Institute of Child Health

The Influence of the Transmembrane Protein EAT-18 upon Nicotinic Acetylcholine Receptors

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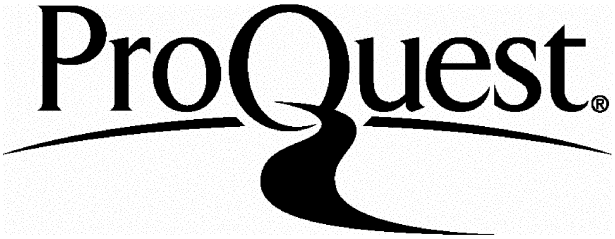

Abstract

Nicotinic acetylcholine receptors (nAChRs) are a diverse family of neurotransmitter-gated ion channels, which are associated with many physiological and pathological conditions. Numerous investigations have been carried out since it was first identified about 100 years ago. However, the mechanism by which this receptor function in variety of species still not entirely clear. A major current problem is that it is difficult to express functional recombinant nicotinic receptor on the surface of expression systems (e.g. in cultured mammalian and insect cell lines). Recent genetic studies have identified some promising candidates molecules, which facilitate more efficient trafficking, assembling or folding of nAChRs.

EAT-18 is a recent identified small molecule with a single transmembrane domain and a short extracellular region, which has previously been reported, associated with a defect in *C. elegans* feeding behaviour. Evidence shows that eat-18 is required for α-bungarotoxin binding to most or all pharyngeal nicotinic receptors. The current project aims to examine the influence of co-expression of eat-18 with a variety of nAChRs subunit combinations. eat-18 was subcloned into plasmid vector pRmHa3 and co-transfected with a variety of human (H), rat (R) and *Drosophila* (D) nAChRs subunit combinations (Hα7, Rα7, Rα4/β2, Rα3/β4, Dα7, Dα6, Dα1/β2, Dα3/β2, Dα2/β2, Dα1/α2/β2, Dα3/α2/β2 and Dα3/β1/β2) in *Drosophila* S2 cells. Radioactive non-selective agonist epibatidine and α7-selective antagonist methyllyacconitine (MLA) were used for ligand binding studies. Increase in specific radioligand binding were observed when eat-18 was co-expressed with some subunit combination, but were not statistically significant.

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Last but by no means the least, I sincerely thank my parents for everything!

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Abbreviations

nAChR	Nicotinic Acetylcholine Receptor
NMJ	Neuromuscular Junction
PCR	Polymerase Chain Reaction
α-BTX	α-bungarotoxin
ACh	Acetylcholine
CNS	Central Nervous System
AChBP	ACh-binding Protein
NCA	Non-competitive Allosteric Activator Site
NCB	Non-competitive Negative Allosteric Sites
GABA	γ-Amino Butyric Acid
AD	Alzheimer's Disease
PD	Parkinson's Disease
TS	Tourette syndrome
NAc	Nucleus Accumbens
ACR	Acetylcholine Receptor
ALP	alpha-actin Associated LIM Protein
MQ	milli-Q
LB	Luria-Bertani
5-HT	5-hydroxytryptamine
TE	Tris-EDTA
ddNTPs	dideoxynucleotides
HBS	HEPES-buffered Saline
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
MLA	Methyllyacconitine
C. elegans.	*Caenorhabditis elegans*
CIAP	Calf Intestinal Alkaline Phosphatase
Carbachol	Carbamylcholine Chloride
v/v	Volume to volume
w/v	Weight to volume

rpm	Revolutions per minute
DNA	Deoxyribouncleic acid
RNA	Ribonucleic acid
KDa	Kilodaltons

Nomenclature

EAT-18 refers to the protein form of eat-18 cDNA sequence

eat-18 refers to the cDNA sequence

Hα7 refers to Human α7 nAChR

Rα4β2 refers to Rat α4β2 nAChR

Dα3 refers to *Drosophila* α3 nAChR

ALS refers to *Drosophila* Dα1 nAChR subunit

SAD refers to *Drosophila* Dα2 nAChR subunit

ARD refers to *Drosophila* Dβ1 nAChR subunit

SBD refers to *Drosophila* Dβ2 nAChR subunit

Chapter 1

1.0 Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that belong to a gene superfamily of homologous receptors including GABA (γ-Amino Butyric Acid), glycine and 5-hydroxy tryptamine (5-HT_3) receptors (1;2). These proteins, comprising five membrane-spanning subunits, are involved in fast communication activities between nerve cells and sensory or effector cells. A chemical signal released by the nerve cell and detected by the ion channel receptor is converted into an electrical signal via the opening of the ion channel to allow a transient influx of ions through. The members of the superfamily can be classified according to the nature of the ions that pass through the channel. nAChRs and 5-HT_3 receptors conduct cations and evoke an excitatory response, while glycine and $GABA_A$ receptors conduct anions and are responsible for inhibitory events (e.g. the receptor activation leads to membrane hyperpolarisation).

1.1. Historical review of nicotinic acetylcholine receptors (nAChRs)

The nicotinic acetylcholine receptor (nAChR) was first identified almost 100 years ago (2). Since then, nAChRs have been the subject of numerous investigations (3). In 1970, Changeux and colleagues successfully purified a nAChR from *Torpedo californica* (4), and the first nAChR subunit was sequenced about a decade later (5). The best characterised function of nAChRs is to mediate synaptic transmission at the neuromuscular junction (NMJ) and in the nervous system of both vertebrates and invertebrates (6). However, their presence on non-neuronal cells such as skin keratinocytes, (7), bronchial epithelial cells(7), endothelial cells of the arterial system (8),

macrophages(9) and the surface of the blood fluke, Schistosoma (10), suggests a considerable functional spectrum, which required further exploration.

1.2. nAChR in variety of species

1.2.1. nAChR from the *Torpedo* electric organ

The electric organs of the *Torpedo californica* ray and the fresh water electric eel (*Electrophorous electricus*) are composed of rows or columns of cells termed electrocytes, which are modified muscle cells. The nAChR isolated from these modified muscle cells resembles the nAChR isolated from the vertebrate neuromuscular junction (refer to section 1.2.2). The snake α-bungarotoxin (α-BTX), isolated from the venom of the Malayan banded krait, *Bungarus multicinctus* (11) binds almost irreversibly to *Torpedo* electric organ nAChRs to inactivate receptor function and allowed the purification of the nAChR from this rich source of receptors. (4;12) The specific high affinity binding of α–BTX has been proved to be useful for the extensive characterization of the nAChR and helped to revealed many of this receptor biochemical and pharmacological properties (13).

The *Torpedo* electric organ nAChRs have been reported composed of four different subunits designated α, β, γ, δ,arranged as a pentamer structure in the stoichiometry $\alpha_2\beta\gamma\delta$ (14–16) . Each subunit contributes 40-64 Kilodaltons (kDa) to form this pentameric glycoprotein, which has a molecular mass of approximately 290 kDa. The

native *Torpedo* electric organ nAChR pentamers are exists as in a "dimeric" form, where two nAChR pentamers are cross-linked by a disulphide bond(17-19). The five membrane spanning subunits of each pentamer are arranged to Form a ring around a narrow central pore, which comprises the ion channel and is impermeable to ions when the nAChR is in the resting state(16;19). Upon ligand binding and nAChR activation, a conformational change occurs to open the channel pore, allowing the selective passage of ions through the plasma membrane (20;21).

Cloning of the genes encoding the *Torpedo* electric organ α, β, δ and γ subunits (22-24), allowed expression of functional recombinant nAChRs in the oocytes of the South African clawed frog, *Xenopus laevis* (25;26) in an approach that has been widely used in the functional characterization of nicotinic receptors.

1.2.2. nAChR of the vertebrate neuromuscular junction (NMJ)

The nucleotide sequences of the *Torpedo* electric organ subunits were used to construct cDNA probes, and then can be used to screen mammalian libraries and identify genes encoding nAChR subunits expressed at the vertebrate neuromuscular junction (24;27-29). With the help of this approach, mammalian α1, β1, γ and δ subunits and a novel subunit, designated ε, resembling the γ subunit, were identified (30). The muscle nAChR can exist in two forms, where the $\alpha 1_2\beta 1\varepsilon\delta$ form dominant in adult muscle (31). Small changes in the biophysical properties of the two nAChRs subtypes are observed, where channel

conductance is relatively low in foetal muscle ($\alpha 1_2\beta 1\gamma\delta$) and high in adult muscle ($\alpha 1_2\beta 1\varepsilon\delta$) (31).

The nAChRs at the vertebrate neuromuscular junction are involved in chemical signaling between motor neurons and the muscle effector cells.

1.2.3. The invertebrate nAChRs

nAChRs are also important modulators of fast synaptic transmission in invertebrate species. In the insect nervous system, acetylcholine (ACh) appears to be the major excitatory neurotransmitter (32) and nAChRs are present in high density in insect neural membrane extracts (33;34). The majority of sensory pathways and interneurones use ACh as the excitatory transmitter, whereas at NMJ, the transmitter is glutamate(33). The first demonstration of ACh receptors in the insect neurons was shown by locally applied by ACh (35).

1.2.3.1. nAChRs of *Drosophila*

The *Drosophila* nAChR subunits resemble the vertebrate subunits in their predicted membrane topologies and share between 33-50% sequence homology (36). 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 conserved, a fact exploited when raising subunit-specific 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 (37).

The six *Drosophila* subunits: ALS, ARD, SAD, SBD, Dα3 and Dα4 (TABLE 1.2) encode functional nAChR subunits. Expression of *Drosophila* nAChRs was expected to confine to the nervous system. This has been confirmed by *in situ* hybridization studies in late *Drosophila* embryos the transcripts for the four analysed nAChR genes were shown to be expressed throughout the CNS (Central Nervous System) but no expression was detected outside the CNS (38-40).

1.2.3.2. nAChRs of the nematode *C. elegans*

Nematodes have been shown to possess nAChRs (41-46) and much of the work done so far has been focused on gene products which were sensitive to levamisole. Levamisole is an agonist that is more potent than acetylcholine at the nematode muscle nAChRs (46). Levamisole is an antihelmintic agent, which is used to treat adult and larval nematode infections in domestic animals.

Eight nAChRs has been cloned: ACR-2, ACR-3, ACR-16, DEG-2, DEG-3, LEV-1, UNC-29 and UNC38 (TABLE 1.3) (47) till now. These eight 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 *Torpedo* α subunit, which implies a common evolutionary origin for these proteins (43).

1.2.4. Neuronal nAChRs

Since the cloning of the first neuronal nAChR subunit from a rat adrenal medulla phaeochromocytoma (PC12) cDNA library (48;49), twelve vertebrate neuronal-type subunits, designated α2-α10 and β2- β4 have been identified and cloned (49-51) (TABLE 1.1).

Neuronal nAChRs show considerable diversity in subunit composition with evidence for nAChRs containing one, two, or more different subunit subtypes in the pentameric structure (47). Expression of combinations of the different neuronal subunits in heterologous expression systems is one approach used to ascertain the possible subunit composition of native nAChRs.

