained when either ALS, ARD, SAD or SBD were used as a template were ligated into the TA cloning vector. The TA clones were sequenced and the number of non-specific products was reduced and again all four of the previously identified subunits were identified from at least one of the libraries. There was still no evidence of potential new *Drosophila* nAChR subunits.

The next strategy used the reverse TM4 degenerate primer (5'-A(G/T)CCAGAGAAACAAGCGATCCA(A/G/T)(C/G/T)ACCAT- 3') and a forward primer designed to the TM2 region. The forward primer sequence was designed to a highly conserved region of the TM2 in ARD, SBD and $\beta 2$. The β subunits were

selected as a basis to design this primer because both ALS and SAD have been shown to couple with rat $\beta 2$ or $\beta 4$ structural subunits to form a functional receptor which suggests that there may be further *Drosophila* non- α subunits to be identified. The degenerate forward primer 5' - YTMKGWATTWSSATWYTSSTGTCG- 3' , based on the sequences of the β subunits was used. (The single letter code for 2 or more nucleotides is as follows: R = A or G, Y = C or T, M = A or C, K= G or T, S = C or G, W = A or T, H = A or C or T, B = C or G or T, V = A or C or G, D = A or G or T and N = A, C, G or T).

Similar PCR conditions used for the previous strategies were used and both SBD and ARD were isolated from one or more of the cDNA libraries but there was no evidence of novel *Drosophila* nAChR subunits.

The final PCR screening strategy used to screen the libraries was using a forward primer to the cDNA library vector, either 5' -AATTAACCCTCACTAAAGGG- 3' or 5' -TAATACGACTCACTATAGGG - 3' and a reverse primer to a conserved region in rat nicotinic subunits 5' -CAITKYTGIIIRTCRAAIGGRAA- 3' which is upstream of TM1. The PCR conditions used were 95°C for 30 seconds, 50 or 60°C for 30 seconds and 72°C for 60 seconds.

2.28 Transfection of cultured cell lines

Exponentially growing S2 cells were transfected by a modified calcium phosphate method (Chen and Okayama, 1987). The DNA was made up to a final volume of 500 μ l in 0.1 X TE [10 mM Tris.Cl (pH 7.4) and 1mM EDTA (pH 8.0)] with a total amount of DNA in the region of 20 - 30 μ g. The ratio of plasmid encoding nAChR cDNA and plasmid conferring hygromycin resistance (pCOHygro) was 2:1. 500 μ l 2X HEPES-buffered saline (HBS), (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄.2H₂O, 12 mM dextrose and 50 mM HEPES) was slowly added with gentle mixing followed by 62 μ l of 2M CaCl₂ and incubated for 30 min to allow the

precipitate to form. Exponentially growing S2 cells were harvested and spun at 900g for 3 min. The supernatant was discarded and the cell pellet washed with 3 ml phosphate buffered saline (PBS). The cells were then resuspended in the calcium phosphate-DNA precipitate and incubated at room temperature for 20 min. To each tube 9 ml prewarmed medium was added and the cells plated out in 25 cm² flasks. The cells were incubated for 6 h at 25°C and were then harvested, centrifuged at 900g for 3 min, resuspended in fresh prewarmed medium and replated in flasks for 24 - 48 h at 25°C.

Stably transfected cells were selected by growth in M3 medium containing 300 µg/ml hygromycin B (Pettinger *et al.*, 1953). A control flask of cells, not transfected with pCOHygro, was established in order to establish the time course required for untransfected cell death in selection medium.

Expression of the nAChR subunits in the clonal or polyclonal S2 cell lines was assayed by nicotinic radioligand binding. Expression of the nAChR subunits was induced by the addition of CuSO₄ to the growth medium of semi-confluent transfected S2 cells to a final concentration of 0.6 mmol l⁻¹ (CuSO₄ was prepared as a 30 mmol l⁻¹ stock). Induction of expression is by the inducible metallothionein promoter on the pRmHa3 plasmid expression vector.

HEK-293 cells were transiently transfected by a modified calcium phosphate DNA co-precipitation method (Chen and Okayama, 1987) and assayed 24 h after transfection. Plasmid encoding nAChR cDNAs in the mammalian expression vectors pcDNA1 or pcDNA3 (20 µg total amount of DNA added) were mixed with 500 µl 2X BES (50 mM BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl and 1.5 mM Na₂HPO₄·2H₂O, pH 6.96). To this solution 0.25 M CaCl₂ (500 µl) was added slowly. The transfection mixture was allowed to stand at room temperature for 30 min before being added dropwise to exponentially growing HEK-

293 cells in 10 cm² plates. Cells were transfected overnight at 37°C and then the medium replaced with fresh prewarmed medium following a wash with PBS. Transfected cells were then incubated for a further 24 h before harvesting for experiments. For low temperature experiments, cells were transfected overnight at 37°C and then washed with phosphate buffered saline (PBS) and incubated in fresh medium (DMEM (Gibco), 25 mM HEPES, 10 % FCS and 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco), without NaHCO₃) at 25°C for a further 24 h.

2.29 Long-term storage of stably transfected cell lines in liquid nitrogen

Frozen stocks of stably transfected S2 cells and stably transfected HEK-293 cells were harvested by centrifugation at 900g for 3 min. The cell pellet was resuspended in medium containing 10% DMSO that had been chilled on ice for 10 - 20 min. The cells were transferred to cryotubes and cooled slowly to -20°C for up to 24 h. The cells were then transferred to and stored in liquid nitrogen. When required, frozen aliquots were thawed rapidly, added to 10 ml of medium and centrifuged at 900g for 3 min. The supernatant was aspirated and the cell pellet resuspended in 10 ml of fresh growth medium for growth in 10 cm² dishes or 25 cm² flasks.

2.30 Preparation of clonal cell lines

Transfected cell lines were serially diluted to a cell count of 1 cell per 50 µl in conditioned medium, determined with a haemocytometer (Weber Scientific). Conditioned medium was prepared by removing the medium from exponentially growing untransfected cells and centrifuged to remove any cells (1200g for 3 min). Aliquots (50 µl) of the diluted cell suspension were transferred to wells in a 96 well plate. Wells containing more than one cell were discarded and the wells containing only one cell were incubated until confluent (4 - 5 weeks). Wells containing confluent cells were expanded into 24 well, 12 well, 6 well and then into 25 cm² flasks.

2.31 *Drosophila* membrane preparation

Whole *Drosophila* (1.37g) were frozen at -70°C . Frozen *Drosophila* were homogenised in 20 ml ice cold homogenisation buffer (5 mM NaPO_4 , 1 mM EDTA and 300 μl of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (0.5 mM) and phenyl-thio carbamide (10 $\mu\text{g}/\text{ml}$). The sample was centrifuged at 1000g for 10 minutes at 4°C and the supernatant was filtered through glass wool. The eluent was centrifuged again for 10 min and then the supernatant was centrifuged as 40,000g for 25 minutes at 4°C . The pellet was re-suspended in 10 ml homogenisation buffer, re-centrifuged and the resulting pellet was re-suspended (2 mg/ml) in 10 mM phosphate buffer containing protease inhibitors and stored at -70°C .

2.32 Rat brain membrane preparation

Brains from 17 day old rats, provided by Dr. Yvonne Vallis, were placed in 10 volumes of ice-cold buffer A (10 mM Tris-HCl, 0.1 mM EDTA pH 7.5) containing the protease inhibitor cocktail: 1 mM PMSF, 0.1 mg/ml aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin and homogenised. The homogenate was centrifuged at 500g for 10 min at 4°C . The supernatant was then centrifuged at 48,000 x g for 10 min at 4°C . The pellet was washed with 10 volumes of buffer A and re-centrifuged at 48,000 x g. The final pellet was resuspended in buffer A containing the protease inhibitors and the protein concentration determined. The membrane preparation aliquots were stored at -70°C .

2.33 Radioligand binding

For binding studies with [^3H]-epibatidine (Du Pont NEN), [^3H]-methylcarbamyl choline iodide (NEN), [^3H]-acetylcholine iodide (American Radiolabelled Chemicals Inc.) or [^{125}I]- α -BTX (Amersham) on cell membrane preparations, cells were washed twice in phosphate buffered saline (PBS) and resuspended and assayed in 10 mM potassium phosphate buffer (pH 7.2) containing the protease inhibitors leupeptin (2 $\mu\text{g}/\text{ml}$) and pepstatin (1 $\mu\text{g}/\text{ml}$). Cells were incubated with radioligand for 120 min at

4°C in a total volume of 300 µl. Non-specific binding was determined by addition of 1 mM carbachol. Radioligand binding was assayed by filtration onto 0.5% polyethylenimine presoaked Whatman GF/B filters followed by rapid washing with cold 10 mM potassium phosphate buffer using a brandel cell harvester. Radioactivity was measured by scintillation counting.

For binding studies with [¹²⁵I]-α-BTX (Amersham) on intact cells, cells were washed twice in phosphate buffered saline (PBS) and resuspended in Hank's buffered salt solution (Gibco) containing 0.5% bovine serum albumin (BSA). Cells were incubated with radioligand for 90 min at 25°C. Non-specific binding was determined by addition of 1 mM carbachol and 1 mM nicotine. Samples were harvested as described above but with Whatman GF/A filters. Filters were assayed in a Wallac model 1261 γ-counter.

Curves for equilibrium binding were fitted by equally weighted least squares (CVFIT program; David Colquhoun, University College London). The estimated Hill coefficients did not differ significantly from 1, so the data were refitted using the Hill-Langmuir equation (with $n_H=1$) to estimate equilibrium constants.

2.34 SDS-polyacrylamide gel electrophoresis of proteins

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by (Davis, 1964; Ornstein, 1964; Laemmli, 1970), was carried out to separate polypeptides using an 8% resolving gel (21.1 ml H₂O, 8 ml 40% acrylamide, 10 ml 1.5M Tris (pH 8.8), 0.4 ml 10% SDS, 0.4 ml 10% ammonium persulphate and 24 µl TEMED) with a 5% stacking gel (5.8 ml H₂O, 1 ml 40% acrylamide, 1 ml 1.5M Tris (pH 6.8), 80 µl 10% SDS, 80 µl 10% ammonium persulphate and 8 µl TEMED), total gel size was 15cm by 20cm and 0.15cm thick. Protein samples were resuspended with loading buffer (final concentration of constituents: 50 mM Tris.Cl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10%

glycerol and 100 mM dithiothreitol for reducing conditions). Broad range molecular weight markers (Rainbow coloured markers or [^{14}C] Methylated proteins from Amersham) were diluted into loading buffer. After the gel had polymerised the wells were washed with deionised water to remove any unpolymerised acrylamide and the gel was mounted in the gel electrophoresis apparatus. Tris glycine running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS) was added to the upper and lower buffer reservoirs. The samples were loaded and the gels run at 55 mA for an hour and then run overnight at 8 mA. The gel was fixed (25% methanol and 10% acetic acid) for 30 min and then washed briefly in deionised water. The gel was then transferred to a minimal volume of Amplify (Amersham) and gently rocked for 30 min followed by a brief rinse with deionised water. The gel was then placed on damp Whatman 3 MM paper, covered with saran wrap and dried under vacuum at 80°C for 2 h. The dried gel was then exposed to X-ray film at -70°C with an intensifying screen.

2.35 Metabolic labelling and immunoprecipitation

Cells were metabolically labelled following growth in methionine-free medium (DMEM or M3) for 20 min (in either 10 cm plates or 25 cm² flasks), by labelling with 250 μCi Pro-mix (Amersham), a mixture of [^{35}S]-methionine and [^{35}S]-cysteine, in methionine free-medium (typically 2.5 ml) for at least 3 h. DMEM or M3 medium containing 30 mg/L methionine and 10% FCS was added to HEK or S2 cells respectively (typically for 3 h). Cells were washed three times with PBS and scraped from the plates or detached from the flasks into 1 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 5 mM EDTA, 1% Triton X-100) and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide and 10 $\mu\text{g/ml}$ each of leupeptin, aprotinin and pepstatin). Solubilisation and all subsequent steps were performed at 4°C. After an overnight solubilisation, non-solubilised material was pelleted by centrifugation at 14,000 g for 15 min. The cell lysate was then precleared by a 30-min incubation with 30 μl of a 1:1 mixture of protein G-agarose (Calbiochem) in lysis buffer. Antibodies were added to the cell lysate,

incubated for 4 h and immunoprecipitated by the addition of 50 μ l of protein G-agarose for a further 3 h. Samples were washed twice with 1 ml of lysis buffer containing 1 M NaCl, washed once with lysis buffer containing 500 mM NaCl, and then washed again with lysis buffer containing 150 mM NaCl. Typically, the entire sample derived from a 10 cm plate or a 25 cm² flask was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.36 Sucrose gradient centrifugation

Cells were washed with PBS and resuspended in 300 μ l lysis buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.6), 5 mM EDTA and 1% Triton) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide and 10 μ g/ml each of leupeptin, aprotinin and pepstatin). Solubilisation and all subsequent steps were performed at 4°C. After 2 h solubilisation, non-solubilised material was pelleted by centrifugation at 14,000 g for 15 min. 250 μ l of the supernatant was gently layered onto a 5 ml 5 - 20% linear sucrose gradient prepared in lysis buffer, using a linear gradient maker connected to a peristaltic pump, in ultracentrifuge tubes (Beckman, Ultra Clear Centrifuge Tubes). Gradients were centrifuged at 40,000 rpm to $\omega^2t = 9.00 \times 10^{11} \text{ rad}^2 / \text{s}$ in a Beckman XL- 80 ultracentrifuge using an SW - 55 Ti rotor at 4°C. Sixteen fractions of 320 μ l were taken sequentially from the top of the gradient and transferred to 4.5 ml assay tubes (SLS) and stored on ice. An equal volume of radioligand in lysis buffer containing protease inhibitors, was added to each fraction. Fractions were incubated on ice for 2 h and then harvested using a Brandell cell harvester with rapid filtration onto pre-soaked Whatman GF/B filters and rapid washing with 10 mM phosphate buffer (4°C). Radioactivity was measured by scintillation counting using a Beckman LS6500 Multi purpose Scintillation Counter.

2.37 Western blots

Cells were washed with PBS, pelleted and resuspended in 20 μ l PBS and 40 μ l loading buffer (100 mM Tris.Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM dithiothreitol for reducing conditions) was added and the solution sonicated for approximately 2 min. The solution was centrifuged at 13,000 g for 5 min prior to loading onto the gel. SDS-PAGE was carried out as described in Section 2.34. The gel was run at 55 mA for 2-3 h with rainbow markers (Amersham). The gel was then placed on a tray in buffer AP (25 mM Tris, 190 mM Glycine and 20 % methanol) and the stacking gel removed. The gel was soaked for 15 - 30 min. The gel holder for Western transfer via electroblotting was then set up. Onto the presoaked filter a piece of filter paper was positioned and the gel placed on top of this. The nitrocellulose was placed on top of the gel, to lay between the gel and the anode, and this in turn was covered with a piece of wet filter paper and the other fibre pad and placed in the cassette. The transfer was carried out at 100 v / 360 m Amps for 2.5 - 3 h.

The nitrocellulose was transferred to blocking solution (0.1% Tween, 7.5% milk powder and 1% FCS) and placed on a shaker overnight at 4°C. The nitrocellulose was then washed 5 times in PBS + 0.1% Tween (two 5 min washes, once for 15 min and then two further 5 min washes). The nitrocellulose was then incubated with primary antibody in a total volume of 7 ml (0.1 % Tween and 7.5 % milk powder) for 2 h at room temperature. The nitrocellulose was washed with PBS + 0.1 % Tween (three 5 min washes followed by a 15 min wash).

The secondary antibody labelled with horse radish peroxidase (HRP) was incubated with the nitrocellulose for 1 - 2 h in a total volume of 7 ml (0.1 % Tween and 7.5 % milk powder). The excess antibody was then washed off by three 5 min washes in PBS + 0.3 % Tween and then three 5 min washes in PBS + 0.1 % Tween.

The nitrocellulose membrane was covered with 8 ml of a 50:50 mix of detection solution I and detection solution II from the ECL detection kit (Amersham) and incubated for 1 min. Excess solution was drained off and the membrane wrapped in saran wrap. The membrane was exposed to autoradiographic film (Hyperfilm-ECL) repeatedly for various times from 15 sec to 1 h. If a high background was observed the membrane was washed again in PBS + 0.3% Tween, incubated in solution I and II for 1 min and re-exposed to autoradiographic film.

2.38 Expression in *Xenopus* oocytes

Oocytes were removed from mature *Xenopus laevis* and treated with 2 mg/ml collagenase type 1A (Sigma) in low calcium (<20 nM) standard oocyte saline (SOS) (NaCl 100 mM, KCl 2 μ M, CaCl₂ 1.8 μ M, MgCl₂ 1 μ M, HEPES 5 μ M; pH 7.6) for 40 min at room temperature and then manually defolliculated. Plasmid cDNA (20nl) was injected into the oocyte nucleus using a digital microdispenser (Drummond "Nanojet"). Following injection, oocytes were incubated in sterile SOS supplemented with 50 μ g/ml gentamycin sulphate, 100 u/ml penicillin, 100 μ g/ml streptomycin and 2.5 mM sodium pyruvate at 18°C. Electrophysiological studies were performed 3-5 days after injection. Oocytes were secured in an experimental chamber (of approximate volume 90 μ l) and perfused continually with SOS (5 ml/min). Oocytes were impaled with two 2M KCl -filled glass microelectrodes of resistance 2-5 M Ω . Membrane currents were amplified and monitored using an Oocyte Clamp OC-725C voltage clamp amplifier (Warner Instrument Corp.). In all experiments, the clamped holding potential was -100 mV. Agonists were bath-applied in SOS and full dose response curves were carried out on each oocyte. Mean dose response curves were from a minimum of 3 oocytes.

2.39 Intracellular Ca²⁺ measurements

Fluorescent ratiometric intracellular calcium measurements were performed on fura2AM-loaded populations of cells (approximately 1 X 10⁷ cells) using a Perkin-

Elmer LS-50B fluorescence spectrometer fitted with a stirred cuvette holder. Cell monolayers or single cell suspensions were washed with Hanks' balanced salt solution (HBSS; Gibco). The cell pellet was resuspended in 5 ml HBSS containing 4 μ M fura-2 acetoxymethyl ester (Molecular Probes) and incubated at 25°C in the dark for 45 min. After loading, the cells were washed in HBSS three times and resuspended in approximately 6 ml high calcium buffer (25 mM HEPES, 35 mM sucrose, 75 mM CaCl_2 and pH to 7.4 with KOH). The excitation wavelength was rapidly alternated between 340 and 380 nm by means of a fast filter accessory (Perkin Elmer). Emitted fluorescence was detected at 510 nm. A 340 nm / 380 nm ratio was calculated every 40 ms and data averaged over four ratio data points. Agonists were added directly to the cuvette and maximum responses were elicited by addition of 10 % Triton-X 100 to permeabilise the cells to calcium.

2.40 Materials

Radioligands: (+/-) epibatidine [5,6-bicycloheptyl- ^3H]- (2.07 TBq/mmol) and methylcarbaryl choline iodide,[N-methyl- ^3H]- (2.886 TBq/mmol) from NEN, acetylcholine iodide [methyl ^3H] (2.77 GBq/mmol) were obtained from American Radiolabelled Chemicals Inc, (3-[^{125}I]iodotyrosyl) α -Bungarotoxin (7.4 TBq/mmol) from Amersham Life Sciences. Pro-Mix L-[^{35}S] *in vitro* cell labelling mix was from Amersham Life Science as were the [^{14}C] methylated protein markers and the Rainbow coloured protein molecular weight markers.

Chemicals and reagents: Agarose, LMP-agarose, Glycine, Tris, Tryptone, Lambda Hind III DNA markers from Gibco. Amberlite, Ammonium persulfate, BES, Carbamylcholine Chloride (Carbachol), 40 μ M dNTPs, EDTA, Levamisole, TES, Yeast Extract, Mineral oil and Noble Agar from Sigma. Calcium Chloride, DMSO, Ethidium Bromide, Glycerol, Glycine, Potassium Hydrogen Carbonate, Sucrose, Sodium Chloride, Triton and Urea from BDH. Liquid Scintillation Cocktail from Beckman.

HGA I and NE buffers I and II from New England Biolabs. *Bam*HI, *Bst*XI, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Not*I, *Pvu*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, *Xho*I, *Xma*I, T4 DNA ligase, ligation buffer, T4 DNA polymerase, PCR Markers, alkaline phosphatase, CIP buffer, Taq DNA polymerase, and buffers A, B, C, D, E, F, G, H and MC from Promega. Pfu DNA polymerase from Strategene.

Wizard DNA Clean-UP system from Promega. QIAGEN Maxi prep kits and QIAprep-spin column kits for plasmid mini preparations were obtained from QIAGEN. Protein G-Sepharose was from Pharmacia Biotech. Albumin bovine fraction V from BDH. Amplify from Amersham. Scientific imaging film from Kodak. Prism-Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and phenol/water/chloroform from Applied Biosystems.

Buffers and Solutions: G418 (Geneticin), Lipofectamine Reagent, Optimem I, Penicillin - Streptomycin 10,000 units/ ml - 10,000 µg/ml, Hanks Balanced Salt Solution, DMEM and Nutrient Mixture Ham's F-10 from Gibco. Sheilds and Sang M3 insect cell medium from Sigma. 10 X TBE from Bio - Whittaker. ACRYL/BIS 29:1 solution and ACRYL/BIS 19:1 Solution were from Amresco.

Disposables: 96 well, 24 well, 12 well, and 6 well plates, 25cm² and 75cm² tissue culture flasks, and 1.8ml cryotubes were from Nunclon. 6 and 10 cm² Tissue Culture Dishes were from Becton Dickenson. 10 and 25 ml Blow-out pipettes were from Sarstedt as were the 15 ml Falcon tubes. 50 ml Centrifuge tubes were from Nunc. 250 and 500 ml 0.22 µm Stericup filtration systems were from Milipore. Fast Turn Cap Poly Q Vials from Beckman.

3: Cloning and plasmid expression vectors

3.1 Introduction

A central goal of this research project was the expression of nAChRs from cloned subunit cDNAs by heterologous expression. The nAChRs from *Drosophila*, *C.elegans* and rat were investigated in various expression systems including the *Drosophila* S2 cell line, the mammalian HEK-293 cell line and *Xenopus* oocytes.

3.2 Cloning of a full-length *Drosophila* SBD cDNA

The *Drosophila* nAChR β subunit cDNA clone (SBD), isolated previously (Sawruk *et al.*, 1990b), contains the entire coding region of the mature SBD protein but lacks part of the N-terminal signal sequence. To facilitate expression studies with SBD, a full-length SBD cDNA was constructed as part of this research project. The complete 5' coding region of the SBD mRNA was cloned from a *Drosophila* cDNA library (in plasmid pNB40), (Brown and Kafatos, 1988), by PCR using a primer designed to the 5' cloning site of the cDNA library plasmid (5'-ATATTGTCGTTAGA-ACGCGGCTAC -3') and to the known coding region of the SBD cDNA sequence (5'-GCTGAGACAATTTTCAGACCTAACC- 3'). The resultant PCR product was cloned into plasmid pCRII (Invitrogen) and sequenced to establish that the band was the N-terminal signal sequence of SBD.

To generate a full-length SBD cDNA, the 5' SBD cDNA PCR fragment was ligated to the original SBD cDNA. This was achieved by PCR with the forward primer 5' -CCAAAAAGCTCGAGCTCACAGCAACAAAATGTGGCATTGG- 3' which was designed to introduce the restriction sites *Xho*I and *Sac*I (underlined) to facilitate subcloning into the expression vectors pRmHa3 and pBluescript and the reverse internal SBD primer 5' - GCTGAGACAATTTTCAGACCTAACC- 3'. The PCR product obtained was digested with the restriction enzymes *Xho*I and *Acc*III which cuts SBD internally. The pBluescript-SBD (lacking the 5' signal sequence) was also digested with *Xho*I and *Acc*III. The digested PCR product was subcloned

directionally into the *Xho*I and *Acc*III cut pBluescript-SBD. The full length SBD sequence was checked by restriction mapping and by full-nucleotide sequencing.

The full-length SBD cDNA was excised from pBluescript by restriction digestion with *Sac*I (one *Sac*I site introduced by PCR at the 5' end and a *Sac*I site 3' to the SBD insert in pBluescript) and subcloned into CIP treated *Sac*I cut pRmHa3. The full-length SBD construct was also subcloned into pcDNA3 and details of the subcloning are shown in Table 3.1. The constructs were checked by restriction enzyme mapping and full nucleotide sequencing.

The nucleotide and deduced amino acid sequences of the 5' end of the SBD cDNA are presented in Figure 3.1. The deduced amino acid sequence of the full length SBD protein was analysed to determine the predicted site of proteolytic cleavage by the algorithm of von Heijne (von Heijne, 1986). Interestingly, the most favoured position for cleavage of this putative signal sequence was eight amino acids before the position predicted in the other three *Drosophila* nAChR subunits. The full-length SBD sequence has been submitted to the EMBL database and has the accession number Y14678.

