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Pharmacological Evaluation of Choline on α4β2 Neuronal Nicotinic Acetylcholine Receptors

Ladislas Bizimana

A thesis submitted in fulfilment of the requirements for the degree of Master of Philosophy

Faculty of Pharmacy

The University of Sydney

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Declaration

The work described in this thesis was conducted under the supervision of Dr Thomas Balle and Prof. Mary Collins (Faculty of Pharmacy, The University of Sydney, Sydney).

This thesis was edited by Cherry Russell, PhD, retired Associate Professor, Faculty of Health Sciences, The University of Sydney.

I declare that the work in this thesis has not previously been submitted or accepted for the award of any other degree. It contains no material that has been written or published by another person, except where acknowledgement is made.

Ladislas Bizimana

September, 2015

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List of abbreviations

Abbreviation	Term
α-BXT	α-Bungarotoxin
AC	Adenyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChBP	Acetylcholine Binding Protein
ADHD	Attention-Deficit Hyperactivity Disorder
AD	Alzheimer’s Disease
Asp	Aspartic acid amino acid
ATP	Adenosine triphosphate
CaMK	Ca^{2}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CDP-Choline	Cytidine 5-diphosphocholine
ChAT	Choline acetyltransferase
CHT	High-affinity choline transporter
CF	Ca^{2+} Free ND96 Solution
CNS	Central Nervous System
CREB	cAMP response element-binding protein

cRNA	Complementary ribonucleic acid
CTP	Cytidine Triphosphate
C-terminal	Carboxy terminal
Cys	Cysteine amino acid
Cys-loop	Cysteine-Loop
DHBE	Dihydro-β-erythroidine
EC_{50}	Agonist concentration that elicits 50% maximum response or antagonist that inhibits 50% spontaneous current
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPSC	Excitatory postsynaptic currents
ERK	Extracellular signal-regulated kinases
GABA	γ-Aminobutryic Acid
Gly	Glycine amino acid
Gln	Glutamine amino acid
GTP	Guanosine Triphosphate
HEK	Human embryonic kidney cell lines
His	Histidine amino acid
HS	High-sensitivity stoichiometry
I_{ACh}	ACh induced current
IC_{50}	Antagonist concentration that inhibit 50% of the current elucidated by the agonist

I_{Max}	Maximum current response
JAK2	Janus kinase 2
LBD	Ligand binding domain
Leu	Leucine amino acid
LGIC	Ligand-gated ion channel
Ls	*Lymnae stagnalis*
LS	Low sensitivity stoichiometry
M1	First transmembrane domain
M2	Second transmembrane domain
M3	Third transmembrane domain
M4	Fourth transmembrane domain
mAChR	Muscle nicotinic acetylcholine receptor
Met	Methionine amino acid
MIR	Main immunogenic region
MLA	Methyllycaconitine
nAChR	Neuronal nicotinic acetylcholine receptor
NAM	Negative allosteric modulators
ND96	Frog ringer buffer
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
N-terminal	Amino terminal
OR2	Oocyte Releasing Buffer 2

PAM	Positive allosteric modulators
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PEMT	Phosphatidylethanolamine methyltransferase
PET	Positron emission tomography
Phe	Phenylalanine amino acid
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
SAM	S-adenosyl methionine
Ser	Serine amino acid
TH	Tyrosine hydroxylase
Thr	Threonine amino acid
Trp	Tryptophan amino acid
Tyr	Tyrosine amino acid
Val	Valine amino acid
VAChT	Vesicular acetylcholine transporter
WHO	World Health Organization

Conference Presentations

‘Choline selectively activates and modulates the low-sensitive α4β2 neuronal nicotinic acetylcholine receptors’

L. Bizimana[1], N. Absalom[1], M. Collins[1], P. K. Ahring[1], T. Balle[1]

[1]Faculty of Pharmacy, The University of Sydney, NSW.

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Abstract

Choline is the precursor of acetylcholine, the endogenous neurotransmitter responsible for activation of muscarinic and nicotinic acetylcholine receptors. At the same time, choline is also the metabolite of acetylcholine following synaptic degradation by acetylcholine esterase. It is thus not surprising that choline is an important biomolecule and, in fact, an essential nutrient because its biosynthesis is insufficient to meet the demands of the body. Choline has also been found to directly activate some nicotinic acetylcholine receptors, which suggests that it may play a role in neurotransmission beyond being a precursor/metabolite. The present thesis evaluates pharmacological effects of choline on α4β2 neuronal nicotinic acetylcholine receptors (nAChRs).

Using electrophysiological techniques, the study investigates effects of choline on currently known α4β2 nAChRs stochiometries — (α4)3(β2)2 and (α4)2(β2)3. Choline was found to activate (α4)3(β2)2 nAChRs with EC_{50} of 0.4mM and a maximal efficacy of 4%. In contrast, it did not produce any response on (α4)2(β2)3, suggesting that an α4α4 interface is required in order to observe choline effect. When co-applied with ACh we revealed that low concentrations of choline potentiated currents induced by 1 µM and 10 µM ACh, but these effects were inhibited by 1mM choline or higher. In addition, the co-application of choline with 100 µM ACh showed inhibition with an IC_{50} value of 26mM but no potentiation was observed. Preincubation of choline on (α4)3(β2)2 nAChRs demonstrated that ACh responses on these receptors were decreased up to 60% of their maximum efficacy. To further explore the effects of choline we tested the compound on mutant receptors engineered to only have α4α4 or α4β2-like binding sites. These engineered receptors are triple mutation (3M) induced on complementary interface of either α4 or β2 native subunits and are denoted $(\alpha4)3(\beta2^{3M})2$ or $(\alpha4^{3M})3(\beta2)2$ respectively. The $(\alpha4)3(\beta2^{3M})2$ mutant receptor bears one native α4α4 site and two α4α4-like sites whereas $(\alpha4^{3M})3(\beta2)2$ mutant has two native α4β2 sites and one α4β2-like site. On these mutant constructs, choline activated the $(\alpha4)3(\beta2^{3M})2$ receptors with EC_{50} of 0.3mM and a maximum response of four percent as compared to 1mM ACh efficacy. In contrast, choline had no effect on $(\alpha4^{3M})3(\beta2)2$ nAChRs, highlighting the importance of the α4α4 site for choline mediated response.

Overall our results show that at least one α4α4 binding site is required to notice choline activity on α4β2 nAChRs. Choline also can modulate ACh-mediated activation of nAChRs. Low concentrations of choline enhance ACh evoked response, but this response is inhibited by high concentrations of choline. This study definitely suggests that choline may contribute to balance ACh-mediated signalling of α4β2 nAChRs.

Chapter 1

Introduction

This thesis reports findings from an investigation of Choline effects on α4β2 neuronal nicotinic receptors. This chapter begins by locating the study's focus in relation to relevant previous research and existing knowledge of the cholinergic system, with particular attention to the neuronal nicotinic acetylcholine receptors. This is followed by a statement of the study's aim and objectives.

1.1 The Cholinergic System

In the central nervous system (CNS), the neurons that contain choline acetyltransferase (ChAT) enzyme and use acetylcholine (ACh) (Figure 1) as the endogenous neurotransmitter are called cholinergic neurons [1]. The cholinergic system is composed of two main classes of receptors—muscarinic and nicotinic receptors—so-called because of their high affinity with the natural alkaloids muscarine and nicotine, respectively [2]. Muscarine receptors are metabotropic receptors, which act by coupling to G proteins and which mediate neurotransmission slowly. In contrast, nicotinic receptors are ionotropic receptors belonging to the cys-loop ligand-gated ion channel superfamily. Ionotropic receptors mediate rapid response in neurotransmission since, in contrast to metabotropic receptors, they do not need G proteins as second messengers.

Figure 1. Structure of acetylcholine

The significance of nicotinic receptors in modulating biological function stems from their ability to translate the binding of ACh to receptor motion that will select and allow ion flow through the channel and induce a cellular response [2, 3]. Since its discovery in 1914, ACh has been recognised as an endogenous signaling compound which is synthesised in the cytoplasm of the nerve terminal from choline and acetyl-CoA through the action of ChAT. Specifically, choline has a dual origin [3]. One of these is as an essential nutrient taken from a diet rich in choline or related compounds. Another source consists of denovo biosynthesis from phosphatidylcholine, which represents 95% of the total choline in mammalian tissue. Once synthesised, ACh is stored in synaptic vesicles until a nerve impulse reaches the presynaptic terminal, resulting in the release of ACh in the synaptic cleft to transfer the message to the postsynaptic terminal. The action is rapidly terminated by the Acetylcholinesterase to allow repolarisation. ACh is then metabolised into choline and acetate (Figure 2).

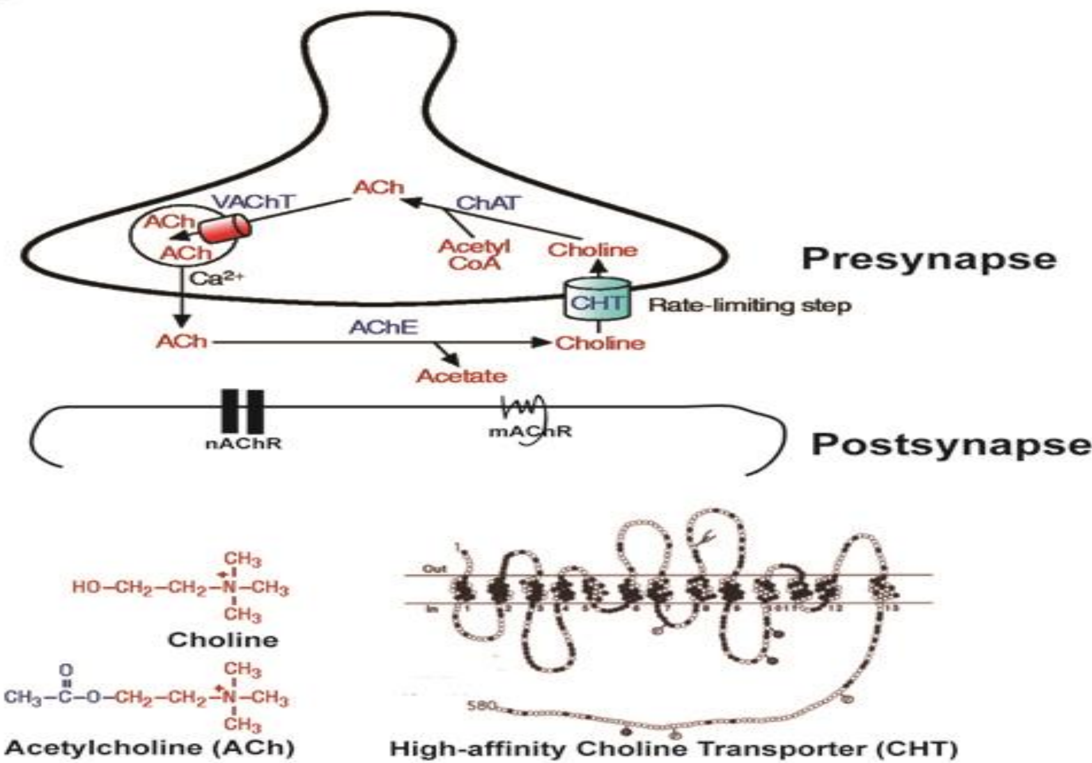

Figure 2. ACh synthesis and metabolism
Choline and Acetyl-CoA reaction is catalysed by ChAT. The synthesised ACh is taken by vesicular ACh transporter (VAChT) and stored in synaptic vesicles. ACh release is initiated by an action potential on presynaptic neuron that opens a voltage-gated calcium channel and Ca^{2+} influx stimulates the release of ACh from presynaptic vesicles into the synaptic cleft. The released ACh activates the receptors on postsynaptic terminal nAChR or mAChR and is rapidly hydrolysed by the enzyme acetylcholinesterase (AChE) into Choline and acetate. Choline is then taken up by the high-affinity choline transporter (CHT)[4].

The nicotinic receptors mediate synaptic transmission at both the neuromuscular junction and the neuronal synapse. The muscle and neuronal nicotinic receptors (nAChRs) have been further subdivided into up to 17 known nAChRs subunit genes in the human genome [5]. The muscle type receptors include genes encoding for α1, β1, γ, δ, and ε subunits whereas neuronal nicotinic receptors consist of those encoding for α2 to α10 and β2 to β4 subunits. From these subunits, a functional receptor is made of five assembled subunits, which results in many possible subunit combinations and stochiometries (Table 1) thus explaining the diverse physiological function of nicotinic receptors.

Table 1 nAChRs Subtypes, Subunits and their Possible Combination
Adapted from Millar, 2003 [5]

Receptor subtype	Subunits	Subunit combinations
Muscle type	α1, β1,δ, ε, γ	α1, β1, γ, δ α1, β1, ε, δ
Neuronal (αBtx[a]-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 (αBtx-sensitive)	α7, α8	α7 α8 α7β2 α7β3 α5α7β2 α5α7β4
Sensory epithelia	α9, α10	α9 α9α10

[a] αBtx: αBangarotoxin (a snake toxin used to classify the nAChRs)

1.2 Neuronal Nicotinic Acetylcholine Receptors

The neuronal nicotinic acetylcholine receptors (nAChRs) belong to an important subclass of cholinergic receptors in the CNS, where they mediate the rapid responses of ACh. nAChRs mediate excitatory responses and also regulate the release of a number of other neurotransmitters including glutamate, dopamine and noradrenaline, which are important neurotransmitters in memory formation and consolidation [6].

The nAChRs consist of various α and β subunits which make up the pentameric receptor (Table 1). The activation of nAChRs by the binding of ACh results in an influx of Na^+ into the cell and an efflux of K^+, leading to a depolarisation of the postsynaptic neuron and the initiation of a new action potential [7]. Continued application of ACh on the nAChRs leads to the receptor's desensitisation. This mechanism is believed to be a result of phosphorylation by either cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC).

nAChRs are expressed widely in the CNS. They are mainly present at presynaptic neurons, where they mediate neurotransmitter release, but they also modulate postsynaptic effects on dendrites and are implicated in cell excitability and neuronal integration [8]. These processes play important physiological roles including arousal, anxiety, and varied cognitive functions. It is therefore not surprising that nAChR dysfunction or imbalance can lead to cognitive impairment that is a common characteristic in, for example, Alzheimer's disease (AD), schizophrenia and attention deficit hyperactivity disorder (ADHD) [9].