In the majority of cases, combinations of both α and β subunits appear necessary for assembly of functional nAChRs. When expressed in *Xenopus* oocytes, the α2, α3 and α4 subunits each assemble into functional ion channels upon co-expression with β2 or β4 subunits (49;52-55). The α5 subunit does not form functional nAChRs when expressed in pair-wise combination with any β subunit (55;56), but may participate in the formation of nAChRs containing more than two different types of subunit, such as α3β4α5 and α4β2α5 (47). Similarly, while the β3 subunit does not participate in the formation of functional channels when expressed in pair-wise combinations of subunits, β3 may participate in nAChRs complexes containing more than one type of β subunit, such as α4β2β3β4 nAChRs (57-59). Heterologous expression of the chick α6 subunit in mammalian cells suggests the formation of functional α6β2 and α6β4 nAChRs (60),

while expression of human subunits in *Xenopus* oocytes suggests α6 assembles more efficiently into complexes that contain more than one type of α or β subunit, such as α3β4β6 and α6β2α5 nAChRs (59;60). Each heteromeric nAChR subtype appears to be stimulated by the application of nicotine, though none of the previously mentioned combinations shows sensitivity to α-BTX (50;61).

The α7, α8 and α9 subunits are capable of forming homomeric complexes that are sensitive to α-BTX when expressed alone in *Xenopus* oocytes (56;62-66). Early studies suggested that the pharmacological properties of the α7 homomer were not affected by the co-injection of any of the α3, α5, β2, β3 or β4 subunits (56;63;67), but more recent data has suggested that *in vivo*, α7 may co-assemble with subunits such as β2, β3 or α5 (67-71). In the chick nervous system, the α7 and α8 subunits are able to co-assemble to form heteromeric nAChRs in addition to homomeric nAChRs in addition to homomeric nAChRs (66;71;72). The α10 subunit does not form functional ion channels when expressed alone or in combination with any neuronal β subunit in *Xenopus* oocytes, but does appear to co-assemble with the α9 subunit (51;73).

1.3. Nicotinic acetylcholine receptor (nAChR) structure

Among different kinds of nicotinic acetylcholine receptor in different species, the neuronal nicotinic receptor is the best characteristized. However, at the meantime other types of nicotinic receptors are not fully characterized so far.

1.3.1. Primary structure of subunits

Biochemical investigations with *Torpedo* receptor (74) and with neuronal receptors (75) have established for more than two decades. Both peripheral (e.g. muscle type) and neuronal nAChRs are comprised of hetero-oligomers consisting of five membrane spanning subunits which form a barrel like structure in the membrane around a central ion channel (76) (FIG. 1.1).

The clearest evidence that nAChRs are pentamers comes from electron microscopy of tubular crystals prepared from *Torpedo* electric organ post-synaptic membranes. By analyzing the data from many separate images it became possible to obtain a three-dimensional structure for this receptor at 4.6 Å resolution(20). At this resolution it is clear that the *Torpedo* nAChR forms a pentameric structure. It is also possible to identify areas of secondary structure, such as a transmembrane α-helical segment believed to line the ion-channel pore (20).

While the well-documented difficulties associated with crystallization of transmembrane proteins has, so far, precluded the generation of an atomic resolution structure of

nAChRs, an intriguing insight into nAChR structure has come from X-ray diffraction studies with a soluble ACh-binding protein (AChBP) identified recently in the snail *Lymnaea stagnalis* (77). The AChBP, which is also a pentamer, shows considerable sequence similarity to the nAChR extracellular N-terminal ligand-binding domain (78). Significantly, amino acids, which are known to be involved in formation of the nAChR binding site, are conserved in the AChBP (77;78).

Several possible models have been proposed for the orientation within the membrane of nAChR subunit polypeptides. The consensus model, supported by extensive experimental evidence, is illustrated in FIG 1.1. Each nAChR subunit contains a large (≈ 200-amino-acid) N-terminal domain (analogous to the AChBP) and four hydrophobic proposed transmembrane domains. The second of these hydrophobic domains contain amino acids that influence channel conductance and are presumed to line the channel pore (79).

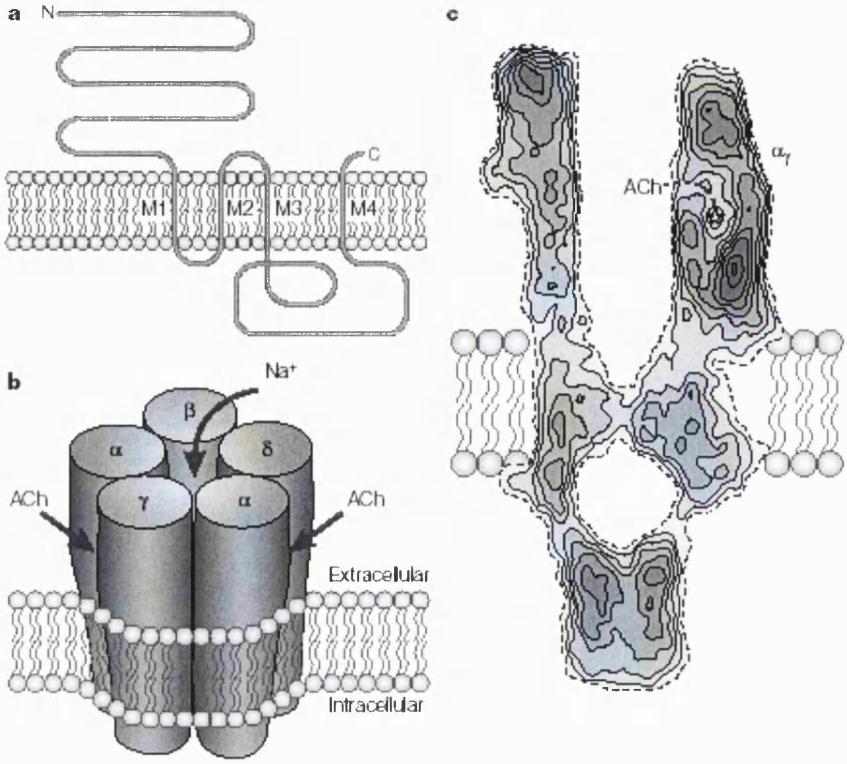

FIG 1.1 Structure of the nicotinic acetylcholine receptors. a. The threading pattern of receptor subunits through the membrane. b. A schemtation of the quaternary structure, showing the arrangement of the subunits in the muscle-type receptor, the location of the two acetylcholine (ACh-) binding sites (between an a- and a g- subunit, and an a- and a d- subunit), and the axial cation-conducting channel. c. A cross-section through the 4.6-A structure of the receptor determined by electron microscopy of tubular crystals of Torpedo membrane embedded in ice. Dashed line indicates proposed path to binding site. The figure was adapted from (17).

1.3.2. Ligand binding sites

A diverse range of compounds is known to be pharmacologically active at nAChRs. The drugs acting at nAChRs can be divided into three main classes: (I) agonists, (II) antagonists and (III) allosteric ligands, both activating and inhibitory. These compounds produce their effects by action at one of a number of ligand binding sites that exist on the receptor-ion channel complex.

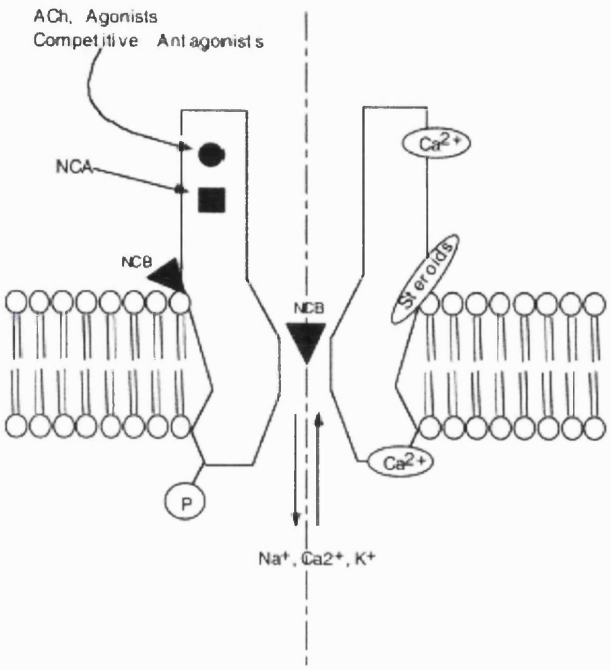

FIG 1.2 Schematic cross section of a nicotinic receptor showing the ion channel, the Ach binding site and multiple allosteric sites distributed throughout the extracellular part of the protein. Allosteric sites shown include the non-competitive allosteric activator site (NCA); non-competitive negative allosteric sites (NCB); binding sites for Ca^{2+} and steroids and phosphorylation sites (P). The picture was adapted from (80).

The reconstitution of functional receptors isolated by biochemical purification of proteins from *Torpedo* electric organ constituted the first demonstration that these proteins can form both the ligand-binding site and ionic pore. From investigations in the 1980s on the kinetics of ionic current through the single channel, it has been accepted that two agonist-binding sites exist. The occupancy of these two sites, in a positive cooperative way, stabilizes the receptor channel in the open state (81-84). Using the method of the fluorescence resonance energy transfer, between a receptor-bound fluorescent agonist and two membrane-fluorescent probes, the ACh-binding sites were estimated to be 25 Å below the extracellular apex of the nAChR. Later, high-resolution electron microscopy

technique results were in agreement with the proposed location for the ACh-binding sites in putative 'pockets' and approximately 30 Å above the membrane surface (16;20;85;86).

With the availability of specific chemical compounds and the amino acid sequences of the different nAChR subunits, important advances concerning our knowledge of the ACh-binding site have been made. For instance, photo-affinity experiments carried out with the muscle-type receptor have shown that the ligand-binding site must be at the interface between the αs and their adjacent δ or γ subunits (FIG. 1.2) (1;87;88).

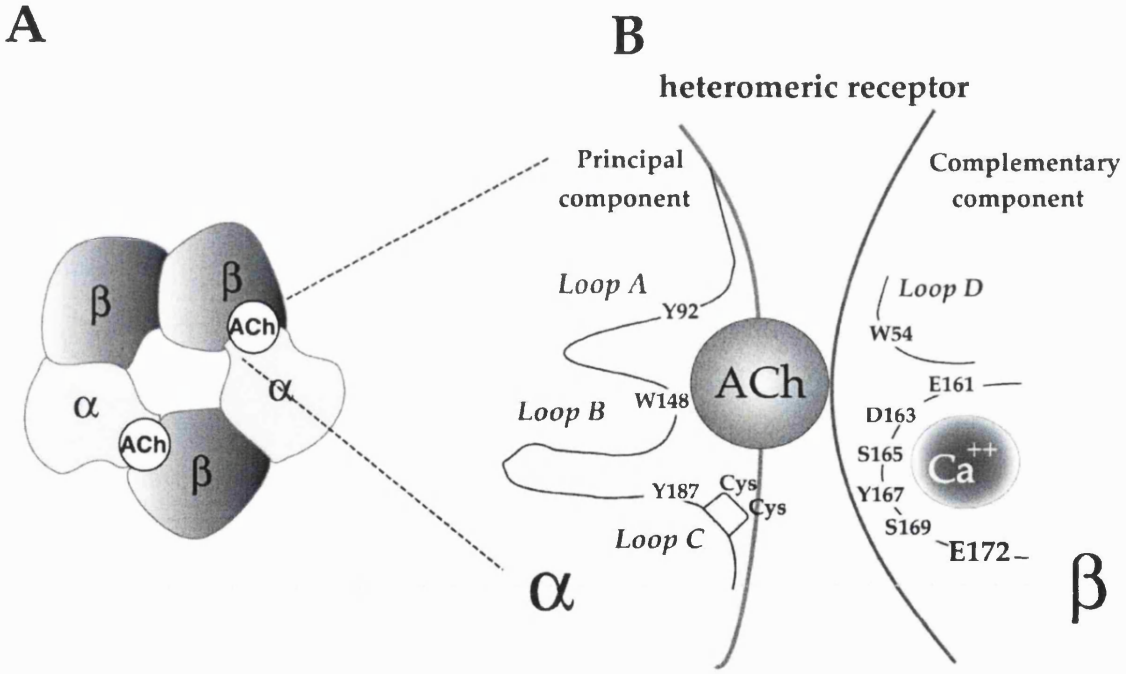

FIG 1.3. Schematic representation of the nAChR. A: Heteropentameric complex of neuronal nicotinic α and β subunits. The ACh-binding site is localised at the interface of α and the adjacent subunit. B: Representation of the principal component (α subunit) with its three loops A, B, C and the two loops D, E from the complementary component (β subunit). The E loop was shown to participate in the Ca^{2+}- binding region involved in the calcium potentiation [adapted from (61)].