3.3 Subcloning of nAChR subunits into plasmid expression vectors

For expression in the *Drosophila* S2 cell line the inducible expression vector pRmHa3 was chosen (Bunch *et al.*, 1988; Bunch, 1989). pRmHa3 has been used successfully to express other receptors and ion channels such as *Drosophila* GABA, muscarinic and dopamine receptors (Millar *et al.*, 1994; Millar *et al.*, 1995; Han *et al.*, 1996; Hardie *et al.*, 1997). The four *Drosophila* nAChR subunit cDNAs were subcloned into pRmHa3 (Figure 3.2) and the mammalian expression vector, pcDNA3 (Invitrogen) shown in Figure 3.3. The rat subunits α 3, α 4, β 2, β 3 and β 4 in the cloning vector pcDNAIneo were subcloned into pRmHa3. The orientation of the insert with respect to the 5' *Drosophila* metallothionein promoter and the 3' alcohol

A

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gagttgcgattggactctccaaccgtgaggttggtattaaacggaaactcctagcaaaatgtgttaagtttagttaaaaaaccttcaattataagcaataag
      10      20      30      40      50      60      70      80      90     100

              M W H W S L L C V F L L V P L A N S T A P I S
tagcacaaaaagctagagaaaaacagcaacaaaatgtggcattggagtttggtgtgtgttttctacttggtccgttggcgaattccacggctccaataag
      110     120     130     140     150     160     170     180     190     200

      F E A N P D T K R L Y D D L L S N Y N R L I R P V V N N T E T L T
ttttgaggcaaattccggacaccaaactgtctctatgatgatttggtgagcaactacaatcgattgattaggcccggttggaacaacacggagaccctgacg
      210     220     230     240     250     260     270     280     290     300