Support for the involvement of the nAChR system in nicotinic addication comes from the over-representation of tobacco smokers in schizophrenic [9] and ADHD patient groups [10], which suggests that patients alleviate attention deficit symptoms by smoking cigarettes. More direct evidence from the use of nicotine patches confirms that it is nicotine, the main

psychoactive ingredient in tobacco, that is responsible for the effects and, combined with results from animal models, there is solid evidence for pro-cognitive effects of nicotine and other nicotinic agonists [11].

1.2.1 Physiological function of nAChRs

The broad distribution of nAChRs in the CNS suggests that these receptors play a major role in brain physiology. nAChRs functions include cognitive performance, addiction, attention, etc. For example, cholinergic cells located in the basal forebrain are thought to have a role in memory and arousal [1].

There is evidence that α4 and β2-containing nAChRs are involved in the central dopaminergic system. It was found that β2-containing nAChRs reinforce and contribute to nicotine response, which stimulates DA release [12]. In an electrophysiology study, Piciotto et al. showed that mesencephalic dopaminergic neurons from mice lacking the β2 subunit no longer respond to nicotine, and that self-administration of nicotine is attenuated in these mutant mice.

A number of studies highlight the crucial role of nAChRs in learning and memory. Kumari et al. [13] studied human behavioural performance and blood oxygenation level-dependent regional brain activity using functional magnetic resonance imaging. Their study involved administration of either nicotine or saline to healthy nonsmoking men who were then given an "n-back" task. This is a working memory test in which subjects are asked to check

different characteristics of a stimulus and to report when the presented stimulus is the same as the one presented in previous trials [14].

The results indicated better performance in terms of response accuracy over all trials after the administration of nicotine rather than the placebo [13]. An increased response after taking nicotine was observed in areas activated by the task such as anterior cingulate, superior frontal cortex, superior parietal cortex, parahippocampal gyrus and cerebellum. Poor learning and performance was also revealed in mice that lacked β2 nAChRs [15]. Another study conducted by Levin highlighted the importance of α4β2 nAChRs in memory [16]. He induced working memory deficits by blocking ventral hippocampal α4β2. These deficits were reversed by chronic systemic nicotine administration. This therapy did not, however, reverse similar working memory deficits induced on ventral hippocampal α7.

Another study investigated the effect of mecamylamine (a non-competitive nicotinic antagonist) on attention processes such as alerting, orienting and executive control in 12 healthy male subjects using a magnetic resonance imaging scanner [17]. The results confirmed an association between the activation of some neural networks with attention components and a reduced response time to the task following administration of mecamylamine. Although mecamylamine is a non-selective nAChRs antagonist, its effects indicate that blocking the nAChRs has an impact on cognition and attention and that nAChRs may have significance in other CNS-related pathologies in general.

1.2.2 nAChRs dysfunction and neuropathologies

A number of CNS pathologies were reported to be directly or indirectly linked to nAChRs dysfunction. Disorders such as AD, ADHD, depression and anxiety, schizophrenia, and Parkinson diseases (PD) were found to be associated with physiological nAChRs abnormalities [18-23]. The following section discusses different neuronal pathologies and the role of a4b2 nAChR subtype in particular.

I.2.2.1 Alzheimer's disease

Alzheimer's disease is the most common neuro-degenerative disorder [24]. It is the leading cause of dementia contributing to 50-70% of cases [25]. Itscharacteristics include memory and cognitive loss. These AD symptoms are thought to result from a deficiency in some nAChRs subtypes. Both in *vitro* and in *vivo* studies have shown a deficit in some nAChRs subtypes in AD patients. For example, measuring nAChRs in the AD patient's brain using PET and an analysis of an AD brain tissue indicated an important reduction of α4 subtype up to 47% [26, 27]. Another study compared two groups—non-smoking patients with AD and non-smoking healthy individuals—by measuring levels of α4β2 nicotinic acetylcholine receptor binding. This study showed a significant alteration in the amount of α4β2 receptors in the group with AD [22].. It has been reported that 50% or more of α4β2 nAChRs are lost from the neocortex and hippocampus of patients with advanced AD [28].

This remarkable loss of α4β2 nAChRs in patients with AD justifies research into the development of molecules that target those receptors. Overall, the evidence cited above indicates that deficits in α4β2 nAChRs might be early phenomena of AD, which emphasises the importance of nAChRs as a potential target for drug development and research.

1.2.2.2 Nicotinic addiction

Nicotine is the main addictive compound in tobacco smoking. Tobacco smoking has been identified by the World Health Organisation (WHO) as the second leading risk for mortality worldwide [29]. According to the WHO, six million people die every year as a result of tobacco smoking. Despite its harmful effects, smoking continues to increase particularly in low and middle income countries and it remains a growing risk factor for mortality. Nicotine addiction has been a major problem for individuals who have attempted to quit smoking [30]. At the molecular level, nicotine has high affinity with α4β2 nAChRs [31, 32]. These receptors are assumed to be highly associated with smoking addiction due to their abundance and wide distribution in the brain. The implication of α4β2 nAChRs in nicotine addiction has also been reported in several studies. One study aiming to evaluate the role of α4β2 nAChRs in nicotinic addiction was performed in monkeys [31]. These α4β2 receptors were measured at baseline using radioligand 2-[18F] fluoro-A-85380 (2-FA) and PET. Motivation to self-administer nicotine was also measured. Findings suggested that the monkeys with a higher motivation to self-administer nicotine also had the lowest expression levels of α4β2 nAChRs. Other studies highlight the role of α4β2 nAChRs agonists in relief from tobacco craving and withdrawal symptoms resulting from smoking cessation [32]. For instance, in a double blind randomised clinical trial using varenicline, which is an α4β2 nAChRs partial agonist, varenicline was shown to be more effective in management of tobacco addiction and improving withdrawal symptoms.

In summary, the studies cited above show that α4β2 nAChRs play a major role in expressing nicotinic addiction and molecules targeting those receptors could be exploited as potential drugs for smoking cessation.

1.2.2.3 Attention deficit hyperactivity disorder

ADHD is one of the most common childhood psychiatric disorders and can also persist into adulthood [33]. Children with this disorder have abnormal behavioural symptoms such as inattention, impulsivity and hyperactivity. Among adults, the common characteristics of the disorder include forgetfulness, difficulty completing tasks, problems with prioritisation, and inability to self-monitor behaviour [34].

Evidence shows that nicotinic addiction has high correlation with ADHD and that α4β2 nAChRs are affected. For example, the likelihood of being a regular smoker was examined in a longitudinal retrospective study involving self-reports from patients with ADHD symptoms. The extent to which ADHD symptoms predicted the age at onset of regular smoking and number of cigarettes smoked was also evaluated. Results confirmed a linear relation between ADHD and smoking outcome measures ($p<0.001$) [35]. Another controlled clinical trial has demonstrated that ABT-894, an α4β2 nAChRs agonist, is efficacious in reducing ADHD symptoms in adults in 243 participants α4β2 agonist [36]. This suggests that molecules that activate α4β2 nAChRs are potential useful therapy of ADHD.

1.2.2.4 Depression and anxiety

Many scientific papers have reported a connection between nicotine dependence and depression. There is also evidence of overrepresentation of depressed individuals among smokers and that people who quit smoking often experience immediate depressive symptoms as a result of nAChRs desensitisation [37-41].

A partial nAChRs agonist, lobeline, which is used for smoking cessation and which has also been reported to have dual effects of potentiating and inhibiting ACh on α4β2 nAChRs [42], was examined for its anxiolytic effects in mice using elevated plus-maze and marble-burying tests. Results showed that lobeline has anxiolytic potential effects and suggest that nAChR antagonists may be helpful in reducing anxietyin humans [19]. Lobeline was further tested for its antidepressant effects on mice using forced swim test, tail suspension test, and novelty suppressed feeding test. The behavioural and neuroendocrine results showed that a pretreatment of lobeline on mice demonstrated an antidepressant activity including reduced immobility time and feeding latency, significant attenuation of plasmatic corticosterone levels, and reduction of norepinephrine and serotonin levels in the prefrontal cortex and hippocampus [43]. These observations indicate that molecules known to have affinity with a4b2 nAChRs may exert their anxiolytic effects by partly interacting with nAChRs, which could be an interesting avenue to explore for further development of novel depression and anxiety therapies.

1.2.2.5 Schizophrenia

In a study assessing 78 schizophrenic outpatients using the Brief Psychiatric Rating Scale, Goff et al. compared current smokers and nonsmokers. Their results showed that 74% of patients were current smokers who reported a mean of 19 cigarettes consumed per day. Current smokers also reported a higher number of previous hospitalisations and higher doses of neuroleptics than nonsmokers [44]. A body of evidence has confirmed that nAChRs are decreased in schizophrenics and that the hippocampus, the brain region rich in α4β2, is the most affected area.[20]. In a study, density of α4β2 nAChRs in postmortem schizophrenics' brains was examined using [^{3}H] cytisine as a radioligand. The findings showed a 30%

decrease in those receptors in the post-mortem striatum of patients with schizophrenia compared to controls [45]. This study indicates that α4β2 nAChRs are affected in patients with schizophrenia and that smoking seems to be a helpful mechanism for relief from schizophrenic symptoms. This suggests the possibility that α4β2 agonists other than nicotine might be useful in managing schizophrenia.

1.2.2.6 Parkinson's disease

α4β2 nAChRs are believed to have a relevant relation with the dopaminergic system, which is thought to be deficient in PD patients. For example, using cyclic voltammetry to measure neuronal firing as a result of dopamine release, Perez et al. evaluated the role of α4β2 receptors in dopamine release by varying damage in nigrostriatum, a critical region for PD [46]. Their findings suggest that single-pulse–stimulated α4β2 nAChRs mediated dopamine release decreases to a similar extent with increasing nigrostriatal damage. Another study assessed the levels of α4β2 nAChRs using a radioligand in patients with PD with depressive or mild cognitive symptoms and compared the results to those from healthy volunteers. The results showed a general reduction of α4β2 nAChRs in patients with PD and depression or cognitive symptoms. However, reduction of the number of α4β2 nAChRs binding to radioligand was observed in patients with PD and severe or mild cognitive or depressive symptoms [23]. Altogether these data suggest that molecules directed to α4β2 nAChRs could be important in therapy for PD.

In summary, several neuronal disorders are associated with nAChRs disyfunction. The variability of these disorders is an indication of the complexity and diversity of nAChRs as well as their broad dispersion in CNS.

1.2.3 Subdivision and structure of nAChRs

1.2.3.1 The subdivision of nAChRs

One of the methods used to characterise nAChRs in the CNS is based on radioligand binding studies of the brain [47]. These studies showed the existence of two main classes of nAChRs. One class was determined on the basis of sensitivity to the snake toxin αBungarotoxin (αBgtx). Bgtx-sensitive nAChRs had affinity with αBgtx at nM level. Another class, referred as to AChRs, had affinity with classical agonists such as ACh and epibatidine at nM levels, but no affinity with αBgtx.

Subsequently, molecular cloning studies showed the existence of 12 subunits, α2 to α10 and β2 to β4. These combine into αBgtx-sensitive (α7 to α10) and αBgtx-insensitive receptors (α2 to α6 and β2 to β4) [48, 49]. This variety of nAChRs suggests their diverse function although they share a common basic structure, which is described in the following section.

1.2.3.2 The structure of nAChRs

The nAChRs are pentameric ligand-gated ion channels made of transmembrane protein complexes [8]. These proteins complexes mediate fast synaptic transmission in the CNS. The variation in structure of nAChRs subtypes imparts their unique characteristics and functions [50]. These characteristics and functions also depend on the types of subunits assembled to form the receptor, the receptor's location [51], the type and number of binding sites and the ligands to which they bind. These properties are discussed in this section and are important for understanding their implications of nAChRs in diseases and the design or development of new molecules.

The most detailed information about the structure of nAChRs has been provided by recent 4 Ǻ electron images of the receptor in the postsynaptic membranes of the marbled electric ray *Torpedo marmorata [52].* The receptor consists of a ring of five subunits, Each subunit is made up of an N-terminal extracellular domain on which a ligand binding domain (LBD) is located, four hydrophobic transmembrane segments (M1-M4) and two hydrophilic loops (M2-M3), a large loop that varies in size and sequence between subunits and at which phosphorylation sites are located, and a C-terminal end with a hydrophilic extracellular segment (Figure 3) [47, 50, 53].

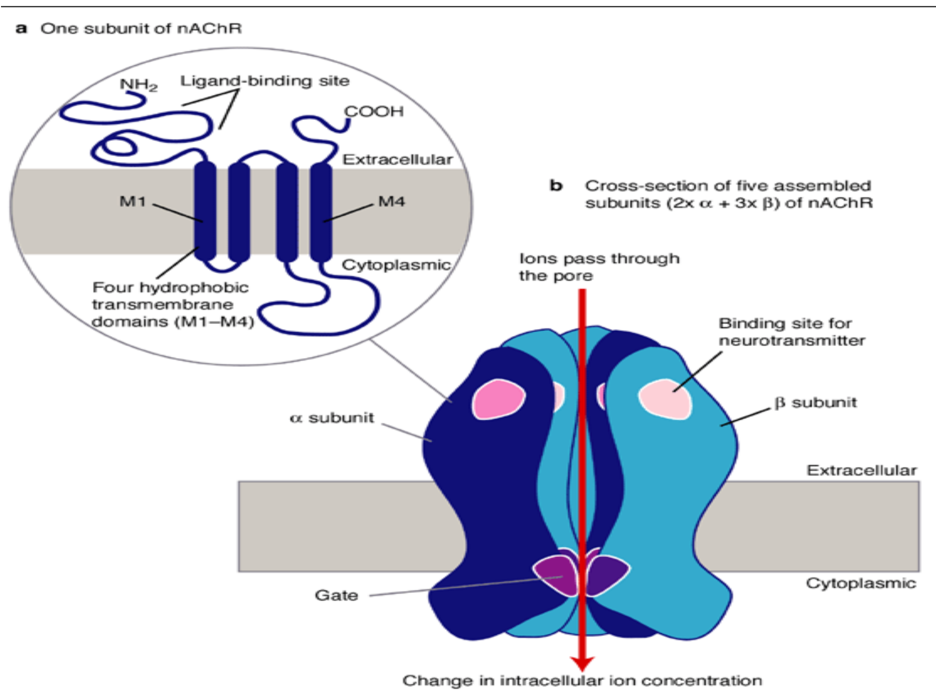

Figure 3. Schematic illustration of nAChR.
It shows subunit components (a) which include an extracellular N-terminal, four hydrophobic transmembrane domains (M1 to M4) and extracellular C-terminal. Five assembled subunits form a pentameric receptor (b) showing the location of binding sites, the direction of ions from the extracellular domain through the pore and their passage through the receptor gate towards the cytoplasm.