Six loops termed A-F have been identified in the formation of the ACh-binding site. The α subunit, which harbours the principal component of the binding site, comprises the A,

B and C loops, while the complementary component of the adjacent subunit (δ, γ or ε for the muscle and β for the neuronal) comprises the D, E and F loops (FIG. 1.3). The important participation of tyrosine residues to the ACh-binding pocket has been identified by the combination of site-directed mutagenesis and electrophysiological investigation of the chick α7 receptor subtype (89). Modelling of the putative three-dimensional structure of the nAChRs (90;91) all confirmed that the ACh-binding site must reside at the interface between two subunits and that the ligand must penetrate into a gorge to form appropriate chemical bridges and initiate the transduction. Modelling of the extracellular domains with the known crystal structure of the copper-binding proteins was also attempted (92). The very recent identification of ACh binding protein, a molecule that efficiently binds ACh and that is secreted from the glial cells of snails, allowed the first crystallisation of a protein that resembles the N-terminal domain of the nAChRs. X-ray diffraction analysis of this structure confirmed the predictions made from the various models and the existence of the A, B, C, and E loops in the formation of the binding pocket (77).

1.4. Transition states of nicotinic receptor

Nicotinic receptors can exist in at least one of four interconvertible functionally distinct conformational states at any one time. These states can be interpreted in terms of the "conformational scheme" of Katz and Thesleff (1957) (93) and consist of: (I) a resting state **R**, (II) an activated state **A**, where the channel opens on a microsecond to millisecond timescale when activated but which has a low affinity for Ach (10-1 μM) and nicotinic ligands (74). Binding of ligands to the nAChR structure either at the ACh site or

any of the allosteric sites can modify the equilibrium between the different conformational states of the receptor at any one time. Additionally, ligands binding to the nAChR can be considered to differentially stabilize the conformational state to which they preferentially bind (93).

1.5. Nicotinic acetylcholine receptors (nAChRs) subunit diversity

A total of 17 nAChR subunits have been identified in vertebrate species (TABLE 1.1.). Since five subunits co-assemble to generate a functional nAChR, the potential nAChRs with very many different subunit combinations. It appears that native nAChRs are assembled into functional pentamers with a relatively restricted number of subunit combinations. It is clear that assembly of nAChRs, like that of other oligomeric ion channels, is a tightly regulated and ordered process, which requires appropriate subunit-subunit interactions (94).

Data presented in TABLE 1.1 are derived from either electrophysiological study, which have demonstrated functional expression from defined subunit combinations, or from studies with native nAChRs (e.g. by immunoprecipation). The extent of nAChR subunit diversity and evidence for subunit co-assembly in two model invertebrate species, the insect *Drosophila* and the nematode *C.elegans*, is presented in TABLE 1.2 and 1.3, respectively. Analysis of genome sequence data suggests the presence data suggests the presence of 10 nAChR subunits in *Drosophila* (95).

Receptor subtype	Subunits	Subunit combinations

Muscle-type	α1, β1, δ, γ, ε	α1, β1, γ, δ
		α1, β1, ε, δ
Neuronal (α–BTX-Insensitive)	α2–α6, β2–β4	α2β2
		α2β4
		α3β2
		α3β4
		α4β2
		α4β4
		α6β2
		α6β4
		α2α5β2
		α3α5β2
		α3α5β4
		α3α6β2
		α3α6β4
		α3β3β4
		α4α5β2
		α5α6β2
		α6β3β4
		α3α5β2β4
		α3α6β3β4
		α4α5α6β2
		α4β2β3β4
Neuronal (α–BTX-sensitive)	α7,α8	α7
		α8
		α7β2
		α7β3
		α7β8

		α5α7β2
		α5α7β4
Sensory epithelia	α9,α10	α9
		α9α10

TABLE 1.1 It is common for vertebrate nAChRs to be classified as either 'muscle-tpye' or 'neuronal' depending on whether they are expressed at the neuromuscular junction or within the central or peripheral nervous system. The most recently identified vertebrate nAChR subunits (α9 and α10) are expressed primarily in the mechanosensory hair cells of sensory epithelia (the coclea and vestibular labyrinth) and, consequently, do not fall conveniently into the convential classification of muscle-type and neuronal nAChRs. α-BTX, α-bungarotoxin [adapted from (47)]

Subunit combinations	Alternative *Drosophila* subunit nomenclature	Experimental technique
Dα1 (+Vertebrate β2)	ALS (+Vertebrate β2)	Electrophysiology/ binding
Dα2 (+Vertebrate β2)	SAD (+Vertebrate β2)	Electrophysiology/ binding
Dα3 (+Vertebrate β2)	Dα3 (+Vertebrate β2)	Electrophysiology/ binding
Dα4 (+Vertebrate β2)	Dα4 (+Vertebrate β2)	Binding
Dα1, Dα2 (+Vertebrate β2)	ALS, SAD (+Vertebrate β2)	Electrophysiology
Dα1, Dα2, Dβ2	ALS, SAD, SBD	Co-precipitation
Dα3, Dβ1	Dα3, ARD	Co-precipitation
Dβ1, Dβ2	ARD, SBD	Co-precipitation

TABLE 1.2 Ten putative nAChR subunit genes have been identified within the complete genome sequence of the model insect species D. Melanogaster. Seven genes encode α subunits (Dα1/ALS, Dα2/SAD, Dα3-Dα7) and three β subunits (Dβ1/ARD, Dβ2/SEB and Dβ3). No combination of cloned Drosophila nAChR subunits is able to reliably generate a functional nAChR in heterologous expression systems, a problem which has severely hindered their characterization (97). It has, however, been possible to generate recombinant nAChRs by co-expression of Drosophila (and of other insect) nAChR a subunits with the vertebrate β2 subunit (and with other vertebrate non-α subunits). The table summarizes studies conducted with Drosophila/ vertebrate nAChR subunit combination and those with native Drosophila nAChRs. ALS, α-like subunit, ARD, acetylcholine receptor of *Drosophila*; SAD, second α subunit of *Drosophila*; SBD, second β subunit of Drosophila [Adapted from (47)].

Subunit combinations	Experimental technique
ACR-2/UNC-38	Electrophysiology

ACR-3/UNC-38	Electrophysiology
ACR-16	Electrophysiology
DEG-3/DES-2	Electrophysiology
LEV-1/UNC-29/UNC-38	Electrophysiology

TABLE 1.3 The complete genome sequence of the nematode *C. elegans*, an important model system for molecular genetic studies, has revealed a large number of putative nAChR subunits. Estimates of the number of possible subunits range from 27 to 42 (98:99). Proposed non-a subunits are: ACR-2, ACR-3, ACR-22, LEV-1, UNC-29. Alternative nomenclature has been used for some *C. elegans* nAChRs, for example Ce21 (ACR-16) and ACR-4 (DES-2) Subunit nomenclature reflects either the phenotype of genetic mutants (DEG, neuronal degeneration; DES, suppression of neuronal degeneration phenotype; LEV, resistance to levamisole; UNC, unco-ordinated movement) or follows a chronological system with the abbreviation ACR (acetylcholine receptor) [adapted from (47)].

1.6. Distribution of native neuronal nAChRs

In comparison to muscarinic receptors, neuronal nAChRs are expressed in relatively low density in the human brain. In addition, their pattern of distribution is relatively homogenous and is not restricted to the well-defined brain cholinergic pathways. The neuroanatomical distribution of various nAChR subtypes and subunit mRNA has been fairly extensively characterized in rodent and chick brain but has been less well characterized in human brain (TABLE 1.4). Functional neuronal nAChRs can be located on axon terminals, where they play a well-documented role in modulating synaptic transmission, but they can also be found on cell bodies and/or dendrites, where they may mediate direct postsynaptic effects (50;96-99). Activation of presynaptic nAChRs is known to facilitate the release of several neurotransmitters including GABA, glutamate, dopamine and acetylcholine (ACh) itself (99). Numerous functional studies have recently enhanced our understanding of the role of these receptors in the central nervous system (80;87;100;101).

Brain region	β2	β3	β4	α3	α4	α5	α7
Cortex		+	+			+	
Prefrontal	+			++	+		++
Motor	+			++	+		+++
Entorhinal	+			++	+		+
Cingular	+			+	+		
Temporal	+			+	+(+)		
Thalamus		+	+			+	
Dorsomedial	+			+++			++
Lateroposterior			+++				
Reticular			++			+(+)	
Ventro-posterolateral	+			+++			
Geniculate bodies						++	
Hippocampus	+(+)			+			++
Dentate gyrus	+(+)			+			++
Caudate putamen	+(+)	+	+			+	++
Cerebellum	+	+	+	+	+(+)	+	

TABLE1.4 Distribution of nicotinic receptor subunit mRNA in the human brain [table adapted from: (80)]. Data was from (102-108).

1.7. 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: cortex, thalamus and interpenduncular nucleus as well as substantial binding in the amygdala, septum, brain stem motor nuclei and locus ceruleus (109). The mesolimibic system has been implicated in nicotine addiction (110).

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 neurons in the hippocampus (a centre for learning and memory) which has rich cholinergic innervations and dense nAChR expression (111). 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 (AD) and other dementia's such as Parkinson's disease (112;113) and the ability of nicotine to enhance certain forms of memory suggests a role for nicotinic agents in the treatment of some of he deficits due to this condition. The diversity of nAChRs potentially allows the development of selective compounds, which have fewer side effects than current treatments. Cholinergic transmitter replacement therapy forms the mainstay of AD treatment and is based on theory that low levels of ACh are responsible for the cognitive decline associated with the disease. Classically, replacement therapy has involved the use of cholinesterase inhibitors such as tacrine, donepezil and rivastigmine, which prevent the breakdown of ACh released from cholinergic neurons, thereby increasing thee concentration of transmitter available to interact with receptors. These drugs have moderate palliative effects on symptoms as well as having some ability to slow disease

progression (114-116).

The mechanism by which cigarette smoking may have a protective effect against Parkinson's Disease (PD) is still under exploration. It may involve nicotine facilitating dopamine release from neuron in the nigrostriatal region which is the area depleted of dopamine containing neurons in Parkinson's disease. Also there are some research indicated that nicotine counteracted the locomotor effects MPTP in animal models of PD (117;118).

Gilles de la Tourette syndrome (TS) is characterized by repetitive motor and vocal tics. A number of studies have reported that administration of nicotine by means of gum or transdermal patches potentiate the action of neuroleptics and are effective in ameliorating the symptoms of TS (119;120). However, there is no direct evidence of nAChR involvement in the condition.