      V W L G L K L S Q
87 gtttggtaggtctgaaattgtctcagc
      310     320

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B

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ALS:                MGSVLFAAVFIALHFATGGLANPD*AKRLYDDLLSNYNRLIRPVGN...
ARD:                MESSCKSWLLCSILVLVAFSLVSASEDEERLVRDLFRGYNKLIRPVQN...
SAD:                MAPGCCTTRPRPIALLAHIWRHCKPLCLLLVLLLLCETVQANPD*AKRLYDDLLSNYNRLIRPVS...
SBD:                MWHWSLLCVFLLVPLANSTAPISFEANPD*TKRLYDDLLSNYNRLIRPVVN...

```

Fig. 3.1: Nucleotide and amino acid sequence of the *Drosophila* SBD nAChR subunit. A: The nucleotide sequence is numbered from the 5' end of the cDNA clone and extends to the position of the 3' PCR priming site (underlined). B: The deduced amino acid sequences of the N-terminal regions of the three previously published full-length *Drosophila* nAChR subunits (ALS, ARD and SAD) are shown together with the newly determined SBD sequence. The putative N-terminal signal sequences, predicted by applying the algorithm of von Heijne (1986) are underlined. Asterisks indicate conserved amino acids.

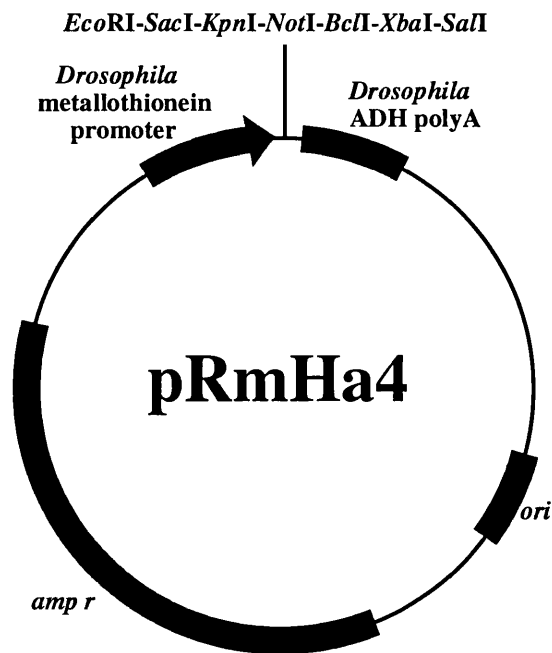
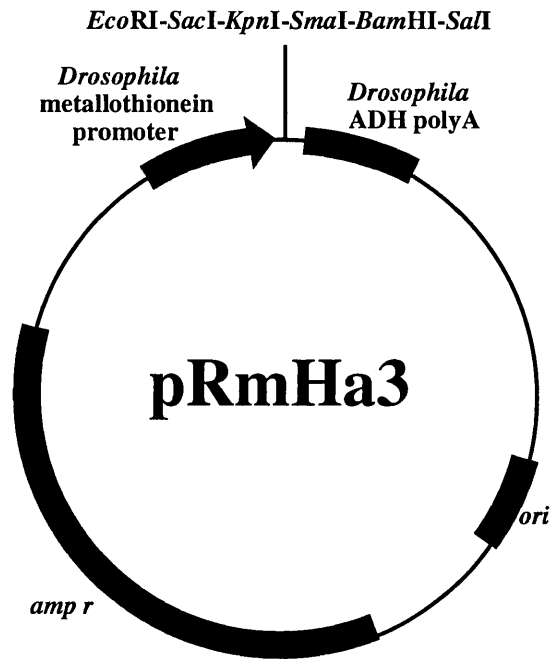


Fig. 3.2: The expression vector pRmHa3 (top) is based on the bacterial plasmid pUC18 and contains the promoter, metal response element and transcriptional start site from the metallothionein gene. This is followed by the unique cloning sites *EcoRI*, *SacI*, *KpnI*, *SmaI*, *BamHI*, and *SalI* and the polyadenylation signal from the *Drosophila melanogaster* alcohol dehydrogenase gene. It also contains the origin of replication (*ori*) and the beta-lactamase gene which confers resistance to ampicillin (*amp r*) from pUC18. The expression vector pRmHa4 (bottom) is a modified version of pRmHa3, created during this project, with the unique cloning sites *EcoRI*, *SacI*, *KpnI*, *NotI*, *BclII*, *XbaI* and *SalI*.

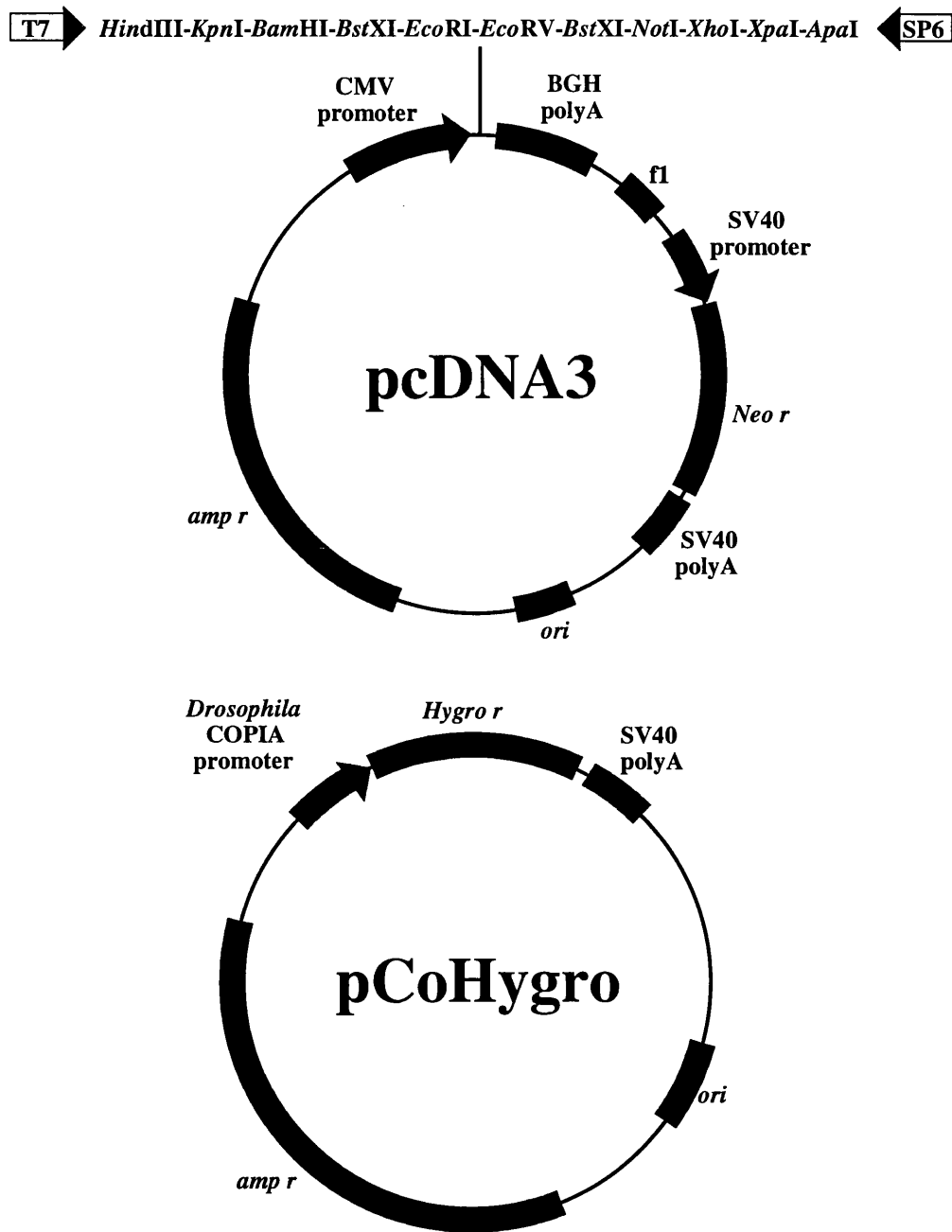


Fig. 3.3: The expression vector pcDNA3 (top) is designed for eukaryotic expression. It has: the human cytomegalovirus (CMV) promoter for high level transcription, a polyadenylation signal and transcription termination from the bovine growth hormone gene to enhance RNA stability. The SV40 origin is for episomal replication. The multiple cloning site has the unique sites *HindIII*-*KpnI*-*BamHI*-*BstXI*-*EcoRI*-*EcoRV*-*BstXI*-*NotI*-*XhoI*-*XpaI*-*ApaI* and is flanked by the T7 and SP6 RNA promoters. pcDNA3 contains the ampicillin resistance gene for selection and maintenance in *E. coli* and the the neomycin resistance gene for selection of transfected cell lines. The pCoHygro vector (bottom) contains the hygromycin resistance gene (*hygro r*) followed by the control element for polyadenylation (SV40 poly A) from DSPhygro (van der Straten *et al.* 1989). The hygromycin gene is under the control of the constitutively active COPIA 5'LTR promoter.

dehydrogenase polyadenylation site in pRmHa3 and the 5' T7 promoter and 3' SP6 promoter in pcDNA3 was confirmed by restriction mapping and nucleotide sequencing.

Expression of the *C. elegans* nAChR subunits was investigated in the S2 cell line and this required subcloning the four subunits (ACR-2, LEV-1, UNC-29 and UNC-38) from their original cloning vectors into the *Drosophila* expression vector pRmHa3.

The subcloning strategies for all the nicotinic subunits and other cDNAs examined during this project are summarised in Tables 3.1 - 3.4. A more detailed subcloning strategy is provided in the Methods Section. *Drosophila* nAChR subunit cDNAs were also subcloned into the mammalian expression vector pcDNA3 for expression studies in mammalian HEK-293 cells. Several subunits were also subcloned into the pGEMHE expression vector to enable *in vitro* transcription of mRNA prior to expression in *Xenopus* oocytes.

Care was taken, to optimise initiation codon flanking sequences during subcloning. Comparison of the consensus sequence flanking translational start sites in many species, including *Drosophila*, has been undertaken (Cavener, 1987) and has shown that the sequence ATCATGG is optimal. The presence of ATG sequences occurring 5' to the main start codon may also have an effect on translation especially in eukaryotic systems. Any nAChR subunits obtained with ATGs preceding the main start codon, were modified during subcloning to remove upstream ATGs and to optimise the consensus signal.

Table 3.1 Summary of subcloning *Drosophila* nAChR subunits into pRmHa3 and pcDNA3.

Subunit	Subcloned from	Restriction enzymes	Subcloned into	Restriction enzymes
SAD	pBluescript	<i>KpnI, SmaI</i>	pRmHa3	<i>SmaI</i>
ALS	pBluescript	<i>KpnI, NotI</i>	pRmHa4	<i>KpnI, NotI</i>
ARD	pBluescript	<i>KpnI, BamHI</i>	pRmHa3	<i>KpnI, BamHI</i>
ARD	pRmHa3	<i>BstXI</i> (Blunt)	pRmHa3	<i>SmaI</i>
SBD	pBluescript	<i>EcoRI</i>	pRmHa3	<i>EcoRI</i>
SBD*	pBluescript	<i>SacI</i>	pRmHa3	<i>SacI</i>
ALS	pRmHa4	<i>KpnI, XbaI</i>	pcDNA3	<i>KpnI, XbaI</i>
ARD	pRmHa3	<i>KpnI, BamHI</i>	pcDNA3	<i>KpnI, BamHI</i>
SAD	pBluescript	<i>KpnI, NotI</i>	pcDNA3	<i>KpnI, NotI</i>
SBD*	pBluescript	<i>XbaI, XhoI</i>	pcDNA3	<i>XbaI, XhoI</i>

Table 3.2 Summary of subcloning rat nAChR subunits into pRmHa3.

Subunit	Subcloned from	Restriction enzymes	Subcloned into	Restriction enzymes
$\alpha 3$	pcDNA1/Neo	<i>NotI, EcoRI</i>	pRmHa3	<i>NotI, EcoRI</i>
$\alpha 5$	pcDNA1/Neo	<i>NotI, EcoRI</i>	pRmHa3	<i>NotI, EcoRI</i>
$\beta 3$	pcDNA1/Neo	<i>NotI, EcoRI</i>	pRmHa3	<i>NotI, EcoRI</i>
$\beta 4$	pcDNA1/Neo	<i>NotI, SacI</i>	pRmHa3	<i>NotI, SacI</i>

Tables 3.1 and 3.2 show a summary of the subcloning of the *Drosophila* and rat nAChR subunits from their original cloning vectors (pcDNA1/neo or pBluescript) into the *Drosophila* expression vector pRmHa3 or the mammalian expression vector pcDNA3. The restriction enzymes used to excise the subunit cDNA and to cut the vector are listed. The SBD* denotes that the subcloning is with the full length SBD clone.

Table 3.3 Summary of subcloning *C. elegans* subunits into pRmHa3.

Subunit	Subcloned from	Restriction enzymes	Subcloned into	Restriction enzymes
UNC-38	pBluescript	<i>SacI, SalI</i>	pRmHa3	<i>SacI SalI</i>
UNC-29	pBluescript	<i>HindIII, BamHI</i>	pRmHa3	<i>SmaI</i>
LEV-1	pBluescript	<i>KpnI, SacI</i>	pRmHa3	<i>KpnI, SacI</i>
LEV-1	pRmHa3	<i>HindIII, XhoI</i>	pRmHa3	<i>SmaI</i>
ACR-2	pBluescript	<i>EcoRI</i>	pRmHa3	<i>EcoRI</i>
ACR-2	pRmHa3	<i>HgaI</i>	pRmHa3	<i>EcoRI</i>

Table 3.4 Summary of subcloning of miscellaneous ion channel subunit cDNAs.

Subunit	Subcloned from	Restriction enzymes	Subcloned into	Restriction enzymes
LCCH3	PSP64	<i>EcoRI, BamHI</i>	pRmHa3	<i>EcoRI, BamHI</i>
TRPL	pBluescript	<i>EcoRI</i>	pRmHa3	<i>EcoRI</i>
Locust α L-1	pBluescript	<i>EcoRI</i>	pcDNA3	<i>EcoRI</i>

Tables 3.3. and 3.4 summarise the subcloning of the *C. elegans* nAChR subunits from their original expression vector pBluescript and the subcloning of the other channel cDNAs from their cloning vectors into pRmHa3 and pcDNA3. The restriction enzymes used to excise the subunit cDNA and to cut the vector are listed.

3.4 Summary of subcloning of cDNAs encoding miscellaneous ion channel subunits

During the course of the project, several associated projects were started which also involved subcloning various subunits into the expression vectors pRmHa3 and pcDNA3. These are summarised in Table 3.4

The first of these was the *Drosophila* GABA receptor β subunit LCCH3 (Henderson *et al.*, 1993) which was subcloned into the expression vector pRmHa3 for stable expression in S2 cells with the GABA α subunit RDL (Millar *et al.*, 1994) and with the subunit encoded by the dieldrin resistant form of the *rdl* gene RDL(A302S) (Buckingham *et al.*, 1996). In this study wild type and insecticide-resistant homooligomeric GABA receptors of *Drosophila melanogaster* were stably expressed in the *Drosophila* cell line.

The putative integral membrane protein TRP (transient receptor potential), which is required for phototransduction in the *Drosophila*, was cloned in 1989 (Montell and Rubin, 1989). More recently a transient receptor potential-like gene (TRPL) has been cloned (Phillips *et al.*, 1992). The S2 cell line was used for stable transfections with heterologous cDNA for TRPL alone or in combination with the *Drosophila* muscarinic receptor (DM1). My involvement in this project has been limited to subcloning and the generation of stable *Drosophila* S2 cell lines.

The locust α L-1 subunit was subcloned from its cloning vector pBluescript into the *Drosophila* expression vector pRmHa3. The α L-1 subunit had been shown to form functional homomeric channels when injected into *Xenopus* oocytes (Marshall *et al.*, 1990; Amar *et al.*, 1995) which is activated by nicotine and antagonised by α -BTX, mecamylamine and *d*-tubocurarine. The α L-1 subunit was subcloned into pRmHa3 for expression studies in S2 cells to investigate whether α L-1 could form homomeric channels in these cells.

3.5 Discussion

The successful subcloning of the nicotinic subunits into the *Drosophila* and the mammalian expression vectors pRmHa3 and pcDNA3 allowed the expression of these subunits in S2 cells, mammalian cells and oocytes. One motivation for examining expression of *Drosophila* nAChR subunits in a *Drosophila* cell line was the hope that this would provide a more native cellular environment than is provided by either the mammalian cell line or *Xenopus* oocytes. The results are described in the following chapters.

Previous attempts to express the *Drosophila* SBD subunit from the partial cDNA clone (Sawruk *et al.*, 1990b) have employed a chimeric cDNA construct containing a foreign signal sequence. Since the foreign signal sequence is expected to be proteolytically cleaved to generate the mature SBD protein, the foreign leader peptide should not affect the functional properties of the SBD subunit. However, several reports have identified the N-terminus of neurotransmitter-gated ion channel subunits as being important for protein folding (Sumikawa, 1992; Verrall and Hall, 1992; Kuhse *et al.*, 1993). By the construction and expression of a full-length SBD cDNA clone it should be possible to examine whether the previously reported difficulties in expressing functional *Drosophila* nAChRs can be attributed to the non-native signal sequence of the chimeric SBD protein. The expression of the full-length SBD cDNA clone is investigated in S2 cells (Chapter 4) and in HEK-293 cells (Chapter 6).

The subcloning of the ion channel cDNAs locust α L-1, the *Drosophila* GABA receptor subunits RDL, RDL^r, LCCH3 and the *Drosophila* TRP protein into the expression vector pRmHa3 has been described in this Chapter. Radioligand binding to these proteins expressed in the *Drosophila* S2 cell line and functional data are described in Chapter 5.

4: Expression of cloned nAChR subunit combinations in *Drosophila* S2 cells

4.1 Introduction

The use of S2 cells has been reported previously for the successful functional expression of *Drosophila*, GABA, muscarinic acetylcholine and dopamine receptors (Millar *et al.*, 1994; Millar *et al.*, 1995; Han *et al.*, 1996). In this study the expression of *Drosophila* nAChR subunits was examined in this cell line by the expression of heterologous proteins in an attempt to attain functional nAChRs.

Stable cell lines were generated by co-transfecting nAChR subunit cDNAs cloned into the expression vector, pRmHa3 (Bunch *et al.*, 1988), together with the selectable marker plasmid, pCOHygro (van der Straten *et al.*, 1989). Stable cell lines were isolated by growth in medium containing hygromycin B. A series of stably transfected *Drosophila* S2 cell lines were generated in which all four cloned *Drosophila* nAChR subunits were co-expressed in various combinations. The *Drosophila* subunits were also co-expressed with rat nAChR subunits. The stably transfected S2 cell lines are summarised in Table 4.1. The expression of the cloned subunits was under the strict control of the inducible metallothien promoter, which could be induced by addition of 0.6 mM CuSO₄ 24 h prior to the experiment.

4.2 Time course for induction of transfected subunits with 0.6 mM CuSO₄

Previous studies using the *Drosophila* expression vector pRmHa3 used 0.6 mM CuSO₄ for 24 h to induce expression in the S2 cell lines before harvesting the cells for assay (Millar *et al.*, 1994; Millar *et al.*, 1995). To ascertain whether this was an optimal time course for these experiments, a CuSO₄ time course experiment using the polyclonal cell SAD/β2 cell line was carried out. The results of the time course experiment, 0 - 72 h, are shown in Figure 4.1.

4.3 CuSO₄ concentration curve for the induction of S2 cells

The polyclonal SAD/β2 cell line was used to perform a CuSO₄ dose response curve ranging from 0 - 1.2 mM the results are shown in Figure 4.2. Previous studies using

Stably transfected S2 cell line	[³ H] Epibatidine binding
α4	-
β2	-
α4/β2	6.5 ± 2.3
ARD/α3	-
SBD/α3	-
ALS/α4	-
SAD/α4	-
ARD/α4	-
SBD/α4	-
ALS/β2	1.0 ± 0.4
SAD/β2	0.9 ± 0.4
ARD/β2	-
SBD/β2	-
ALS/β3	-
SAD/β3	-
ALS/β4	0.07 ± 0.03
SAD/β4	0.08 ± 0.02
ALS	-
SAD	-
ARD	-
SBD	-
ALS/SAD	-
ALS/ARD	-
ALS/SBD	-
SAD/SBD	-
SAD/ARD	-
ARD/SBD	-
ALS/SAD/ARD	-
ALS/SAD/ARD/SBD	-

Table 4.1: Summary of S2 cell lines stably transfected with combinations of *Drosophila* and rat nAChR subunits. The data shows presence or absence (-) of specific binding with 3 nM [³H]-epibatidine. The binding is represented as B_{\max} (pmol/mg of membrane protein) and are mean ± SEM values from at least three separate determinations derived from full binding curves.

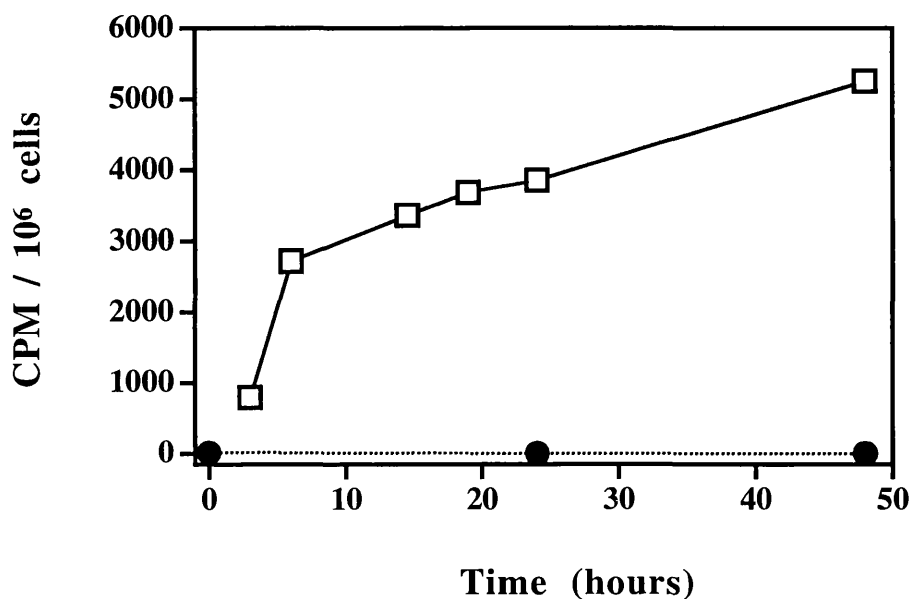


Fig. 4.1: Time course for CuSO₄ induction of polyclonal SAD/β2 S2 cell lines. Graph shows 3 nM [³H]-epibatidine binding to polyclonal SAD/β2 cells (—□—) induced with 0.6 mM CuSO₄ for 0 - 48 h. 3 nM [³H]-epibatidine binding to untransfected S2 cells is also shown (.....●.....).

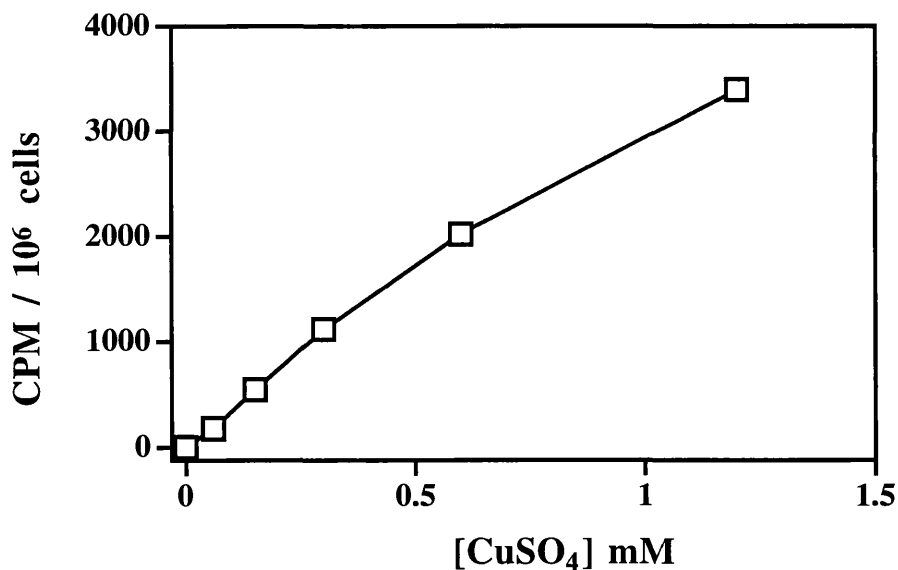


Fig. 4.2: Concentration curve for CuSO₄ induction of the polyclonal SAD/β2 S2 cell line with CuSO₄ for 24 h. Graph shows 3 nM [³H]-epibatidine binding to polyclonal SAD/β2 cells induced with 0.03-1.2 mM CuSO₄ for 24 h. Induction levels measured by specific 3 nM [³H]-epibatidine binding determined in the presence or absence of 1 mM carbachol.

the expression vector pRmHa3 used a concentration of 0.6 mM CuSO₄ for a 24 h induction period (Millar *et al.*, 1994; Millar *et al.*, 1995; Han *et al.*, 1996).

A concentration of 0.6 mM CuSO₄ was selected for all subsequent experiments because it induced a high level of expression as shown by the specific [³H]-epibatidine binding to the polyclonal cell line. An induction time of 24 h was also used for most experiments. Higher concentrations could have been used as could a longer time course, however since CuSO₄ can have toxic effects (Bunch *et al.*, 1988), the concentration and induction time were kept to lowest levels that produced reasonable expression levels.

4.4 Nicotinic radioligand binding to untransfected S2 cells and *Drosophila* membrane preparations

The nicotinic radioligands were used to establish the absence of endogenous nAChRs in untransfected S2 cells. Radioligand binding studies with [³H]-epibatidine, [³H]-methylcarbamylcholine, [³H]-nicotine, [³H]-ACh and [¹²⁵I]- α -BTX did not show any specific binding on untransfected S2 cells which suggests that S2 cells do not have endogenous nAChRs.

Experiments were performed with the nicotinic radioligands: [³H]-epibatidine (10 nM), [³H]-methylcarbamylcholine (30 nM), [³H]-nicotine (30 nM), [³H]-ACh (30 nM) and [¹²⁵I]- α -BTX (10 nM) to determine the levels of non-specific binding in transfected and untransfected S2 cells. Epibatidine was found to have the lowest level of non-specific binding. Methylcarbamyl choline also had low levels of non-specific binding whereas there was significant non-specific binding with [³H]-nicotine, [³H]-ACh and [¹²⁵I]- α -BTX.

[³H]-epibatidine (Badio and Daly, 1994) has been shown previously to be a particularly high affinity ligand for vertebrate nAChRs such as rat $\alpha 4/\beta 2$ (Sullivan *et*

al., 1995), $\alpha 3/\beta 4$ (Flores, 1996), human $\alpha 3/\beta 2$, $\alpha 3/\beta 2/\alpha 5$ (Wang *et al.*, 1996) and chick $\alpha 6$ when co-expressed with human $\beta 4$ in oocytes (Gerzanich *et al.*, 1997). There was no data available of the affinity of [^3H]-epibatidine for the *Drosophila* nAChRs. Therefore, binding studies were performed with *Drosophila* membrane preparations and the results are shown in Figure 4.5 and Table 4.2. The radioligand [^3H]-epibatidine was chosen to screen the transfected S2 cell lines because of its high affinity and low non-specific binding.

4.5 [^3H]-epibatidine binding to stably transfected S2 cell lines

Binding of [^3H]-epibatidine to membrane preparations of S2 cell lines expressing *Drosophila* and rat nAChRs was examined. Various combinations of rat nAChR subunits could be identified in stably transfected in S2 cells by [^3H]-epibatidine binding e.g. $\alpha 4/\beta 2$ or $\alpha 3/\beta 4$. When S2 cells were transfected with a single subunit such as $\alpha 4$ or $\beta 2$, no specific [^3H]-epibatidine binding could be detected. When S2 cells stably transfected with various combinations of *Drosophila* nAChR subunits were tested, no specific [^3H]-epibatidine binding could be detected for any combination including the cell line expressing all four cloned subunits ALS, ARD, SAD, and SBD. Table 4.1 summarises the nAChR subunit combinations expressed in stably transfected S2 cell lines that show specific [^3H]-epibatidine binding (3 nM). Data are mean \pm SEM values from at least three separate determinations derived from full saturation binding curves.

Due to the lack of [^3H]-epibatidine binding with the *Drosophila* nAChR subunit combinations, stably transfected S2 cells expressing *Drosophila*/rat hybrid receptors were studied. When either of the two cloned *Drosophila* α subunits (ALS or SAD) was co-expressed with the rat $\beta 2$ subunit in *Drosophila* S2 cells, high levels of specific binding with [^3H]-epibatidine were obtained. No binding was detected when the rat $\beta 2$ (or any other rat neuronal nAChR subunit) was expressed alone in S2 cells. We therefore conclude that the binding of [^3H]-epibatidine depends upon the co-

Table 4.2: Summary of nicotinic radioligand binding to stably transfected S2 cell lines.

	$K_d \pm \text{SEM}$ (nM)	B_{max} (pmol/mg of membrane protein
	$[^3\text{H}]$ -Epibatidine	
SAD/ β 2	0.23 ± 0.11	0.88 ± 0.38
SAD/ β 4	< 1 nM	0.09 ± 0.03
ALS/ β 2	0.19 ± 0.06	0.99 ± 0.41
ALS/ β 4	< 1nM	0.07 ± 0.03
α 4/ β 2	1.16 ± 0.17	1.58 ± 0.16
<i>Drosophila</i> mem. prep.	1.55 ± 0.61	0.72 ± 0.06
	$[^3\text{H}]$ -Methylcarbamyl choline	
SAD/ β 2	5.54 ± 0.79	0.50 ± 0.19
ALS/ β 2	1.72 ± 0.26	0.75 ± 0.12
α 4/ β 2	2.94 ± 0.43	1.46 ± 0.43

Table 4.2. Mean K_d values and B_{max} values derived from saturation binding experiments in polyclonal S2 cells expressing *Drosophila* and rat subunits. The data are mean \pm SEM values from at least 3 separate determinations derived from full binding curves.

assembly of the *Drosophila* and rat nAChR subunits. Specific binding was also observed when either ALS or SAD were co-expressed with the rat $\beta 4$ nAChR subunit. In cell lines co-expressing either of the rat subunits $\alpha 3$ or $\alpha 4$ with either of the *Drosophila* β nAChR subunits ARD or SBD, no specific [^3H]-epibatidine binding was detected.

4.6 Saturation binding curves for ALS/ $\beta 2$ and SAD/ $\beta 2$ polyclonal cell lines

In an attempt to characterise these receptors further, full dose response curves to [^3H]-epibatidine and [^3H]-methylcarbamyl choline were conducted on the polyclonal S2 cell lines SAD/ $\beta 2$ and ALS/ $\beta 2$ and the results are shown in Figures 4.3 and 4.4. The mean data for the K_d values and B_{max} values are summarised in Table 4.2. The pharmacological properties of these subunit combinations resemble those reported previously when the two *Drosophila* α subunits were co-expressed with the chick $\beta 2$ in *Xenopus* oocytes (Bertrand *et al.*, 1994). Bertrand *et al.* showed that ALS and SAD could form functional channels in oocytes when co-expressed with the chick $\beta 2$ subunit with the respective EC_{50}s of 0.18 μM and 35 μM for ACh.

4.7 [^{125}I]- αBTX binding to stably transfected S2 cells

Specific high-affinity cell surface [^{125}I]- αBTX binding could be detected with the S2-ALS/ $\beta 2$ cell line, but not with the S2-SAD/ $\beta 2$ cell line. In this respect the pharmacological properties of these subunit combinations resemble those reported by Bertrand *et al.* (1994) when the two *Drosophila* α subunits were co-expressed with the chick $\beta 2$ in *Xenopus* oocytes. 1nM [^{125}I]- αBTX binding on whole cell preparations was measured, non-specific binding was determined with 1 mM nicotine and 1 mM carbachol, the results are shown in Figure 4.6. The binding was carried out using stably transfected S2 cell lines: ALS, ALS/ $\beta 2$, ALS/ $\beta 4$, SAD/ $\beta 2$ and untransfected S2 cells.