Assembly of different subunits to form a pentameric ion channel results in a funnel- shaped complex (Figure 4) made up of hydrophobic residues on the outer side and hydrophilic residues on the inner which is suggested to have a role in ion selectivity and permeation. This ion channel contains an ion filter that determines which cations to traverse the channel [49, 54].

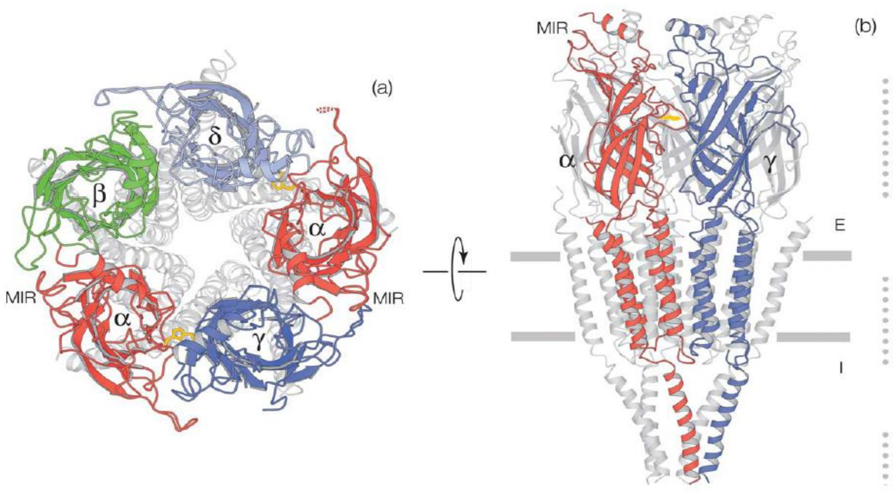

Figure 4.*Torpedo marmorata's* nicotinic receptor.
As viewed (a) from the synaptic cleft and (b) parallel with the membrane plane. The ligand-binding domain is highlighted in (a) and only the front two subunits are highlighted in (b) (a, red; b, green; g, blue; d, light blue). Also shown are the locations of aTrp149 (gold), the main immunogenic region (MIR), a small region on the extracellular part of α subunit and major target of the anti-nAChR antibodies as well as the membrane (horizontal bars; E, extracellular; I, intracellular). Adapted from Unwin [52].

Although nAChRs have many structure similarities, there exist differences in ligand binding and affinity on various subtypes [55]. One reason is the variation in the number of subunits assembled and the number of binding sites in the targeted nAChRs subtype. The typical example is α4β2 nAChR subtype, which is reported to be predominant in the brain and to exist in two different/alternate stochiometries. These characteristics are relevant in studying their function and designing molecules targeting α4β2 subtype.

1.2.3.3 The α4β2 nAChR structure and its relevance

The α4β2 subtype is assembled from α4 and β2 subunits in a pentameric arrangement with two currently known alternate stochiometries, (α4)2(β2)3 and (α4)3(β2)2 [56]. It is the most abundant nAChR subtype in the brain and is estimated at around 90% of nAChRs expressed in the CNS in the rodent brain. This receptor subtype is the main target for several ligands targeting the nAChRs [50, 57].

The α4β2 nAChRs are widely dispersed in the brain. These receptors have been localised thanks to radio-ligand binding studies. For instance, a study using [^{3}H] cytisine, an agonist with high affinity at nAChRs, in conjuction with poyclonal antisera, mainly localised α4β2 in the rat cortex [57], A similar study also demonstrated that expression of α4β2 is most important in the thalamus, cerebral cortex and dorsal hippocampus [51, 58].

It is currently known that the α4β2 receptor can assemble in two different stoichiometries, (α4)2(β2)3 and (α4)3(β2)2, containing either two or three α4 and three β2 or two β2 subunits respectively [56]. In addition, (α4)2(β2)3 bears only two identical agonist binding sites in α4:β2 interfaces whereas (α4)3(β2)2 has a third agonist binding site in the α4: α4 interface in addition to two previously known sites in α4: β2 interfaces [59]. Table 2 summarises key features of the two stochiometries.

Pharmacologically, the two stochiometries differ in the way they respond to ACh and other agonists. The (α4)2(β2)3 receptor responds with high sensitivity to ACh with a monophasic dose response curve. In contrast, the (α4)3(β2)2 receptor is less sensitive to ACh and responds to ACh in a biphasic manner [59].

The specific location of each of the α4β2 stochiometries in the brain is not clear due to limitations in existing techniques as well as lack of new methods to study the anatomical

distribution of individual stoichiometry [60]. For example, a recent study of two radioligands— [^{3}H]epibatidine and [^{3}H]NS3573—known to differentiate (α4)3(β2)2 from (α4)2(β2)3 stochiometries based on EC_{50} values was unable to distinguish both stochiometries as the two radioligands presented in the same way, with a very narrow range of affinity, and showed a single binding site model [61]. This difficulty restrains the localisation of individual stoichiometry using such radioactively labeled ligands. Moreover, it is unlikely that it will be possible to separate (α4)2(β2)3 and (α4)3(β2)2 using other techniques such as antibody labeling or in *situ* hybridisation with cRNA probes or mass spectrometry because the two stochiometries share important structural similarities and are difficult to separate [59, 60].

Table 2. Brief Comparison of (α4)2(β2)3 and (α4)3(β2)2 Stochiometries

(α4)2(β2)3	(α4)3(β2)2
Composed of two α4 subunits and three β2 subunits	Composed of three α4 subunits and two β2 subunits
Bears two identic binding sites at α4:β2 interface	Bears two identic binding sites at α4:β2 interface and one binding site at α4:α4 interface
High sensitivity to ACh, EC_{50}= 1.6μM Concentration–response data: Fit very well to a monophasic Hill equation,	Low sensitivity Concentration–response data: Best fit biphasic Hill equation, with both high EC_{50_1}= 0.95μM and low EC_{50_2}= 83nM ACh sensitivity components.

The organisation and structure of the binding sites in the (α4)3(β2)2 and (α4)2(β2)3 receptors are discussed in detail in the next section. Emphasis is on the presence of the additional binding site in (α4)3(β2)2, which offers an opportunity for development of novel therapeutics targeting these receptors. The work presented in this thesis focuses on the binding properties of nAChR constructed with α4 and β2 receptor subunits.

1.2.3.4 nAChRs agonists binding sites

Structural studies suggest that the agonist binding site on nAChRs is located at the interface between two adjacent subunits in LBDs at the extra cellular N terminal. In heteromeric receptors, it is believed that the principal (+) part of the binding site is always formed by the α-residues, which binds to the cationic end of agonists[62]. This binding site consists of a cation-π interaction with one of several conserved aromatic residues and typically a hydrogen bond from the $N^{+}H$ of the drug to a backbone carbonyl. The complementary (-) part is formed by residues of the neighboring non α subunit. Previous work has shown that it involves a hydrogen bonding interaction with the hydrogen bond acceptor of agonist[63]. Thus the agonist binding sites on (α4)2(β2)3 nAChRs are located between the principal interface of α subunits and the complementary interface of β2subunits (Figure 5). In the case of two neighboring α subunits such as in (α4)3(β2)2 receptors both principal and complementary interfaces are on each of the α-α interfaces and the number of binding sites is equal to the number of α subunits [64].

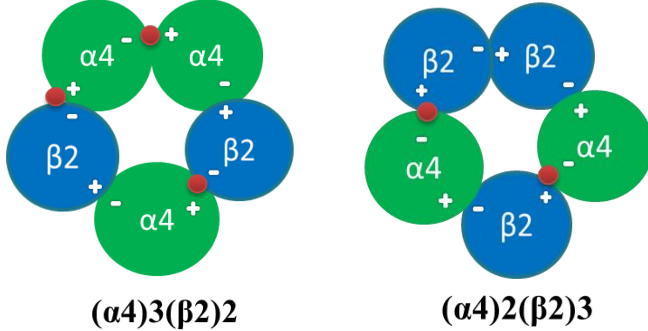

Figure 5. Illustration of (α4)2(β2)3 and(α4)3(β2)2 receptors.
The principal (+) and complementary (-) interfaces are shown as well as binding site(●).

An early description of nAChRs binding sites was based on radiolabelling experiments on the *torpedo* [49, 65]. The results led to exploration of the ACh binding sites, which were found to be expressed by aminoacids located in the LBD**.** Although the study of nAChRs from *torpedo* has elucidated the organisation and full length of nAChRs, the crystal structure of AChBP has shown a unique and well-suited model to study the receptor-ligand interaction [62]. In fact, AChBP has a binding pocket for agonist that is most similar to the binding pocket of nAChRs.

The AChBP has very close similarities to nAChRs alpha subunits, and most residues involved in ligand binding within nAChRs are conserved. As a result AChBP nAChRs share common agonists including ACh and antagonists such as d-tubocurarine [62, 66]. It is suggested that the ligand binding site on AChBP nAChRs is made of residues in a cleft formed by a series of loops named A to F. It is believed that the residues from loops A- C contribute to the principal interface of the binding site and residues from loops D-F contribute to the complementary interface.

One of the most recent studies has reported the crystal structure of ACh bound to AChBP from *Lymnaea stagnalis* (Figure 6). This study has shown that the ligand relevant residues implicated in ACh binding include Tyr89, Trp143 and Tyr185 residues from respectively loops A, B and C on the principal binding interface, cation-π interaction between ACh and Trp53 from loop D on the complementary side and ACh hydrogen bond mediated by water molecule to Leu102 and Met114 residues of the AChBP [67].

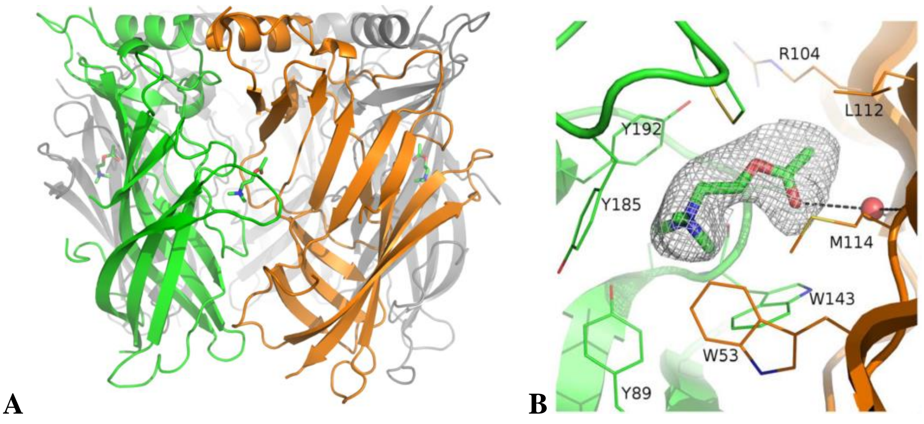

Figure 6: Structure of ACh bound to *Ls-AChBP*.
(A) Side view of cartoon representation of the *Ls-AChBP* with ACh (green stick). The ACh molecule is located between the principal side of the binding pocket, (+) interface (green) and the complementary side, (-) interface (orange); and
(B) ACh bound to *Ls-AChBP*, as reported here, with principal side-chain carbon atoms in green and complementary side chain carbon atoms in orange. Adapted from Olsen et al.[67].

1.2.3.5. Additional nAChRs binding sites

A recent study found that (α4)3(β2)2 receptors have an agonist binding site in α4α4 interface in addition to two other known binding sites in α4β2 interface [59]. This additional binding site has particular characteristics and structure. For instance, it was documented that ligands of a particular size could not bind to the α4α4 binding site of the (α4)3(β2)2 nAChRs, which can result in decreased efficacy [68]. It is also argued that the binding site in a α4α4 interface needs to be occupied by an agonist to observe a full response. Moreover, Harpsoe et al. have demonstrated that three residues found in the α4α4 interface are different from residues in the α4β2 interface [59]. Illustration of these three key bind site residues distinguishing α4α4 and α4β2 binding sites has helped to understand the pharmacology profiles of the α4β2 nAChRs agonists. The three residues were found to be His142, Gln150, and Thr152 in α4, and Val136, Phe144, and Leu146 in β2.

Interestingly, they were able to convert α4α4 (-) interface into α4β2 (-) like interface and *vice versa* by inducing triple mutation (3M) of the above-mentioned residues [59]. The mutations—H142V, Q150F and T152L on (-) α4 side make an α4α4 binding site resemble an α4β2, and the mutations—V136H, F144Q and L146T on (-) β2 interface make an α4β2 binding site resemble an α4α4. Therefore, the $(\alpha4)3(\beta2^{3M})2$ receptor contains one native α4α4 site and two α4α4-like sites whereas the $(\alpha4^{3M})3(\beta2)2$ receptor contains two native α4β2 sites and one α4β2-like site (Figure 7). The engineered receptors are denoted $\alpha4^{3M}\beta2$and $\alpha4\beta2^{3M}$ receptors, indicating that they contain three mutations in α4 and β2 subunits, respectively.

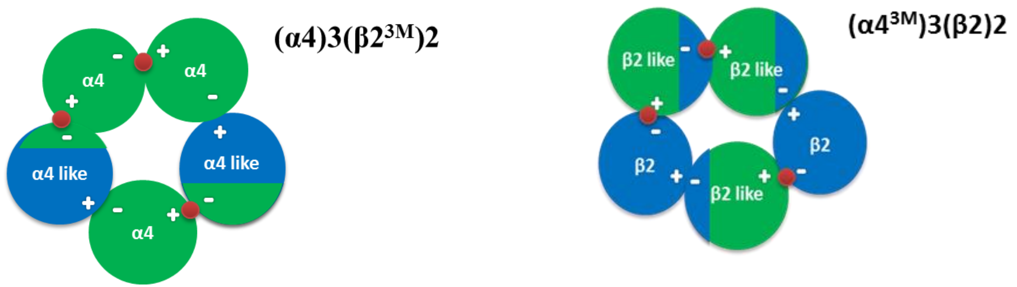

Figure 7. Illustration of engeneered (α4)3(β23M)2 and (α43M)3(β2)2 receptors.
A triple mutation (3M) on (-) interfaces is shown by a small differently coloured section on the receptor. The binding site is shown as a red dot (●).