The possible involvement of nAChRs in schizophrenia was suggested by the high percentage of smokers present in the schizophrenic population compared to the general population, 90% compared to 33% (121). Furthermore, the number of [^{3}H] cytosine and [^{125}I]BTX binding sites in CA3 region of the hippocampus in postmortem schizophrenic brains was significantly reduced compared to control brains, indicating a deficit in nAChR number in schizophrenia (122).

nAChRs are apparently involved in the pathophysiology of both anxiety disorders and

depression. Nicotine administration has been observed to have anxiolytic effects in humans and in animal models of anxiety (123). This action can be blocked by administration of the non-competitive nAChR antagonist mecamylamine and by the benzodiazepine inverse agonist flumazenil.

One major potential use of drugs that act on the nAChRs is 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 (124).

The antihelmintic agents morantel, pyrantel and levamisole have been shown to have an action at the ACh receptors of the nematode Ascaris suum (125). They are thought to bring about their therapeutic effect by acting at ACh receptors to increase conductance and depolarization of the muscle bag membrane resulting in maintained muscle contraction.

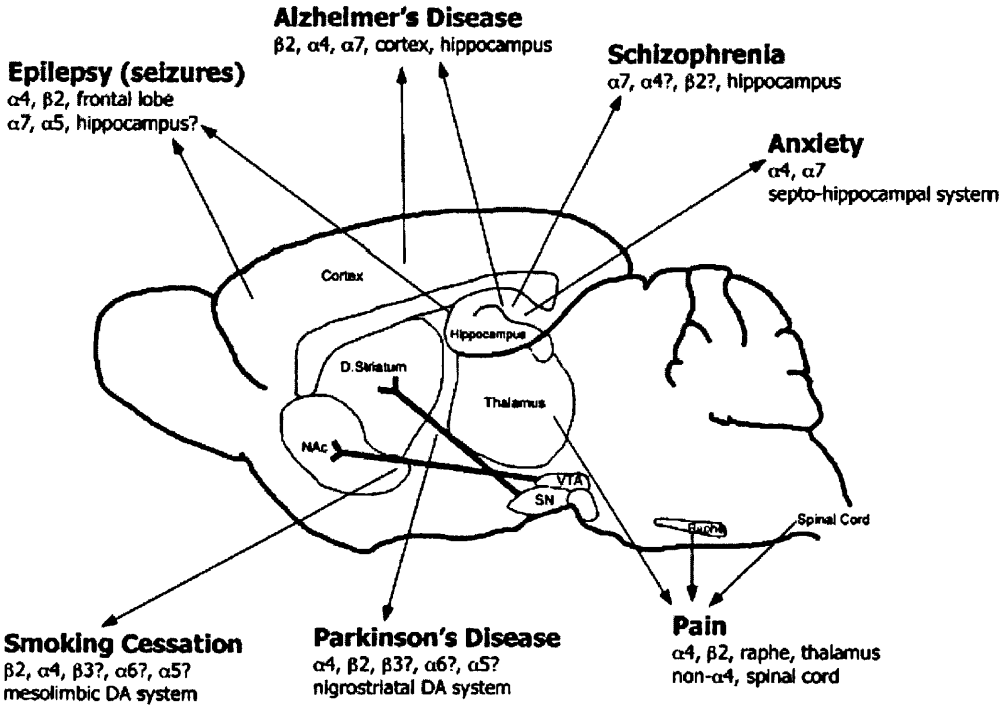

FIG.1. 4 Sagittal brain section showing the putative anatomical loci and nAChR subunit compositions implicated in the etiology of several neurological and psychiatric disorders. The possible targets for the therapeutic actions of nicotine in these disorders, as well as in smoking cessation and treatment of pain are also shown. NAc, nucleus accumbens (126).

1.8. Genetic analysis of nicotinic signaling on worms

The nicotinic acetylcholine receptor is among the most thoroughly characterized molecules in the nervous system, and its role in mediating fast cholinergic neurotransmission has been broadly conserved in both vertebrates and invertebrates. However, the accessory molecules that facilitate or regulate nicotinic signaling remain mostly unknown. One approach to identify such molecules is to use molecular genetics in a simple, experimentally accessible organism to identify genes required for nicotinic signaling and to determine the molecular identify of the mutant genes through molecular cloning. Because cellular signaling pathways are often highly conserved between different animal species, the information gained from studies of simple organisms has

historically provided many critical insights into more complex organisms, including humans. Genetic screens essentially make no prior assumptions about the types of molecules involved in the process being studies; thus, they are well suited for identifying previously unknown components of cell signaling pathways. The sophisticate genetic tools available in organisms such as the nematode *C. elegans* and the fruit fly *Drosophila melanogaster* have also proven extremely powerful in elucidating complex biologic pathways in the absence of prior biochemical information and for assessing a molecule's *in vivo* function of in the context of an intact nervous system.

C.elegans is particularly well-suited to molecular studies of nervous system function: it has a simple nervous system containing 302 neurons, and the position, cell lineage, and synaptic connectivity of each of these neurons is precisely known(127).

At least 40 genes with sequence similarity to nicotinic receptor subunits have been identified in the *C.elegans* genome; although at present only 27 of these have been shown to be authentic nicotinic receptor homolog (127).

With the sequencing of the *C. elegans* genome, the known nematode nAChR gene family has grown considerably. Using reverse transcription- polymerase chain reactions, the transcription of 17 of the predicted nAChR subunit gene (14α and 3 non-α) has been demonstrated an ACR (acetylcholine receptor) number (128). Together with 10 other subunit genes described earlier (deg-3, des-2 or acr-4, acr-4, acr-16, acr-5, acr-2, acr-3,

lev-1, unc-29, unc-38 and unc-63) (129), this is now the largest known family (at least 27 members) of nAChR subunit genes in a single species (128).

In *Caenorhabditis elegans*, the ric-3 gene is required for the maturation of multiple nicotinic acetylcholine receptors (nAChRs), whereas other neurotransmitter gated channels expressed within the same cells are unaffected by the presence of RIC-3 (130). Mutations in ric-3 were identified initially in screens for mutants resistant to the acetylcholinesterase inhibitor aldicarb (131). Loss-of-function mutations in ric-3 also confer resistance to levamisole (132) and suppress the cell-death phenotype of deg-3 dominant mutations (133). Importantly, ric-3 mutants exhibit abnormalities in pharyngeal pumping, which lead to defects in feeding (FIG 1.5). Rapid pharyngeal pumping in involves excitatory neurotransmission through a nicotinic acetylcholine receptor; electrophysiological recording demonstrated that this cholinergic neurotransmission was greatly impaired in ric-3 mutants. Both the levamisole-sensitive and the levamisole-insensitive nAChRs (but not the GABA receptor) in the body muscle NMJ were severely defective in ric-3 mutants (133). Thus, ric-3 appeared to encode a molecule that is specifically required for the activity of multiple nicotinic receptors. This finding inspired the further study about more promising candidates.

EAT is a group of proteins closed linked with the *C. elegans* feeding (pharyngeal pumping) activities. 16 members in this family have been identified so far (TABLE 1.3). The members in this protein family encode a wide range of different type of protein. A recent published paper suggests a promising new candidate, eat-18. The study has

demonstrated that mutations in eat-2 and eat-18 cause the same defect in *C. elegans* feeding behavior: the pharynx is unable to pump rapidly in the presence of food. EAT-2 has been proved to be a nicotinic acetylcholine receptor subunit that functions on the pharyngeal muscle. It is localized to the synapse between pharyngeal muscle and the main pharyngeal excitatory motor neuron MC, and it is required for MC stimulation of pharyngeal muscle. However, eat-18 encodes a small protein that has no homology to previously characterized proteins. It has a single transmembrane domain and a short extracellular region. Allele-specific genetic interactions between eat-2 and eat-18 suggest that EAT-18 interacts physically with the EAT-2 receptor. While eat-2 appears to be required specifically for MC neurotransmission, eat-18 also appears to be required for the function of other nicotinic receptors in the pharynx. In eat-18 mutants, the gross localization of EAT-2 at the MC synapse is normal, suggesting that it is not required for trafficking. These data indicate that eat-18 could be a novel component of the pharyngeal nicotinic receptor (134).

Gene	Introduction	Reference
eat-1	It encodes both a homolog of mammalian alpha-actin associated LIM protein (ALP) and a homolog of mammalian Enigma protein.	(135)
eat-2	It encodes a beta subunit of the nicotinic acetylcholine receptor (nAChR) superfamily, which encodes ligand-gated ion channels that regulate fast action of acetylcholine at neuromuscular junctions and in the nervous system.	(134)
eat-3	It encodes a mitochondrial dynamin-related protein that is orthologous to human OPA1.	(136;137)
eat-4	It encodes an ortholog of the mammalian BNPI vesicular glutamate transporter that affects chemotaxis, feeding, foraging and thermotaxis.	(138)
eat-5	It encodes an innexin, expressed in pharyngeal muscle cell groups pm4 and pm5 and that is required for synchronized pharyngeal muscle contractions.	(139)

eat-6	It encodes an ortholog of the alpha subunit of a sodium/potassium ATPase, which in turn affects Na+, K(+)-ATPase activity of membranes by affecting the level of the phosphorylated intermediate of the Na+, K(+)-ATPase. EAT-6 also affects relaxation of the pharyngeal muscles.	(140)
eat-7	Uncloned locus that appears to affect the initiation of many activities that includes movement, feeding, and defecation when an external stimulus absent, and also affects life span.	(141)
eat-8	The mutation of eat-8 would result in animals that exhibit only brief and rare pharyngeal pumping and also display slightly loopy movement. However, the molecular identity of eat-8 is not yet known.	(142)
eat-9	It is required for a normal speed and rhythm of pharyngeal pumping.	(143)
eat-10	It was identified in screens for genes required for normal development and function of the excitable cells of the pharynx.	(136)
eat-13	It is required for normal retention of food during the pharyngeal pumping cycle.	(144)
eat-14	Uncloned locus that affects relaxation of the pharyngeal grinder muscle.	(136)
eat-15	Link with the abnormal pharyngeal pumping.	(136)
eat-16	It encodes an RGS protein that affects movement, pharyngeal pumping, egg laying, and synaptic transmission.	(145)
eat-17	Uncloned locus that affects feeding by affecting posterior movement of bacteria in the pharyngeal corpus and isthmus.	(144)
eat-18	EAT-18 is expressed in pharyngeal muscle, is required for neurotransmission by the MC pharyngeal neuron.	(134)
eat-20	It encodes a paralog of the C. elegans and Drosophila gene crb-1 and crumbs, expressed in pharynx, head neurons, hypodermis, and developing embryos.	(144)

TABLE 1.5 Brief summarize the function of the members in EAT protein family.

1.9. The aim of this study

The project is to study the influence of a small protein, EAT-18, upon neuronal nicotinic acetylcholine receptors (nAChRs).

Chapter 2

2.0 Methods

2.1 Plasmid constructs and subcloning

2.1.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) thermocycling was performed in a Peltier Thermal Cycler, PTC-225 (MJ Research). Typical reactions were performed in 20 µl volumes and contained 10-20 ng plasmid DNA, 250 µM dNTPs, 0.25µM forward and reverse primers and 2.5 U *Pfu* polymerase (Stratagene) in 1X *Pfu* polymerase reaction buffer. *Pfu* polymerase was only used for the generation of PCR fragments for use in subcloning reactions.

Typical reactions for the generation of fragments for use in subcloning involved a 5 min denaturation step at 95°C, followed by thermocycling with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds, repeated for 30 cycles. A final extension step was carried out at 72°C for 5 minutes.