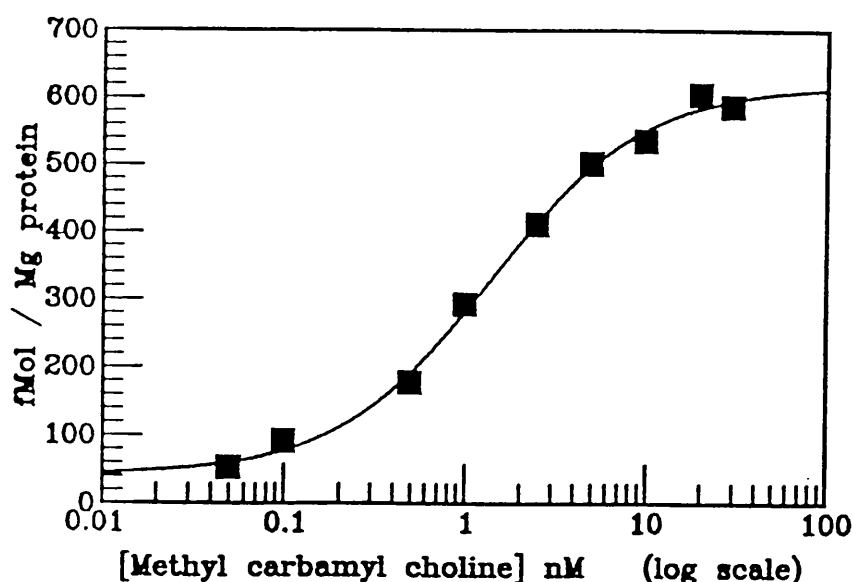
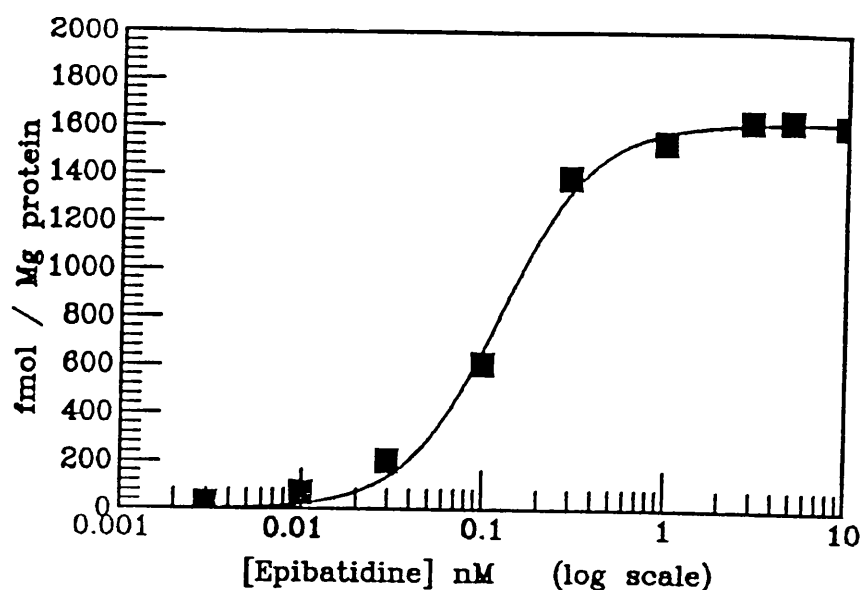


Fig. 4.3: Saturation binding curves for $[^3\text{H}]$ -epibatidine (top panel) and $[^3\text{H}]$ -methylcarbamylcholine (bottom panel) to polyclonal S2 cells transfected with the ALS and $\beta 2$ nAChR subunits. Full saturation binding curves were obtained between 0.003 and 30 nM ligand, in the presence or absence of 1 mM carbachol. The graphs show a representative saturation binding curve from each data set. Each point is a mean of triplicate counts with error bars. The K_d value and B_{\max} value were calculated from a mean of at least $n=4$ binding curves. The K_d values for $[^3\text{H}]$ -epibatidine and $[^3\text{H}]$ -methylcarbamylcholine binding to the polyclonal ALS/ $\beta 2$ cell line are 0.19 ± 0.06 nM and 1.72 ± 0.26 nM respectively.

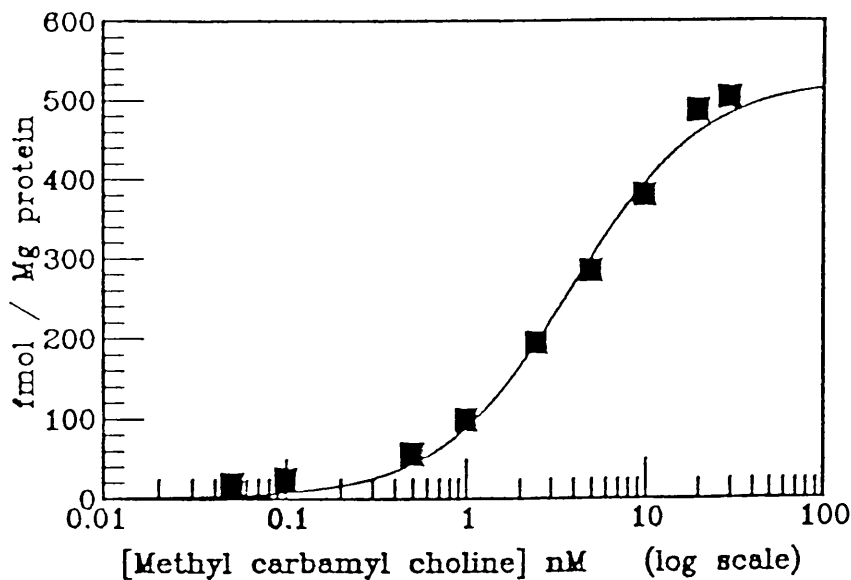
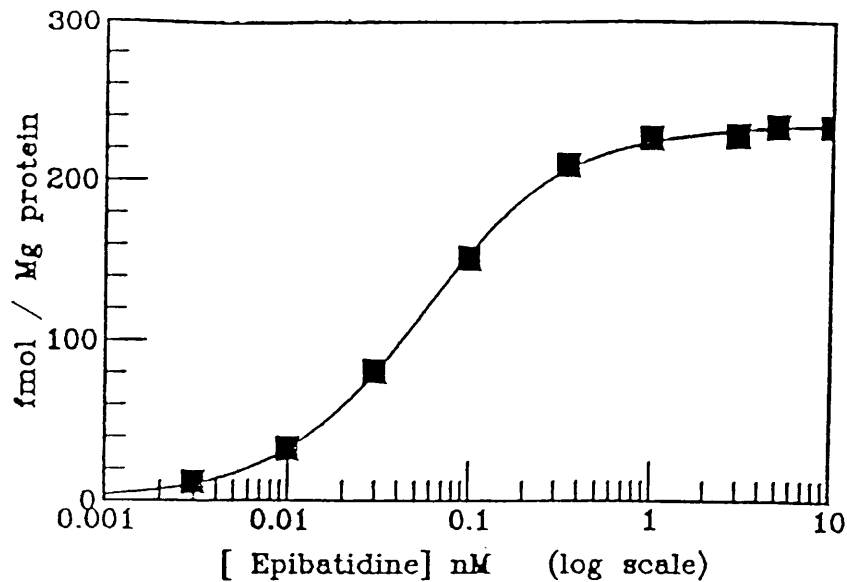


Fig. 4.4: Saturation binding curves for [³H]-epibatidine (top panel) and [³H]-methylcarbamylcholine (bottom panel) to S2 cells transfected with the SAD and β 2 subunits. Full saturation binding curves were obtained between 0.003 and 30 nM ligand, in the presence or absence of 1 mM carbachol, on the polyclonal S2 cell line expressing the SAD and rat β 2 nAChR subunits. The graphs show a representative curve from each data set. Each point is the mean of triplicate counts with error bars. The K_d value and B_{max} value were calculated from a mean of at least $n=4$. The K_d values for [³H]-epibatidine and [³H]-methylcarbamylcholine binding to the polyclonal SAD/ β 2 cell line are 0.23 ± 0.11 nM and 5.54 ± 0.79 nM respectively.

4.8 Radioligand binding studies on stably transfected S2 cell lines expressing *C. elegans* nAChR subunits

Stable cell lines were generated by co-transfecting *C. elegans* nAChR subunit cDNAs cloned into the expression vector pRmHa3 (Bunch *et al.*, 1988) together with the selectable marker plasmid pCOhygro (van der Straten *et al.*, 1989). A series of stably transfected *Drosophila* S2 cell lines were generated in which various combinations of the four *C. elegans* subunits were expressed. No specific binding was observed with any combination of subunits with any of the four radioligands tested (see Table 4.3). A *C. elegans* membrane preparation was kindly supplied by Dr. David Sattelle and low levels of specific [³H]-ACh and [³H]-epibatidine binding were observed (see Table 4.3).

4.9 Binding and functional studies with the ion channel subunit cDNAs for locust α L-1, RDL and TRPL

The α L-1 subunit had been shown to form functional homomeric channels when injected into *Xenopus* oocytes (Marshall *et al.*, 1990; Amar *et al.*, 1995) which is activated by nicotine and antagonised by α -BTX, mecamylamine and *d*-tubocurarine. In this study the α L-1 subunit was subcloned into pRmHa3 for expression studies in S2 cells to investigate whether α L-1 could form homomeric channels in these cells. To date, no specific [³H]-epibatidine binding has been observed in stably transfected S2 cells expressing the α L-1 subunit. It is possible that the apparent inability of the subunit to generate homo-oligomeric channels in *Drosophila* S2 cells is related to the apparent host-cell specific folding of vertebrate homo-oligomeric channels reported recently by others working in this group (Cooper and Millar, 1997; Cooper and Millar, 1998).

In this study, the stably transfected S2 cell lines: RDL, LCCH3, RDL/LCCH3, RDL(A302S) and RDL(A302S)/LCCH3 were established to assess the effect of co-expression of RDL with the β like subunit LCCH3. The cell lines were screened for

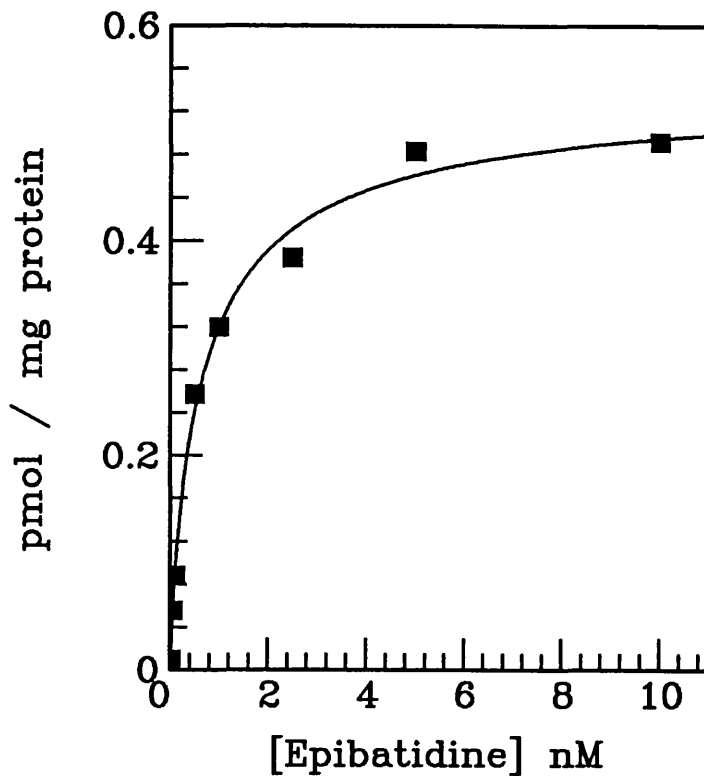


Fig. 4.5: Saturation binding curve for [^3H]-epibatidine to whole *Drosophila* membrane preparations. Full saturation binding curves were obtained between 0.01 and 10 nM ligand, in the presence or absence of 1 mM carbachol to determine non-specific binding. The graph is a representative curve, each point is the mean of triplicate counts with error bars. The K_d value and B_{\max} value were calculated from a mean of 3 full saturation binding curves. The K_d value for [^3H]-epibatidine is 1.55 ± 0.61 nM and the B_{\max} calculated at 0.72 ± 0.06 pmol/mg of membrane protein.

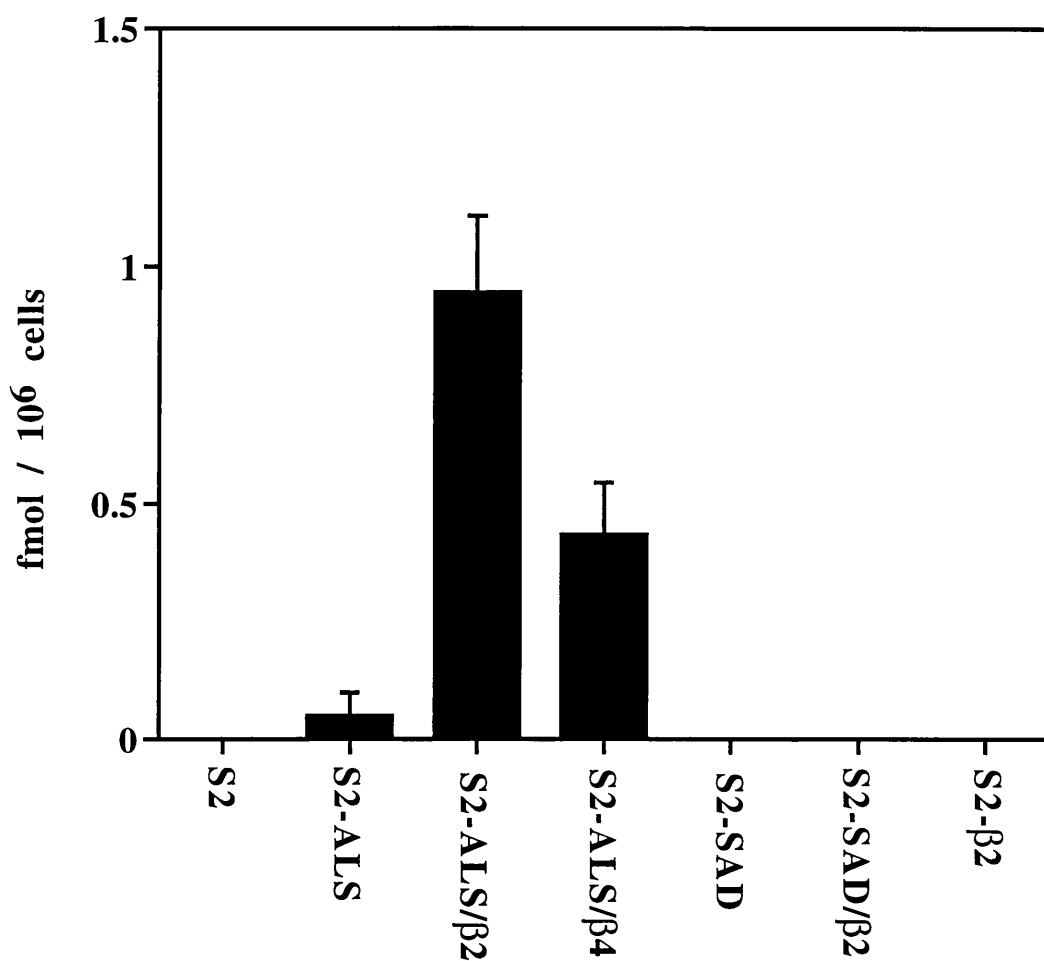


Fig 4.6: Cell surface [¹²⁵I]-αBTX binding to untransfected and stably transfected S2 cell lines. Graph shows specific [¹²⁵I]-αBTX binding (1 nM) to six stably transfected S2 cell lines and untransfected S2 cells. Non specific binding was determined in the presence of 1 mM nicotine and 1 mM carbachol. The graph shows the mean of n=4-5 binding assays with standard error bars.

Table 4.3: Nicotinic radioligand binding to S2 cells expressing *C. elegans* nAChR subunits.

Cell line	3 nM [³ H]- epibatidine	30 nM [³ H]- methylcarb	30 nM [³ H]-nicotine	30nM [³ H]-ACh
ACR-2	-			
LEV-1	-			-
UNC-29	-			
UNC-38	-			
ACR-2/LEV-1	-			
ACR-2/UNC-29	-			
ACR-2/UNC-38	-			
LEV-1/UNC-29	-			
LEV-1/UNC-38	-			
UNC-29/UNC-38	-			
UNC-38/Rat β2	-	-	-	-
LEV-1/UNC-29/ UNC-38	-			
LEV-1/UNC-29/ UNC-38/ACR-2	-	-	-	-
<i>C.elegans</i> membrane preparation	15.95 ± 4.9	-	-	39.06 ± 11

Table 4.3: Nicotinic radioligand binding to stably transfected S2 cell lines and *C. elegans* membrane preparation. Specific binding of ligands is shown in fmol per mg of protein. (-) No specific radioligand binding detected.

binding with the ligands [^3H]-muscimol and [^3H]-4-n-propyl-4'-ethylbicycloorto-benzoate (EBOB). The S2-RDL and S2-RDL(A203S) cell lines established in this study showed GABA-induced currents that were reduced by picrotoxin and dieldrin when tested by Dr. Steve Buckingham, Cambridge (Buckingham *et al.*, 1996).

The S2 cell line was used for stable transfections with heterologous cDNA for TRPL alone or in combination with the *Drosophila* muscarinic receptor (DM1). My involvement in this project has been limited to subcloning and the generation of stable *Drosophila* S2 cell lines. Whole cell recordings were made on the TRPL and TRP stably transfected S2 cell lines by Dr. Roger Hardie, Cambridge and has resulted in a recent publication (Hardie *et al.*, 1997). Estimates of 60 - 70 pS for channel conductance were confirmed directly in patch clamp recordings of single TRPL channels in S2 cells.

4.10 Discussion

The co-expression of the four cloned *Drosophila* nAChR subunits in the S2 cell line failed to result in functional nAChRs or receptors that could be detected by nicotinic radioligand binding. The construction of the full-length SBD cDNA clone and its expression with the other *Drosophila* nAChR subunits also failed to form receptors detectable by nicotinic radioligands or functional nAChRs. The expression of a full-length clone has excluded the possibility that previous difficulties in expressing functional *Drosophila* nAChRs is due to the non-native signal sequence of the chimeric SBD protein.

By the stable expression of *Drosophila* and rat nAChR subunits in the S2 cell line it has been established that both the cloned *Drosophila* α subunits acquire the ability to bind nicotinic radioligands when co-expressed with the rat $\beta 2$ subunit. Surface expression of the ALS/ $\beta 2$ combination was detected with [^{125}I]- α BTX binding.

In this study the ability of the two cloned *Drosophila* α subunits to co-assemble with either the rat $\beta 3$ or $\beta 4$ subunit was examined. After stable expression in *Drosophila* S2 cells, specific [^3H]-epibatidine binding could be detected with the ALS/ $\beta 4$ and SAD/ $\beta 4$ subunit combinations but not with either ALS/ $\beta 3$ or SAD/ $\beta 3$. As with the $\beta 2$ containing cell lines, surface [^{125}I]- αBTX binding with S2-ALS/ $\beta 4$ cells, but not S2-SAD/ $\beta 4$ cells could be detected (see Figure 4.6). By the stable expression of *Drosophila* and rat nAChR subunits in a *Drosophila* cell line, it has been established that both of the cloned *Drosophila* α subunits acquire the ability to bind radioligands when co-expressed with either the rat $\beta 2$ or $\beta 4$ subunits but not the rat $\beta 3$ subunit. In this respect the *Drosophila* α subunits resemble the rat $\alpha 2$, $\alpha 3$ and $\alpha 4$ subunits which generate functional nAChRs when co-expressed with $\beta 2$ or $\beta 4$ subunits, but fail to do so with the $\beta 3$ subunit (Deneris *et al.*, 1989; Duvoisin *et al.*, 1989; Luetje and Patrick, 1991).

Drosophila cell lines were constructed in which the two cloned *Drosophila* β subunits (ARD and SBD) were co-expressed with either the rat $\alpha 3$ or $\alpha 4$ subunits, no specific nicotinic radioligand binding was detected with any of these subunit combinations (see Table 4.1) despite evidence that the *Drosophila* β subunits do co-assemble with the rat α subunits (see Chapter 5).

Despite high levels of specific radioligand binding in cells transfected with several combinations of *Drosophila* and rat nAChR subunits, no specific nicotinic radioligand binding in *Drosophila* or mammalian cells transfected with *Drosophila* nAChR subunits was detected in the absence of vertebrate β subunits (see Table 4.1). The data suggest that co-assembly with other, as yet unidentified *Drosophila* nAChR subunits, is likely to be required for the generation of functional *Drosophila* nAChRs and this is investigated further in Chapter 7.

The S2 cell lines expressing the *C.elegans* nAChR subunits did not show any signs of expression when screened with the nicotinic ligands available, nicotine, epibatidine, methylcarbamylcholine or ACh. By this time over 20 potential nAChR subunit candidates had been identified from the genome mapping project. The work on the *C. elegans* cloned subunits for this project was postponed until specific radioligands such as [³H]-levamisole became available and to wait until all the potential nAChR subunits clones had been isolated from the genome mapping project. With more specific ligands to the *C. elegans* nAChRs and an idea of how many potential nAChR subunits there are, then a better strategy to assess and investigate the heterologous expression of *C. elegans* nAChRs in various cell lines could be established.

The locust (*Schistocerca gregaria*) nAChR subunit α L1 has been shown previously, by expression studies in *Xenopus* oocytes, to form functional homomeric nAChRs which resemble *in vivo* insect receptors (Marshall *et al.*, 1990; Amar *et al.*, 1995). The channels are opened by nicotine and antagonised by α -BTX, *d*-tubocurarine and mecamylamine. In this study, there was no evidence of a homomeric nAChR in S2 cells stably transfected with the α L1 subunit, screened by radioligand binding with nicotinic ligands. This result suggests that the nature of the host cell may be important for the expression of locust nAChR subunit α L1.

The importance of the host cell environment has been indicated recently by others working in this group, for the expression of homomeric α 7 nAChRs in nine cell lines (Cooper and Millar, 1997) and α 8 nAChRs in HEK-293 cells, SH-SY5Y cells and the rat pituitary cell line GH₄C₁ (Cooper and Millar, 1998). The expression of α 7 has been shown to differ in the extent of correctly folded protein in different isolates of the rat pheochromocytoma (PC12) cell line (Blumenthal *et al.*, 1997). The expression of homo-oligomeric α 9 receptors may also be host cell specific, although there is no direct evidence, the absence of any published reports of α 9 expressed in mammalian cell lines whereas functional α 9 receptors have been expressed in

Xenopus oocytes (Elgoyhen *et al.*, 1994), supports this idea. It is possible that the expression of the homo-oligomeric locust α L-1 receptor is also dependent on the host cell environment. A further illustration of the importance of the host cell has been provided by recent evidence from this laboratory which demonstrated that the ion channel properties of the hetero-oligomeric nAChRs α 3/ β 4 has also been shown to be host cell specific indicating that this phenomenon is not restricted to homo-oligomeric receptors (Lewis *et al.*, 1997).

The reasons for the host cell dependency of the folding, assembly and functional properties of these ion channels are still unclear. There are many possible reasons for the differences including: different post-translational modifications such as receptor phosphorylation, different ionic conditions or host cell specific chaperone proteins. There are numerous possible explanations but the actual mechanism are still to be elucidated.

GABA is the main inhibitory neurotransmitter in vertebrate brain, in the insect CNS and at the insect NMJ (Sattelle, 1990). Three *Drosophila* GABA subunits have been cloned RDL (ffrench-Constant *et al.*, 1991), LCCH3 which shows most similarity to GABA receptor β subunits (Henderson *et al.*, 1993) and GRD which is encoded by the *grd* gene (GABA and glycine-like receptor of *Drosophila*) (Harvey, 1994). RDL has been shown to form robust functional homo-oligomeric channels when expressed in *Xenopus* oocytes which show a similar pharmacology to native insect receptors (ffrench-Constant *et al.*, 1993; Buckingham *et al.*, 1994). A mutated form of RDL in which there is a single base substitution from alanine to serine at position 302, RDL(A302S), in the second transmembrane domain has been shown to confer resistance to the insecticide dieldrin at native and heterologously expressed receptors (ffrench-Constant *et al.*, 1993). During this project I was involved in the subcloning of the RDL(A302S) (RDL^r) subunit into the pRmHa3 expression vector establishing the stably transfected *Drosophila* S2 cell lines including S2-RDL^r. The stably transfected S2 cell line S2-RDL had been established previously (Millar *et al.*, 1994).

Electrophysiological studies carried out by Dr. Steve Buckingham, Cambridge, showed that both RDL and RDL(A302S) form functional homo-oligomeric channels when expressed in S2 cells and *Xenopus* oocytes (Millar *et al.*, 1994; Buckingham *et al.*, 1996). The insecticide resistant form of the receptor showed decreased sensitivity to picrotoxin and dieldrin.

The *Drosophila* photoreceptor TRPL was successfully subcloned into the *Drosophila* expression vector pRmHa3 and the stably transfected S2 cell lines TRP, TRPL, TRP/TRPL, TRP/DM1, TRPL/DM1 and DM1 were established. The S2 cells expressing TRP and TRPL, when tested by our collaborators, showed similarities with the light-induced currents in the photoreceptors. The results indicated that the channels encoded by the *trpl* gene are responsible for a component of the response to light *in situ* (Hardie *et al.*, 1997).

5.0 Assembly of nAChR subunits in *Drosophila* S2 cells

5.1 Introduction

Radioligand binding studies (described in Chapter 4) provided evidence that the *Drosophila* α subunits (ALS and SAD) were capable of co-assembly with either the rat $\beta 2$ or $\beta 4$ subunits when expressed in *Drosophila* S2 cells. The co-expression and co-assembly of *Drosophila* nAChR subunits was investigated further by techniques such as sucrose gradients, metabolic labelling and immunoprecipitation. Sucrose density centrifugation was used to estimate the size of the protein complexes of the ALS/ $\beta 2$ and SAD/ $\beta 2$ receptors to assess whether the subunits combine into pentamers. Antibodies to $\beta 2$ and to individual subunits and tagged subunits were used to analyse co-assembly of the subunits in the S2 cell line.

5.2 ALS/ $\beta 2$ and SAD/ $\beta 2$ subunit combinations examined by sucrose gradient centrifugation

Sucrose gradient centrifugation has been widely used to estimate the size of polypeptide and protein complexes. The technique has been used previously to investigate the assembly of the *Torpedo* nAChR, expressed in a mouse fibroblast cell line (Claudio *et al.*, 1987). Using [125 I]- α BTX, a single radioligand peak was shown to migrate at 9S, precisely where the native monomeric form of the *Torpedo* nAChR migrates. A similar peak has been shown with the nAChR $\alpha 7$ subunit in SH-SY5Y and PC-12 cells (Cooper and Millar, 1997) and the $\alpha 8$ nAChR subunit in GH $_4$ C $_1$ cells (Cooper and Millar, 1998).

Sucrose gradient centrifugation was used in this study to assess whether ALS/ $\beta 2$ or SAD/ $\beta 2$ combinations assembled into a complex of the size expected of a pentameric nAChR. Rat $\alpha 4/\beta 2$ and $\alpha 3/\alpha 5/\beta 4$ receptors, expressed in S2 cells, were sedimented on a 5 - 20% sucrose gradient using conditions that had been previously used to determine the pentameric ($\alpha_2\beta\gamma\delta$) muscle-type nAChRs (shown in Figure 5.1). The rat $\alpha 4/\beta 2$ combination did not show a typical sucrose gradient with a 9S peak characteristic of a pentameric complex. There was evidence of aggregation with the

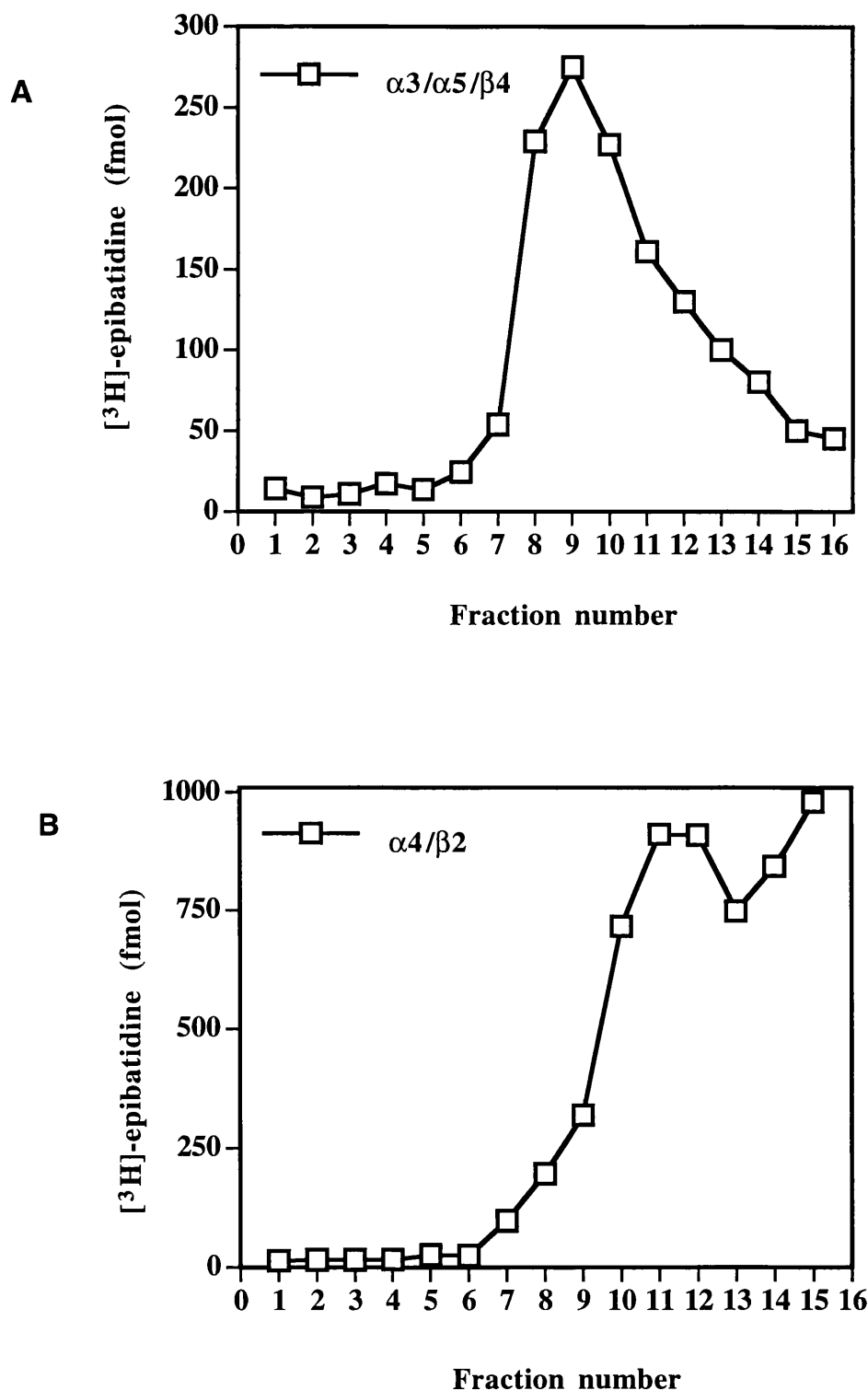


Fig. 5.1: Sucrose gradient sedimentation of rat nAChR subunit combinations $\alpha 3/\beta 4/\alpha 5$ (A) (cell line established by Dr. P. Harkness) and $\alpha 4/\beta 2$ (B) in stably transfected S2 cell lines. The cells were solubilised in lysis buffer containing 1% Triton X-100 and sedimented on a linear 5-20% sucrose gradient. Fractions were collected from the top of the gradient (fractions 1-16) and then incubated on ice for 2 h with [³H]-epibatidine (3 nM). The samples were harvested on a Brandell Harvetser and counted on a scintillation counter.

$\alpha 4/\beta 2$ combination. The $\alpha 3/\alpha 5/\beta 4$ combination has a sucrose gradient profile with a more typical 9S peak with no evidence of aggregation. Detergent solubilised ALS/ $\beta 2$ and SAD/ $\beta 2$ polyclonal S2 cell lines were centrifuged and individual fractions from the gradient were assayed for [^3H]-epibatidine binding. A [^3H]-epibatidine binding peak, corresponding to a sedimentation coefficient of $\sim 9\text{S}$, was observed in the ALS/ $\beta 2$ S2 cell line (shown in Figure 5.2).

There was no such peak with the SAD/ $\beta 2$ construct indicating an intrinsic property of the hybrid SAD/ $\beta 2$ receptor. Specific [^3H]-epibatidine binding was observed with the SAD/ $\beta 2$ complex after solubilisation in 1% Triton X-100. However, unlike ALS/ $\beta 2$, the SAD/ $\beta 2$ complex was not stable enough to withstand the centrifugation. The polyclonal SAD/ $\beta 2$ cell line was incubated in lysis buffer with 1% Triton X-100 at 4°C for an identical time period for the SAD/ $\beta 2$ sample to be run on a sucrose gradient. The polyclonal SAD/ $\beta 2$ cell line was also incubated in lysis buffer containing 5 - 20% sucrose with 1% Triton X-100. When binding assays were performed on the SAD/ $\beta 2$ samples from the gradient and those incubated in identical buffer, at the same temperature and for an identical time, the samples from the gradient showed no specific binding with [^3H]-epibatidine whereas the samples that were not sedimented by centrifugation showed high levels of specific [^3H]-epibatidine binding (100 - 130 fmol per sample equivalent to a fraction taken from the gradient). The solubilisation of SAD/ $\beta 2$ in lysis buffer containing 1% Triton X-100 was not the reason for the loss of binding. The results suggest that the association between SAD and $\beta 2$ is unstable when sedimented in detergent and even when less stringent solubilising techniques such as 0.1% Triton X-100, 1% or 0.1% lubrol were used the SAD/ $\beta 2$ complex still dissociated.

5.3 Metabolic labelling and immunoprecipitation

The transfected cell lines containing nAChRs subunits were radioactively labelled by the direct incorporation of the radioactive amino acids [^{35}S]methionine and

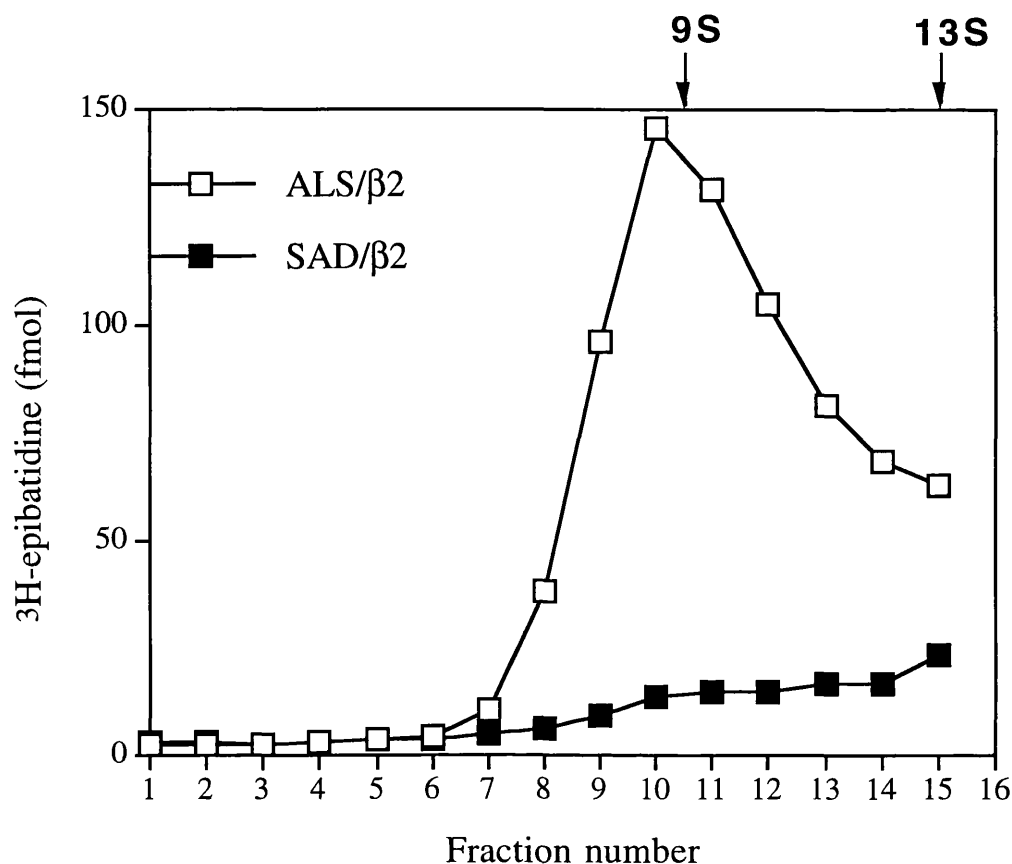


Fig. 5.2: Sucrose gradient sedimentation of ALS/β2 and SAD/β2 stably transfected S2 cell lines. In both cases, cells were solubilised in lysis buffer containing 1% Triton X-100 and sedimented on a linear 5-20% sucrose gradient. Fractions were collected from the top of the gradient (fractions 1-15) and then incubated for 2 h on ice with 3 nM [³H]-epibatidine. The samples were harvested on a Brandell Harvester and counted on a scintillation counter. The positions of the native nAChR electric organ pentameric 'monomer' (9S) and disulphide-linked dimer (13S) are shown.

[³⁵S]cysteine into the newly synthesised polypeptides by metabolic labelling. In these experiments, immunoprecipitation studies were carried out to investigate subunit assembly in the transfected cell lines. Monoclonal and polyclonal antibodies specific for single subunits of heterologous ion channels were used to investigate the co-assembly of the subunits. Subunits were assumed to be co-assembled when antibodies specific for one subunit co-precipitated other subunits.