An agonist binding on nAChRs is followed by a rapid opening of the transmembrane ion channel, which consequently leads to membrane depolarisation [69]. The result of the receptor activation and the opening of the ion channel pore is the entrance of cations primarily Ca^{2+}. By converting acute nAChR stimulation into sustained cellular events, Ca^{2+} signals are the crucial link between nACh receptors and the downstream processes that influence on many neuronal functions[69, 70]. The entry of Ca^{2+} contributes to maintaining

the membrane potential depolarization provoked by an agonist. In turn, levels of intracellular Ca^{2+} regulate nAChR activity.

An increased amount of Ca^{2+} is followed by a chain of reactions or cell signalling (Figure 5) resulting in gene expression for different effects including learning, memory and addiction. [71, 72].

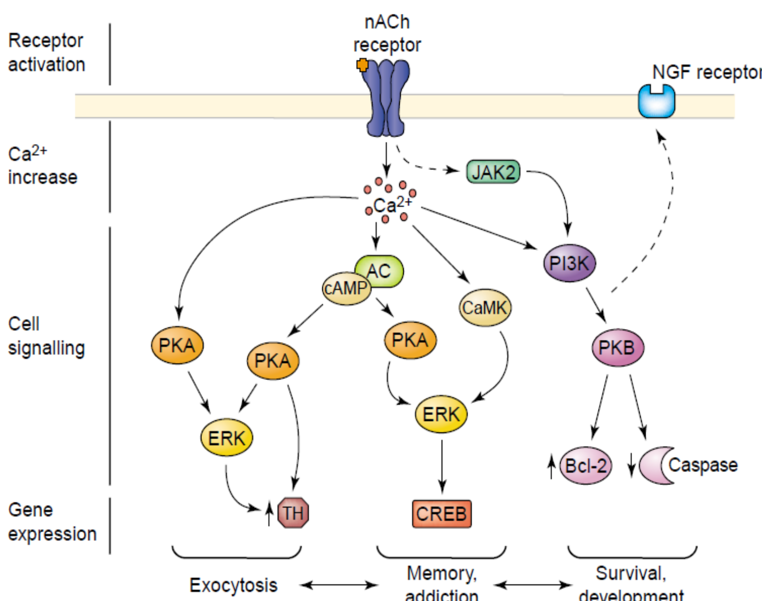

Figure 8. Key signalling molecules in Ca^{2+}dependent nAChR-mediated processes.
Ca^{2+} from nACh receptor activation can activate adenylyl cyclase (AC), protein kinase A (PKA), PKC, Ca^{2+}–calmodulin-dependent protein kinase (CaMK) and phosphatidylinositol 3-kinase (PI3K). In turn, these phosphorylate downstream targets, such as extracellular signal-regulated mitogen-activated protein kinase (ERK), which leads to the activation of transcription factors such as the cAMP response element-binding protein (CREB) and increases in expression of genes that encode, for example, tyrosine hydroxylase (TH) and nerve growth factor (NGF) receptors. The lipid signalling cascade that is initiated by PI3K, through phosphorylation of PKB (Akt) is credited with modulating the relative activities of neuroprotective and apoptotic factors, such as Bcl-2 and caspases, respectively. Adapted from Dajas-Bailador and Wonnacott [72].

1.2.4 Neuronal nicotinic acetylcholine functional states

The nAChRs were found to exist in at least one of three interchangeable states; active state, onset desensitisation and resting states; that define the level of affinity of the receptor for its agonist or antagonist [73].

First, the active state, or state A, is referred to as the open ion channel state. Second, the states named I and D —which are, respectively, two fast onset and slow onset desensitisation states—represent a closed ion channel [72]. The third state is the resting state R. Agonists were found to have higher affinity with the receptor in state A than in state R. In fact in the presence of an agonist, the receptor is more stable in the desensitised state whereas it is more stable in the resting state in the absence of an agonist. In contrast, antagonists have higher affinity with the resting receptor than in the active state. However, I and D states are characterised by respectively high-affinity and very high-affinity binding profiles for both agonists and atagonists [74].

Activation and desensitisation transitions for heteropentameric receptors are reported to vary with the molecules bound to the receptors [49, 51, 64, 75]. For instance, the desensitisation state was studied on rat α4β2 nAChRs by applying ACh and Nicotine [74]. These α4β2 receptor full agonists in a concentration range of 0.1-100 μM produced a similar onset time constant of 5 seconds. However, the fast recovery for receptors desensitised by ACh was 50% compared to 10% for nicotine which means that nicotine showed a longer lasting desensitisation than ACh.

These findings and reported structure differences in α or β subunit in active and desensitised states explain the existence of two different populations of α4β2 nAChRs which respond differently to the binding of ACh with high or low affinity [73, 74]. Overall, the nAChRs exist in different functional states, and characteristics of these states can be different for

various ligands bound on the receptors. In this thesis, functional states of α4β2 receptors following the application of selected compounds will be discussed.

1.2.5 nAChRs ligands

Compounds that bind nAChRs have continued to emerge since the discovery of two well-known molecules that have high affinity with nAChRs, namely, nicotine occurring in nature as an active compound of *Nicotiana tabacum,* and ACh which was the first neurotransmitter to be discovered [76, 77].

The nAChRs ligands originate from diverse sources including endogenous proteins such as ACh, many plant alkaloids such as nicotine, cytisine, d-Tubocurarine, and methyllcaconitine and those of animal origin such as epibatidine (a poisonous alkaloid found on the skin of the frog *Epipedobates anthonyi*), the conotoxins in the venom of the marine cone snail, and synthetic ligands such as tetramethylammonium (TMA) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) [78].

1.2.5.1 nAChRs agonists

Because of their diversity, the nAChRs subtypes vary in their sensitivity to agonists. A typical example is the differential sensitivity of high sensitive receptor (HS) or (α4)2(β2)3 stoichiometry to ACh and to nicotine, which is about 100 and 10 times higher, respectively, than the low sensitive receptor (LS) or (α4)3(β2)2 stoichiometry [79]. Similarly, other agonists like epibatidine and cytisine were reported to be more potent at HS than at LS receptors. This nonequivalent selectivity and efficacy can partially explain the existence of different agonist binding sites on nAChRs and the complexity involved in designing more selective agonists [68]. Moreover, ligands may behave as full agonists on one nAChR subtype and partial agonists on the other. For example, choline fully and partially activates α7

and α3β2 respectively [80]. Nicotine as a classic nAChRs agonist and the structures of some other agonists are discussed below.

1.2.5.1.1 Nicotine

Nicotine (Figure 7) is the main psychoactive constituent of *Nicotiana tabacum*, a very common drug known for its addictive properties [76, 81]. It has been demonstrated that nicotine interacts with nicotinic receptors in the brain and has particularly high affinity with the α4β2 nAChRs subtype.

Figure 9. Nicotine structure

Pyridine and pyrrolidine rings in nicotine are believed to play an important role in binding to the nAChRs [82]. Nicotine binding was evaluated using AChBP (Figure 8), which is the best identified model for nAChRs.

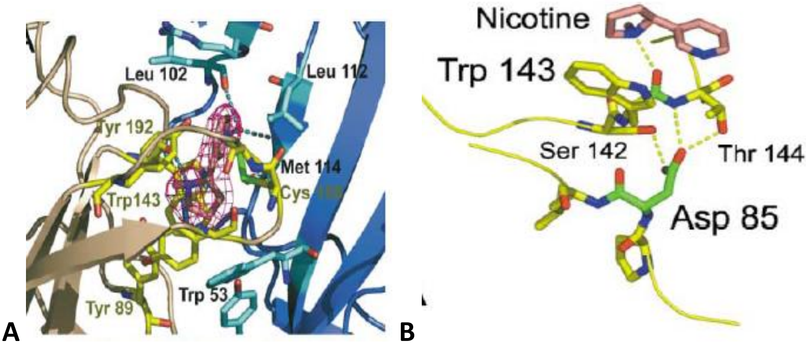

Figure 10. Nicotine binding to AChBP
(A) The nicotine binding site shown with ligand in ball and stick, electron density (SigmaA weighted) superimposed, and residues in the binding site in light yellow (principal side) and light blue (complementary side).
(B) Contribution of Asp85 to ligand binding: The charge compensation of (A) nicotine (pink carbons) could be aided by the stabilisation of the Trp143 carbonyl oxygen by an Asp85 (green) charged hydrogen bond. The yellow dashed lines indicate the hydrogen bonds. Adapted from Celie et al. [82].

It was suggested that the high affinity of nicotine to nAChRs could be a result of close packing of its aromatic groups and that hydrogen bonds between the nicotine pyridine ring and residues Leu102 and Met114 at the side chain as well as the nicotine pyrrolidine ring and carbonyl group of Trp143 [82].

Chronic application of nicotine to nAChRs has been shown to be a cause of receptor desensitisation and up-regulation [81, 83-86]. It was found that the number of $(\alpha4)2(\beta2)3$ nAChRs subtypes is increased two-fold by chronic application of nicotine. This up-regulation is thought to be a result of multiple mechanisms and is believed to be the cause of nicotine addiction. In fact, the desensitised and increased number of nAChRs in nicotine smokers leads to an increased need for nicotine to keep the receptors occupied and counteract withdrawal symptoms [87]. Although nicotine is reported to be a risk factor for serious diseases in smokers, including lung cancer and cardiovascular disease, its activity on nAChRs is partly associated with beneficial effects such as improving cognition and increasing alertness [9, 88, 89].

Drug discovery research suggests nicotine as a lead compound for potential drugs for neurodegenerative diseases like Alzheimer's and Parkinson's. For instance, studies of nicotine's structure have demonstrated that modifying the linker between pryrodine and pyrrolidine resulted in safer, more potent and higher affinity nAChRs ligands than nicotine [90-92]. Some of the nicotine-derived compounds such as 2-methyl-3-(2-(S)-pyrrolidinylmethoxy), pyridine dihydrochloride salt (ABT-089) and (S)-3-Methyl-5-(1-Methyl-2-Pyrrolidinyl) Isoxazole (ABT-418) have displayed significant anxiolytic and cognition-enhancement effects.

In short, nicotine analogs with partial agonist or antagonist properties at α4β2 nAChRs could be useful in the treatment of nicotine dependency [93]. In fact, a full agonist such as nicotine administered simultaneously with a partial agonists would not produce an agonist maximum effect as some receptors are occupied by a partial agonist [94]. By contrast, a full antagonist will stop an agonist from binding on the receptor. This is significant when considering the fact that α4β2 receptor is important for normal cognitive functions in different parts of the brain, so the complete antagonism might result in adverse effects on other brain functions. Thus, partial agonists could theoretically offer benefits over either full agonists or full antagonists**.**

1.2.5.2 nAChRs antagonists

There is evidence that nAChRs antagonists have potential theurapeutic uses in controlling neuronal disorders, but less work has been done to discover novel competitive or non-competitive antagonists of the brain nicotinic receptors [95]. Most of the available nAChRs antagonists seem, however, to be non-selective. This lack of selectivity of nAChRs antagonists, in addition to the beneficial effects, is linked to unwanted effects that could limit their use. For instance, mecamylamine , which is a non-competitive antagonist at both central

and peripheral nAChRs, has been shown to relieve the symptoms of smoking withdrawal [96, 97]. It is not surprising that the non-selectivity of mecamylamine has led to other clinical interests such as antidepressant activity [98]. This example and others given below indicate that there is a continuing need for more selective antagonists of the nAChRs.

1.2.5.2.1 Dihydro-β-erythroidine(DHβE)

The ability to selectively antagonise nAChRs subtypes may permit potential isolation of the desired effects in clinical applications. For example, cognitive impairment is a clinical condition that can be controlled by acute administration of dihydro-β-erythroidine(DHβE) and low doses of methyllycaconitine (MLA), which are selective antagonists for α4β2 and α7 nAChRs respectively [95, 99, 100].

DHβE is an alkaloid from the plant *Erythrina americana* that has displayed high affinity and selective potent inhibition effects for α4β2 nAChRs [50, 101]. It has been widely studied and serves as a reference antagonist for the α4β2 nAChR. Nowadays, structural studies are using DhβE as a model for the discovery of novel antagonists [102]. Recently, for example, two structurally related isolated alkaloids, erythravine and 11 α-hydroxyerythravine (Figure 9) from *Erythrina mulunga* were found to have inhibition effects on α4β2 nAChRs at nanomolar concentrations [103].

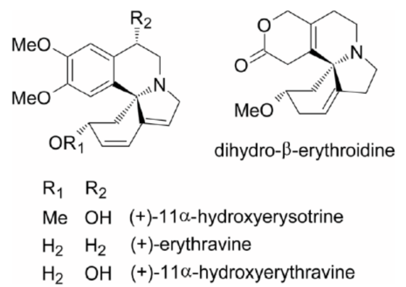

Figure 11. DHβE and related alkaloids

1.2.5.3 nAChRs modulators

The revelation of benzodiazepines as positive allosteric modulators on $GABA_A$ receptors motivated scientists to explore various compounds that would allosterically modulate nAChRs. For instance, galanthamine and physostigmine were successfully identified as compounds useful in AD therapy with a mechanism similar to that of benzodiazepines [104]. These compounds are suggested to increase the likelihood of ion channel opening induced by the agonist or simply to reduce the desensitisation period of the receptor [105]. Molecules such as galanthamine and desformylflustrabromine have shown interesting results in preventing nAChRs desensitisation by nicotine thus reducing nicotinic addiction and AD [104, 105]

Ligands such as desformylflustrabromine, which was found to potentiate ACh response on the α4β2 subunit at more than 265% [106], are identified as allosteric modulators. Positive allosteric modulators (PAM) are ligands that potentiate nAChR agonist response in opposition to negative allosteric modulators (NAM) which reduce the nAChRs agonist response [107]. Allosteric modulators are therefore defined as compounds that by themselves do not activate the receptor, but potentiate or inhibit the agonist response by interacting with the binding site other than the known agonist binding site.

In addition to the classic agonist binding sites on nAChRs, other binding sites were found in other location different from known LBD [108]. These previously unknown binding sites are referred to as modulators binding sites. In studies using chimeric and point-mutated receptors techniques, it was found that a compound encoded NS206 binding site was linked to the α4-subunit transmembrane domain, which is different from the classic agonist binding site

located on the extracellular N-terminal. These observations suggest the existence of other distinct binding sites in the α4β2 nAChR subunit.