2.1.2 Restriction digestion of DNA

Typically, 2-3 µg plasmid DNA was subjected to digestion with 5-10 U restriction enzyme in a 20 µl reaction volume containing 1X reaction buffer compatible with the specific enzyme. Digests were incubated for 1 hour at the incubation temperature required for optimal enzyme activity (usually 37°C, but some enzymes require incubation at 25°C or 55°C). For double digests in which the reaction buffers of the two enzymes were incompatible, digestion with the first enzyme was carried out in a 20µl reaction

volume for 1 hour. The reaction mix was then diluted to 60µl with milli-Q (MQ) water, the second reaction buffer added to a 1X final concentration and the digestion continued with the second enzyme for 1 hour.

2.1.3 Dephosphorylation of DNA

When subcloning strategies involved digestion with a single enzyme, 5'-phosphate groups were removed from the digested DNA using calf intestinal alkaline phosphatase (CIAP; Promega) to prevent re-ligation of the plasmid vector. Typically, 2-3 µg digested plasmid DNA was incubated with 0.2 U CIAP for 30 min at 37 °C. A further 0.2 U CIAP was added and the samples incubated again at 37°C for 30 min. For dephosphorylation of DNA with 5' recessed or blunt ends, DNA was incubated with CIAP at 37°C for 15 minutes, then at 56°C for 15 minutes to ensure accessibility of the recessed ends. A second aliquot of CIAP was added and the samples incubated a 37°C for a further 15 minutes, then at 56°C for 15 minutes.

2.1.4 Agarose gel electrophoresis and DNA purification

Agarose gel electrophoresis allows the visualisation and separation of digested plasmid DNA or PCR products according to molecular weight. Restriction enzyme digests or PCR products were separated by electrophoresis through 1% agarose gels (GibcoBRL) and run against 1µg *Hind*III digested Lambda(λ) DNA standard marker (Invitrogen) in order to estimate the relative size of the DNA bands. When the DNA fragments were required for further subcloning steps, low melting point agarose gels were used to allow subsequent purification. DNA bands were excised from the gel with a scalpel and

transferred to microfuge tubes. DNA was extracted from the gel slices using the Wizard™ DNA clean-up system (Promega) according to the manufacturer's instructions. Briefly, the gel slices are dissolved in 1 ml Wizard™ DNA clean-up Resin and forced through a mini-column using a 2ml syringe. DNA bound to the column is washed with 2ml isopropanol and excess isopropanol removed by centrifugation at 13000 rpm for 2min in a bench-top microfuge. The DNA is eluted in 50µl purified DNA was run on a 1% agarose gel against *Hind*III digested λ DNA to assess the yield.

2.1.5 DNA Ligations

Ligation reactions typically contained a molar ratio of vector: insert of 1:3 in a reaction volumn of 10 µl. Background levels of re-ligated vector were assessed through control reactions in which insert was substituted with MQ water. Reactions contained 1mM ATP and 0.5 U T4 DNA ligase (Roche) and were incubated at 16°C overnight.

2.1.6 *E. coli* Transformations

2µl ligation mixture or 1-20 ng plasmid DNA was added to 50µl chemocompetent cells in a 5 ml polypropylene transformation tube (Falcon) pre-chilled on ice. The DNA and competent cells were mixed gently by swirling and incubated on ice for 30 minutes. The cells were subjected to heat-shock at 42°C for 90 seconds and allowed to recover for 2 minutes on ice. 600µl SOC medium (20g/L tryptone, 5g/L yeast extract, 0.5g/L NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 20mM glucose, sterilised by autoclaving at 10 lb./sq. in. for 15 minutes) was added to the cells and the tubes incubated at 37°C with shaking at 200 rpm for 1 hour to allow expression of the plasmid's antibiotic resistance gene. 20-200µl

aliquots of transformation mixture were plated onto Luria-Bertani (LB)- agar plates (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0 using NaOH, 15 g/L agar) containing inhibitory concentrations of appropriate antibiotic (50μg/ml ampicillin ± 10μg/ml tetracycline) and grown at 37°C overnight (~17 hours).

2.1.7 Screening colonies

Individual colonies were picked from the agar plates and screened for the presence of the correct DNA construct either by PCR or by extraction of DNA and subsequent restriction mapping. In a typical PCR of bacterial colonies, thermocycling steps followed a 5 minutes denaturation step to help lyse the bacterial cells and involved denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds, repeated for 30 cycles. A final extension step was carried out at 72°C for 5 minutes.

2.1.8 Small scale preparation of plasmid DNA

Where PCR was an inappropriate screening method, small scale, miniature preparations (mini-preps) of plasmid DNA were extracted via alkaline lysis method (149). 2 ml aliquots of LB medium (10g/L tryptone, 5 g/L yeast extract, 10g/L NaCl, pH7.0 using NaOH) were inoculated with individual bacterial cultures were pelleted by centrifugation at 13000 rpm for 1 minutes in a bench-top microfuge. Pellets were resuspended in 100μl ice-cold Solution I (50mM glucose, 25mM Tris/Cl pH 8.0, 10 mM EDTA pH8.0, autoclaved at 10 lb./sq. in. for 15 minutes) and lysed with the addition of 200μl Solution II (0.2 M NaOH, 1% SDS), mixing gently by inversion. 150μl ice-cold Solution III (per 100 ml: 60ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml MQ water) was

added and mixed to halt the lysis reaction and induce precipitation of chromosomal DNA. Samples were incubated on ice for 5 min and centrifuged at 13000 rpm, 5 minutes. The supernatant was phenol:chloroform (1:1, pH=8.0) extracted and ethanol precipitated, incubating in 2X volumes of 99% ethanol for 2 minutes at room temperature. Samples were centrifuged for 5 minutes at 13000 rpm and the DNA pellets washed in 1 ml ice-cold 70% ethanol. Air-dried pellets were resuspended in 50µl Tris-EDTA (TE) buffer, pH 8.0, containing 10µg/ml RNAase A (DNAse-free, Sigma). Positive clones were identified by restriction mapping and subsequent agarose gel electrophoresis.

2.1.9 Large-scale preparation of plasmid DNA

Single *E. coli* colonies containing plasmid DNA were used to inoculate 250ml LB medium cultures for large-scale plasmid DNA purification using Qiagen™ Plasmid Maxi Kit purification columns. Bacterial cultures were pelleted by centrifugation in a Beckman J2-M1 centrifuge for 10 minutes at 6000 rpm (6000 x g), 4°C using a JA-14 rotor. Pellets were resuspended in 10ml ice-cold Buffer P1 (50 mM Tris-Cl pH 8.0, 10mM EDTA, 100µg/ml RNase A) and lysed with incubation in 10ml Buffer P2 (200 mM NaOH, 1% (w/v) SDS) for 5 minutes at room temperature. Lysis was terminated and precipitation initiated by additional of 10ml ice-cold Buffer P3 (3M potassium acetate, PH 5.5), incubating on ice for 20 minutes. Samples were centrifuged at 13000 rpm (>20000 x g) for 30 minutes at 4°C in a JA-14 rotor. Sample supernatants were added to the Qiagen™ purification columns to extract the plasmid DNA following equilibration of the columns through addition of 10 ml Buffer QBT (750 mM NaCl, 50mM MOPS pH= 7.0, 15% (v/v) isopropanol). DNA bound to the columns was washed twice in 30 ml Buffer QC (1M

NaCl, 50 mM Tris-Cl pH= 8.5, 15% (v/v) isopropanol via centrifugation at 10500 rpm (> 15000 x g) for 30 minutes at 4°C in a JA-17 rotor. DNA pellets werewashed in ice-cold 70% ethanol and resuspended in 1ml sterile MQ water. DNA purity and concentration was determined by measurement of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a BIORAD SmartSpec™ 3000 spectrophotometer. A_{260}= 1 represents approximately 50μg/ml double stranded DNA. An A_{260}:A_{280} ratio of 1.6-2.0 indicates a pure DNA preparation. Ratios significantly less than 1.6 indicate protein contamination and ratio greater than 2.0 indicate salt impurities. Maxi prep DNA of positive clone was analysed by restriction mapping and sequencing (Section 2.4).

2.1.10 Plasmid expression vectors

The expression vector pRmHa3 is based on the bacterial plasmid pUC18 and contains the promoter, metal response element and transcriptional start site from the metallothonein gene. This is followed by the unique cloning sites *EcoR*I, *Sac*I, *Kpn*I, *Sma*I, *BamH*I, and *Sal*I and the polyadenylation signal from the *Drosophila melanogaster* alcohol dehydrogenase. It also contains the origin of replication (ori) and the beta- lactamase gene, which confers resistance to ampicillin (amp r) from pUC18 (FIG 2.1).

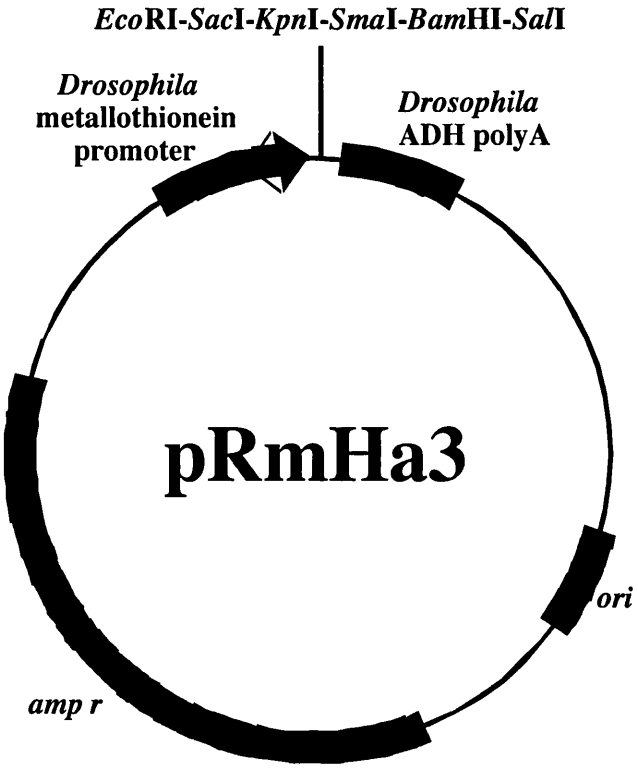

FIG 2.1 The picture brief indicate the main features of the transfection vector pRmHa3.

2.1.11 Plasmid constructs

The Rα3, Rα4, Rα7, Rβ2 and Rβ4 subunit cDNAs was provided by Dr. Jim Patrick, Baylor College of Medicine, Houston. Then Rα7 cDNA was excised from pcDNA and subcloned into pRmHa3 by Dr. Sandra T. Cooper, Department of Pharmacology, UCL. Rα4 and β2 were subcloned into pRmHa3 by Dr. Neil Millar, Pharmacology Department, UCL; Rat β4, α3 and *Drosophila* α3, β4, SAD, SBD, ARD, ALS were provided and subcloned into pRmHa3 by Dr. Stuart J. Lansdell, Pharmacology Department, UCL. eat-18 and eat-2 constructs were provided by Dr. James P. McKay, Department of Molecular Biology, University of Texas Southwestern Medical Center. Both Eat-18 and Eat-2 were excised from pDH105 *Eco*RI site of pDH105 and subcloned into the *Eco*RI sites of

pRmHa3. *C.elegans* ric-3 construct was provided by Dr. Sarah Halevi (Department of Physiology, Hebrew University-Hadassah Medical School, Israel) and subcloned into pRmHa3 by Anne Doward (Department of Pharmacology, UCL).