The stably transfected S2 cell line containing the rat nAChR subunits $\alpha 4$ and $\beta 2$ was investigated by metabolic labelling and immunoprecipitation to investigate the co-assembly of the two subunits. Co-assembly of the rat nAChR subunits was proven biochemically by co-precipitation with the monoclonal antibodies (mAbs) mAb299 and mAb290, which are specific for the rat $\alpha 4$ and $\beta 2$ subunits, respectively (Whiting and Lindstrom, 1988). The stably transfected rat $\alpha 4/\beta 2$ S2 cell line was metabolically labelled and immunoprecipitation experiments conducted with mAb290 and mAb 299, the results are shown in Figure 5.3.

Co-assembly of the *Drosophila* and rat nAChR subunits was shown in this study by co-precipitation with the monoclonal antibodies (mAbs) mAb299 and mAb290 (Whiting and Lindstrom, 1988) and the results are shown in Figure 5.3. With mAb290 (raised against the rat $\beta 2$ subunit), co-precipitation of the rat $\alpha 4$ subunit and the two *Drosophila* α subunits was observed. Co-assembly of SBD and $\beta 2$ was detected with mAb290 although the intensity of the band corresponding to the $\beta 2$ subunit was significantly greater than that of SBD. This may be due to: the precipitation of unassembled or aggregated $\beta 2$ protein by mAb290, differences in relative stoichiometries of $\beta 2$ and SBD in assembled complexes, dissociation of $\beta 2$ /SBD complexes during the solubilisation or wash procedures used in this technique. In addition, the SBD protein may form non-specific associations with $\beta 2$ aggregates which may not represent a genuine assembly intermediate. With mAb299 (raised against the rat $\alpha 4$ subunit), the rat $\beta 2$ subunit and the *Drosophila* SBD subunit were co-precipitated. Thus, SBD appears to be able to co-assemble with $\alpha 4$, despite the inability of this subunit combination to bind nicotinic radioligands. Co-precipitation of the two *Drosophila* α subunits (ALS or SAD) with rat $\alpha 4$ was not detected. When the *Drosophila* ARD subunit was co-expressed with either the rat $\alpha 4$ or $\beta 2$ subunit, little or no $\alpha 4$ or $\beta 2$ subunit was revealed in the immunoprecipitates. It is possible, therefore, that ARD is capable of co-assembling with both $\alpha 4$ and $\beta 2$ subunits, but that the co-

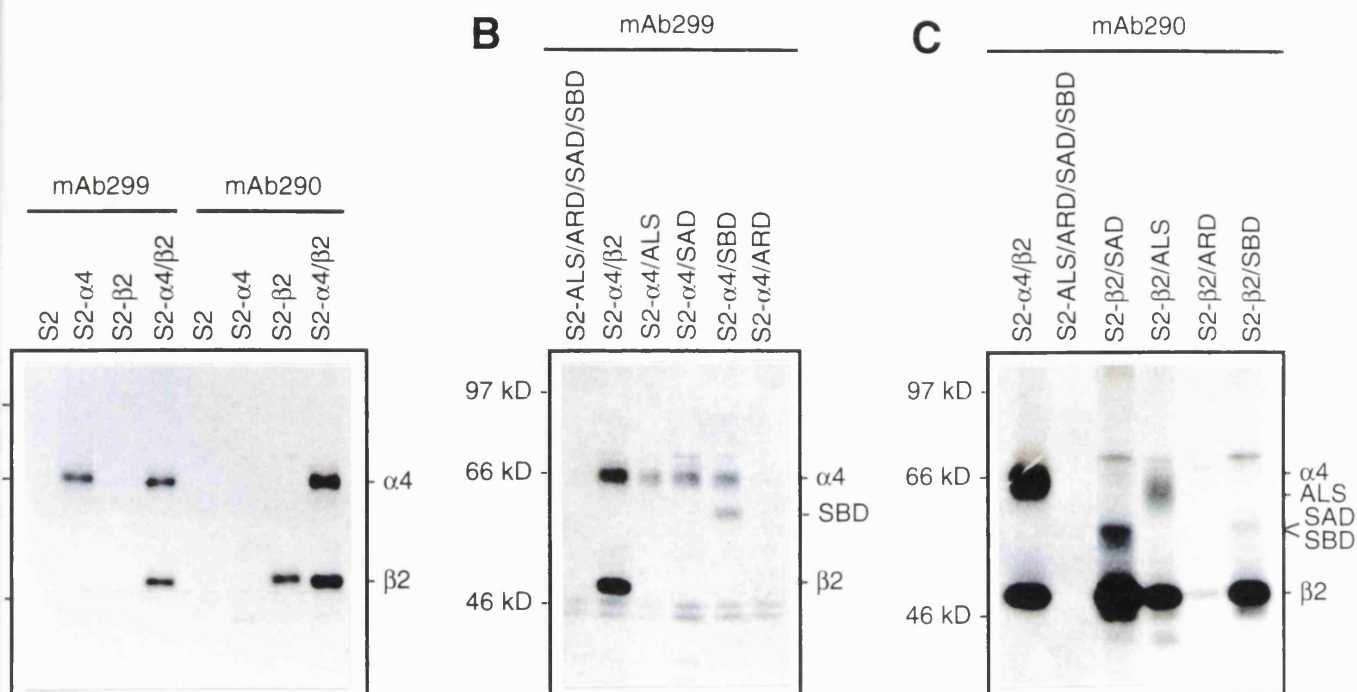


Fig 5.3: Co-immunoprecipitation of *Drosophila* and rat nAChR subunits stably expressed in *Drosophila* S2 cells. A: mAb299 (raised against the rat $\alpha 4$ subunit) immunoprecipitated the $\alpha 4$ subunit, co-precipitated the $\beta 2$ subunit (when co-expressed with $\alpha 4$), and there was no cross reactivity with the $\beta 2$ subunit. mAb290 (raised against the $\beta 2$ subunit) immunoprecipitated the $\beta 2$ subunit, co-precipitated the $\alpha 4$ subunit (when co-expressed with $\beta 2$), and there was no cross reactivity with $\alpha 4$ subunit. B: mAb299 co-precipitated the *Drosophila* SBD (β -like) subunit but not the ALS or SAD (α -like) subunits. No $\alpha 4$ subunit could be detected when co-expressed with ARD. mAb299 did not cross react with any of the four *Drosophila* subunits. C: mAb290 co-precipitated the *Drosophila* ALS and SAD subunits and, to a lesser extent, the SBD subunit. No $\beta 2$ subunit could be detected when co-expressed with ARD. mAb290 did not cross react with any of the four *Drosophila* subunits.

The figures show a representative co-immunoprecipitation which was repeated to give at least $n=3$ for each combination.

assembled subunit complex is rapidly degraded due to the production of incorrectly folded subunits. This interpretation is supported by immunoprecipitation studies which show that when either the rat $\alpha 4$ or $\beta 2$ subunits are expressed alone they can be detected at levels similar to the levels observed when they are co-expressed with subunits other than ARD (panel A Figure 5.3).

5.4 Immunoprecipitation with monoclonal antibodies to ALS and SAD

Two monoclonal antibodies to the *Drosophila* ALS and SAD nAChR subunits, (mAb D4) and mAb C3 respectively, were kindly supplied to us by Dr. Eckart Gundelfinger, University of Hamburg, Germany (Schuster *et al.* 1993, Jonas *et al.* 1994). These antibodies were used for immunoprecipitation studies on the polyclonal cell lines ALS, ALS/ $\beta 2$, SAD, SAD/ $\beta 2$ and untransfected S2 cells. This was carried out in parallel with the same cell lines using the $\beta 2$ antibody mAb 270. The blots obtained using the monoclonal antibodies to ALS and SAD showed no specific band, whereas the same cell lines with anti $\beta 2$ revealed specific $\beta 2$ bands and co-precipitation with both ALS and SAD. Neither of the two monoclonal antibodies raised against the *Drosophila* nAChR subunits appeared to be suitable for use in the immunoprecipitation studies. In order to examine the co-assembly of the subunits, it was therefore necessary to introduce a foreign epitope tag into the individual subunits to which a mAb was available and perform immunoprecipitation studies epitope-tagged subunits.

5.5 Introduction of a FLAG-epitope to *Drosophila* nAChR subunits

The four *Drosophila* nAChR subunits were epitope tagged with the FLAG-epitope (Hopp *et al.*, 1988). The FLAG-epitope is an eight amino acid sequence that was incorporated into the nAChR subunit cDNAs by subcloning synthetic oligonucleotides into suitable restriction enzyme sites. The full cloning strategy for each subunit is described in the Materials and Methods (Section 2.19) and a summary is shown in Figure 5.4. Figure 5.5 shows the site of insertion of the FLAG-epitope

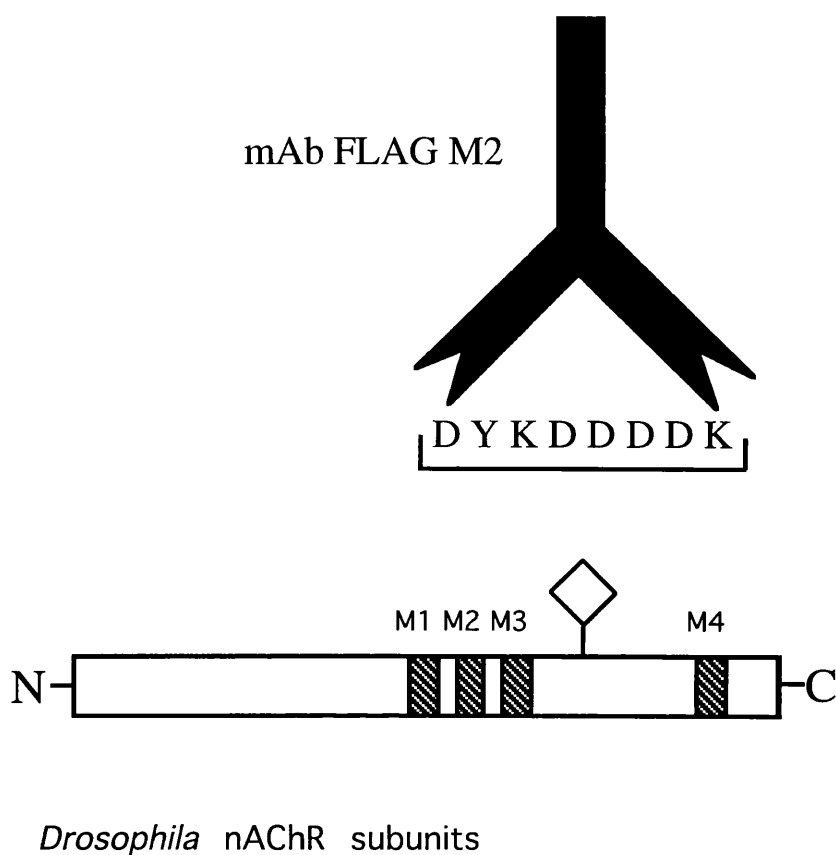


Fig 5.4: Epitope-tagging the *Drosophila* nAChR subunits ALS, ARD, SAD and SBD. The *Drosophila* subunits in the cloning vector pRmHa3 were cut at a unique restriction enzyme site within the cytoplasmic loop between TM3 and TM4. The eight amino acid FLAG epitope was incorporated into the nAChR subunit cDNAs by subcloning of synthetic oligonucleotides.

ALS-FLAG

YLLFTMMLVTL SVVVTIAVL NVNFRSPVTHRMAPWVQRLF IQILPKLLC
IERPKKEEPEEDQPPEVLTDVYHLPPDVKFVNYDKRFSGDYGI PALPA
SHRFDLAAAGGISAHCFAPPLPSSLPLPGADDDLFSPSGLNGDISPGC
CPAAAAAAAADLSPTFEKPYAREMEKTIEGSRFIAQHVKNKDKFESVEE
DWKYVAM**DYKDDDDKGM**VLDRL**FLWIFAIACVVGTALIILQA**

ARD-FLAG

YLLFTFIMNTVSILVTVII IWNFRGPRTHRMPMYIRSIFLHYLPAFLF
MKRPRKTRLRWMMEMPGMSMPAHPPSYGSPAELPKHISAIGGQSKME
VMELSDLHHPNCKINRKVNSSGGLGLGDGCRRESESSDSILLSPEASKA
TEAVEFIAEHLRNEDLYIQTRWDKYVAM**DYKDDDDKGM**VIDRL**OLYIF**
FIVTTAGTVGILMDA

SAD-FLAG

YLLFTMLLVGLSVVITII IHYRKPSTHKMRPWIRSFFIKRLPKLLLM
RVPKDLLRLDLAANKINYGLKFSKTKFGQALMDEMOMNSGGSSPDSLRRM
QGRVGAGGCNGM**DYKDDDDKGM**HVTTATNRF SGLVGALGGGLSTLSGYN
GLPSVLSGLDDSLSDVAARKKYPFELEKAIHNVMF IQHHMQRQDEFNAE
DQDWGFVAMVMDRL**FLWLFMIA SLVGTFVILGEA**

SBD-FLAG

YLLFTMILVSLSVWTTVCVI NIHFRSPSTHNMSRLVRKLFLHFMPKLMM
MRRTQYTLPDYDDSTPSNGYTNEIDVRDSISDFPSEFKDSQDGAYDNGM
DYKDDDDKGMQNSVSDSNV IPRNLTPPEVLQALRAVRF IAQHIKDADKDN
EIVEDWK FVSMVLDRL**FLWLFTLSCVFGTLAICQS**

Fig. 5.5: Amino acid sequence alignment of the four *Drosophila* nAChR subunits ALS, ARD, SAD and SBD between the third and fourth putative transmembrane domains. The figure shows the site of insertion of the FLAG epitope (shown in bold and with a line below the sequence). The third and fourth transmembrane domains are boxed and in bold type.

into each of the four *Drosophila* nAChR subunits. The subunits were digested with a unique enzyme within the region between TM3 and TM4 of the encoded protein, within the cytoplasmic loop. This site was chosen in the hope that it would minimise problems of subunit misfolding. Work being carried out simultaneously in the group had also shown that the introduction of a FLAG-epitope into this region of the rat $\alpha 7$ subunit did not prevent folding into a conformation capable of being transported to the cell surface and binding nicotinic radioligands (Cooper and Millar, 1997).

5.6 Immunoprecipitation studies using a mAb to the FLAG-epitope

Stable S2 cell lines were established with combinations of the FLAG-tagged *Drosophila* subunits, co-expressed with either rat nAChR subunits or untagged *Drosophila* nAChR subunits. The cell lines are summarised in Table 5.1. The cells were metabolically labelled and the subunits were immunoprecipitated with the anti-FLAG mAb.

Figure 5.6 shows the individual epitope-tagged *Drosophila* nAChR subunits expressed singularly in stably transfected S2 cells. Immunoprecipitation revealed specific bands for each tagged subunit except the ALS-FLAG construct. Since the tagged ALS subunit could not be detected, the ALS-FLAG construct was re-sequenced and checked by restriction mapping. No sequencing errors could be detected which suggests that the FLAG-epitope may cause the production of an incorrectly folded ALS protein which is rapidly degraded.

To investigate subunit co-assembly, the FLAG-tagged subunits were co-expressed in stable polyclonal S2 cells with the rat $\beta 2$ subunit. The cells were metabolically labelled and immunoprecipitated using the mAbFLAG-M2 and the anti- $\beta 2$ antibody mAb290. The results are shown in Figure 5.7. mAbFLAG-M2 immunoprecipitated the FLAG-tagged ARD, SAD and SBD subunits but not the FLAG-tagged ALS

Table 5.1: Summary of stably transfected S2 cell lines expressing epitope-tagged *Drosophila* nAChR subunits.

S2 cells lines expressing epitope-tagged <i>Drosophila</i> nAChR subunits.	S2 cells lines co-expressing epitope-tagged <i>Drosophila</i> nAChR subunits and the rat $\beta 2$ subunit.	S2 cells lines co-expressing epitope-tagged and untagged <i>Drosophila</i> nAChR subunits.
S2-ALS-FLAG	S2-ALS-FLAG/ $\beta 2$	S2-ALS-FLAG/ARD
S2-ARD-FLAG	S2-ARD-FLAG/ $\beta 2$	S2-ALS-FLAG/SAD
S2-SAD-FLAG	S2-SAD-FLAG/ $\beta 2$	S2-ALS-FLAG/SBD
S2-SBD-FLAG	S2-SBD-FLAG/ $\beta 2$	S2-ARD-FLAG/ALS
		S2-ARD-FLAG/SAD
		S2-ARD-FLAG/SBD
		S2-SAD-FLAG/ALS
		S2-SAD-FLAG/ARD
		S2-SAD-FLAG/SBD
		S2-SBD-FLAG/ALS
		S2-SBD-FLAG/ARD
		S2-SBD-FLAG/SAD

Table 5.1: Summary of S2 cell lines stably transfected with combinations of epitope-tagged *Drosophila* nAChR subunits co-expressed with either the rat $\beta 2$ subunit or untagged *Drosophila* nAChR subunits. The cell lines were used for metabolic labelling and immunoprecipitation studies. Other stable S2 cell lines used in these studies are listed in Table 4.1

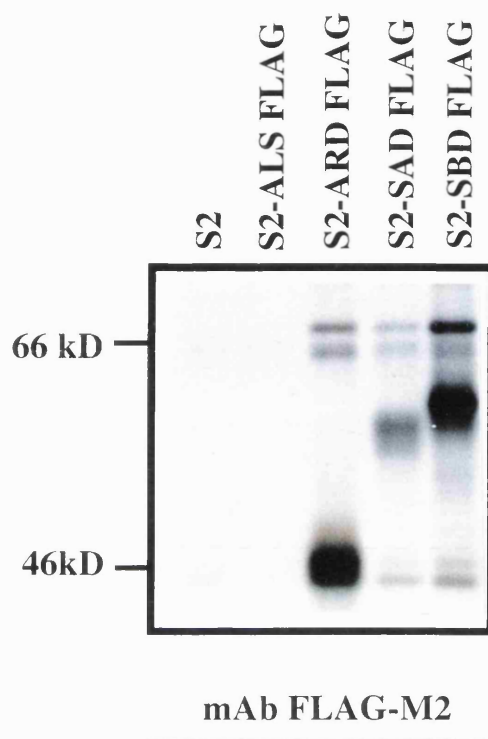


Fig 5.6: Immunoprecipitation of epitope-tagged *Drosophila* nAChR subunits stably expressed in *Drosophila* S2 cells. The epitope-tagged ARD, SAD and SBD subunits were immunoprecipitated by mAbFLAG-M2 (Hopp, 1988) but the epitope-tagged ALS subunit could not be detected.

The figures show a representative co-immunoprecipitation which was repeated to give at least n=3 for each combination.

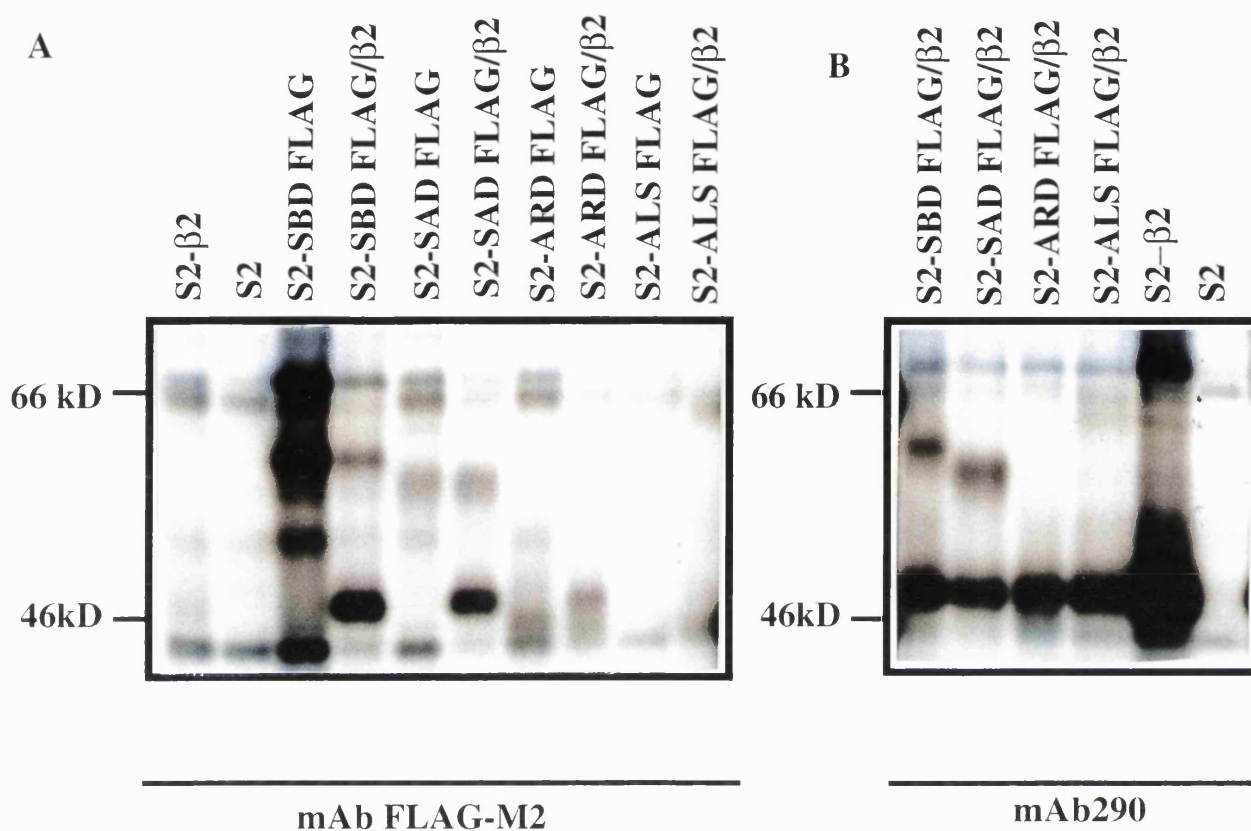


Fig 5.7: Co-immunoprecipitation of epitope-tagged *Drosophila* nAChR subunits and the rat nAChR $\beta 2$ subunit stably expressed in *Drosophila* S2 cells. A: The epitope-tagged ARD, SAD and SBD subunits, but not the epitope-tagged ALS subunit, were immunoprecipitated by mAbFLAG-M2 (Hopp, 1988). The $\beta 2$ subunit was co-precipitated by mAbFLAG-M2 when co-expressed with epitope-tagged ARD, SAD or SBD and there was no cross reactivity with the $\beta 2$ subunit. B: mAb290 (raised against the rat $\beta 2$ subunit) immunoprecipitated the $\beta 2$ subunit, co-precipitated the epitope-tagged ARD, SAD and SBD subunits but not the epitope-tagged ALS subunit and did not cross react with the epitope-tagged *Drosophila* subunits.

The figures show a representative co-immunoprecipitation which was repeated to give at least $n=3$ for each combination.

subunit and did not cross react with the rat $\beta 2$ subunit. The $\beta 2$ subunit was co-precipitated by mAbFLAG-M2 when co-expressed with epitope-tagged ARD, SAD or SBD. This result is shown in Figure 5.7. Epitope-tagged ARD, SAD and SBD subunits, when co-expressed with the $\beta 2$ subunit, were co-precipitated by mAb290. mAb290 did not cross react with the epitope-tagged subunits. This result is shown in Figure 5.7. The FLAG-tagged ALS subunit was not precipitated by mAbFLAG-M2 or mAb290 when expressed alone or in combination with the $\beta 2$ subunit.

To investigate the co-assembly of the *Drosophila* nAChR subunits, FLAG-tagged subunits were expressed in various paired combinations with a non-tagged *Drosophila* nAChR subunit in stably-transfected S2 cell lines. Co-assembly between the subunits was investigated using mAbFLAG-M2 and the results are shown in Figures 5.8 and 5.9. The FLAG-tagged ALS nAChR subunit was not immunoprecipitated when expressed alone or when expressed in combination with ARD or SAD or SBD (Figure 5.8 A). The epitope-tagged ARD subunit was immunoprecipitated by mAbFLAG-M2 and SBD and SAD were co-precipitated when co-expressed with the epitope-tagged ARD subunit (Figure 5.8 B). Figure 5.9 A shows that the ARD and SAD were co-precipitated when co-expressed with the epitope-tagged SBD subunit. ARD and SBD were co-precipitated when co-expressed with the epitope-tagged SAD subunit which is shown in Figure 5.9 B. mAbFLAG-M2 did not cross react with any of the four *Drosophila* subunits.

5.7 Western blots with polyclonal antibodies to LEV-1, UNC-29, UNC-38

Three polyclonal antibodies to the *C. elegans* nAChR subunits LEV-1, UNC-29 and UNC-38 were kindly provided by Dr. D. Sattelle and were used for Western blotting to investigate the expression of these three subunits in stably-transfected S2 cells and also to investigate the cross-reactivity of the antibodies to the other *C. elegans* subunits. The antibodies were each tested on stably-transfected S2 cell lines expressing the single nAChR subunits LEV-1, UNC-29, UNC-38 or ACR-2. The

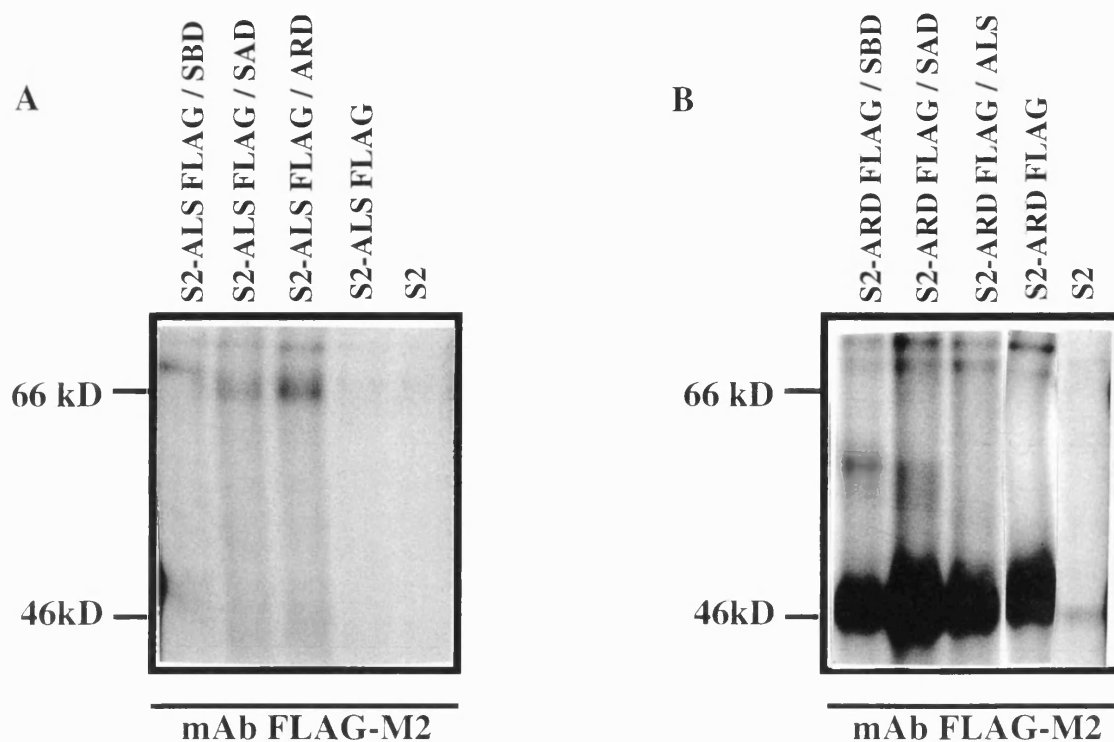


Fig 5.8: Co-immunoprecipitation of epitope-tagged *Drosophila* nAChR subunits stably expressed in *Drosophila* S2 cells. A: The epitope-tagged ALS subunit was not immunoprecipitated by the mAbFLAG-M2 (Hopp, 1988). The *Drosophila* nAChR subunits ARD, SAD and SBD were not co-precipitated with the epitope-tagged ALS subunit. B: The epitope-tagged ARD subunit was immunoprecipitated by the mAbFLAG-M2 and SBD and SAD were co-precipitated when co-expressed with the epitope-tagged ARD subunit. mAbFLAG-M2 did not cross react with any of the four *Drosophila* subunits.

The figures show a representative co-immunoprecipitation which was repeated to give at least n=3 for each combination.

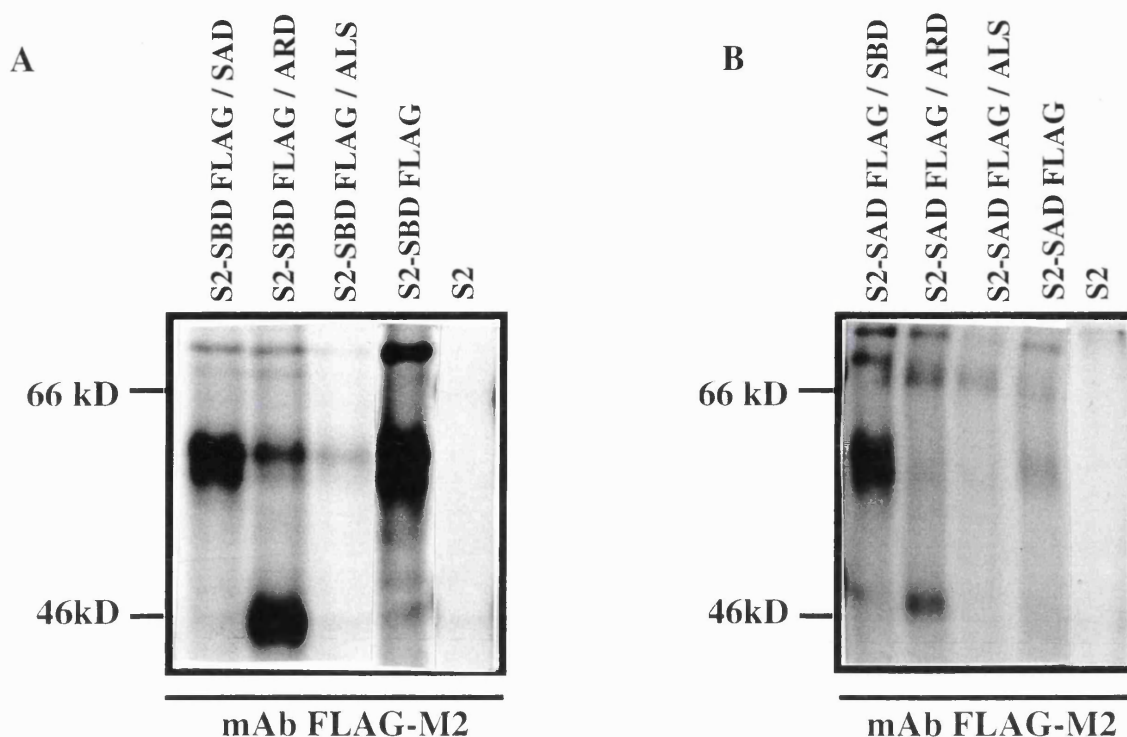


Fig. 5.9: Co-immunoprecipitation of epitope-tagged *Drosophila* nAChR subunits stably expressed in *Drosophila* S2 cells. A: The epitope-tagged SBD subunit was immunoprecipitated by mAbFLAG-M2 (Hopp, 1988). The *Drosophila* nAChR subunits ARD and SAD were co-precipitated when co-expressed with the epitope-tagged SBD subunit. B: The epitope-tagged SAD subunit was immunoprecipitated by mAbFLAG-M2. The *Drosophila* nAChR subunits ARD and SBD were co-precipitated when co-expressed with the epitope-tagged SAD subunit.

The figures show a representative co-immunoprecipitation which was repeated to give at least n=3 for each combination.

polyclonal antibodies did not show any specific bands but did have many non-specific interactions which prevented the antibodies being used successfully for Western blot analysis (data not shown).

5.8 Immunoprecipitation with polyclonal antibodies to LEV-1, UNC-29, UNC-38

The three polyclonal antibodies used for Western Blotting were then used in a metabolic labelling experiment to investigate whether the antibodies would be suitable for co-assembly studies in S2 cells. All three antibodies gave non-specific bands and no specific protein bands could be detected above background when tested on stably transfected S2 cell lines expressing single *C. elegans* nAChR subunits LEV-1, UNC-29, UNC-38 or ACR-2 (data not shown).

5.9 Discussion

The co-assembly of the rat nAChR subunits $\alpha 4$ and $\beta 2$ was shown in stably-transfected S2 cells using the monoclonal antibodies mAb299 and mAb290 (Whiting and Lindstrom, 1988). In this study mAb299 and mAb290 were used successfully to show the co-assembly of the rat $\alpha 4$ and $\beta 2$ subunits by metabolic labelling and immunoprecipitation.

Due to the lack of specific antibodies to the four *Drosophila* nAChR subunits, co-assembly of the *Drosophila* nAChR subunits was investigated using epitope-tagging and mAbFLAG-M2 to the FLAG-epitope. Co-assembly of *Drosophila* nAChR subunits with the rat nAChR subunits was investigated using the mAbs to the rat $\alpha 4$ and $\beta 2$ subunits.

The co-expression of the four *Drosophila* subunit cDNAs in S2 cells failed to generate a radioligand binding site whereas a site was formed by the co-expression of the rat nAChR subunits $\alpha 4$ and $\beta 2$. Radioligand binding sites were generated by the

co-expression of *Drosophila* α subunits with rat β subunits and the co-assembly of these subunit combinations were investigated using mAbs to the rat subunits.

The *Drosophila* α subunits ALS and SAD both co-assembled with the rat β 2 subunit as did the β subunit SBD, but to a lesser extent. There is evidence from co-immunoprecipitation studies that both of the *Drosophila* β subunits are capable of co-assembly, to some extent, with both rat α and β subunits.

When the *Drosophila* ARD subunit is co-expressed with rat nAChR subunits there appears to be a rapid degradation of the rat nAChR subunits, which may indicate the production of a misfolded subunit complex. When the *Drosophila* ARD subunit was co-expressed with either the rat α 4 or β 2 subunit, little or no α 4 or β 2 subunit was revealed in the immunoprecipitates. It is possible therefore that the ARD can co-assemble with both rat α 4 and β 2 subunits, but the subunits are degraded owing to the production of incompletely folded protein complexes. This interpretation is supported by immunoprecipitation studies showing that when either rat α 4 or β 2 are expressed alone, they can be detected at levels similar when they are co-expressed with subunits other than ARD. There is evidence from the expression of muscle nAChR subunits that misfolded subunit complexes do not exit from the ER but are rapidly degraded (reviewed by Green and Millar 1995). The band corresponding to the ARD protein in the co-immunoprecipitation studies (figures 5.6 - 5.9) runs at a size approximately 48 kD which is considerably smaller than the predicted size of the mature protein which is approximately 58 kD. The size difference observed when ARD or the epitope-tagged ARD subunit is co-expressed with other subunits may be explained by partial degradation of the ARD protein.

When ARD is coexpressed with the epitope-tagged SBD or SAD subunits there is evidence of co-assembly. The ARD does not appear to be degraded in these combinations which indicates that when co-expressed with *Drosophila* rather than rat nAChR subunits the ARD subunit may be less likely to misfold and be degraded.

Due to the lack of useful monoclonal antibodies, co-assembly of *Drosophila* nAChR subunits was investigated by the introduction of epitope-tags. The results show that epitope-tagged ARD, SAD and SBD are co-precipitated by mAb290 when co-

expressed with the rat $\beta 2$ subunit, indicating that there is co-assembly with these subunits. The $\beta 2$ subunit is co-precipitated by mAbFLAG-M2 when co-expressed with the epitope-tagged ARD or SAD or SBD subunit.

The epitope-tagged ALS subunit was not precipitated by mAbFLAG-M2. The four *Drosophila* subunits were epitope-tagged between TM3 and TM4 (as shown in Figure 5.5). The tag was introduced into SAD and SBD at a site approximately mid way between the two transmembrane domains. ALS and ARD were epitope-tagged at a site close to TM4. It is not clear why the tagged ALS subunit appears to misfold since the ARD-FLAG construct appears to be folded correctly.

The results show that the SAD subunit can co-assemble with ARD and SBD and that when co-expressed the ARD and SBD subunits can co-assemble. The results with the epitope-tagged ALS subunit do not allow any interpretation as to its co-assembly with the other *Drosophila* nAChR subunits. The co-expression of the epitope-tagged ARD, SAD or SBD subunits with the untagged ALS subunit do not suggest that co-assembly of these subunits occurs. This is in contrast to a previous study which showed co-assembly of the *Drosophila* ALS and ARD subunits in *Drosophila* head membrane preparations (Schloss *et al.*, 1988; Schloss *et al.*, 1991).