The modulators binding sites are of great importance in understanding receptors' behaviour and providing an opportunity for rational drug design. Compounds that bind on those binding sites were found to have diverse pharmacological properties including receptor selectivity and agonist potentiation or inhibition.

An increasing number of studies has focused on nAChRs modulators. A recent study has shown that two compounds, desformylflustrabromine and galantamine, both of which are PAM at α4β2 nAChR, decreased nicotine self-administration in a rat model, which suggests a possible target for nicotinic addiction therapy [109]. Table 3 displays some examples of known compounds that modulate nAChRs.

Table 3. Modulators of nAChRs

Examples as adapted from Pandya, A. and J.L. Yakel [110]

Zn^{2+}	Potentiates α2, α3, α4 containing nAChR, Significantly potentiates of α4β4, but inhibits α3β2 and α7 receptors Modest potentiation of **α4β2** receptors.
17-β-estradiol	Naturally synthesised steroids (progesterone, testosterone, estradiol, corticosterone). 17-β-estradiol exerts allosteric effects on heteromeric **α4** containing nAChR; the effects are generally inhibitory at low concentrations.
Galantamine	Galantamine which is AChE inhibitor has been shown to potentiate **α4β2**, α3β4, and α6β4 nAChRs at concentrations between 0.1 and 1mM and at concentrations >10 mM galantamine was found to inhibit responses.
Desformylflustrabromine (dFBr)	PAM of **α4β2** nAChR. dFBr is one of the few known compounds capable of discriminating α4β2 from other nAChR subtypes to produce selective potentiation of responses.

In conclusion, the discovery of molecules that modulate α4β2 nAChR represents an important step towards a therapy for neuronal disorders [104, 105, 109, 111]. The use of nAChRs allosteric modulators increases the probability of receptor opening induced by agonists and decreases receptor desensitisation [104, 105]. Furthermore, the use of a modulator has potential clinical benefits as the response on nAChRs is reached with a much lower concentration of the agonist, thus minimising the agonist's unwanted effects.

This thesis explored reported effects of choline in modulating ACh responses on nAChRs. This modulation of ACh effects by choline was investigated with particular focus on α4β2 receptors. A general overview of knowledge about choline is presented below.

1.2.6 Choline and its derivatives

1.2.6.1 Choline biochemistry

The chemical name of choline is trimethyl-beta-hydroxy-ethylammonium (Figure 10). It is a quaternary ammonium compound which is known to be widely distributed and has a prominent biochemical role in the cells [112, 113]. Most importantly, choline is required in the synthesis of the phospholipids in cell membranes, cholinergic neurotransmission, transmembrane signalling, and lipid-cholesterol transport and metabolism. It is believed that the cells die from apoptosis due to lack of choline.

Figure 12. Choline structure.
(trimethyl-beta-hydroxy-ethylammonium).

In physiological conditions, choline concentrations in the human brain range between 10 and 20 μM in the normal condition and may increase up to 100 μM in pathological conditions such as AD and seizures [114-116]. Other experiments have shown that free choline levels in rat brains vary between 30 and 60 μM [3]. It has also been found that there was no variation

of choline concentration in the brain as a result of oral administration of choline [117], which suggest that the brain has a source of choline other than diet.

Choline in the cells has a dual origin, as a nutrient and by denovo biosynthesis. Although several papers have categorised food rich in choline and identified possible consequences of choline-deficient diets, such as hepatic steatosis, there is no evidence that adult cholinergic neurons require dietary choline to synthetise ACh [112, 113]. Rather, it has been demonstrated that the reuptake of intrasynaptic choline is the limiting factor controlling ACh synthesis by blocking the reuptake process using hemicholinium-3. However, no food or endogenous molecule has shown the same ability [118, 119].

Endogenous choline exists in the form of phosphatidylcholine, which accounts for 95% of the total choline in mammalian tissue [120]. The remaining 5% is present as various species such as choline itself, phosphocholine, glycerophosphocholine, cytidine 5-diphosphocholine (CDP-Choline) and acetylcholine. The phosphatidylcholine form is biosynthesised in the mitochondria through the sequential methylation of phosphatidylethanolamine by S-adenosyl methionine (SAM) in the methionine cycle by phosphatidylethanolamine methyltransferase (PEMT). In the cholinergic neurons, choline is derived from three main sources (Figure 11) [3, 121]. In the first source, choline is brought into neurons by a low affinity transport mechanism, maintaining equilibrium between circulating choline in extracellular fluid and choline in other brain cells. The second source consists of choline formed by ACh hydrolysis by AChE in the synaptic cleft; choline is then taken up by a high affinity transport mechanism. The third source is believed to be choline-related bases, and the mechanism consists of base exchange or phospholipase mediated hydrolyses.

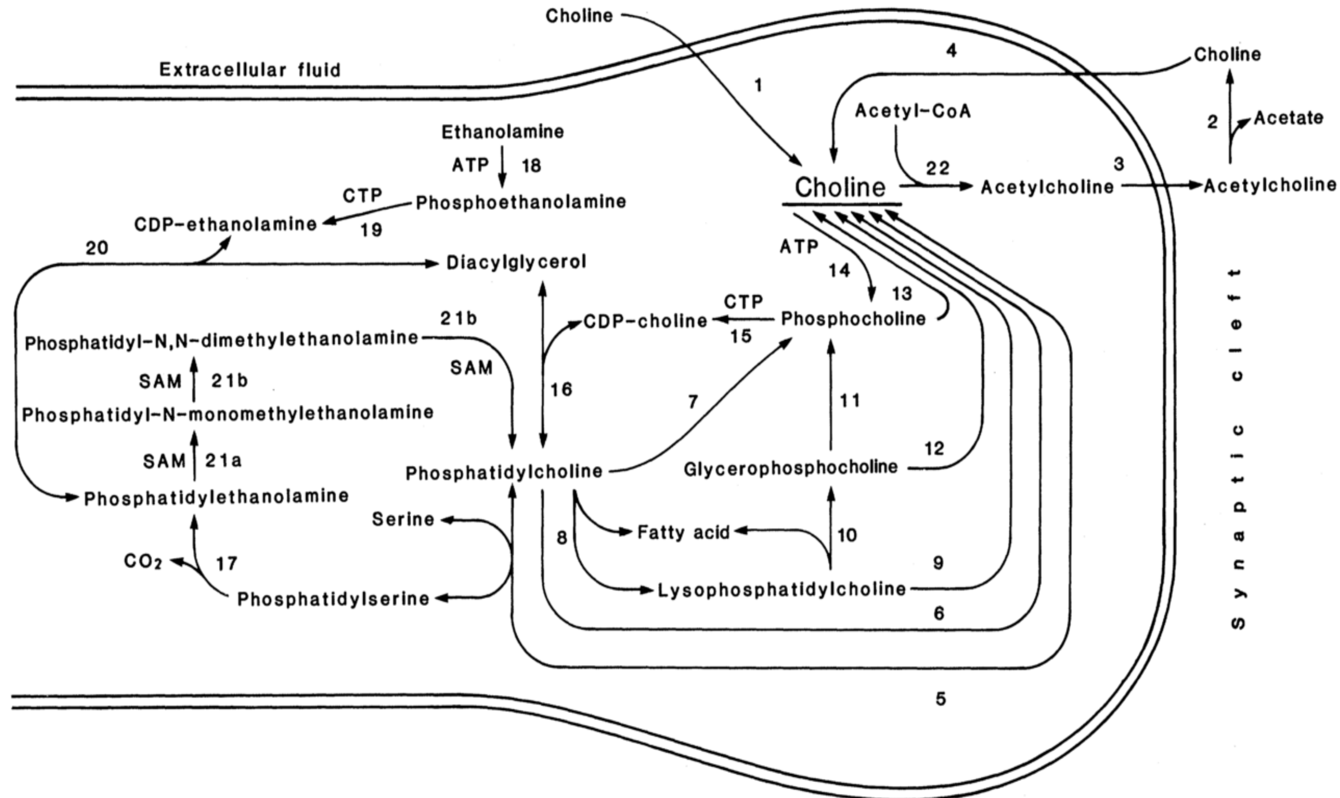

Figure 13. Sources of choline for neurons in the brain.
Choline from extracellular fluid is transported in neurons by a low affinity transport mechanism (1). Choline resulting from hydrolysis of AChE in the synaptic cleft is taken up by a high affinity transport mechanism (2). Choline is liberated by base exchange (5) or phospholipase mediated hydrolyses (6 to 13). This figure was adapted from Blusztajn and Wurtman [3].

The high affinity transport of choline from the synaptic cleft predominantly occurs at presynaptic cholinergic nerve terminals, with an average affinity for choline between 0.1-10 μM [118, 119, 122]. The low-affinity transport, which is mainly for phospholipid synthesis, has an average affinity for choline between 30-100 μM and is a sodium-independent carrier mediated uptake. By contrast, the high-affinity transport is a sodium-dependent carrier-mediated uptake and the most important source of choline for the cholinergic neuron to synthetise ACh.

Taken together, these findings show that choline is essential to maintain normal conditions of various functions particularly protecting neurons and a normal cholinergic neurotransmission. Cholinergic neurons also have a mechanism for reproducing choline, which partly maintains its constant level in the brain. The brain levels of choline were reported to be much higher in infants and, most importantly, choline is known to have a prominent role in early CNS development, memory and cognition.

1.2.6.2 Choline and nervous system development

In the first days of life, high levels of plasma choline are reported but these levels decrease significantly with increasing age [123, 124]. For example, the serum free choline level was measured in humans and was found to be 35.1±1.1 µmol/L at birth, decreasing gradually to 10.9±0.6 µmol/L a year after birth. It was also reported that plasma concentration in newborns was three times higher than in mothers aged between 22 and 36 [123, 125].

High levels of choline in fetuses, newborns and breast milk explain the exceptional importance of choline [123, 125-127]. The choline supply is particularly sensitive during critical periods of nervous system development, with evidence of effects on cognition [120, 128-130]. To demonstrate choline's role in cognitive performance, studies using animal models have shown that prenatal choline depletion is highly correlated with memory deficits [128, 130]. For example, male rats were exposed to choline deficient, sufficient or supplemented diet during the embryonic stage and thereafter given a controlled diet [131]. At the age of 70 days, the rats were given a task to locate eight baited arms of a 12 arm radial maze. The results showed that rats who had been exposed to sufficient choline made many more choices than rats who had received the deprived or supplemented choline diet.

All these data show that choline enhances cognition and memory function in early age, but this role is not yet clearly established in adults. However, it is known that choline activates nAChRs [132] through which cognition and memory functions are partly expressed. Although it is not yet known whether choline may activate nAChRs under physiological circumstances, some evidence supporting the interaction of choline with different nAChRs subtypes has been reported.

I.2.6.3 Choline and nicotinic receptors

The role of choline in the early CNS development has been widely discussed in the literature [130]. These functions, particularly learning and memory, are known to be mediated by nAChRs [22]. However, little is known about the specific mechanism through which choline influences cognition and its interaction with receptors in the brain[132]. Early study demonstrated the agonistic effect of choline on cholinergic receptors, but its potency was perceived to be too low to impact the CNS [132]. Moreover, a number of quaternary ammonium compounds including choline were reported to have pharmacological activities similar to those of muscarine and nicotine actions in the CNS.

Choline was found to be a full and selective agonist of the α7 nAChR subtype and a weak agonist for α3β4 (Table 4); no activation was seen on α4β2 subtypes [80]. Nevertheless, the choline regulating effects of nAChRs have been investigated and the inhibitory potency of choline on α3β4 was found to be higher than on α4β2 [133, 134]. Moreover, a dual effect of choline co-applied with ACh was observed on α4β4 nAChRs [135]. It was shown that a low concentration of choline potentiated responses evoked by 1 μM ACh, but inhibited by 300 μM and higher concentrations of choline (Table 4). This is believed to result from the competitive and noncompetitive effects of both ligands. Most recent data have shown that

choline regulates hippocampal network activity, which is mediated in part by α7 nAChR [136]. Another study has revealed that choline induces α4 and β2 nAChR subunits upregulation, and it is believed that this upregulation could be a response to altered metabolic and inflammatory conditions [137].

Table 4. Reported effects of Choline on different nAChRs subtypes

	Choline effects		
nAChR subtype	**Effect/potency**	**Potentiation**	**Inhibtion**
Rat α7 [80]	Selective full agonist, EC_{50} = 1.6 mM		
Bovine α7 [134]	A weak full agonist, EC_{50} = 0.43 mM.		
Bovine α3β4 [134]	Voltage-dependant inhibition; IC_{50} = 0.97 mM		
Rat α3β4 from hippocampal and dorsal striatal slices [133]		ACh Potentiation by choline <3 μM	ACh Inhibition by choline >3 μM, IC_{50} of 15μM
Rat α4β4 expressed in *Xenopus* oocytes [135]		Choline enhanced responses evoked by 1 μM of ACh	ACh 300 μM was inhibited by choline, IC_{50} = 0.87mM
Rat α4β2 from hippocampal and dorsal striatal slices [133]			ACh 100μM was inhibited by 200 μM to 10mM of choline, IC_{50}=372 μM

In summary, the data reported above suggest that choline has the ability to interact with nAChRs and has major effects on the developing brain. It is particularly noted that free choline present in synaptic fluid following neuronal depolarisation may or may not attain levels sufficient to activate the nAChRs. Although interaction of choline with brain receptors including α4β2 is reported, the effect of choline on different stochiometries of α4β2 nAChRs is not yet known. In the present study, choline concentrations relatively comparable to those found in the brain were administered on α4β2 nAChRs in order to produce more robust scientific evidence of the effects of choline, which could lead to better understanding of its role in the brain.

1.3 Aim and Significance of the Study

The overall aim of this study was to evaluate choline activity on different stochiometries of α4β2 nAChRs in the presence or absence of ACh using electrophysiology techniques. As previously noted, choline plays two roles: as precursor and metabolite of ACh. It is believed that the two molecules cohabit in the synaptic cleft once the release of ACh is stimulated. It is therefore hypothesised that certain amounts of choline compete with ACh on postsynaptic nAChR. Understanding the effect of choline on specific nAChR and its competition with ACh on the same receptors would be an important contribution to current knowledge. Such information is essential to understand nAChR signaling in *vivo* and for the development of new ligands and may eventually translate into the design of novel therapeutics.