2.2 Sequencing

Fluorescence-based cycle sequencing was carried out using the ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, template DNA (0.1-10μg) and specific primer are mixed with 8μl Terminator Ready Reaction Mix in 20μl reaction volumes. Terminator Ready Reaction Mix contains BigDye® (dye-labelled ddNTP) terminators, FS. AmpliTaq® DNA polymerase, unlabelled dNTPs, $MgCl_2$ and buffer. FS. AmpliTaq® DNA polymerase is a variant of *Taq* DNA polymerase, with a point mutation in the active site that reduces discrimination for dideoxynucleotides (ddNTPs). Thermocycling steps involve denaturation at 96°C for 30 seconds, annealing at 50°C for 15 second and extension at 60°C for 4 minutes, for 25 cycles. Reactions are ethanol/sodium acetate precipitated using 2μl sodium acetate, pH= 5.2 and 50μl 99% ethanol per 20μl reaction, incubated on ice for 10 minutes and centrifuged at 13000 rpm, 15 minutes in a microfuge. Pellet are washed in 250 μl 70% ethanol, air-dried and resuspended in 10μl formamide. ABI PRISM® 3100-*Avant* Genetic Analyzer (ABI Applied Biosystems). Reactions were run on a 50 cm capillary (ABI Applied Biosystems) array using POP 6 polymer (ABI Applied Biosystems). Data was extracted using 3100-*Avant* Data Collection Software Version 1.0 (ABI Applied Biosystems).

2.3 Transfections of cultured cell line

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 440µl in 0.1X TE [10 mM Tris. Cl (pH7.4) and 1mM EDTA (pH=8.0)] with a total amount of DNA in the region of 20-30 µg. 500µl 2X HEPES-buffered saline (HBS), (280 mM NaCl, 10mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12mM dextrose and 50mM HEPES) was slowly added with gentle mixing followed by 62 µl of 2M $CaCl_2$ and incubated for 30 minutes to allow the precipitate to form. Exponentially growing S2 cells harvested and spun at 900g for 3 minutes. The supernant 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 minutes. To each tube 9 ml prewarmed medium was added and the cells plated out in $25cm^2$ flasks. The cells were incubated for 6 hours at 25°C and were then harvested, centrifuged at 900g for 3 minutes, resuspended in fresh prewarmed medium and replated in flasks for 24-48 hours at 25 °C.

Expression of the nAChR subunits was induced by the addition of $CuSO_4$ to the growth medium of semi-confluent transfected S2 cells to a final concentration of 0.6 mmol l^{-1} ($CuSO_4$ was prepared as a 30 mmol l^{-1} stock). Induction of expression is by the inducible metallothionein promoter on the pRmHa3 expression vector.

2.4 Radioligand binding

Reaction conditions for equilibrium radioligand binding, including the transfected cell line, the radioligand and the compounds used to determine non-sepecific binding all depended on the particular cDNA combinations being assayed. Samples were harvested using a Brandel Cell Harvester (Model M-36, Semat, UK) in ice-cold 10mM potassium phosphate buffer (for membrane preparations) onto GF/B glass fibre filters (Whatman) pre-soaked for 2h in 0.5% (w/v) polyethylenimine (PEI). Filters were equilibrated for 24 h in 5ml "Ready Safe" scintillation cocktail (Beckman) and counted for radioactivity in a scintillation counter.

For radioligand binding assays with S2 cells transiently transfected with combination of subunits, cell membranes were incubated with 30nM [^{3}H]-epibatidine (PerkinElmer Life Sciences, Boston; specific activity 56.2 Ci/mmol), 30nM [^{3}H]-methyllyacconitine ([3H]-MLA; Tocris Cookson Ltd, Avonmouth, UK; specific activity 26 Ci/mmol). Non-specific binding was typically determined by addition of 1mM nicotine and 1mM carbamylcholine chloride (carbachol).

For binding studies with [^{3}H]-epibatidine (Du Pont NEN) and [^{3}H]-MLA on cell membrane preparations, cells were washed twice in phosphate buffered saline (PBS) and resuspended and assayed in 10mM potassium phosphate buffer (pH=7.2) containing the protease inhibitors leupeptin (2μg/ml) pepstatin (1μg/ml). Cells were incubated with radioligand for 120 min at 4°C in a total volume of 300μl. Non-specific binding was determined by addition of 1mM carbachol. Radioligand binding was assayed by filtration onto 0.5% polyethlenimine presoaked Whatman GF/B filters followed by rapid washing

with cold 10mM potassium phosphate buffer using a brandel cell harvester. Radioactivity was measured by scintillation counting.

Chapter 3

3.0 Results

3.1. Subclone the eat-18 and eat-2 cDNAs into pRmHa3

eat-18 and eat-2 cDNAs were excised from pDH105 provided by Dr. James McKay as described in section 2.1.11 and subsequently subcloned into pRmHa3. DNA sequencing has been used to confirm these sequences are at the right orientation. After successfully subcloning the eat-18 and eat-2 cDNAs sequence into the pRmHa3 vector, a variety of nicotinic acetylcholine receptors subunits combinations have been co-expressed with EAT-18. The effect of how the EAT-18 might influence the expression of nAChRs has been investigated by radioligand binding studies.

3.2. Specific [^{3}H]-MLA binding to transiently transfected S2 cell lines expressing nAChRs subunits combinations

The radio-labelled α7 selective antagonist methyllycaconitine (MLA) was applied to test whether the new protein EAT-18 can help the homomeric nAChRs increase ligand binding. The results obtained by transient transfect on of these homomeric nAChRs in S2 cells are presented in TABLE 3.1 and FIG 3.1.

Statistically, no significant increase of [^{3}H]-MLA binding has been detected. However, when expressing ric-3 with rat α7 or human α7 nAChR an obvious increase of ligand binding was observed (FIG 3.1).

DNA Plasmid Transfected	[^{3}H]-MLA Binding (fmol/mg)	Number of Binding Assay (n)
S2	19.92±1.55	3
Rα7	15.36±0.85	3

Rα7+eat-18	14.54±2.33	3
Rα7+ric-3	27.75±6.47	3
Rα7+ric-3+eat-18	20.69±9.73	3
Hα7	25.75±0.54	3
Hα7+eat-18	19.92±5.33	3
Hα7+ric-3	60.94±6.92	3
Hα7+ric-3+eat-18	74.24±18.86	3
Dα7+eat-18	16.02±14.25	3
Dα6+eat-18	8.98±12.53	3

TABLE 3.1 Summary of [^{3}H]-MLA binding to cell membranes prepared from of S2 cells transiently transfected with homomeric nAChRs. Number of independent experiments performed also presented. Data are means (± standard error) of 3 independent experiments performed in triplicate and are listed as fmol/mg of protein.

3.2. Specific [^{3}H]-Epibatidine binding to transiently transfected S2 cell line expressing nAChRs subunits combinations

Previous work has shown that transient transfection of Rα4, β2, D*rosophila* ALS, SAD, ARD, SBD into S2 cells gives no [^{3}H]-Epibatidine (30nM) binding. Also, no [^{3}H]-Epibatidine (30nM) binding can be detected when co-expressing Dα3/ARD, Dα3/SBD, SAD/SBD, ARD/SBD and ALS/SAD/ARD nAChR subunits together in S2 cells. (150; 151) However, when transfecting S2 cells with rat α4/β2, ALS/β2(rat), SAD/β2(rat), Dα3/β2(rat) and Dα3/β4 subunit combination specific binding of [^{3}H]-Epibatidine can be detected (150;151). The data in this series of experiments is consistent with these previously published data. The non-selective agonist [^{3}H]-Epibatidine was applied on a variety of heteromeric combinations of nAChRs in order to explore the potential influence that EAT-18 can exert on level of nicotinic radioligand binding. The ligand binding data is shown in TABLE 3.2 and FIG 3.2.

DNA Transfected	[^{3}H]-Epibatidine Binding	Number of Binding Assay (n)
S2	3.68±3.00	8
eat-18	2.57±1.74	3
eat-2	10.39±129.06	3
α4β2	899.85±246.12	6

α4β2+eat-18	1115.42±20.71	6
ALSβ2	38.00±14.7	5
ALSβ2+eat-18	31.46±44.17	5
α3β4	88.40±51.6	3
α3β4+eat-18	115.19±11.04	3
Rα7	32.46±2.63	2
Rα7+eat-18	6.32±5.07	2
Hα7	11.11±7.37	2
Hα7+eat-18	3.42±46.34	2
Dα3β2	135.66±37.76	3
Dα3β2+eat-18	142.05±8.18	3
SADβ2	124.73±19.38	3
SADβ2+eat-18	146.21±44.75	3
ALS/SAD/SBD+eat-18	2.76±1.84	3
Dα3/SAD/SBD+eat-18	2.37±1.29	4
Dα3/ARD/SBD+eat-18	0.69±0.85	3

TABLE 3.2 summary of [^{3}H]-Epibatidine binding to cell membranes prepared from of S2 cells transiently transfected with heteromeric nAChRs subunit combinations. Number of independent experiments performed also presented. Data are means (± standard error) of several independent experiments performed in triplicate and are listed as fmol/mg of protein.

3.4. Comparison of the ligand binding level on nAChRs subunits combinations expressed with or without EAT-18

It is clear that in FIG 3.2, Rα4/β2, Rα3/β4, *Drosophila* ALS/β2 and Dα3/β2 transient tranfected to S2 cells did show certain level of ligand binding when compared with other nAChRs subunits combinations (FIG 3.3- FIG 3.5). And even though rat and human α7 nAChRs cannot form a functional receptor correctly. However, when co-expressing with ric-3 an obvious increase of ligand binding level can be detected (FIG 3.6, FIG 3.7).

The levels of ligand binding on the nAChRs expressed with EAT-18 were normalized to the readings of nAChRs expressed without EAT-18 in order to minimise the variation from each independent experiments. Statistically, the nAChRs subunit combinations co-expressed with EAT-18 do not appear to be having significant difference between the ligand binding level. (FIG 3.3 to FIG 3.7)

Even though an obvious increase of [^{3}H]-MLA binding level has been observed when rat α7 or human α7 nAChRs are co-expressed with ric-3 and ric-3 + eat-18, the differences are still not significant ($p < 0.05$). (FIG 3.8, FIG 3.9)

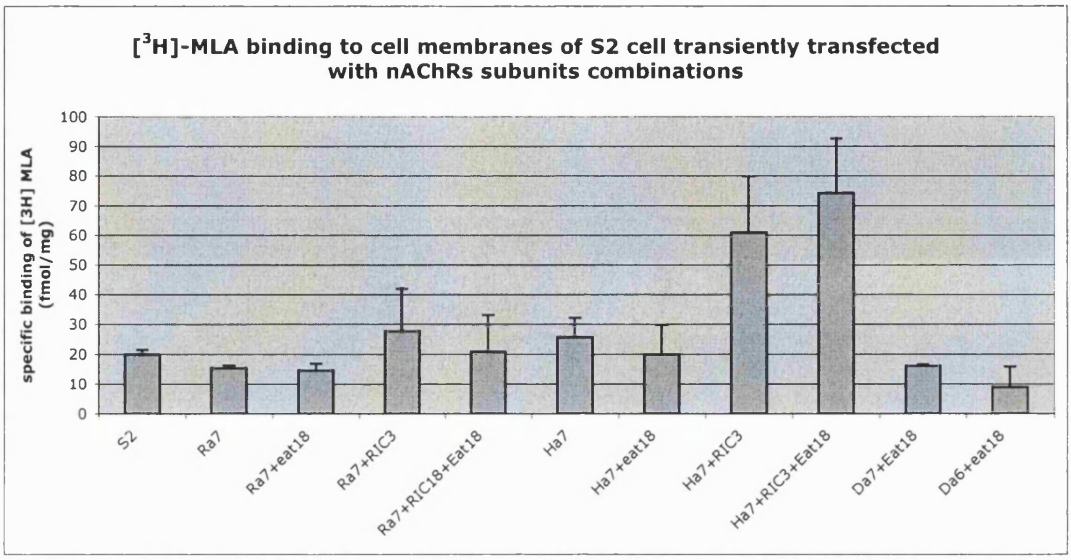

FIG 3.1 Specific [^{3}H]-MLA binding to cell membranes of S2 cells transiently transfected with Human, Rat, *Drosophila* homomeric nAChR combinations (Rα7, Hα7, Dα7, Dα6). Rat and Human α7 nAChRs are co-expressed with ric-3 and ric-3+eat-18. Data are presented as means (± standard error) of three independents experiments preformed.