By the stable expression of *Drosophila* and rat nAChR subunits in a *Drosophila* cell line it has been established that both of the *Drosophila* α subunits acquire the ability to bind nicotinic radioligands when co-expressed with either rat $\beta 2$ or $\beta 4$ subunits. The results in this chapter from the sucrose gradient centrifugation studies show that the *Drosophila* α subunits co-assemble with the rat $\beta 2$ subunit and that ALS/ $\beta 2$ assembles into a complex of the size expected of a pentameric nAChR.

6.0 Temperature-sensitive expression of cloned *Drosophila* nAChR subunits

6.1 Introduction

Although robust expression of functional nAChRs from cloned *Drosophila* nAChR subunits has not been achieved to date, the cloned *Drosophila* nAChR α subunits ALS and SAD have been reported to form functional channels when co-expressed with the chick nAChR β 2 subunit in *Xenopus* oocytes (Bertrand *et al.*, 1994). In the present study, the human embryonic kidney (HEK-293) cell line was co-transfected with either the ALS and rat β 2 subunit cDNAs, or the SAD and rat β 2 nAChR subunit cDNAs. This mammalian cell line has been used successfully for the expression of several vertebrate neurotransmitter-gated ion channels including nAChR such as rat α 3/ β 4 (Stetzer *et al.*, 1996), rat α 4/ β 2 (Gopalakrishnan *et al.*, 1995) and the cell line has been used extensively for the characterisation of the GABA_A receptors (Pritchett *et al.*, 1988).

6.2 Expression of nAChR subunits in HEK-293 cells

HEK-293 cells were transfected with combinations of *Drosophila* and rat nAChR subunits. Despite functional expression in *Xenopus* oocytes of ALS/ β 2 (this study) and SAD/ β 2 (Bertrand *et al.*, 1994) there was no detectable specific binding of nicotinic radioligands observed in HEK-293 cells transiently co-transfected with either ALS and rat β 2 or SAD and rat β 2 nAChR subunit cDNAs. In contrast, when the rat α 4 and β 2 subunits were co-expressed in HEK-293 cells, high levels of specific nicotinic radioligand binding were obtained (Table 6.1). The cells in this experiment were maintained at 37 °C (Lansdell *et al.*, 1997).

6.3 Expression of nAChR subunits in oocytes

In order to confirm that the cDNA constructs used to transfect HEK-293 cells are capable of generating functional nAChRs, the plasmids constructs were injected into *Xenopus* oocyte nuclei. Functional nAChRs were detected in oocytes in response to bath applied ACh by two-electrode voltage clamp recording. A dose-response curve for ACh from oocytes injected with pcDNA3-ALS and pcDNAIneo- β 2 is shown in

Table 6.1: Binding of [³H]-epibatidine to transiently transfected HEK-293 cells.

Cell line	B_{\max} (pmol/mg of membrane protein)
HEK-rat α 4	-
HEK-rat β 2	-
HEK-rat α 4 / rat β 2	0.5 ± 0.2
HEK-ALS / rat β 2	-
HEK-SAD / rat β 2	-
HEK-ALS/SAD/ARD/SBD	-

Table 6.1. Binding of [³H]-epibatidine (3nM) to transiently transfected HEK-293 cells. Results are the mean \pm s.e. (n=3) expressed as pmol/mg of membrane protein.. Cells were transfected and incubated at 37 °C.

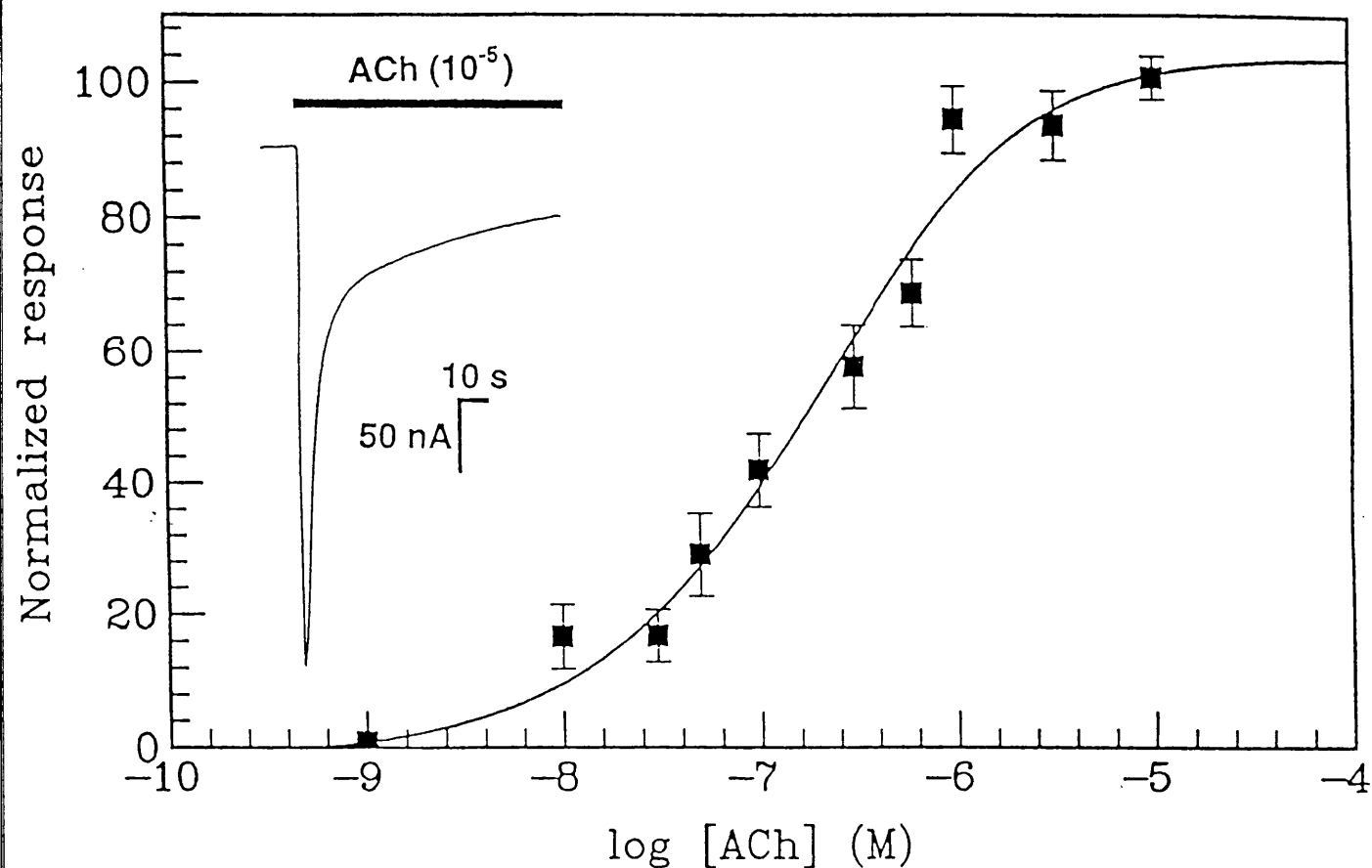


Fig. 6.1: Dose-response curve for bath-applied ACh with oocyte-expressed nAChRs. The *Drosophila* ALS (in pcDNA3) and rat $\beta 2$ subunits (in pcDNA1neo) were co-expressed in *Xenopus* oocytes by nuclear injection. Mean \pm SEM (bars) peak amplitudes, in response to bath-applied ACh at 10^{-5} M in the presence of $5 \mu\text{M}$ atropine. A full set of data points was derived from six separate oocytes. Inset: response to bath-applied ACh (10^{-5} M).

Figure 6.1. This data gave an estimated EC_{50} of 0.18 μ M for ACh which agrees well with the value reported by Bertrand *et al.* for ALS and chick $\beta 2$ co-expression (Bertrand *et al.*, 1994). This confirms that *Drosophila* α and vertebrate β subunits can co-assemble into functional nAChR channels (Lansdell *et al.*, 1997) and that the plasmid constructs used for expression studies in HEK-293 cells are capable of generating functional nAChRs in *Xenopus* oocytes.

6.4 Temperature-sensitive expression of *Drosophila* / rat hybrid receptors in HEK-293 cells

Despite the absence of detectable radioligand binding in HEK-293 cells after transfection with either ALS and rat $\beta 2$, or SAD and rat $\beta 2$, these subunit combinations acquired the ability to bind nicotinic radioligands when transfected cells were grown at a lower temperature. HEK-293 cells transfected with either the ALS and rat $\beta 2$ cDNAs, or the SAD and rat $\beta 2$ cDNAs (and incubated at 37°C overnight) were transferred to 25°C for a further 24 h. Cells treated in this way showed specific [³H]-epibatidine binding (Figure 6.2). However, although the correct folding of *Drosophila* nAChR subunits appears to be temperature-sensitive, co-expression of all four cloned *Drosophila* nAChR subunits in HEK-293 cells at either 37°C or 25°C, failed to generate functional channels or receptors that could be detected by specific radioligand binding (Lansdell *et al.*, 1997).

6.5 Investigation into region of temperature-sensitivity of ALS using ALS / rat $\alpha 4$ chimeric constructs

In order to identify regions of the *Drosophila* nAChR subunit responsible for the temperature-sensitive folding, a series of chimeric constructs were designed using the ALS and $\alpha 4$ subunit cDNAs (Figure 6.3). The chimeric cDNAs were then transiently expressed in HEK-293 cells with the rat $\beta 2$ subunit at 37°C. Transfected cells were then incubated at either 37°C or 25°C for 24 h. Radioligand binding with [³H]-epibatidine was performed 24 h after transfection.

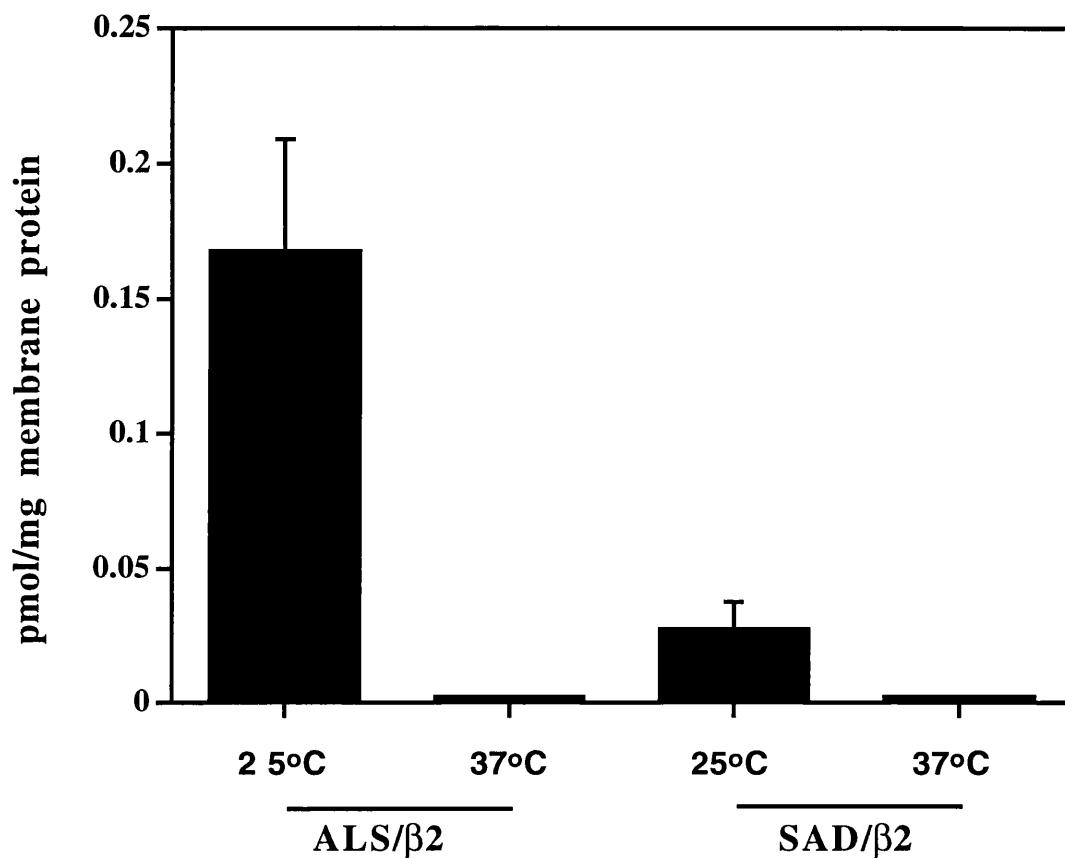


Fig. 6.2: Temperature-sensitive co-expression of *Drosophila* neuronal nAChR α subunits and the rat β 2 subunit. Binding of [3 H]- epibatidine (3 nM) to transiently transfected HEK-293 cells revealed that expression of *Drosophila* nAChR subunits is temperature-sensitive. Cells incubated at 25°C but not at 37°C acquired the ability to bind the nicotinic radioligand. The data suggests that the *Drosophila* subunits misfold when expressed at 37°C.

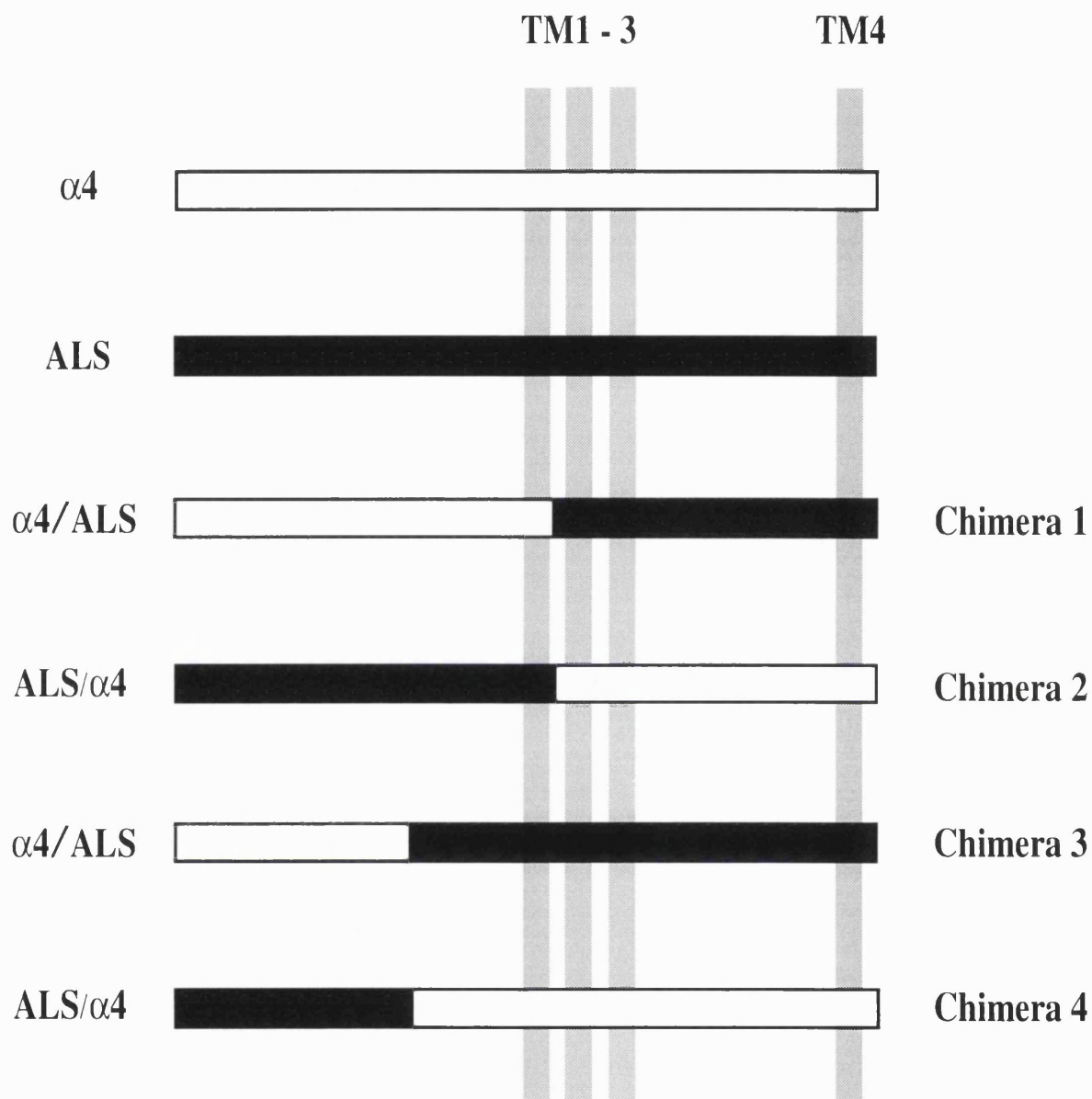


Fig. 6.3: A schematic representation of the four *Drosophila* ALS and rat $\alpha 4$ chimeric constructs designed to investigate the temperature-sensitive expression of the *Drosophila* ALS subunit. The chimeras were co-expressed with the rat $\beta 2$ subunit in the S2 cell line at 25°C and in the HEK-293 cell line at both 25°C and 37°C. Cells were screened for expression of the chimeric nAChR by [3 H]-epibatidine binding. The region of the chimeric cDNA encoding the ALS subunit is shown in black.

A chimeric cDNA (Chimera 1) was constructed which contained the coding region of the rat $\alpha 4$ cDNA up to and including TM1 (0-830) and the *Drosophila* ALS cDNA from the TM1 to end of the coding region (2100-3070). The region of ALS from TM1 to the end of the coding sequence was amplified by PCR, introducing the restriction sites *Dra*III and *Sal*I using the primers 5'-TGGCGAGA-AGATCACACTGTGCATCAGC-3' and 5'-GGCTCATCGTATATTGTCGACGATCATC-3'. The PCR conditions used were 96°C for 30 sec, 55°C for 45 sec and 72°C for 180 sec for 25 cycles with a ramp of 0.5°C / second. The PCR product was digested with the enzymes *Dra*III and *Sal*I and subcloned directionally into *Dra*III and *Sal*I cut pRmHa3- $\alpha 4$. Chimera 1 was excised from pRmHa3 and subcloned directionally into *Eco*RI and *Xba*I cut pcDNA3.

A chimeric cDNA (Chimera 2) was constructed which contained the coding region of the *Drosophila* ALS cDNA up to and including TM1 (1180-2100) and the rat $\alpha 4$ cDNA from TM1 to the end of the coding region (820-1950). This was constructed by two step PCR. ALS was amplified using a 5' primer to the vector pRmHa3 5'-CAATGTGCATCAGTTGTGGTCAGC-3' (oligo 43) and an internal primer to ALS with an $\alpha 4$ overhang 5'-CCCAGCGACTCTGGCGAGAAGATCTCGCTCTGC-3'. $\alpha 4$ was amplified using a 3' vector primer 5'-TCTAGCATTTAGGTGACACTATAG-3' (SP6 primer) and an internal $\alpha 4$ oligo with an ALS overhang 5'-CCTTCAGAGTGTGGCGAGAAGGTCACACTGTGC -3'. The PCR conditions used are as described for Chimera 1. The two PCR products were gel purified and then mixed in equal amounts. The whole chimera was then amplified by PCR using the two vector primers oligo 43 and SP6. The resultant PCR band was then gel purified, restriction digested and subcloned directionally into *Kpn*I and *Xba*I cut pcDNA3.

The third chimeric construct cDNA (Chimera 3) was constructed which contained the coding region of the rat $\alpha 4$ cDNA to a region preceding TM1 (0 - 535) and then the

Drosophila ALS cDNA to the end of the coding region (1790 - 3590). This was constructed by two step PCR. $\alpha 4$ was amplified by PCR using a 5' vector primer 5'-TAATACGACTCACTATAGGG-3' (T7 primer) and an internal primer to $\alpha 4$ with an ALS overhang 5' - GGATCCTGGACCTACGATGGTTATATGGTGGACTTC -3'. ALS was amplified using a 3' vector primer 5'-AGGAGAAGAATGT-GAGTGTGCATC-3' (oligo 41) and an internal ALS primer with an $\alpha 4$ overhang 5'-ACCATCGTAGGTCCAGGATCCAACTTCATGGTACA-3'. The PCR conditions used were as described for Chimera 1. The two PCR products were gel purified and then mixed in equal amounts. The whole chimera was then amplified by PCR using the two vector primers oligo 41 and T7. The resultant PCR band was then gel purified, restriction digested and subcloned directionally into *Kpn*I and *Xba*I cut pcDNA3.

A chimeric cDNA (Chimera 4) was constructed which contained the coding region of the *Drosophila* ALS cDNA to a region preceding TM1 (1180 - 1790) and then the rat $\alpha 4$ cDNA to the end of the coding region (535 - 1950). This was constructed by two step PCR using the conditions described for Chimera 1. ALS was amplified using a 5' primer to the pRmHa3 5'-CAATGTGCATCAGTTGTGGTCAGC-3' (oligo 43) and an internal ALS primer introducing an $\alpha 4$ overhang 5'-GCACAGTGTGACCTTCTCGCCAGAGTCGCTGGGCAG -3'. $\alpha 4$ was amplified using a 3' vector primer 5'-TCTAGCATTTAGGTGACACTATAG-3' (SP6 primer) and an internal primer to $\alpha 4$ with an ALS overhang 5'-CCCAGCGACTCTGGCGAGAAGGTCACACTGTGCATC -3'. The two PCR products were gel purified and then mixed in equal amounts. The whole chimera was then amplified by PCR using the two vector primers oligo 43 and SP6. The resultant PCR band was then gel purified, restriction digested and subcloned directionally into *Kpn*I and *Xba*I cut pcDNA3.

6.6 [³H]-epibatidine binding to chimeric *Drosophila*/rat nAChRs expressed in S2 and HEK-293 cells

Chimera 1 when co-expressed with the rat nAChR β 2 subunit in stably transfected S2 cells showed specific [³H]-epibatidine binding (Figure 6.4). The chimera was subcloned into the mammalian expression vector pcDNA3 and transiently co-transfected with the rat β 2 subunit into HEK-293 cells. The transfected cells were then screened for [³H]-epibatidine binding at 37°C and 25°C (Figure 6.5). Specific binding was detected at both temperatures indicating that the region of ALS involved in the temperature-sensitivity is located in the N-terminal region.

Transiently-transfected HEK-293 cells lines were also established which co-expressed either Chimera 2, 3 or 4 with the rat β 2 nAChR subunit. None of resulting transfected cell lines showed any specific [³H]-epibatidine binding at either 37°C or 25°C suggesting that these chimeric constructs are incapable of folding into a conformation capable of binding nicotinic radioligands at either temperature.

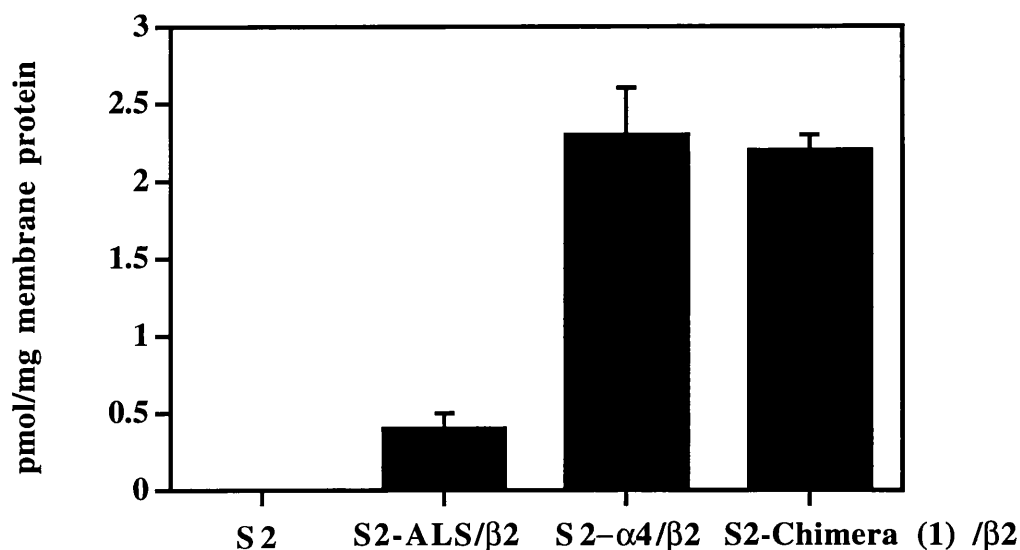


Fig. 6.4: Co-expression of the rat $\beta 2$ nAChR subunit with either ALS, $\alpha 4$ or the $\alpha 4$ /ALS chimera-1 construct in the *Drosophila* S2 cell line. Binding of [3 H]-epibatidine (3nM) to stably transfected S2 cell lines (n=3) indicated that the chimeric construct co-assembled with the $\beta 2$ nAChR subunit forming a complex which bound [3 H]-epibatidine.

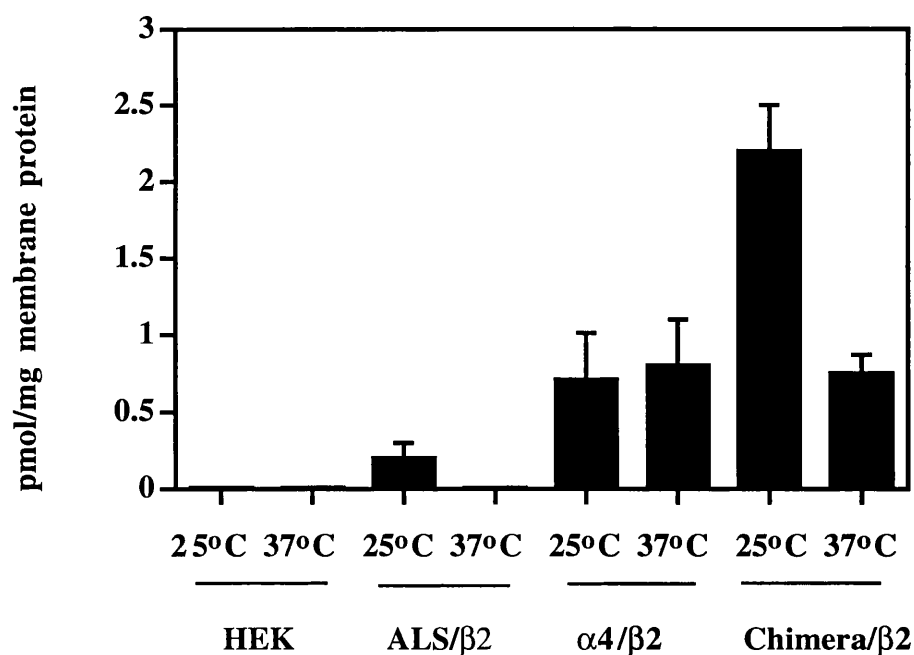


Fig. 6.5: Co-expression of the rat $\beta 2$ nAChR subunit with either ALS, $\alpha 4$ or the $\alpha 4$ /ALS chimera-1 construct in the HEK-293 cell line at 25°C and 37°C. Binding of [3 H]-epibatidine (3nM) to transiently-transfected cell lines (n=3) indicated that the chimeric construct co-assembled with the $\beta 2$ nAChR subunit forming a complex which bound [3 H]-epibatidine at both temperatures.

6.7 Discussion

It has been reported previously that the functional expression of the four cloned *Torpedo* nAChR subunits in mammalian fibroblast cells is temperature-sensitive (Claudio, 1987). It has also been possible to show that this temperature-sensitivity is a property which is intrinsic to the *Torpedo* nAChR subunits by the expression of *Torpedo*-rat hybrid nAChRs in a rat myoblast cell line (Paulson and Claudio, 1990). The rat myoblast cell line normally expresses an endogenous AChR at 37°C. Both the fibroblast and myoblast cell lines expressed exogenous *Torpedo* subunits at 37°C and 26°C but neither expresses an assembled *Torpedo* subunit at 37°C. At 26°C, however, the cell lines expressing all *Torpedo* subunits express assembled *Torpedo* nAChRs and in the myoblast cell lines expressing the *Torpedo* α subunit *Torpedo*-rat hybrid nAChRs were expressed. The results were inferred that the temperature-sensitivity is directly attributable to *Torpedo* subunits and not specific to the fibroblast cell line. This study has shown a similar temperature-sensitivity of *Drosophila* nAChR subunits.

Other cloned *Drosophila* neurotransmitter receptors have been expressed successfully in both mammalian cells (at 37°C) and in the *Drosophila* S2 cell line, including a muscarinic AChR (Blake *et al.*, 1993; Millar *et al.*, 1995) and a dopamine receptor (Han *et al.*, 1996). It is possible therefore that the misfolding of *Drosophila* nAChR subunits reflect differences between the folding of ionotropic and G protein-coupled receptor proteins.

The results in this study did not reproduce in a mammalian cell line (grown at 37°C) the successful co-expression in *Xenopus* oocytes of the *Drosophila* nAChR α subunits (ALS and SAD) with the vertebrate neuronal nAChR $\beta 2$ (Bertrand *et al.*, 1994; Lansdell *et al.*, 1997). However, by growing transfected mammalian HEK-293 cells at a lower temperature (25°C), the *Drosophila* nAChR α subunits acquired the ability to bind nicotinic radioligands. This is interpreted as demonstrating that the

Drosophila nAChR subunits were misfolding when expressed at 37°C. This interpretation is supported by the observation that these subunits co-assembled with the rat $\beta 2$ subunit into a conformation which generated an agonist binding site when co-expressed in either *Drosophila* S2 cells (at 25°C) or in *Xenopus* oocytes (at 18°C). Further studies with *Drosophila* nAChR subunits heterologously expressed in the mammalian HEK-293 cell line will be conducted at 25°C. The use of chimeric constructs has in part identified the temperature-sensitive region of ALS to a region N-terminal to the second transmembrane domain. Further investigation into the ALS/ $\alpha 4$ chimeric constructs is necessary to establish which region(s) of the ALS cDNA confer temperature-sensitivity.

7.0 Novel *Drosophila* and *C. elegans* nAChR subunits

7.1 Introduction

Despite expression in what might be considered a more native cellular environment, *Drosophila* nAChR subunits (as assayed by radioligand binding) could not be detected in the *Drosophila* S2 cell line unless *Drosophila* nAChR subunit cDNAs were co-expressed with vertebrate nAChR subunits. The results strongly suggest that the inability of these *Drosophila* nAChR subunits to generate functional channels in the absence of vertebrate subunits is due to a requirement for co-assembly with, as yet unidentified *Drosophila* nAChR subunits. I was aware of several attempts to identify novel *Drosophila* nAChR cDNAs by conventional hybridisation methods

. For this reason PCR techniques were employed in an attempt to identify novel nAChR cDNAs in several *Drosophila* cDNA libraries using probes designed to various regions of cloned *Drosophila* and rat nAChR subunits.

7.2 The novel *Drosophila* nAChR subunit D α 3 (ADR)

During the course of my project we became aware of a novel (fifth) nAChR subunit cDNA which had been cloned from *Drosophila*. Although this subunit (D α 3 or ADR) is unpublished we were provided with this cDNA clone in the expression vector pBluescript by Dr Bertram Schmitt, Frankfurt.

This novel subunit has been classified as an α nAChR subunit because it has two adjacent cysteine residues at positions equivalent to cysteine residues 192 and 193 in the *Torpedo* nAChR α subunit. The nucleotide sequence for this subunit had not been fully elucidated. Uncertainties, especially in the intracellular loop between TM3 and TM4, were evident. The third intracellular loop is much longer than in the other *Drosophila* nAChR subunit cDNAs.

7.3 Subcloning the novel *Drosophila* nAChR subunit D α 3 into the *Drosophila* expression vector pRmHa3

The pBluescript-D α 3 construct contained a 5' untranslated region which had an upstream ATG close to the signal sequence. Due to the lack of a complete nucleotide sequence and the lack of suitable restriction sites to subclone the D α 3 subunit from pBluescript into pRmHa3 in such a way to remove the upstream ATG and to optimise the start codon consensus sequence, the D α 3 was subcloned by PCR.

Two PCR oligonucleotides of 26 and 32 bases in length were designed to the 5' and 3' ends of D α 3. The primers were designed to introduce an *Eco*RI and a *Bam*HI site at the ends of the PCR product which could be used to subclone the D α 3 fragment directly into the pRmHa3 expression vector.

The PCR primers 5'- CGGAATTCGAGATGAAGTGGTTTCAAGTGACC- 3' and 5'- CGGGATCCGCTATCCTCCGTCTCTGG -3' were used to amplify the D α 3 subunit from the pBluescript-D α 3 cDNA. The conditions used were: 30 s at 95°C, 45 s at 55°C and 180 s at 72°C for 25 cycles with a ramp of 0.5°C/second using Pfu DNA polymerase (Stratagene). The resultant PCR product was digested with *Eco*RI and *Bam*HI and purified from a low melting point agarose gel. The PCR product was subcloned into the plasmid expression vector pRmHa3 which had been digested with *Eco*RI and *Bam*HI. The pRmHa3-D α 3 construct was checked for correct orientation of the cDNA by restriction mapping and nucleotide sequencing.

7.4 Radioligand binding studies with stably transfected S2 cell lines expressing the novel *Drosophila* nAChR subunit D α 3

Stably transfected S2 cell lines were established expressing various nAChR subunit combinations: D α 3 alone, D α 3 with rat β 2, D α 3 with rat β 4 and all five cloned *Drosophila* subunits D α 3/ALS/ARD/SAD/SBD. Membrane preparations of the each of cell lines were assayed by [³H]-epibatidine binding (3 nM) in the presence or

absence of 1 mM carbachol to determine non-specific binding. No specific binding was observed with any of the S2 cell lines expressing D α 3 in any of these combinations (data not shown).

7.5 Metabolic labelling and immunoprecipitation

The expression and co-assembly of the D α 3 nAChR subunit with the rat β 2 nAChR subunit was investigated by co-precipitation studies using the monoclonal antibody mAb290 which is specific for the rat β 2 subunit. This antibody had been used successfully to show co-assembly of the *Drosophila* α subunits ALS and SAD with β 2 (Figure 5.3). The results of the co-precipitation experiment are shown in Figure 7.1. The rat β 2 subunit was immunoprecipitated by mAb290. The *Drosophila* D α 3 subunit did not cross react with and was not co-precipitated by mAb290 when co-expressed with the rat β 2 subunit.

7.6 Screening *Drosophila* cDNA libraries for potential novel nAChR subunits

The inability of the *Drosophila* nAChR subunits to co-assemble into nAChRs in the absence of vertebrate subunits suggests that this could be due to a requirement for co-assembly with, as yet, unidentified nAChR subunits. In an attempt to resolve this, several *Drosophila* cDNA libraries were screened by PCR using various combinations of primers designed to conserved regions of nAChR subunits. Primers and degenerate primers were designed to the conserved regions of the cloned *Drosophila* TM2, TM3 and TM4 domains. Another PCR screening strategy which was used, employed a forward primer designed to the library vector sequence and a reverse internal primer to a highly conserved region in all previously identified nicotinic subunits. Full details of the primers and techniques used are described in Section 2.27 and one of the strategies used is summarised in Figure 7.2.

Specific and degenerate PCR primers designed to TM3 and TM4 of the four cloned *Drosophila* nAChR subunits were used to screen four *Drosophila* cDNA libraries.

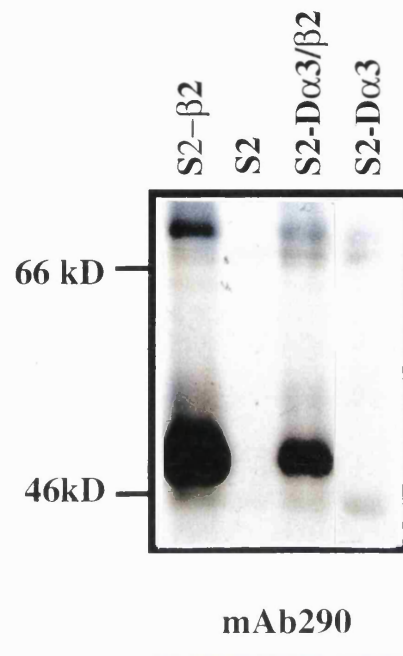