1.3.1 Specific objectives

The study had three specific objectives:

- **a)** To investigate the effect of choline on currently known α4β2 nAChRs stochiometries, (α4)3(β2)2 and (α4)2(β2)3. This will show whether choline activates or not each of those stochiometries. The (α4)3(β2)2 and (α4)2(β2)3 contain respectively one and no α4α4 binding site, therefore this study will determine the impact of α4α4 binding site on the efficacy and potency of choline on those receptors.
- **b)** To determine the efficacy and potency of choline on engineered α4β2 nAChRs. The α4β2 engineered receptors consist of (α4)3($β2^{3M}$)2 and ($α4^{3M}$)3(β2)2, which are receptors containing three point mutations (3M) on complementary α4α4 and α4β2 binding sites where key amino acids involved in receptor binding are different [59]. As explained earlier, mutating the (-) α4 side makes an α4α4 binding site resemble an α4β2, and an α4β2 binding site resemble an α4α4. Therefore, the (α4)3($β2^{3M}$)2 receptor contains one native α4α4 site and two α4α4-like sites whereas the ($α4^{3M}$)3(β2)2 receptor contains two native α4β2 sites and one α4β2-like site. The advantage of using these receptors is is that it allows the effects of the ligand on either the α4α4 or α4β2 binding site to be investigated separately. Experiments on engineered receptors could reveal then choline selectivity between α4α4 and α4β2 binding sites.
- **c)** To investigate the co-application of choline and ACh on (α4)3(β2)2. This will extend our understanding of the physiological role of the α4α4 interface, as well as the choline potentiation or inhibition of ACh responses. The co-application of ACh with choline pre-incubation is also taken into account in order to observe any change in potentiation or inhibition effects.

Chapter 2

Materials and Methods

2.1 Materials

The wild-type rat plasmid cDNA (Table 3) encoding α4 and β2 nAChR subcloned into pSP64 cloning vector from SP6 polymerase promoter (Figure 14) were stored in the molecular biology laboratory of the Faculty of Pharmacy, The University of Sydney. The α4 and β2 nAChR mutants were generously engineered and donated by Denish Indurth, and were stored in similar conditions. All enzymes and compounds choline and ACh used as ligands were obtained from the Faculty of Pharmacy, The University of Sydney. Oocytes of a female *Xenopus laevis*, a species of South African frog, were imported and housed in the Edward Ford Building (Room number 117, Building A27A, The University of Sydney).

Table 5. Plasmid cDNA Vector, RNA Polymerase and Linearisation Restriction Enzyme for each nAChR Subunit

Plasmid cDNA	Plasmid cDNA Vector	Polymerase Promoter	Linearisation Enzymes
α4	pSP64	SP6	*Eco*RI
β2	pSP64	SP6	*HindIII*

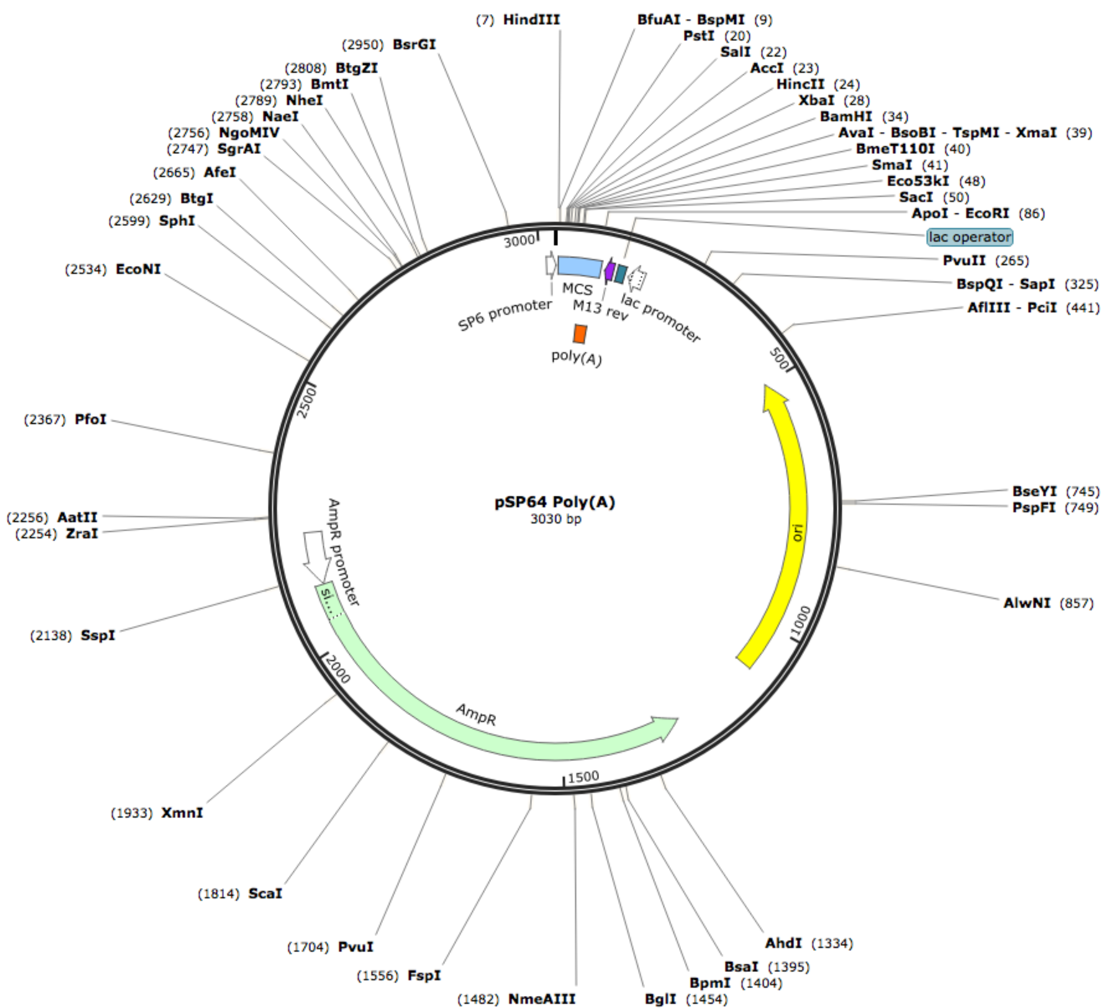

Figure 14. A map of pSP64 Cloning vector for *in vitro* transcription from the SP6 promoter with the addition of a synthetic 30-base poly(A) tail, author: Promega.

2.2 Molecular Biology Methods

2.2.1 Transformation of DNA plasmids

Rat nAChR cDNAs were transfected into one shot Top 10 chemically competent cells and ampicillin-resistant *E.Coli* using aseptic techniques. This was done by adding 1-5 µL of cDNA plasmids (Table 4) to 50 µl *E,Coli* cells and the mixture was incubated on ice (0°C) for 30 minutes. During this period the plasmid cDNAs were adhering to the *E.Coli* cell wall. The cells were then incubated for exactly 30 seconds in the 42°C water bath. This heat-shock creates pores on the cell wall and the plasmids are able to enter the cells [138]. The cells were then placed on ice for 2 minutes to restore the cell wall integrity.

2.2.2 Culturing the transformed *E.Coli* cells

To grow transfected *E. coli* cells, 250 µL of pre-warmed S.O.C medium (bactotryptone 20g/L, bactoyeast exctract 5g/L, NaCl 0.5g/L, 2,5mM KCl (pH 7.0), 10mM $MgCl_2$, 20mM glucose) was aseptically added to the cells and incubated in a shaking incubator (Orbital Mixer Incubater, OM-11; Ratek Instruments, Boronia, Vic., Australia) at 225 rpm at 37°C for an hour. Next, the incubated cells (100 µL) were spread on fresh lysogeny broth (LB)-agar plates containing ampicillin (100 µg/mL) to limit bacterial contamination (*E. coli* cells are ampicillin-resistant) and incubated overnight at 37°C. Isolated *E. coli* colonies were randomly selected from the LB-agar plate and added to LB-broth containing ampicillin (100 µg/mL). The

mixture was then incubated in the shaking incubator overnight (at least 15 hours) at 37°C.

2.2.3 Purification of plasmid DNA

The plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN). *E. coli* cells 5mL were collected by centrifugation at 14,000 rpm for 2 minutes at room temperature in Eppendorf tubes. A cells pellet was formed, the supernatant was discarded and the cell pellet was resuspended in 250 µL of buffer P1 (50mM Tris (pH7.5), 10mM EDTA, 100 µg/mL RNase A), then 250 µL cell lysis solution buffer P2 (200mM NaOH, 1% SDS) was added to solubilise the cell membrane protein and phospholipids, which results in release of the cell content. The cell lysis solution was then neutralised by adding 350 µL of buffer N3 (1.32M CH_3COOK pH4.8). A precipitation was then formed in which denatured and chromosomal DNA were trapped while the plasmid DNA remained in the solution due to their smaller size. The plasmid DNA and the cellular debris were separated by 10 minutes centrifugation at 13000rpm at room temperature. The supernatant obtained, which contained plasmid DNA, was then added into QIAprep spin column and centrifuged for 60 seconds. At the end of this step, the plasmid DNA was bound to the column. The next step was to wash the QIA prep spin column. This was done by adding buffer PE 750 µL (10mM Tris-HCl (pH7.5), 10mM EDTA) and centrifuging for 60 seconds. The flow through was then discarded and the spin column was centrifuged again for 60 seconds to eliminate ethanol residue. Finally, to elute the plasmid DNA, the QIAprep column was placed in a clean 1.5mLcentrifuge tube and elution buffer EB 50 µL (10mM Tris-Cl, pH8.5) was applied to the centre of the column. The column and centrifuge tube

were allowed to stand for 60 seconds, followed by 60 seconds centrifugation at 14000 rpm, and the purified plasmid DNA was collected.

2.2.4 Linearisation of plasmid DNA

The linearisation and purification of plasma cDNA was done to prepare for RNA synthesis. Circular plasmid cDNA was linearised by incubation of plasmids with *Eco*RI and *HindIII* restriction enzymes, respectively, for plasmids containing the α4, β2 inserts at 37 ^{o}C for 2 hours. The completion of linearisation of plasmid DNA was checked by running an electrophoresis gel. To this end, 2 µL of plasmid cDNA was stained with 2 µL of Blue/Orange Loading Dye (Promega ©, Madison, WI, USA) and separated along an agarose gel (~1%; supplemented with tris-acetate ethylenediaminetetraacetic acid (TAE) and ethidium bromide) using a HE-33 Mini Horizontal Submarine Unit (90 mV, 30 to 60 minutes; Hoefer, Inc., San Francisco, CA, USA). The plasmids were then visualised with UV light using GelDoc^{TM} 1000 and Molecular Analyst Software (BioRad, Hercules, CA, USA).

Fragment size was estimated by comparison with a 1 kb DNA ladder (Promega ©, Madison, WI, USA). These fragment sizes were then compared with the predicted fragment sizes, which were calculated from APE-A Plasmid Editor V1.08 for verification of linearised cDNA. The linearised cDNA was then purified by adding 5x volume of buffer PB to the restriction enzyme reaction and mixed well. The mixture was applied to a QIAquick spin column in a provided 2 mL collection tube to bind the DNA, and then the spin column was centrifuged for 60 seconds. The flow-through was discarded. The purity of linearised DNA was maximised by washing the spin columns again with 750 µL of buffer PE, then centrifuged for 60 seconds and the flow

through was again discarded. The spin columns were placed again in the tubes and centrifuged for an additional 60 seconds. The QIAquick columns were put in clean microcentrifuge tubes and the DNA was eluted by adding 50 µL of buffer EB to the centre of the tube and left standing for one minute to increase the concentration of eluted DNA, and the DNA was collected by 60 seconds centrifugation at 14000 rpm.

2.2.5 Synthesis of mRNA

The transcription of α4 and β2 mRNAs from linearised cDNA was done using SP6 polymerase mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA). It involved the reaction of a mixture of 6 µl linearised cDNA with 2 µl of 10x transcription buffer, 10 µl of 2x NTP/CAP ribonucleotide mix (15 mM adenosine triphosphate (ATP), 15 mM cytidine triphosphate (CTP), 15 mM uridine triphosphate (UTP), 3 mM guanosine triphosphate (GTP) and 12 mM Cap Analog) and 2 µL of 10x enzyme mix (including SP6 RNA polymerase and placental RNase inhibitor in 50% glycerol). The reaction mixture was then incubated for 2 hours at 37°C to allow synthesis of mRNA. The reaction was then stopped by adding 30 µL nuclease free water and 30 µL LiCl precipitation solution which resulted in mRNA precipitation. The mixture was chilled for 30 minutes at −20ºC. The mRNA was then pelleted by centrifugation at 4ºC for 15 minutes at 14000rpm, then washed with 500 µL freshly prepared cold 70% ethanol, dried and re-suspended in 15 µl nuclease free water. Purified mRNA was stored at -20°C until needed.

2.2.6 RNA concentration measurement

The concentration of obtained mRNA has to be verified in order to ensure the absence of contaminants and that the ratio to be injected in the cells is correct. The presence of mRNA was verified by heating a sample of mRNA at 94ºC for 1 minute to denature the mRNA with an equal amount of gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18 mM EDTA, 0.025% SDS). This mRNA was run on ~1% agarose gel containing ethidium bromide. The quality and quantity of RNA was checked by comparing its band to a standard DNA ladder.

The quality and concentration of the mRNA was also determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, DE, USA). The 260 nm/230 mm absorbance ratio between 2-2.2 indicates pure mRNA. The pure mRNA was further diluted to the required concentration with nuclease-free water when necessary.

2.3 Electrophysiology Methods

2.3.1 Preparation of *Xenopus laevis* oocytes

The oocytes were obtained by surgically removing ovarian lobes of *Xenopus laevis* under anaesthesia (Tricaine, 850 mg/500 mL), which is done by a trained surgeon on a weekly basis. To get the oocytes ready, the lobes are first rinsed with oocytes releasing buffer 2 (OR2; 82.5mM NaCl, 2mM KCl, 1mM$MgCl_2$, 5mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) hemisodium salt, Ph 7.5) and cut into smaller clusters of cells, then treated with collagenase A (2mg/ml) in OR2 and for two hours at 18°C in a shaking incubator (505; Stuart Scientific, UK). The defolliculated and separated oocytes are obtained and rinsed with ND96 solution

(96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES hemisodium salt, 2.5mM pyruvate, 0.5 mM theophylline, pH7.5). Finally, the best oocytes for use (stage V-VI) were sorted and kept in ND96 solution containing 4 µg/ml kanamycin.