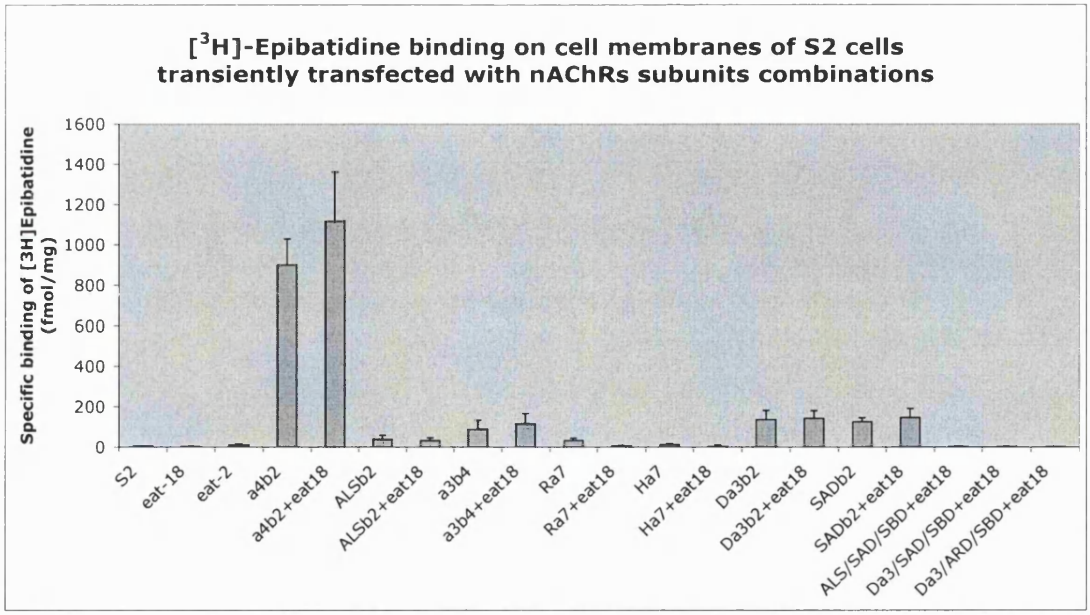

FIG 3.2 Specific [^{3}H]-Epibatidine binding on a variety of heteromeric nAChRs subunits combinations on S2 cell membrane preparation.

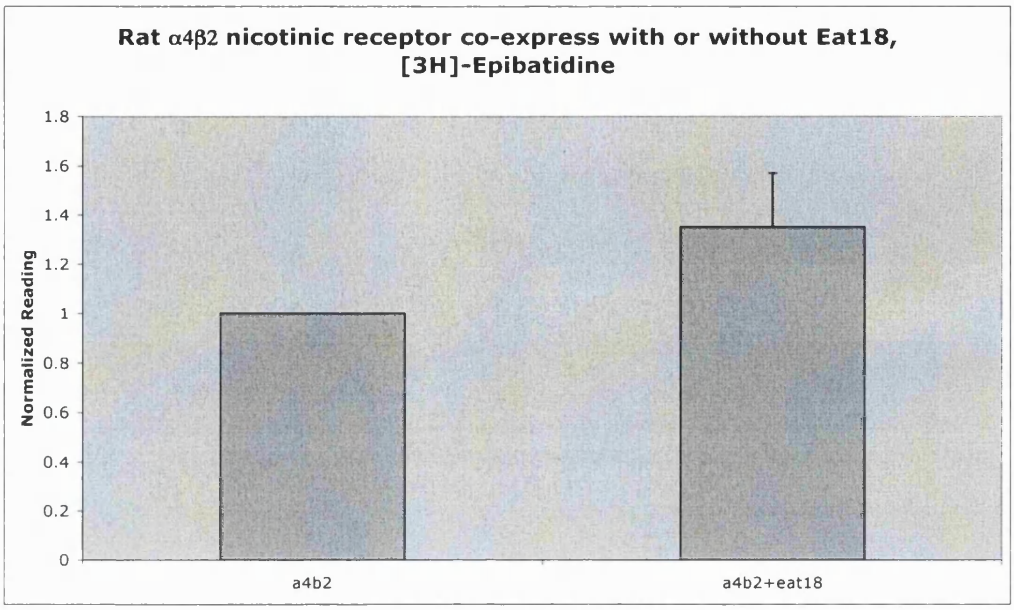

FIG 3.3 Specific [^{3}H]-Epibatidine binding to Rat α4β2 and Rat α4β2+eat-18. Data are means (±standard error) of 6 independent experiments performed in triplicate and presented by normalizing to the α4β2 ligand binding level (α4β2 ligand binding level have been treated equal to one). Significance determined by a two-tailed Student's t test.

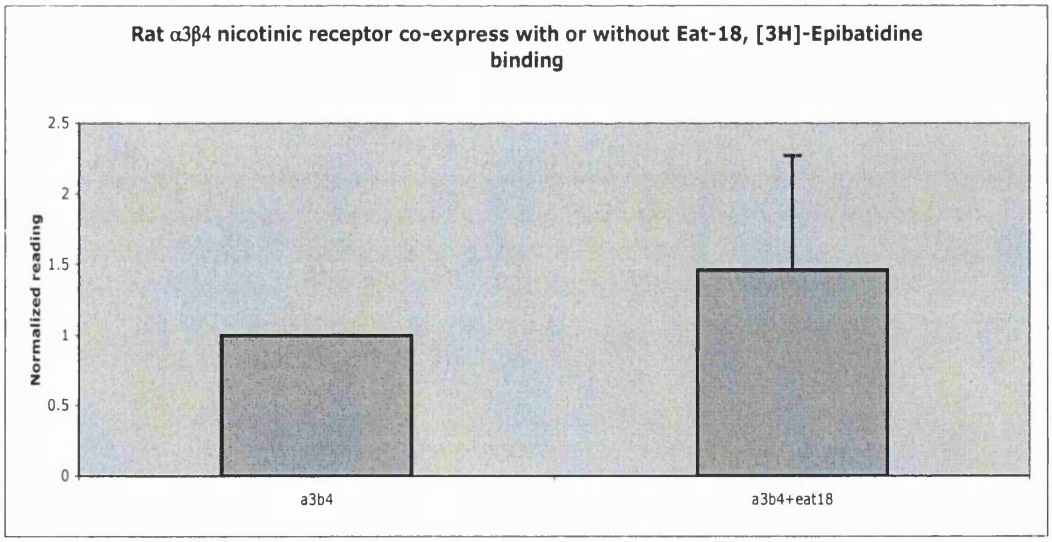

FIG 3.4 Specific [^{3}H]-Epibatidine binding to Rat α3β4 and Rat α3β4+eat-18. Data are means (±standard error) of 3 independent experiments performed in triplicate and presented by normalizing to the Rat α3β4 ligand binding level (Rat α3β4 ligand binding level have been treated equal to one). Significance determined by a two-tailed Student's t test.

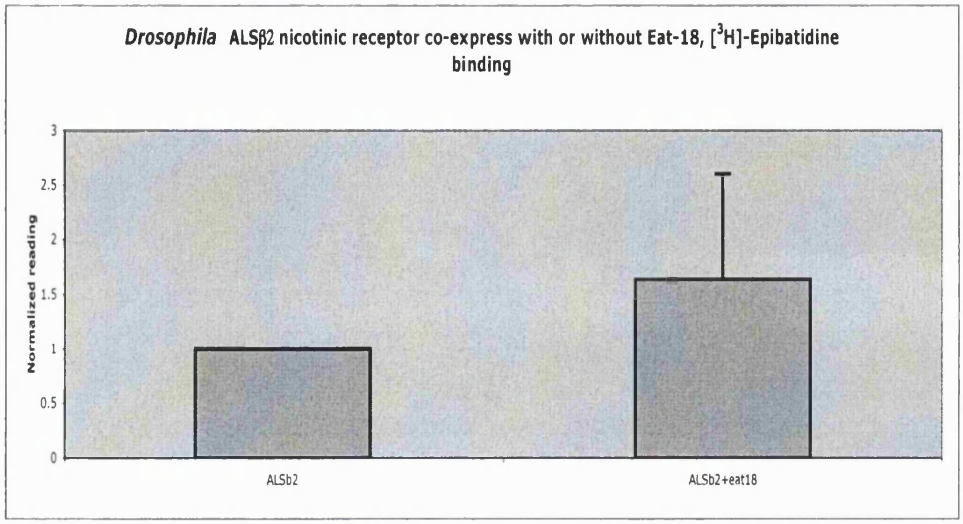

FIG 3.5 Specific [^{3}H]-Epibatidine binding to ALSβ2 and ALSβ2+eat-18. Data are means (±standard error) of 5 independent experiments performed in triplicate and presented by normalizing to the ALSβ2 ligand binding level (ALSβ2 ligand binding level have been treated equal to one). Significance determined by a two-tailed Student's t test.

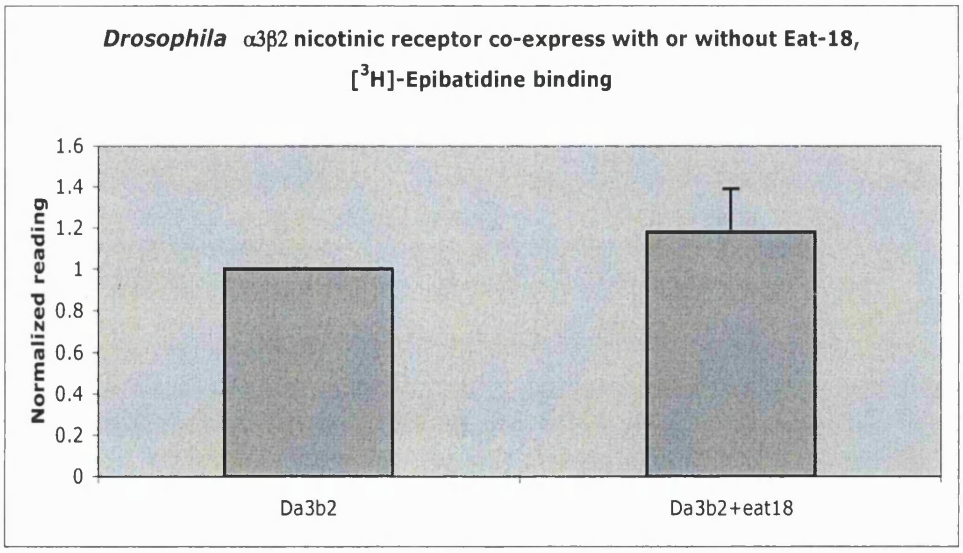

FIG 3.6 Specific [^{3}H]-Epibatidine binding to *Drosophila* α3β2 and *Drosophila* α3β2+eat-18. Data are means (±standard error) of 3 independent experiments performed in triplicate and presented by normalizing to the *Drosophila* α3β2 ligand binding level (*Drosophila* α3β2 ligand binding level have been treated equal to one). Significance determined by a two-tailed Student's t test.