Fig. 7.1: Co-immunoprecipitation of *Drosophila* and rat nAChR subunits stably expressed in *Drosophila* S2 cells. The rat $\beta 2$ subunit was immunoprecipitated by mAb290 (raised against the rat $\beta 2$ subunit). The *Drosophila* D $\alpha 3$ (ADR) subunit did not cross react with and was not co-precipitated by mAb290 when co-expressed with rat $\beta 2$.

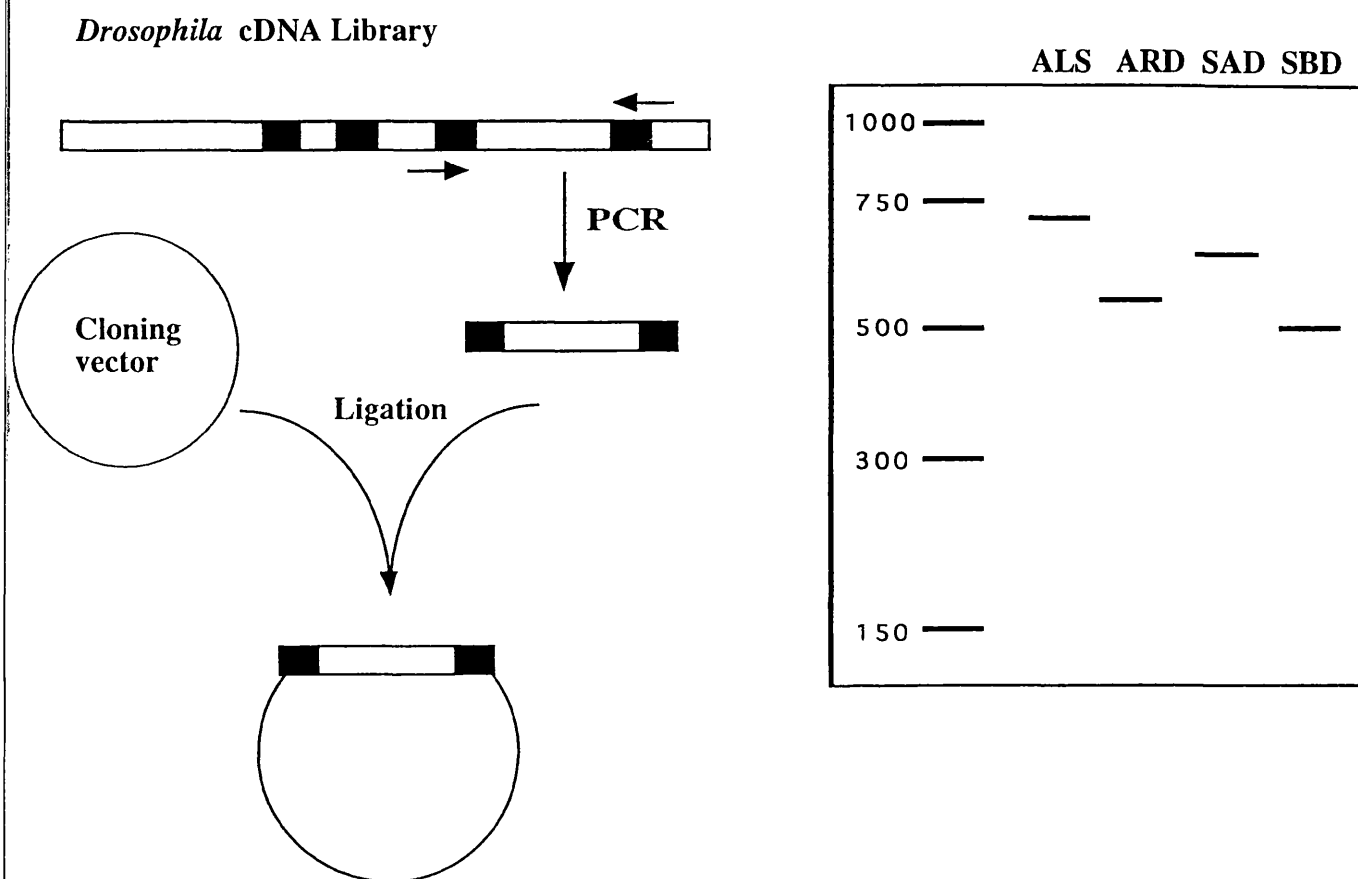


Fig. 7.2: A schematic diagram showing one of the strategies used to screen *Drosophila* cDNA libraries for novel nAChR subunits. Degenerate oligonucleotides were synthesised to the regions of TM3 and TM4 that are highly conserved between nAChR subunits. The cDNA libraries were screened by PCR using these degenerate primers. The PCR products were run on an agarose gel alongside PCR products where the same oligonucleotides had been used to amplify the four previously identified *Drosophila* nAChR subunits (shown diagrammatically). PCR products that were of a different size to those from ALS, ARD, SAD or SBD were ligated into the TA cloning vector and sequenced.

The PCR protocol used was successful, producing bands that were subcloned into the TA cloning vector (Invitrogen) and sequenced. Using this method, the subunits ALS, ARD, SAD and SBD were isolated from the cDNA libraries using the degenerate primers. Other PCR products were shown to be non-specific when they were sequenced and identified by alignment with sequences on the EMBL/Genbank nucleotide database.

ALS and SAD have been shown to co-assemble with the rat $\beta 2$ and $\beta 4$ subunits in the S2 cells, HEK-293 cells and *Xenopus* oocytes (data presented in Chapter 5). These results suggested that there could be another *Drosophila* nAChR structural which is similar to the vertebrate β subunits. Degenerate primers were designed based on the third and fourth transmembrane domains of *Drosophila* β subunits ARD and SBD. Degenerate primers based on the rat $\beta 2$ and $\beta 4$ nAChR subunits were also designed. These were used to screen the *Drosophila* cDNA libraries for novel β like subunits.

The various combinations of primers used to screen the *Drosophila* cDNA libraries were successful in that the four previously cloned *Drosophila* nAChR subunits ALS, ARD, SAD and SBD were isolated. However, no potential novel nAChR like subunits were identified using this protocol.

7.7 A novel *C.elegans* nAChR subunit Ce21

In the latter stages of this project, a novel *C. elegans* nAChR subunit, Ce21, was kindly provided by Dr. Mark Ballivet. Ce21 had been shown to form functional homomeric receptors in when expressed in *Xenopus* oocytes (Ballivet *et al.*, 1996) and appears have most sequence similarity to the vertebrate $\alpha 7$ nAChR subunit. The Ce21 nAChR subunit and the vertebrate $\alpha 7$ nAChR subunit are pharmacologically similar both being activated by ACh and nicotine and both antagonised by dihydro- β -erythrodine and levamisole. Both receptors desensitise quickly upon application of

agonist. Unlike the $\alpha 7$ receptor, the Ce21 receptor is relatively insensitive to methyllycaconitine and to α -BTX.

7.8 Subcloning Ce21 into pRmHa3

Ce21 was excised from its cloning vector pFlip by digestion with *Hind*III and *Bam*HI and the single strand overhangs were removed by treatment with T4 DNA polymerase. The excised Ce21 cDNA was subcloned into *Sma*I cut and alkaline phosphatase treated pRmHa3. The pRmHa3-Ce21 construct was checked for the correct orientation of the cDNA by restriction mapping and nucleotide sequencing.

7.9 Transfection of Ce21 into the *Drosophila* S2 cell line

Polyclonal stable S2 cells lines expressing the Ce21 nAChR alone and co-expressed with the rat $\beta 2$ subunit were established. The polyclonal cell lines were screened for nicotinic radioligand binding with the ligands: [3 H]-epibatidine (3 nM), [3 H]-ACh (30 nM), [3 H]-nicotine (30 nM) and [3 H]-methylcarbamylcholine (30 nM). No specific ligand binding was detected with any of the four ligands tested for Ce21 expressed alone or co-expressed with rat $\beta 2$.

Metabolic labelling and immunoprecipitation with mAb290 was carried out on the stably transfected S2 cell lines: Ce21, Ce21/ $\beta 2$ and $\beta 2$. As shown in Figure 7.3, the Ce21 subunit was not co-precipitated with the $\beta 2$ subunit and did not cross react with mAb290. The *Drosophila* nAChR subunit SAD was successfully co-precipitated by mAb290 when co-expressed with $\beta 2$ in the same experiment as a positive control (data not shown). In addition, the *C. elegans* α nAChR subunit UNC-38 was not co-immunoprecipitated by mAb290 when it was co-expressed with rat $\beta 2$ in S2 cell lines (data not shown).

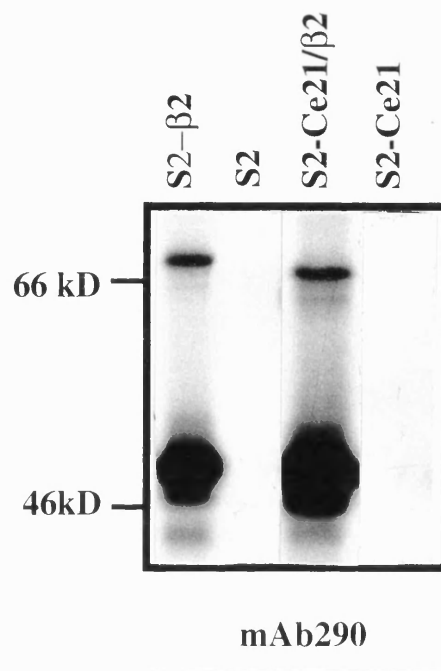


Fig. 7.3: Co-immunoprecipitation of *C. elegans* and rat nAChR subunits stably expressed in *Drosophila* S2 cells. The rat β2 subunit was immunoprecipitated by mAb290 (raised against the rat β2 subunit). The *C. elegans* Ce21 subunit did not cross react with and was not co-precipitated by mAb290 when co-expressed with the rat β2 subunit.

7.10 Transfection of Ce21 into the mammalian HEK-293 cell line

To establish whether the *C. elegans* nAChR Ce21 subunit formed homomeric channels, the subunit was transiently transfected into the mammalian HEK-293 cell line. The transfected HEK-293 cells were screened for specific radioligand binding using the nicotinic ligands: [³H]-epibatidine (3 nM), [³H]-ACh (30 nM), [³H]-nicotine (30 nM) and [³H]-methylcarbamylcholine (30 nM). No specific binding was detected with any ligand tested.

7.11 Transfection of Ce21 into GH₄C₁ cells and SH-SY5Y cells

The Ce21 nAChR subunit has been shown to resemble the vertebrate $\alpha 7$ nAChR subunit (Ballivet *et al.*, 1996). Whereas the homo-oligomeric $\alpha 7$ subunit has been shown to misfold when expressed in some mammalian cell lines, the expression of correctly folded $\alpha 7$ has been demonstrated in GH₄C₁ cells and SH-SY5Y cells (Cooper and Millar, 1997). For these reasons polyclonal GH₄C₁ and SH-SY5Y cell lines expressing Ce21 were established to investigate whether expression of Ce21 is dependent on the host cell. The rat pituitary cell line GH₄C₁ was transfected with the pFlip-Ce21 cDNA alone and also co-transfected with pcDNA1- $\beta 2$ and pFlip-Ce21 using the calcium phosphate precipitation protocol (Section 2.28). Selection of transfected cells by the addition of 0.8 mg/ml geneticin (G-418 sulphate) was initiated after 24 h. The resultant polyclonal cell lines were screened for nicotinic radioligand binding with the same ligands used to screen the S2 and HEK cell lines (Sections 7.9 and 7.10). As with the other cell lines transfected with Ce21 no specific radioligand binding was detected in GH₄C₁ cells. Although low levels of nicotinic radioligand binding could be detected in SH-SY5Y cells (as reported previously, Cooper and Millar, 1997), no elevation in the level of binding was seen in SH-SY5Y cells transfected with Ce21.

Stable cell lines were also established in which Ce21, alone or co-expressed with the rat nAChR $\beta 2$ subunit, in the human neuroblastoma SH-SY5Y cell line. SH-SY5Y

cells were transfected by lipofectamine with pMT3-Ce21 and the vector pcDNA3 ± pcDNA1-β2. The pcDNA3 vector was co-transfected with the other plasmids to confer resistance to G-418 selection. The transfected cells were selected by growth in medium containing 1 mg/ml G-418. The polyclonal cell lines were screened with the radioligands [³H]-epibatidine (3 nM), [³H]-nicotine (30 nM), [¹²⁵I]-αBTX (0.25 - 2.5 nM) and [³H]-methylcarbamylcholine (30 nM). No increase in specific binding above levels of binding to endogenous nAChRs was detected with any nicotinic radioligand in SH-SY5Y cells transfected with Ce21 or co-transfected with the Ce21 and β2 nAChR subunit.

7.12 A *Drosophila* expressed sequence tag encoding a putative nAChR subunit

An expressed sequence tag (EST) nucleotide sequence, part of the Berkeley *Drosophila* Genome Project /HHMI *Drosophila* EST Project (accession number AA540687), was recently deposited in the EMBL/Genbank nucleotide database. This short sequence shows similarity to the *Drosophila* SBD mRNA. The sequence of 512 bases had been isolated from a *Drosophila* Embryo library. The *Drosophila* library is an LD library (made by Ling Hong) using mRNA from 0 - 22 h embryos. The cDNA was made using the Stratagene ZAP-cDNA synthesis kit and subcloned into the Stratagene Uni-Zap XR vector. The clone (LD20458) was obtained from Genome Systems as a bacterial stab. The data on the *Drosophila* EST sequence is unpublished, however, further information can be obtained by contacting the Web site <http://www.fruitfly.berkeley.edu/> or by Email to EST@fruitfly.berkeley.edu.

The clone was streaked onto an LB/agar plate containing 50 µg/ml ampicillin and the individual colonies were verified by nucleotide sequencing. Restriction mapping showed that the EST cDNA insert was approximately 1.4 Kb. The entire cDNA clone was sequenced (on both strands) using a series of oligonucleotide primers. The primers used for sequencing were:

Forward oligonucleotide primers:

5'-AATTAACCCTCACTAAAGGG-3' (Oligo T3 to pBluescript)

5'-AGTCTTCACGTGTTCTTTCTTTTGGT-3' (Oligo 220)

5'-ACGGCGAATGGACCATTTGGAGGAAG-3' (Oligo 226)

5'-ACAAATATCGATCGACTCGTGTCTTC-3' (Oligo 228)

5'-CTTGTTGCGACGAACCATACTTGGAC-3' (Oligo 231)

Reverse oligonucleotide primers:

5'-TAATACGACTCACTATAGGG-3' (Oligo T7 to pBluescript)

5'-AAATGTTAATACAGTTAAAAATGATATT-3' (Oligo 218)

5'-GTGAAGACTAATAAGAATGGAGATAGA-3' (Oligo 219)

5'-GGTCTACAGCGACCGGCTGATGTTCC-3' (Oligo 224)

5'-CTTCCTCCAAATGGTCCATTCGCCGT-3' (Oligo 227)

5'-GCAACGTATTTCCAATCCTCTTTAAC-3' (Oligo 230)

The full nucleotide sequence of the EST clone is shown in Figure 7.4 and the amino acid sequence is aligned with the four *Drosophila* sequences in Figure 7.5. The EST sequence appears to be a partial nAChR clone which is missing the N-terminal region. The EST clone contains the two conserved cysteine residues in positions analogous to 192 and 193 in the *Torpedo* α subunit and by convention should be classified as an α subunit. For the purposes of this thesis I will refer to the subunit encoded by this sequence as D α 4. Compared to amino acid sequences derived from a preliminary nucleotide sequence of D α 3 which was determined during this project, D α 4 shows approximately 86% identity at the amino acid level to the unpublished D α 3 subunit.

7.13 Screening *Drosophila* cDNA libraries for the 5' end of D α 4

Several *Drosophila* cDNA libraries were screened using PCR primers in an attempt to isolate the N-terminal region of the partial nAChR clone D α 4 using the approach which was used successfully to clone the 5' end of the SBD cDNA (Section 3.2).

1	GGCACATGGA	TGAACAACAG	GGCAGCAACG	TTGTTGCTGT	GGGTGTGGAC	50
51	CTATCTGAAT	TTTATATGTC	TGTGGAATGG	GACATTCTCG	AAGTTCCAGC	100
101	TGTTAGGAAT	GAAAAGTTT	ATACTTGTTG	CGACGAACCA	TACTTGACACA	150
151	TCACATTTAA	TATAACAATG	AGAAGGAAGA	<u>CGCTTTTTTA</u>	<u>TACTGTTAAT</u>	200
201	<u>ATAATAATAC</u>	<u>CGTGCATGGG</u>	<u>AATATCATTT</u>	<u>TTAACTGTAT</u>	<u>TAACATTTTA</u>	250
251	<u>CCTTCCATCG</u>	<u>GATAGTGGAG</u>	<u>AAAAGGTCAC</u>	<u>ACTATCTATC</u>	<u>TCCATTCTTA</u>	300
301	<u>TTAGTCTTCA</u>	<u>CGTGTTCCTT</u>	<u>CTTTTGGTGG</u>	<u>TTGAAATTAT</u>	<u>ACCACCAACG</u>	350
351	TCATTGGTTG	TTCCTTTGCT	GGGAAAATAC	CTAATATTTG	CCATGATACT	400
401	<u>TGTGTCTATC</u>	<u>AGTATCTGCG</u>	<u>TGACGGTTGT</u>	<u>TGTTTTGAAC</u>	<u>GTTCAATTTTC</u>	450
451	GTTACACCTCA	AACTCATAAA	ATGGCACCCCT	GGGTAAAGCG	CGTCTTTATT	500
501	GACTTTTTTGC	CAGCGTTTTT	ATTCATTAAA	AGGCCGACGT	ACAACTTTGA	550
551	AACGAGTAAA	TTACTTTTAA	AGGACACACA	TGCTTGCTTT	TATCCTTATT	600
601	ATTCTGCAAC	AAATATCGAT	CGACTCGTGT	CTTCAGGATA	CAACAATAGT	650
651	CTGCCAAAAG	AAGATTTATC	GCAAAGCATT	ACGGCGAATG	GACCATTTGG	700
701	AGGAAGTTGT	CAGGTTTCATG	GCCCAGTGCC	GCCACTCACC	CATTGTAGTT	750
751	CAGACGAAAT	AGCCGCCGTC	CCCAGATATGG	ATATCGGACC	CTTAGGGATG	800
801	AAAAGTCCCA	TTTTAAATAA	TCCAGCATTT	TCCCACTCTA	AATGCCTTCC	850
851	TAAAATTCAC	AAAAGCTGTT	TTTGTGTGCG	CTTTATTGCA	GAGCATACAA	900
901	AAATGCAAGA	GGACTCAACT	AAAGTTAAAG	AGGATTGGAA	ATACGTTGCT	950
951	ATGGTATTGG	ATAGACTTTT	<u>TTTGTGGATA</u>	<u>TTCACGTTGG</u>	<u>CAGTTGTGGT</u>	1000
1001	<u>TGGGACCGCC</u>	<u>GGAATAATTT</u>	<u>TACAAGCTCC</u>	<u>TACGTTATAT</u>	<u>GACGACCGCA</u>	1050
1051	TCCCGATTAT	TGAGCAAAAAG	GATTTGGACT	TTTCAGGAAC	ATCAGCCGGT	1100
1101	CGCTGTAGAC	CTCCACAATA	ACATTATTAT	ATTAAAAGAA	TTTAATTTTA	1150
1151	AACTGAGGTG	TATCAGATTA	TTATTACAAA	ATGAACTTTA	ACTAATATTT	1200
1201	TCTGCAGGAA	AAAATGTTAA	AAATTACATA	TGTGTGTATT	GAAGGCTTTA	1250
1251	AGTAACAAAT	TACCTATTTT	TTATTTTGA	CCACATTTAG	ATATTCCAAA	1300
1301	GTAATTTGGT	ATTTTATAGG	ACTTCAAACC	ACGACATTTT	TTTTATTTAT	1350
1351	CCTTGTCAAC	GAACTTAAAG	AATAA			1376

Fig. 7.4: Nucleotide sequence of the *Drosophila* expressed sequence tag cDNA incomplete (LD20458) identified and preliminary sequencing (512 bp) performed by the Berkeley *Drosophila* Genome Project (Genome Systems, Berkeley). The four proposed transmembrane domains are underlined.

	1				50
ALSMGSVLFAAV	FIALHFATGG	L...ANPDAK
SBDM	WHWSLLCVFL	LVPLANSTAP	ISFEANPDTK
SAD	MAPGCCTTRP	RPIALLAHIW	RHCKPLCLLL	VLLLLCETV.	...QANPDAK
LD2048	-----	-----	-----	-----	-----
ARDMESSCKSWL	LCSILVLVAF	SLVSASEDEE
	51				100
ALS	RLYDDLLSNY	NRLIRPVGNN	SDRLTVKMGL	RLSQLIDVNL	KNQIMTTNVW
SBD	RLYDDLLSNY	NRLIRPVVNN	TETLTVWLGL	KLSQLIEVNL	KNQVMTTNLW
SAD	RLYDDLLSNY	NRLIRPVSNN	TDTVLVKLGL	RLSQLIDLNL	KDQILTNTNVW
LD2048	-----	-----	-----	-----	-----
ARD	RLVRDLFRGY	NKLIRPVQNM	TQKVGVRFGL	AFVQLINVNE	KNQVMKSNVW
	101				150
ALS	VEQEWN DYKL	KWNPDDYGGV	DTLHVPSEHI	WHPDIVLYNN	ADGNYEVTIM
SBD	VKQRWF DYKL	RWDPEEYGGV	EQLYVPSEHI	WVPDIVLYNN	WDGNYEVTLM
SAD	LEHEWQD HKF	KWDPSEYGGV	TELYVPSEHI	WLPDIVLYNN	ADGEYVVTM
LD2048	-----	-----	-----	-----	-----
ARD	LRLVWYDYQL	QWDEADYGGI	GVLRLPPDKV	WKPDIVLFNN	ADGNYEVRYK
	151				200
ALS	TKAILHHTGK	VVWKPPAIYK	SFCEIDVEYF	PFDEQTCFMK	FGSWTYDGYM
SBD	TKATLKYTGE	VFWEPPIYK	SSCEMNVEYF	PYDEQICFMK	FGSWTYNGAQ
SAD	TKAILHYTGK	VVWTPPAIFK	SSCEIDVRYF	PFDQQTTCFMK	FGSWTYDGDQ
LD2048	-----	-----	-----	-----	-----
ARD	SNVLIYPTGE	VLWVPPAIYQ	SSCTIDVTYF	PFDQQTTCIMK	FGSWTFNGDQ
	201				250
ALS	VDLRHLKQTA	DSDN.IEVGI	DLQDYVISVE	WDIMRVPAVR	NEKFYSCCEE
SBD	VDLKHLQIP	GSNL.VQVGI	DLTEFYLSVE	WDILEVPATK	NEEYYPDTLE
SAD	IDLKHISQKN	DKDNKVEIGI	DLREYYPSVE	WDILGVPAER	HEKYYPCCAE
LD2048	VDLRHMDEQQ	GSNV.VAVGV	DLSEFYMSVE	WDILEVPAVR	NEKFYTCCDE
ARD	VSLALYNNKNFV	DLSDYWKSGT	WDIEVPAYL	NVYEGDSNHP
	*		** *	*** **	
	251		TM1		300
ALS	PYLDIVFNLT	LRRKTLFYTV	NLIIPC VGIS	FLSVLVFYLP	SDSGEKISLC
SBD	PFSDITFKLT	MRRKTLFYTV	NLIVPC VALT	FLTVLVFYLP	SDSGEKVTLC
SAD	PYPDIFFNIT	LRRKTLFYTV	NLIIPC VGIS	YLSVLVFYLP	ADSGEKIALC
LD2048	PYLDITFNIT	MRRKTLFYTV	NIIIPC MGIS	FLTVLTFYLP	SDSGEKVTLS
ARD	TETDITFYII	IRRKTLFYTV	NLILPTVLIS	FLCVLVFYLP	AEAGEKVTLG
	** *	*****	* * *	* ** *	*** *
	301	TM2		TM3	350
ALS	ISILLSLTVF	FLLLA EIIPP	TSLTVPLL GK	YLLFTMMLVT	LSVVVTIAVL
SBD	ISILVSLTVF	FLLLA EIIPP	TSLAVPLL GK	YLLFTMILVS	LSVWTTVCVL
SAD	ISILLSQTMF	FLLISEIIPS	TSLALPLL GK	YLLFTMLLVG	LSVVITIIIL
LD2048	ISILISLHVF	FLLVVEIIPP	TSLVPLL GK	YLIFAMILVS	ISICVTVVVL
ARD	ISILLSLVVF	LLLVS KILPP	TSLVLPLIAK	YLLFTFIMNT	VSILVTVIII
	**** *	** *	*** ** *	** *	*

	351				400
ALS	NVNFRSPVTH	RMAPWVQRLF	IQILPKLLCI	ERPKKEEPPEE	DQPPEVLTDV
SBD	NIHFRSPSTH	NMSRLVRKLF	LHFMPKLMMM	RRTQ.....
SAD	NIHYRKPSTH	KMRPWIRSFF	IKRLPKLLLM	RVPKDLLRDL	AAN....KIN
LD2048	NVHFRSPQTH	KMAPWVKRVF	IDFLPAFLFI	KRPTYNFETS	KLLLKDTHAC
ARD	NWNFRGPPTH	RMPMYIRSIF	LHYLPAFLFM	KRPR.....
	* * * *	*	*		
	401				450
ALS	YHLPPDVDFK	VNYDSKRFSG	DYGIPALPAS	HRFDLAAAGG	ISAHCFAEPP
SBD	YTLP.....	DYDDSTPSNG	YTNEIDVRDS	ISD.....
SAD	YGLKFSKTKF	GQALMDEMOM	NSGGSSPDSL	RRMQGRVGAG	GCNGMHVTTA
LD2048	FYPYYSATNI	DRL....VSS	GYNNSLPKED	LSQSITANGP	FGGSCQVHGP
ARDKTRL	RWMMEMPGMS	MPAHPHPSYG	SPAELPKHIS	AIGGKQSKME
	451				500
ALS	LPSSLPLPGA	DDDLFSP.SG	LNGDISPGCC	PAAAAAAAAD	LSPTFEKPYA
SBD	FPSE..FKDS	QDGAYDN.GM	QNSVDSDNVI	P.....RNLT
SAD	TNRFSGLVGA	LGGGLSTLSG	YNGLPVLSG	LDDSLSDVAARKKYP
LD2048	VPPL.....	...THCSSDE	IAAVPMDIG	PLGMKSPILN	NPAFHSKCL
ARD	VMELSDLHHP	NCKINRKVNS	GCELGLGDGC	RRESESSDSI	L.....LS
	501				550
ALS	REMEKTIEGS	RFIAQHVKNK	DKFESVEEDW	KYVAMVLDRM	FLWIFAIACV
SBD	PEVLQALRAV	RFIAQHVKDA	DKDNEIVEDW	KFVSMVLDRF	FLWLFTLSCV
SAD	FELEKAIHNV	MFIQHHMQRQ	DEFNAEDQDW	GFVAMVMDRL	FLWLFMIAVL
LD2048	PKIHKSCFCV	RFIAEHTKMQ	EDSTKVKEDE	KYVAMVLDRM	FLWIFTLAVV
ARD	PEASKATEAV	EFIAEHLRNE	DLYIQTREDW	KYVAMVIDRL	QLYIFFIVTT
		** *	**	** *	* *
	TM4				600
ALS	VGTAIIILQA	PSLH...DQS	QPIDILYSKI	AKKKFELLKM	GSENTL....
SBD	FGTLAIICQS	PSLY...DTR	SPIDRQLSEI	PLRKNNFM.L	PPDIVRQVLT
SAD	VGTFVILGEA	PSLY...DDT	KAIDVQLSDV	AKQIYNLTEK	KN.....
LD2048	VGTAIIILQA	PTLY...DDR	IPI.....I	EQKDLDFSGT	SAGRCRPPQ.
ARD	AGTVGILMDA	PHIFEYVDQD	RIEIIYRGK.
	** *	*	*		

Fig. 7.5. Amino acid sequence alignment of the four previously published *Drosophila* nAChR subunits ALS (Bossy et al. 1988), ARD (Hermans-Borgmeyer et al. 1986), SAD (Bauman et al 1990, Sawruk et al. 1990) and SBD (Sawruk et al. 1990, Lansdell et al. 1997) and the *Drosophila* EST sequence (LD20458). Deduced amino acid sequences have been aligned by the GCG program Pile-up (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). Gaps (.) were introduced to maximise sequence identity. Asterisks show residues that are conserved in all four subunits. The putative transmembrane domains TM1, TM2, TM3 and TM4 are indicated by a line above the sequences. The missing 5' region is shown by dashed lines (-).

Various combinations of forward primers to the library vector, oligonucleotides T3 or T7, and the reverse D α 4 internal primers oligo 218 and oligo 219 (see below) were used. The PCR conditions used were: 95°C for 30 sec, 45°C, 50°C or 55°C for 60 sec and 72°C for 120 sec for a total of 30 cycles. The PCR products were subcloned into the TA cloning vector pCR2.1 (Invitrogen) and sequenced.

A positive TA clone was identified and sequenced in both directions with the primers:

Reverse primers:

5'-AAATGTTAATACAGTTAAAAATGATATT-3' (Oligo 218)

5'-GTGAAGACTAATAAGAATGGAGATAGA-3' (Oligo 219)

5'-CAGGAAACAGCTATGACCATGATTAC-3' (Oligo 225 to vector pCR2.1)

Forward Primers:

5'-CAGGAAACAGCTATGAC-3' ('M13 reverse' oligo to the vector pCR2.1)

5'-ACTTTCACAGCTTATAGATGTGAACC-3' (Oligo 232)

5'-GGTCGAGCAGTCGTGCTATGATTACA-3' (Oligo 233)

A PCR product was obtained which extended further towards the 5' end of the D α 4 gene. By comparison to other *Drosophila* nAChR subunits, it appears that the 5' end of this PCR fragment ends approximately 10 - 20 amino acids from the start of the signal peptide. The sequence of the isolated N-terminal region of D α 4 identified the clone as an incorrectly spliced isoform of the D α 4 subunit in which an exon that encodes 61 amino acids is missing. Sequencing of the D α 4 gene (rather than the cDNA) has not yet been performed to confirm the existence of the missing intron, but comparison with other *Drosophila* nAChR cDNAs strongly suggests that this is an incorrectly spliced isoform of D α 4. This incorrectly spliced variant does not maintain the original reading frame after the splice site and is therefore unlikely to be translated into a functional nAChR subunit. The amino acid alignment with ALS, ARD, SAD and SBD is shown in Figure 7.6. The full length D α 4 subunit has not been isolated to date but work to isolate the clone is ongoing.

	1				50
ALSMGSVLFAAV	FIALHFATGG	L...ANPDAK
SBDM	WHWSLLCVFL	LVPLANSTAP	ISFEANPDTK
SAD	MAPGCCTTRP	RPIALLAHIW	RHCKPLCLLL	VLLLLCETV.	...QANPDAK
Dα4 ESTKR	LNSSGNPDAK
ARDMESSCKSWL	LCSILVLVAF	SLVSASEDEE
					*
	51				100
ALS	RLYDDLLSNY	NRLIRPVGNN	SDRLTVKMGL	RLSQLIDVNL	KNQIMTTNVW
SBD	RLYDDLLSNY	NRLIRPVVNN	TETLTVWLGL	KLSQLIEVNL	KNQVMTTNLW
SAD	RLYDDLLSNY	NRLIRPVSNN	TDTVLVKLGL	RLSQLIDLNL	KDQILTTNVW
Dα4 EST	RLYDDLLSNY	NKLVRPVVNT	TDVLKVCIKL	KLSQLIDVNL	KNQIMTTNLW
ARD	RLVRDLFRGY	NKLIRPVQNM	TQKVGVRFGL	AFVQLINVNE	KNQVMKSNVW
	** *	* * * * *	* *	* * * *	* * *
	101				150
ALS	VEQEWNDYKL	KWNPDDYGGV	DTLHVPSEHI	WHPDIVLYNN	ADGNYEVTIM
SBD	VKQRWFDYKL	RWDPEEYGGV	EQLYVPSEHI	WVPDIVLYNN	WDGNYEVTLM
SAD	LEHEWQDHKF	KWDPSEYGGV	TELYVPSEHI	WLPDIVLYNN	ADGEYVVTM
Dα4 EST	VEQSWYDYKL	RWEPKEYGGV	HMLHVPDHI	WRPDIVLYN-	-----
ARD	LRLVWYDYQL	QWDEADYGGI	GVLRLPPDKV	WKPDIVLFNN	ADGNYEVRYK
	*	* * * *	*	* * * * *	*
	151				200
ALS	TKAILHHTGK	VVWKPPAIYK	SFCEIDVEYF	PFDEQTCFMK	FGSWTYDGYM
SBD	TKATLKYTGE	VFWPEPAIYK	SSCEMNVEYF	PYDEQICFMK	FGSWTYNGAQ
SAD	TKAILHYTGK	VVWTPPAIFK	SSCEIDVRYF	PFDQQTTCFMK	FGSWTYDGDQ
Dα4 EST	-----	-----	-----	-----	-----
ARD	SNVLIYPTGE	VLWVPPAIYQ	SSCTIDVTYF	PFDQQTTCIMK	FGSWTFNGDQ
	201				250
ALS	VDLRHLKQTA	DSDN.IEVGI	DLQDYVISVE	WDIMRVPAVR	NEKFYSCCEE
SBD	VDLKHLDQIP	GSNL.VQVGI	DLTEFYLSVE	WDILEVPATK	NEEYYPDTLE
SAD	IDLKHISQKN	DKDNKVEIGI	DLREYYPVSVE	WDILGVPAER	HEKYYPCCAE
Dα4 EST	VDLRHMDEQQ	GSNV.VAVGV	DLSEFYMSVE	WDILEVPAVR	NEKFYTCCDE
ARD	VSLALYNNKNFV	DLSDYWKSGT	WDIEVPAYL	NVYEGDSNHP
	*		** *	* * * *	
	251		TM1		300
ALS	PYLDIVFNLT	LRRKTLFYTV	NLIIPC VGIS	FLSVLVFYLP	SDSGEKISLC
SBD	PFSDITFKLT	MRRKTLFYTV	NLIVPCVALT	FLTVLVFYLP	SDSGEKVTLC
SAD	PYPDIFFNIT	LRRKTLFYTV	NLIIPC VGIS	YLSVLVFYLP	ADSGEKIALC
Dα4 EST	PYLDITFNIT	MRRKTLFYTV	NIIIPCMGIS	FLTVLTFYLP	SDSGEKVTLS
ARD	TETDITFYII	IRRKTLFYTV	NLILPTVLIS	FLCVLVFYLP	AEAGEKVTLG
	** *	* * * * *	* * *	* * * * *	* * *
	301	TM2		TM3	350