2.3.2 Injection of mRNA in the oocytes

The injection of mRNA in the oocytes was done using micropipettes made of glass capillaries (0.53 mm I.D.x1.14 mm O.D.; Drummond Scientific Company, PA, USA). The micropipettes were prepared by using a microprocessor-controlled micropipette puller (PUL-100, World Precision Instruments, Inc., FL, USA). Thereafter, the micropipettes were beveled at a tip diameter of approximately 10-20 µm and filled with mineral oil. Pre-mixed α4 and β2 wild-types or mutant mRNA in appropriate ratios were drawn up the micropipette using negative pressure. Appropriate ratios were defined based on previous studies which have demonstrated that different proportions of *α*4 and *β*2 subunit RNA injected into *Xenopus* oocytes produce a predominance of either isoform whose function and pharmacology match concatenated receptors [56, 59, 79, 139]. Thus injected α4 and β2 RNA ratios were 10:1 or 1:10 which favours the production of (α4)3(β2)2 or (α4)2(β2)3 receptors respectively. Using a Nanoliter 2000 injector (World Precision Instruments, Inc., FL, USA), approximately 50 nL of mRNA was injected into the cytoplasm of each oocyte; any possible cross-contamination between different ratios was avoided by using separate micropipettes. Injected oocytes were kept in ND96 solution supplemented with kanamycin 4 µg/ml and incubated in an orbital shaker at 18°C for two days before use in the electrophysiology experiments.

2.3.3 Two-electrode voltage clamp

Electrophysiology studies for the receptors were done using two-electrode voltage clamp recording. This is a set-up of a GeneClamp 500 Amplifier (Axon Instruments, Foster City, CA, USA), a power lab (AD instruments, Sydney, NSW, Australia) and chart version 3.5 program for Macintosh. Two glass microelectrodes (0.94 mm I.D., 1.2 mm O.D., 150 mm length; Harvard Apparatus, Kent, UK) were pulled from the micropipette puller (PP-830, Narishige, Japan), filled with 3 M KCl. Oocytes were placed in a cell bath and transfixed with the two electrodes. Oocytes were then clamped at −60 mV and continuously perfused with calcium-free buffer as Ca^{2+} may enter receptors and disrupt the results (CF; 115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl2, 10 mM HEPES).

2.3.4 Ligands application and data recording

The potency and efficacy of ligands were tested on stable oocytes with receptor expression. The receptors' expression was tested by applying the control solution, 1mM Ach, on the clamped oocyte and then observing the inward induced current with a maximum that depends on the receptors' stoichiometry and type expressed in the oocyte. The oocyte was under continual perfusion of calcium-free buffer to keep it alive. The solution of a ligand to be tested was then allowed to run on the oocyte until a maximal response, shown by the maximum induced current, was obtained.

The cell stability and response consistency were verified by 1mM ACh every second ligand application, and an interval of 10 min washing time was considered after each dose application to minimise receptor desensitisation. Increasing concentrations of ACh were applied to wild types and mutants of each α4β2 nAChR stoichiometries,

and the dose response curves were constructed to confirm their pharmacological profile. Pharmacological activity of choline was then evaluated. Increasing concentrations of choline were applied to wild types α4β2 and mutants, and induced currents were recorded in Labchart 7 Reader (ADInstrument). Then choline was co-applied with 1µM, 10 µM and 100 µM ACh on the (α4)3(β2)2 stoichiometry. Lastly, the effect of 300 µM choline pre-incubation on increasing concentrations of ACh was tested on (α4)3(β2)2 nAChR.2.3.5 Analysis of electrophysiological data

Electrophysiology data were collected as currents elicited by ligand applied to the cells. Induced current (I) of the ligand was compared to the current elicited by maximum response (Imax) of the control (ACh 1mM), and the average proportion I/Imax of currents induced on four to six different oocytes was reported. The proportion I/Imax was plotted as a function of ACh concentration and fitted by the least squares method to the sigmoid concentration-response (variable slope) equation (or Hill equation) (see equation 1) in GraphPad Prism 6 (GraphPad Software, CA, USA). Statistical analyses were determined by using a paired student's t-test. A p-value for EC_{50} and Hill coefficient (nH) of <0.05 was determined to be statistically significant.

$$\textbf{(a)}\quad I = \frac{I_{max}[A]^{n_H}}{(EC_{50})^{n_H} + [A]^{n_H}} \qquad \text{or} \qquad \textbf{(b)}\quad I = \frac{I_{50}[D]^{n_H}}{(IC_{50})^{n_H} + [D]^{n_H}}$$

Equation 1. The Sigmoidal Concentration-response Equation.
(a) Where [A] is the concentration of the agonist compound, EC_{50} is the concentration that activates 50% of the maximum response. IMax is the maximum current elicited by ACh on that cell.
(b) Where [D] is the concentration of the antagonist compound, and IC_{50} is the concentration that inhibits 50% of the maximum response. nH is the Hill coefficient, a measurement of co-operativity of agonist binding.

Chapter 3

Results

The main objective of the project was to evaluate the pharmacological activity of choline and choline in combination with ACh on α4β2 nAChRs. To this end, compounds were tested on two stoichiometries of the α4β2 nAChR, (α4)2(β2)3 and (α4)3(β2)2. Subsequently, choline was tested on receptors engineered to have either α4-α4-like binding sites or α4-β2-like binding sites in order to evaluate the effect of choline at the individual interfaces. This chapter begins by presenting results from ACh as well as choline application on wild types $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ receptors. Then the results of choline co-application with fixed concentrations of ACh on (α4)3(β2)2 nAChRs are presented, followed by the results from the investigation of ACh choline effects on mutant receptors, specifically $(\alpha4)3(\beta2^{3M})2$ and $(\alpha4^{3M})3(\beta2)2$. Finally, results from experiments evaluating the effects of ACh on (α4)3(β2)2 after choline pre-incubation are reported. The relevance of the results in a broader physiological context and the contribution of the research to the knowledge base of neuroscience will be discussed in Chapter4.

3.1 Experimental Data Presentation

In the research experiments, two electrode voltage clamp measurements were used to record changes in ion channel conductance following application of different concentrations of ligands. Peak current amplitudes were measured and plotted against concentration. The concentration-response curves were constructed and are shown in

Figures. The data characteristics of the concentration-response curve presented include population size (n), maximum efficacy (I_{max}) and potency (EC_{50} which is the concentration required to produce 50% of the maximum efficacy) along with the significance shown in terms of the negative logarithmic value of EC_{50} and standard error (pEC_{50}±SE). Illustrative current traces are also presented as raw data to show the changes in current in response to ligand application on the receptors.

3.2 ACh on Wild Types α4β2 nAChRs

ACh was first tested on both (α4)2(β2)3 and (α4)3(β2)2 receptors to confirm that the oocytes were expressing uniform receptor from a mix of α4 and β2 RNA in 1:10 or 10:1 ratios. On (α4)2(β2)3 receptors a sigmoidal concentration response curve with an EC_{50} value of 3.4μM (pEC_{50}±SE = 5.5±0.08), n= 5 was obtained and for (α4)3(β2)2 receptors the EC_{50} value was nearly 30 times higher, 100μM (pEC_{50}±SE = 3.9±0.09), n= 4 (Figure 15A). Visual inspection of the ACh response curve for (α4)3(β2)2 showed a prolongation of the curve or "tailing" at low concentrations, which is an indication of biphasic response curve. This curve was therefore fitted to a biphasic Hill equation and statistical analysis that the best fit was obtained with ACh potencies of EC_{50_1}=1.64 μM and EC_{50_2}=154 μM,(Figure 15B) for respectively (α4)2(β2)3 and (α4)3(β2)2 receptors. These values are similar to those previously reported by Harpsoe et al. on (α4)3(β2)2 with α4:β2 expression ratio of 20:1 or 100:1 [59]. These results show that our receptor populations, (α4)2(β2)3 / (α4)3(β2)2, were uniform and can therefore be used for testing other ligands and that 1 mM ACh can be considered as a standard maximal control for α4β2 nAChRs agonists.

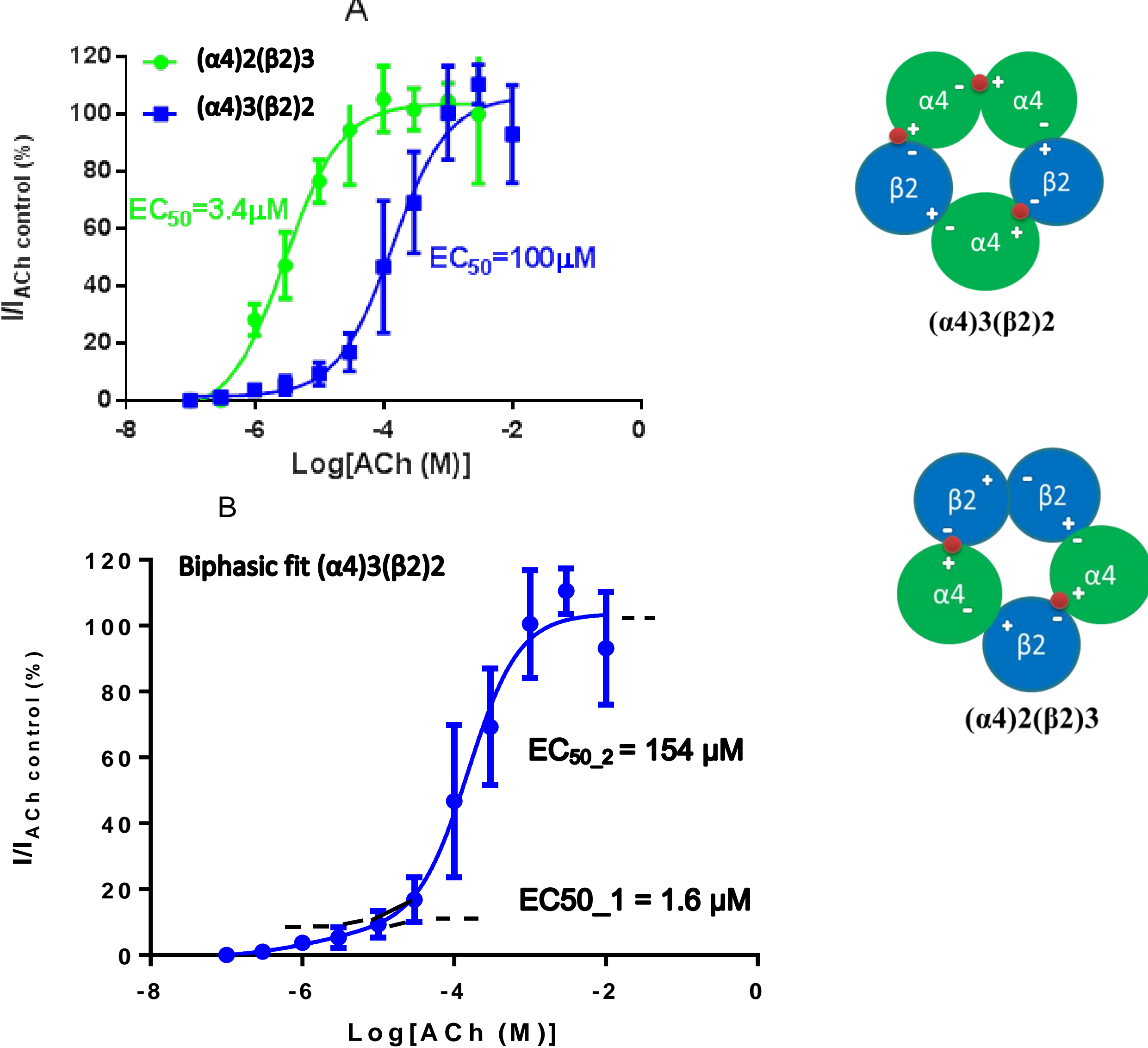

Figure 15. Concentration-response relationships for ACh on α4β2 nAChRs stoichiometries.

ACh on (α4)2(β2)3 (green) and (α4)3(β2)2 (blue) nAChRs fitted to a monophasic (A) and biphasic concentration response curve (B) from ACh on (α4)3(β2)2. Receptor populations were obtained by injecting α4 and β2 cRNA into *Xenopus laevis* oocytes in a 1:10 or 10:1 ratio to yield (α4)2(β2)3 and (α4)3(β2)2 nAChRs, respectively. Peak current amplitudes were measured by two electrode voltage clamp electrophysiology, normalised to the control response (1 mM ACh) and fitted by nonlinear regression to a monophasic or biphasic Hill equations using GraphPad Prism 6. Regression results (Log EC_{50}, I_{max}) are summarised in Table 7 on page71.

3.3 Choline on Wild Types α4β2 nAChRs

In an attempt to evaluate the agonist activity of choline on α4β2 nAChRs, increasing choline concentrations were applied to wild type (α4)3(β2)2 and (α4)2(β2)3. On one hand, choline was tested on (α4)3(β2)2, resulting in inwards currents (Figure 17A) which were then measured and the data were analysed to obtain a concentration response curve (Figure 16). Results from non-linear regression analysis showed that choline is a weak partial agonist with about four percent of the ACh induced maximal response, whereas EC_{50} values revealed that choline is four times less potent than ACh. This is evident when choline and ACh response curves are plotted on the same figure (Figure 13B). Choline efficacy is 30 times lower than ACh efficacy and EC_{50} values of 0.1mM (pEC_{50}±SE = 3.9±0.09) and 0.4mM (pEC_{50}±SE = 3.35±0.48), (n=6) for ACh and choline respectively, show that choline is 4 times less potent. In contrast, choline had no activity on (α4)2(β2)3 receptors when applied in concentrations up to 10 mM (Figure 17B). This suggests that choline does not activate (α4)2(β2)3 receptors, which may partially explain previously reported data indicating that choline does not have any activity on α4β2 hippocampal neurons [80].