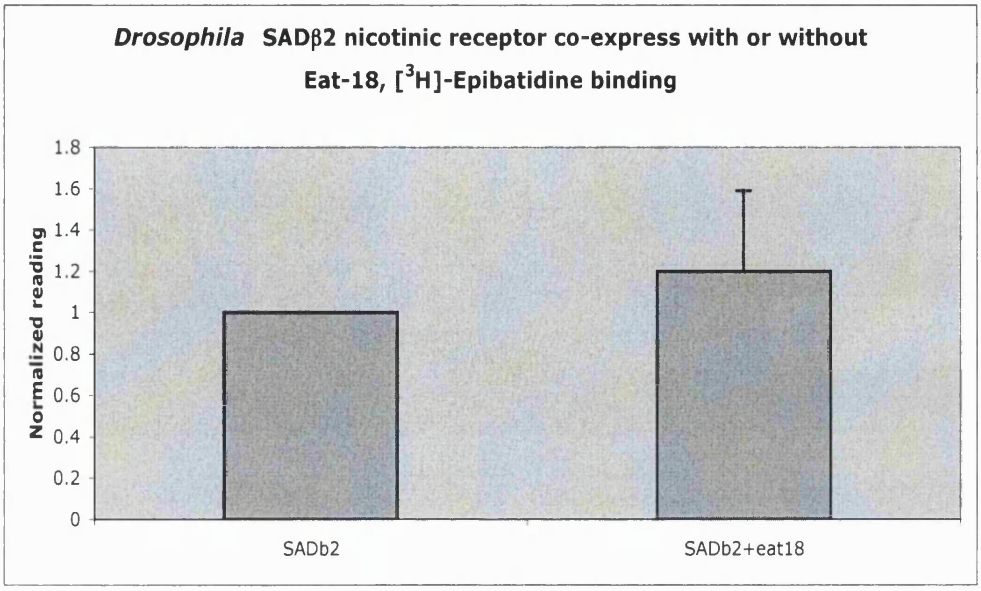

FIG 3.7 Specific [^{3}H]-Epibatidine binding to SADβ2 and SADβ2+eat-18. Data are means (±standard error) of 3 independent experiments performed in triplicate and presented by normalizing to the SADβ2 ligand binding level (SADβ2 ligand binding level have been treated equal to one). Significance determined by a two-tailed Student's t test.

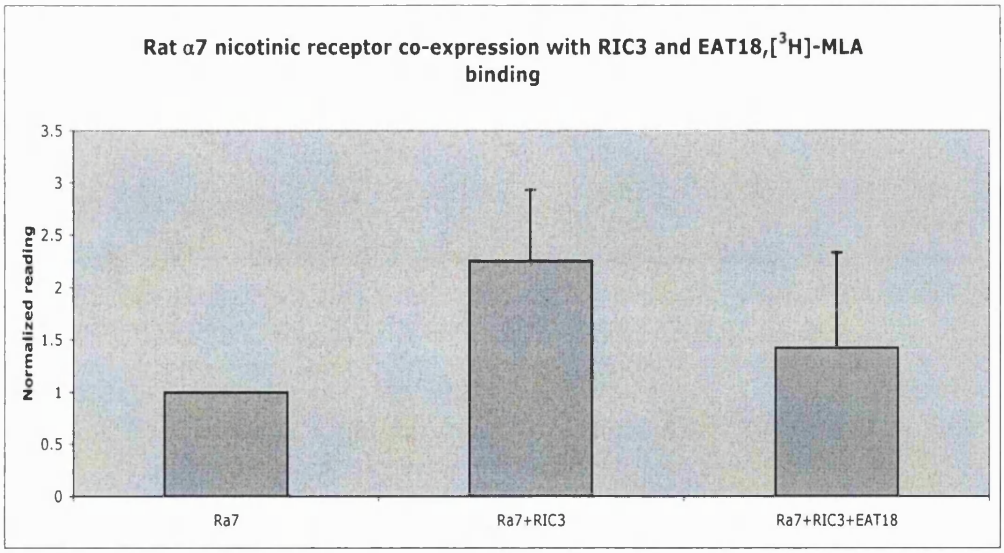

FIG 3.8 Specific [^{3}H]-MLA binding to cell membranes of S2 cells expressing nAChR and associated protein. S2 cell were transiently transfected with Rα7, Rα7 with ric-3 and Rα7 with ric-3+eat-18. Data are means (±standard error) of three independent experiments performed in triplicate and are presented by normalizing to the [^{3}H]-MLA binding level of Rα7. The Rα7 was treated as 1. Significance determined by a two-tailed Student's t test.

binding level of Rα7. The Rα7 was treated as 1. Significance determined by a two-tailed Student's t test.

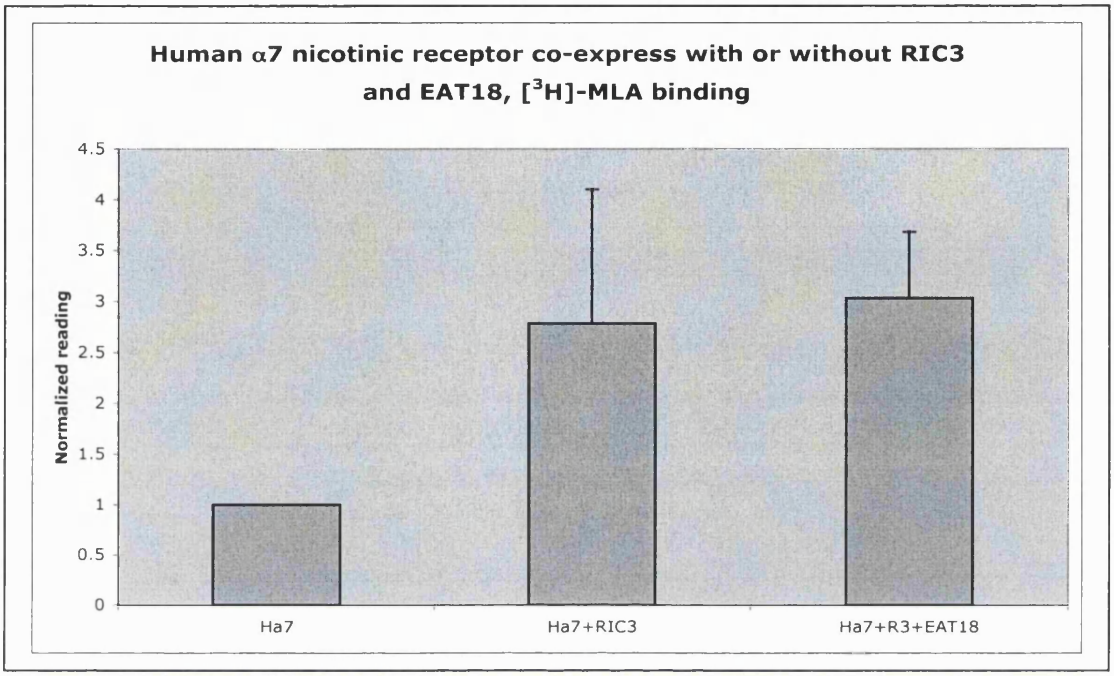

FIG 3.9 Specific [^{3}H]-MLA binding to cell membranes of S2 cells expressing nAChR and associated protein. S2 cell were transiently transfected with Hα7, Hα7 with ric-3 and Hα7 with ric-3+eat-18. Data are means (±standard error) of three independent experiments performed in triplicate and are presented by normalizing to the [^{3}H]-MLA binding level of Hα7. The Hα7 was treated as 1. Significance determined by a two-tailed Student's t test.

Chapter 4

4.0 Discussion

In the year 1995, Leon Avery and his colleagues found that the MC neuron is necessary and probably sufficient for rapid pharyngeal pumping by using laser-ablating subsets of the pharyngeal nervous system. And further experiments suggested the mutations in two genes, eat-2 and eat-18, eliminated MC neurotransmission but not other defects. Evidence also indicated that both gain-of-function and loss-of-function mutations reduced the excitation of pharyngeal muscle in response to the nAChR agonists nicotine and carbachol, which suggest the possibility that eat-18 is required for the function of a pharyngeal nAChR (145). A recently published paper put the study further and found that eat-18 encodes a small protein that has no homology to previously characterized proteins. Experimental evidences indicate that eat-18 could be a component of the pharyngeal nicotinic receptors. The possible functions have been proposed of EAT-18 are: (i) EAT-18 is required for the formation of the acetylcholine-binding site; (ii) eat-18 could be required for inserting the nicotinic receptor into the postsynaptic membrane (137).

Inspired by the fact that ric-3 mutations could affect acetylcholine receptors function properly (136), it is expected that eat-18 could possibly help nAChRs function by certain mechanism. In the present study, the eat-18 cDNA was subcloned into the plasmid vector pRmHa3 as described in chapter 2. A variety of nAChR subunit combinations were co-expressed with eat-18. Radio-ligand binding studies were used to see whether this eat-18 could help to increase level of ligand binding.

As described in Chapter 3, there is no significant difference between the heteromeric and homomeric Rat and *Drosophila* nAChRs expressed with or without eat-18. At the meantime, ric-3 co-expressed with human α7 and rat α7 nAChRs did show obvious increase of the ligand binding level but still do not achieve the statistical significance. And when expressing ric-3 and eat-18 together with the α7 homomeric nAChRs the ligand binding level do not show significant change comparing with the homomeric receptor co-expressed with ric-3 only.

The possible reasons why eat-18 did not show any significant influence upon nAChRs could be: (i) the *C. elegans* protein EAT-18 can only influence *C. elegans* nAChRs function but not *Drosophila*, rat and human nAChRs; (ii) EAT-18 might effect nAChR function by the mechanism other than forming the lignad binding site. So further exploration will be necessary to determine what role does EAT-18 play to affect the nAChRs function properly.

Chapter 5

5.0 Future direction

More *C. elegans* nAChRs subunits should be used in order to examine the possibility that EAT-18 functions by mechanisms other than help enhancing levels of nAChRs detectable by radioligand binding. eat-18-specific epitope tag will be added to carry out the study which would help to determine whether eat-18 has been expressed in the S2 cells since eat-18 antibody is still not available.

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Appendix: Materials used in this project

Radioligands: (+/-) epibatidine [5.6-bicycloheptyl- ^{3}H]- (2.07 TB q/mmol) was obtained from American Radiolabelled Chemicals Inc, [^{3}H]- methyllycaconitine ([^{3}H]-MLA, Tocris Cookson Ltd, Avonmouth, UK, specific activity 26 Ci/mmol).

Chemical and reagents: Agarose, LMP-agarose, Tris, Tryptone, Lambda *Hind* III DNA markers from Gibco. Carbamylcholine Chloride (Carbachol), 40 μM dNTPs, EDTA from Sigma. Calcium Chloride, Ethidium Bromide, Sodium Chloride from BDH. Liquid Scintillation Cocktail from Beckman.

*Eco*RI, T4 DNA ligase, ligation buffer, T4 DNA polymerase, PCR markers, alkaline phosphatase, CIP buffer, and buffer H 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.

Buffers and Solutions: Penicillin –Streptomycin 10,000 units/ml- 10,000 μg/ml, Hanks Balanced Salt Solution from Gibco. Sheilds and Sang M3 insect cell medium from Sigma.