ALS	ISILLSLTVF	FLLLA EIIPP	TSLTVPLL GK	YLLFTMMLVT	LSVVVTIAVL
SBD	ISILVSLTVF	FLLLA EIIPP	TSLAVPLL GK	YLLFTMILVS	LSVWTTVCVL
SAD	ISILLSQTMF	FLLISEIIPS	TSLALPLL GK	YLLFTMLLVG	LSVVITIIIL
Dα4 EST	ISILISLHVF	FLLVVEIIPP	TSLVPLL GK	YLIFAMILVS	ISICVTVVVL
ARD	ISILLSLVVF	LLLVS KILPP	TSLVLPLIAK	YLLFTFIMNT	VSILVTVIII
	* * * *	* * *	* * *	* * *	*

		351			400
ALS		NVNFRSPVTH	RMAPWVQRLF	IQILPKLLCI	ERPKKEEPEE DQPPEVLTDV
SBD		NIHFRSPSTH	NMSRLVRKLF	LHFMPKLMMM	RRTQ.....
SAD		NIHYRKPSTH	KMRPWIRSFF	IKRLPKLLLM	RVPKDLLRDL AAN....KIN
D α 4	EST	NVHFRSPQTH	KMAPWVKRVF	IDFLPAFLFI	KRPTYNFETS KLLLKDTHAC
ARD		NWNFRGPPTH	RMPMYIRSIF	LHYLPAFLFM	KRPR.....
		* * * *	*	*	
		401			450
ALS		YHLPPDVDFK	VNYDSKRFSG	DYGIPALPAS	HRFDLAAAGG ISAHCFAEPP
SBD		YTLP.....	DYDDSTPSNG	YTNEIDVRDS ISD.....
SAD		YGLKFSKTKF	GOALMDEMOM	NSGGSSPDSL	RRMQGRVGAG GCNGMHVTTA
D α 4	EST	FYPYYSATNI	DRL...VSS	GYNNSLPKED	LSQSITANGP FGGSCQVHGP
ARD	KTRL	RWMMEMPGMS	MPAHPHPSYG	SPAELPKHIS AIGGKQSKME
		451			500
ALS		LPSSLPLPGA	DDDLFSP.SG	LNGDISPGCC	PAAAAAAAD LSPTFEKPYA
SBD		FPSE..FKDS	QDGAYDN.GM	QNSVDSDNVI	P.....RNLT
SAD		TNRFSGLVGA	LGGGLSTLSG	YNGLPSVLSG	LDDSLSDVAARKKYP
D α 4	EST	VPPL.....	...THCSSDE	IAAVPMDIG	PLGMKSPILN NPAFSHSHKCL
ARD		VMELSDLHHP	NCKINRKVNS	GCELGLGDGC	RRESESSDSI L.....LS
		501			550
ALS		REMEKTIEGS	RFIAQHVKNK	DKFESVEEDW	KYVAMVLDRM FLWIFAIACV
SBD		PEVLQALRAV	RFIAQHIKDA	DKDNEIVEDW	KFVSMVLDRE FLWLFTLSCV
SAD		FELEKAIHNV	MFIQHMQRQ	DEFNAEDQDW	GFVAMVMDRL FLWLFMIAVL
D α 4	EST	PKIHKSCFCV	RFIAEHTKMQ	EDSTKVKEDE	KYVAMVLDRL FLWIFTLAVV
ARD		PEASKATEAV	EFIAEHLRNE	DLYIQTREDW	KYVAMVIDRL QLYIFFIVTT
			** *	**	** ** *
		<u>TM4</u>			600
ALS		VGTAIIILQA	PSLH...DQS	QPIDILYSKI	AKKKFELLKM GSENTL....
SBD		FGTLAIICQS	PSLY...DTR	SPIDRQLSEI	PLRKNNFM.L PPDIVRQVLT
SAD		VGTFVILGEA	PSLY...DDT	KAIDVQLSDV	AKQIYNLTEK KN.....
D α 4	EST	VGTAIIILQA	PTLY...DDR	IPI.....I	EQKDLDFSGT SAGRCRPPQ.
ARD		AGTVGILMDA	PHIFEYVDQD	RIIEIYRGK.
		** *	*	*	

Fig. 7.6. Amino acid sequence alignment of the four *Drosophila* nAChR subunits ALS (Bossy et al. 1988), ARD (Hermans-Borgmeyer et al. 1986), SAD (Bauman et al 1990, Sawruk et al. 1990) and SBD (Sawruk et al. 1990, Lansdell et al. 1997) and the *Drosophila* D α 4 sequence. The N-terminal region of the D α 4 was isolated by screening a *Drosophila* cDNA library (Novagen) and is truncated approximately 10 amino acids from the start codon. The PCR fragment appears to be incorrectly spliced. The region encoded by the missing exon is shown by dashes (-). Deduced amino acid sequences have been aligned by the GCG program Pile-up (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). Gaps (.) were introduced to maximise sequence identity. Asterisks show residues that are conserved in all five subunits. The putative transmembrane domains TM1, TM2, TM3 and TM4 are indicated by a line above the sequences.

7.14 Discussion

The D α 3 nAChR subunit, when co-expressed with the four previously cloned *Drosophila* nAChR subunits in stably transfected S2 cells, did not show any specific nicotinic radioligand binding. Furthermore, when co-expressed with the rat β 2 or β 4 nAChR subunits in S2 cells no specific binding with the nicotinic radioligands was detected. In this respect D α 3 differs from the two other *Drosophila* α subunits (SAD and ALS) which both exhibit high levels of specific radioligand binding when co-expressed in S2 cells with the rat β 2 or β 4 subunit.

There was no detectable co-expression of the D α 3 nAChR subunit with rat β 2 in co-immunoprecipitation studies using mAb 290. This antibody had been used to show co-assembly of both SAD and ALS with β 2 in S2 cells (Chapter 5). The inability to detect D α 3 by radioligand binding or immunoprecipitation either indicates that D α 3 does not co-assemble with the rat β 2 subunit or alternatively that the D α 3 construct was altered during the PCR subcloning resulting in a failure to express a full length D α 3 protein in S2 cells. Further investigation with this subunit cDNA will be carried out.

The *C. elegans* nAChR subunit Ce21 when expressed alone or co-expressed with the rat β 2 nAChR subunit in S2 cells did not form nAChRs that could be detected by radioligand binding. Ce21 forms homomeric receptors when expressed in *Xenopus* oocytes (Ballivet *et al.*, 1996). The results from immunoprecipitation studies indicated that Ce21 did not co-assemble with the rat β 2 subunit in S2 cells.

The Ce21 subunit was not detected by radioligand binding studies in S2 cells, HEK-293 cells, SH-SY5Y cells or GH $_4$ C $_1$ cells. GH $_4$ C $_1$ cells were used since the vertebrate α 7 subunit had been successfully expressed as a homomeric receptor in these cells. Since Ce21 resembles α 7 in sequence identity and pharmacologically, it was possible that the GH $_4$ C $_1$ cells would be a suitable host cell for the expression of this subunit.

The problems encountered with the expression of the Ce21 nAChR subunit and also the locust α L-1 subunit (Chapter 4) may be due to a combined requirement for a suitable host cell and also a lower temperature similar to the temperature required for the expression of the *Drosophila* nAChR subunits in the mammalian HEK-293 cells (Chapter 6).

The screening of *Drosophila* cDNA libraries using PCR in an attempt to isolate potential novel nAChR subunits did not identify any potential novel clones. The evidence suggests that there other *Drosophila* nAChR subunits to be identified perhaps including a structural subunit similar to the rat β 2 or β 4 nAChR subunits. Another alternative is that there may be a subunit that resembles the rat α 5 nAChR subunit. The vertebrate α 5 subunit does not appear to generate functional receptors when co-expressed in *Xenopus* oocytes in pairwise combinations with β 2, β 3 or β 4 (Boulter *et al.*, 1987; Couturier *et al.*, 1990b). Heterologous expression of the combinations α 4/ β 2 and α 4/ β 2/ α 5 in *Xenopus* oocytes are pharmacologically distinct (Ramirez-Latorre *et al.*, 1996). The α 5 subunit has the pair of adjacent cysteine residues homologous to α 1 positions 192 and 193 but lacks Tyr 190 which is conserved throughout the other α subunits (Kao *et al.*, 1984; Dennis *et al.*, 1988; Abramson *et al.*, 1989; Galzi *et al.*, 1990). Among rat neuronal nAChR α -subunit amino acid sequences, α 5 was the most dissimilar (52%) and surprisingly showed highest sequence homology to the β 3 subunit (68%) (Boulter *et al.*, 1990). These data suggest that the α 5 subunit may have a different role to the other α subunits. The α 5 subunit may have structural role rather than as a ligand binding subunit. If α 5 does function in this way, it is possible that in *Drosophila* nAChRs a subunit equivalent to the vertebrate α 5 subunit exists.

Recently, however, an incorrectly spliced variant of a novel α -like nAChR subunit, D α 4, has been identified. The search for the full length D α 4 clone is in progress and

investigation into the existence of further novel nAChR subunits is underway. This novel partial *Drosophila* nAChR subunit D α 4 is an incorrectly spliced variant of the full length clone. The splice sites match splice sites in the ALS cDNA sequence (Bossy *et al.*, 1988). Ten exons have been identified in the ALS sequence. Four of the seven introns in the ALS coding sequence are perfectly conserved throughout evolution since they occur at precisely the same nucleotide in vertebrate sequences. The other three introns are much less conserved and the coding regions vary in length depending upon the subunit type. One of the putative splice sites in the D α 4 clone, equivalent to position 1629 in ALS, is a highly conserved splice site. The other site, equivalent to position 1810 in ALS, is 15 nucleotides more 5' than its equivalent in the human muscle α subunit (Bossy *et al.*, 1988).

There are only a few reported cases of alternative splicing of nAChRs in the literature. An example is the bovine α 7 subunit in bovine chromaffin cells (Garcia-Guzman *et al.*, 1995). An alternatively spliced isoform of the bovine α 7 subunit in which the exon that encodes the second transmembrane segment was skipped during mRNA processing. The alternative transcript maintains the original reading frame and, following translation, a polypeptide that only differs from the complete α 7 polypeptide in that it lacks the M2 transmembrane region. This transcript was repeatedly observed in RNA samples from different cows at approximately five fold less than the full length transcript. Functional channels were not detected when this construct was injected into oocytes nor were α -BTX binding sites detected on the external membrane. When co-injected with the α 7 cRNA, the shorter isoform inhibited α 7 nAChR expression. The alternative transcript lacks the putative-channel forming region and would not be expected to generate functional homomeric channels when expressed alone. The alternative splice variant in chromaffin cells may have a functional significance in that it could be involved in the inhibition of functional nAChRs and so regulate the number of functional α -BTX-sensitive receptors in chromaffin cells.

The human muscle nAChR α subunit gene is divided into eight introns and nine exons (P1 - P9). An additional 25 amino acids have been observed between P3 and P4 (termed P3A) in the human muscle nAChR α subunit and an analogous sequence corresponding to this novel exon has not been seen in calf, chick or the *Torpedo* electric organ α subunits (Beeson *et al.*, 1990; Morris *et al.*, 1991). When translated these amino acids are located in the extracellular domain of the α subunit between amino acids 58 and 59. A large proportion of antibodies in myasthenia gravis bind to a region on the α subunit in the "main immunogenic region" which includes amino acids 61 - 76 (Barkas *et al.*, 1988). The novel isoform of the muscle α subunit could have a role in the pathogenicity of myasthenia gravis. The exon P3A also contains a cysteine residue which is extracellular and could have a structural role or interactive role with another subunit. There is an equal ratio of the two isoform mRNAs of the α subunit and it is possible that each of the two isoforms could be represented in the AChR pentamer (Beeson *et al.*, 1990).

Two isoforms of the rat $\alpha 4$ subunit have been identified where one has an open reading frame of 1875 base pairs (clone 4-1) and the other 1524 base pairs (clone 4-2) (Goldman, 1987). The clones differ only in 3 amino acids at their C-termini. This could arise from one gene by the alternative splicing of a single primary transcript.

Alternative splice variants have been identified for *Drosophila* GABA receptors. Two *Drosophila* GABA receptor subunits RDL and DRC17-1-2 have been cloned and appear to arise from the alternative splicing of the *rdl* gene (French-Constant *et al.*, 1991; Chen *et al.*, 1994). Both subunits are widely distributed throughout the CNS of *Drosophila* (Hosie and Sattelle, 1996). RDL and DRC17-1-2 differ at only 17 residues mainly in regions of the N-terminal domain which are thought to influence agonist potency. Homologues have been identified in other insect species and mutant forms of these subunits engender resistance to picrotoxin a naturally

occurring antagonist. The splice variants of the *rdl*-gene are unusual in that they can be spliced at two exons which encode regions of the extracellular domain whereas most splice variants of vertebrate GABA receptor subunits differ in their large extracellular loops (French-Constant and Rocheleau, 1993).

The D α 4 cDNA clone isolated from the *Drosophila* cDNA library is missing 61 amino acids which are believed, by comparison to other nAChR subunits, to be critical to agonist binding. It is more likely that this clone represents incorrect splicing rather than an alternatively spliced variant. Whether alternatively spliced variants of *Drosophila* nAChR subunits exist and have physiological roles remains to be elucidated.

8.0 Conclusion

The folding, assembly and functional expression of *Drosophila* nAChRs was investigated by heterologous expression in *Drosophila* S2 cells, mammalian cell lines and in *Xenopus* oocytes. Prior to the start of this project, four *Drosophila* nAChR subunits had been cloned: ALS (Bossy *et al.* 1988), ARD (Hermans-Borgmeyer *et al.* 1986), SAD (Baumann *et al.* 1990, Sawruk *et al.* 1990) and the partial SBD clone (Sawruk *et al.* 1990). As part of this research project a full-length SBD subunit cDNA was cloned and sequenced.

Previous attempts at expression of the four *Drosophila* nAChR subunits in *Xenopus* oocytes failed to generate functional channels. In this study, the expression of *Drosophila* nAChR subunits was investigated using the *Drosophila* S2 cell line which has been used successfully for the expression of *Drosophila* GABA, muscarinic and dopamine receptors (Millar *et al.* 1994, 1995, Han *et al.* 1996). It was hoped that this cell line would provide a more native cellular environment for expression of *Drosophila* nAChRs than either *Xenopus* oocytes or mammalian cell lines. Stably transfected S2 cell lines expressing combinations of *Drosophila* nAChR subunits were established.

The co-expression of the four previously cloned *Drosophila* nAChR subunits in the S2 cell line failed to result in the generation of nAChRs that could be detected by nicotinic radioligand binding. One reason for this could have been the non-native signal sequence of the chimeric SBD protein (Sawruk *et al.* 1990a, 1990b). By the construction and expression of a full-length SBD cDNA the possibility that previous difficulties in expressing robust functional *Drosophila* nAChRs was due to the misfolding of the chimeric SBD protein was eliminated.

By the stable expression of *Drosophila* and rat nAChR subunits in a *Drosophila* cell line it has been possible to establish that both of the cloned *Drosophila* α subunits acquire the ability to bind nicotinic radioligands when co-expressed with either the rat

$\beta 2$ or $\beta 4$ subunit but not with the rat $\beta 3$ subunit. In this respect these *Drosophila* α subunits resemble the rat $\alpha 2$, $\alpha 3$ and $\alpha 4$ subunits which generate functional nAChRs when co-expressed with the $\beta 2$ or $\beta 4$ subunit but fail to do so with the rat $\beta 3$ subunit (Deneris *et al.*, 1989; Duvoisin *et al.*, 1989; Luetje and Patrick, 1991). No radioligand binding was detected when either of the two *Drosophila* β subunits were co-expressed with the rat $\alpha 3$ or $\alpha 4$ subunits. Despite this, however, there is evidence from co-immunoprecipitation studies that both of the *Drosophila* β subunits are capable of co-assembly, to some extent, with both rat α and β nAChR subunits (Chapter 5).

Pharmacological diversity of *Drosophila* nAChRs has been illustrated by the existence of both α -BTX-sensitive and α -BTX-insensitive nAChRs in the nervous system of insects (Breer and Sattelle, 1987), however, little is known about the assembly and subunit stoichiometry of the *Drosophila* nAChRs. Immunohistochemical studies have suggested that the ALS, ARD and SAD subunits are largely co-localised within the *Drosophila* nervous system (Schuster *et al.*, 1993; Jonas *et al.*, 1994).

Co-assembly was investigated in this study using epitope-tagged nAChR subunits with a mAb to the "FLAG" epitope-tag. The results indicated that SAD co-assembled with both ARD and SBD and that ARD also co-assembled with SBD. There was no evidence of the ALS subunit co-assembling with either ARD, SAD or SBD. The epitope-tagged subunit constructed in this study appeared to be degraded possibly indicating misfolding.

Previous immunoprecipitation studies suggested that the *Drosophila* ARD subunit is part of a high-affinity α -BTX-binding nAChR (Schloss *et al.*, 1988) and that in *Drosophila* head membrane preparations ALS and ARD co-assemble to form an α -BTX-binding complex (Schloss *et al.*, 1991).

ALS or SAD co-assemble with the rat nAChR $\beta 2$ and $\beta 4$ subunits to form nicotinic ligand binding complexes in S2 and HEK-293 cells, whereas, specific radioligand binding is not observed when ALS or SAD are co-expressed with other *Drosophila* nAChR subunits. This strongly suggested the existence of further *Drosophila* nAChR subunits to be identified. In this project, *Drosophila* cDNA libraries were screened, by PCR, with degenerate primers designed to conserved regions of other nicotinic receptor subunits in order to identify potential novel *Drosophila* nAChR subunits. During the course of this project a novel α -like subunit, D $\alpha 3$, was identified by another group and although unpublished, I was provided the cDNA clone by Dr. Bertram Scmitt, Frankfurt. Co-expression of this novel subunit with SAD, ARD, SBD and ALS did not result in the formation of a receptor that could be identified by radioligand binding. A sixth potential *Drosophila* nAChR subunit has recently been identified from the Berkeley *Drosophila* Genome Project as an "expressed sequence tag" which was identified as having similarity to the SBD mRNA. Sequencing of the EST cDNA clone revealed that it extended beyond the 3' end of the putative open reading frame but appeared to lack approximately 600 bp at the 5' end. Attempts were made to reclone the 5' end of the EST clone. A PCR product was identified which contained most of the missing 5' region but which, by analogy to the other *Drosophila* nAChR subunit sequences, appeared to be missing an exon in the ligand-binding region. It is likely, therefore, that this represents a cDNA clone derived from an incorrectly spliced mRNA. The isolation of the novel α -like subunits D $\alpha 3$ and the EST sequence (referred to here as D $\alpha 4$) represents another step toward understanding the molecular basis for the diversity of the *Drosophila* nAChRs.

The heterologous expression of *Drosophila* nAChR subunits in mammalian cell lines was also investigated. No specific radioligand binding was detected in HEK-293 cells which were transiently transfected with combinations of *Drosophila* nAChR subunits. In contrast to this, combinations of the rat nAChR subunits such as $\alpha 4/\beta 2$ formed complexes that could be identified by radioligand binding. It was also possible to

show that subunit combinations (such as ALS/ β 2) which appeared to misfold in mammalian cells could generate functional nAChRs in *Xenopus* oocytes (from the same cDNA plasmid constructs). The results of this study showed that the expression of the *Drosophila* nAChR subunits in the mammalian HEK-293 cell line is temperature-sensitive. The co-expression of ALS or SAD with the rat β 2 or β 4 subunits was detected by nicotinic radioligands at 25°C but not at 37°C. A similar phenomenon has been reported for the functional expression of the *Torpedo* nAChR subunits (Claudio, 1987). It is plausible, but still to be investigated, that a similar temperature-sensitivity may explain the problems encountered in expressing other invertebrate nAChR subunits (such as the *C. elegans* nAChR subunits and the locust α L-1 subunit) in mammalian cells.

There is increasing evidence that correct expression of nAChRs is strongly dependent on the nature of the host cell. The folding and assembly of homomeric α 7 or α 8 nAChRs has been shown to be dependent on the nature of the host cell (Cooper and Millar, 1997, 1998) as have the ion channel properties of the hetero-oligomeric nAChRs α 3/ β 4 (Lewis *et al.* 1997). Protein folding and the functional properties of many proteins are dependent upon post translational events such as phosphorylation, glycosylation, fatty acylation and disulphide bond formation. A role for specific chaperone proteins such as BiP and calnexin in the folding and assembly of nAChRs has also been established (Forsayeth *et al.* 1992, Gelman *et al.* 1995). The primary mechanism of regulating cell-specific expression of proteins is likely to be at the level of mRNA synthesis by cell-specific transcription factors. However, it is possible that cell-specific regulation of post translational events such as subunit assembly may provide additional mechanisms for regulating the cell-specific expression of receptors such as the nAChRs.

Future work

The main emphasis of the work following on from this thesis will be to continue screening *Drosophila* libraries for novel nAChR subunits including a full length clone for D α 4. Co-expression studies to investigate the co-assembly of the new subunits D α 3, D α 4 and other potential subunits will be carried out in various expression systems. Further investigation into the temperature-sensitive expression of the *Drosophila* subunits is underway. The essential aim being to generate functional *Drosophila* nAChRs and to elucidate the subunit stoichiometry and to investigate the ability of the subunits to co-assembly.

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