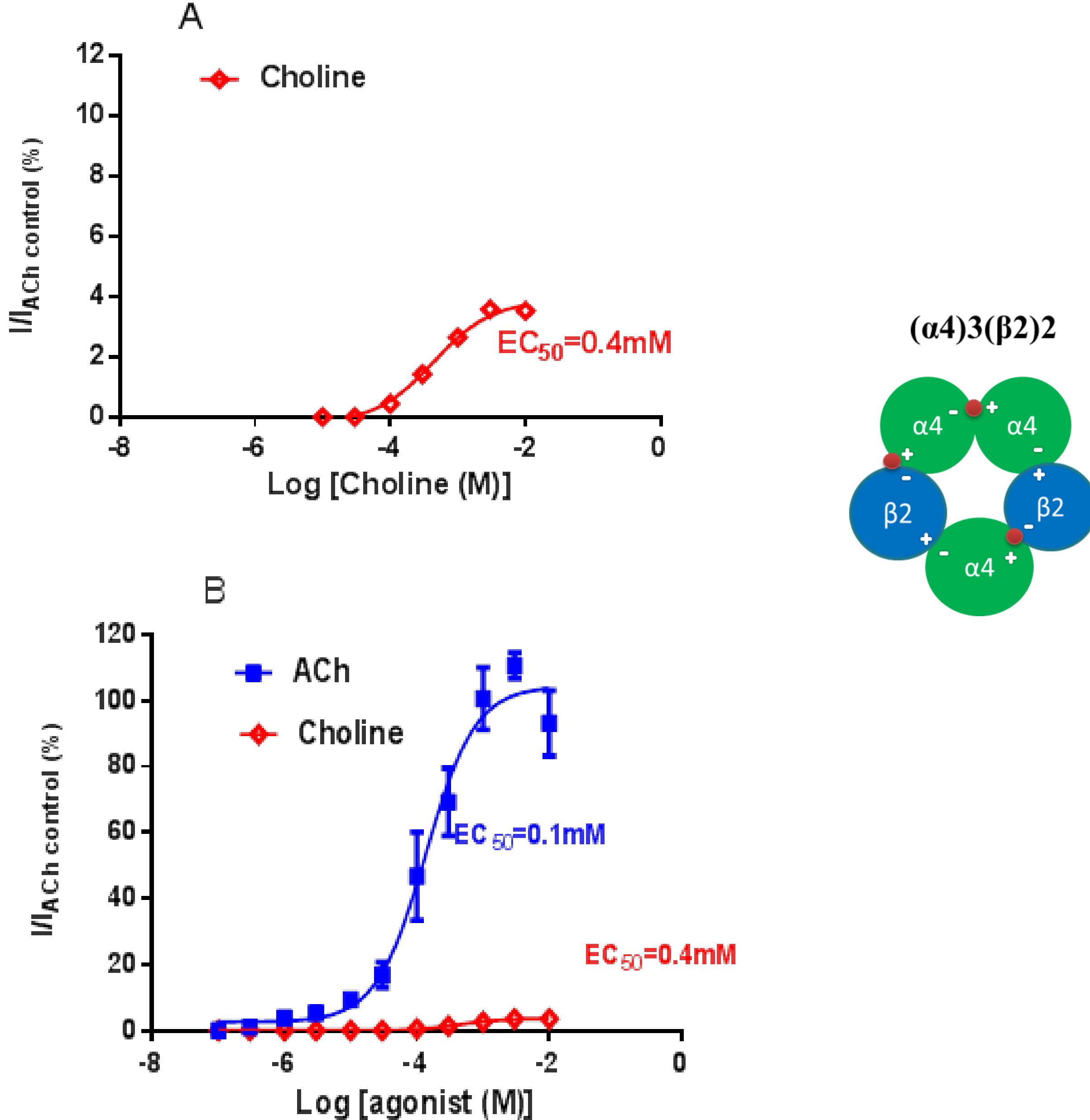

Figure 16. Concentration-response curves for choline alone and for choline compared to ACh on (α4)3(β2)2 nAChRs.
The receptors population, electrophysiological techniques and data analysis are described in the legend to Figure 15. Cartoon on the right side represents (α4)3(β2)2 receptor.

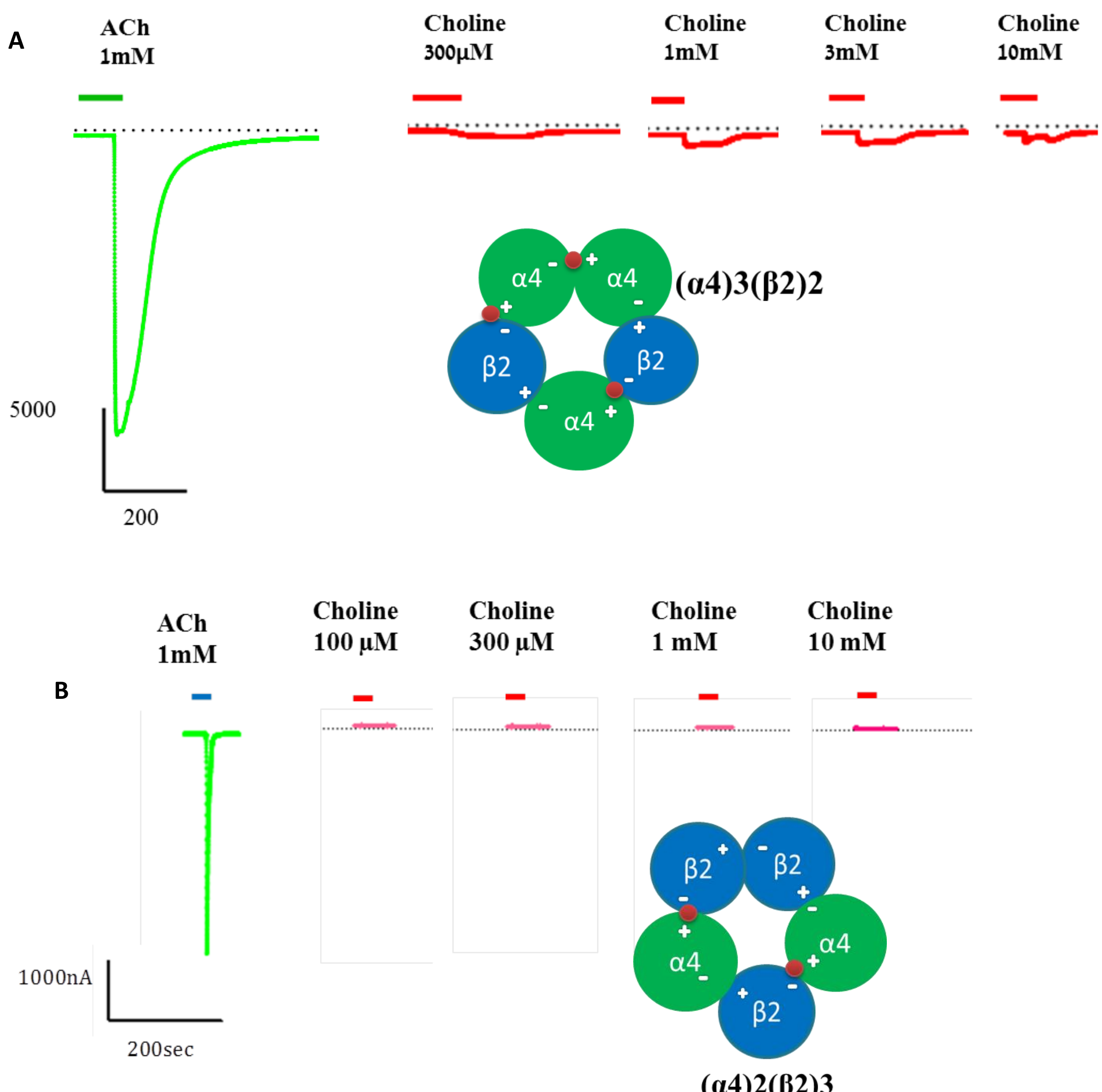

Figure 17. Sample current traces of choline on (α4)3(β2)2 and (α4)2(β2)3 nAChRs expressed in *Xenopus* oocytes.

First, the oocyte was treated with ACh 1mM (control in green) and then increasing concentrations of choline were applied. The control was always applied after every second ligand application and waiting time was 10 minutes before application of the next concentration to allow the signal to return to baseline and to allow remaining traces of ACh to be washed off. The holding potential was -60mV.

No previous studies relating to choline effects on individual and specific α4β2 stochiometries were identified in the literature review. However, a few analytical studies of some nAChRs are comparable to our findings on α4β2 receptors. For example, choline was reported to activate nicotinic receptors in the adrenal medulla, resulting in the secretion of catecholamines [132]. Compared to baseline, secretion of catecholamines was increased after administration of choline 0.5 mM. In the same study, choline was able to displace L-[^{3}H] nicotine from nicotinic receptors in various regions of the brain such as cortex and hypothalamus with an IC_{50} range of 0.5mM to 1.4mM [132], which is the range within which the potency of choline on (α4)3(β2)2 expressed in *Xenopus* oocytes in our experiments was. These results could be significant as α4β2 nAChRs are predominant in parts of the brain studied although it is hard to confirm expression of (α4)3(β2)2 stochiometry due to limitations in currently available techniques [61].

Another study suggested that choline is a weak but selective agonist with full efficacy (Imax = 91% of the maximal amplitude evoked by saturating ACh 3mM) on α7 nAChRs expressed on hippocampal cultured rat neurons with a potency of 1.6mM [80]. However when bovine α7 nAChRs were expressed in *Xenopus laevis* oocytes, choline showed two fold higher potency of 0.6 mM [140]. These two different values may be explained by the fact that the receptors expressed in the oocytes are much more isolated receptors and activated by less concentration of an agonist than the concentration to activate receptors expressed in tissue such as neurons because of the possible presence of receptors other than those of interest [141]. Contrary to α7, choline induced no detectable currents on receptors on hippocampal rat neurons bearing α4β2 nAChRs [80].

In contrast to observations in the literature, our experiments showed that choline does have some effect on α4β2-nAChRs and that the effect is stoichiometry dependent. In fact, choline potencies 400 μM and 600 μM respectively on (α4)3(β2)2 and α7 receptors expressed in *Xenopus laevis* oocytes are quite equivalent. The big difference is that on α7, choline has full agonist efficacy whereas on (α4)3(β2)2 it is a very weak partial agonist. Activity on (α4)3(β2)2 but not (α4)2(β2)3 receptors could indicate that choline requires α4α4 interface to have an effect.

To further explore this possibility, we investigated choline efficacy or potency on receptors that contain either α4β2 or α4α4 binding sites by exploiting a set of mutant receptors engineered to have these features.

3.4 ACh on Engineered α4β2 nAChRs

As in the previous experiments, ACh was used to test and confirm the uniformity of the engineered receptors population expressed in *Xenopus laevis* oocytes. Electrophysiological results of ACh on the engineered receptors, $(\alpha4^{3M})3(\beta2)2$ and $(\alpha4)3(\beta2^{3M})2$, indicated concentration response curves with potencies of, respectively 7.5 μM (pEC_{50}±SE=5.12±0.03), n = 6 and 150 μM (pEC_{50}±SE = 3.79±0.03), n = 6 (Figure 18). These results are similar to those of Harpsoe et al. [59]. Note the equivalence of ACh EC_{50} values on $(\alpha4)3(\beta2^{3M})2$ to the low sensitivity component of wild type (α4)3(β2)2 suggesting the presence of three binding site for ACh as shown in Figure 12B . In fact, the results permit these engineered receptors to be used as standard mutants in this study.

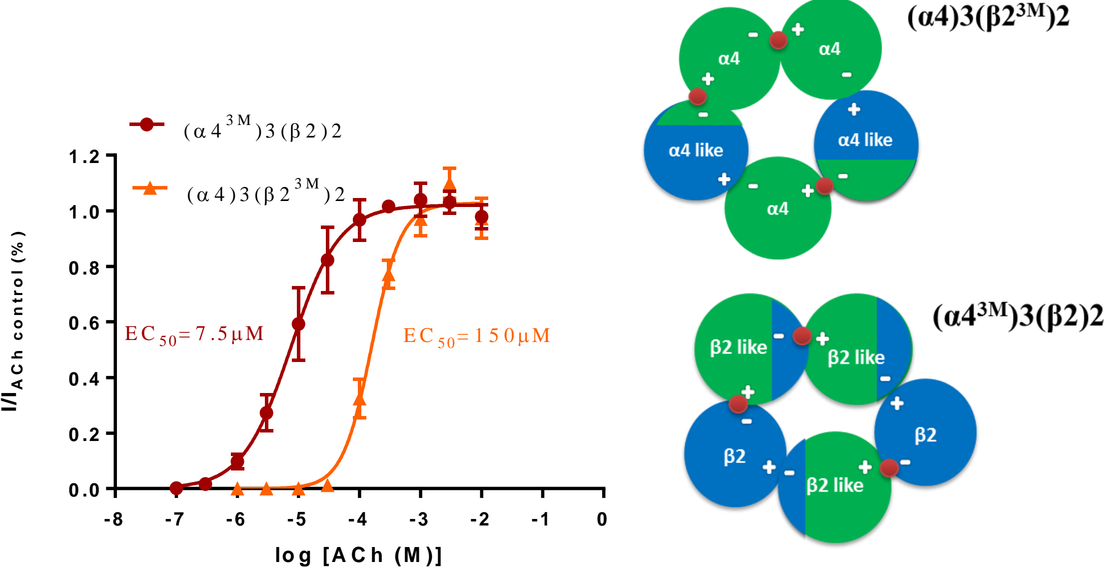

Figure 18. Concentration-response relationship for ACh on (α4^{3M})3(β2)2 and (α4)$_3$(β2^{3M})$_2$ nAChRs expressed in *Xenopus laevis* oocyte.
Receptor populations were obtained by injecting wild-type and engineered mutants (α4^{3M} with β2 and α4 with β2^{3M} into oocytes in 4:1 ratio to yield (α4^{3M})3(β2)2 and (α4)3(β2^{3M})2 nAChRs respectively. 3M denotes H142V, Q150F and T152L mutations on α4 and V136H, F144Q and L146T mutations on β2, respectively. Electrophysiology and data analysis were performed as explained in Figure 15.

3.5 Choline Effects on Engineered α4β2 nAChRs

Choline was tested on both engineered receptors ((α4)3(β2^{3M})2 and (α4^{3M})3(β2)2) and the results were in agreement with those found on wild type receptors, (α4)3(β2^{3})2 and (α4)3(β2)2. On the (α4)3(β2^{3M})2 receptor, which is a receptor with one native and two engineered α4-α4 sites, choline potency was 0.3mM (EC$_{50}$±SE = .07±0.00), n = 6) and the maximum response was 6% compared to the control maximum response (Figure 19A). To put the effect into perspective, the concentration response curves for choline on engineered receptors are plotted together with that of ACh (Figure 19B). This shows that, despite potencies in the same range (0.3 mM for choline and 0.15 mM for ACh), the efficacy of choline is significantly lower than that of ACh.

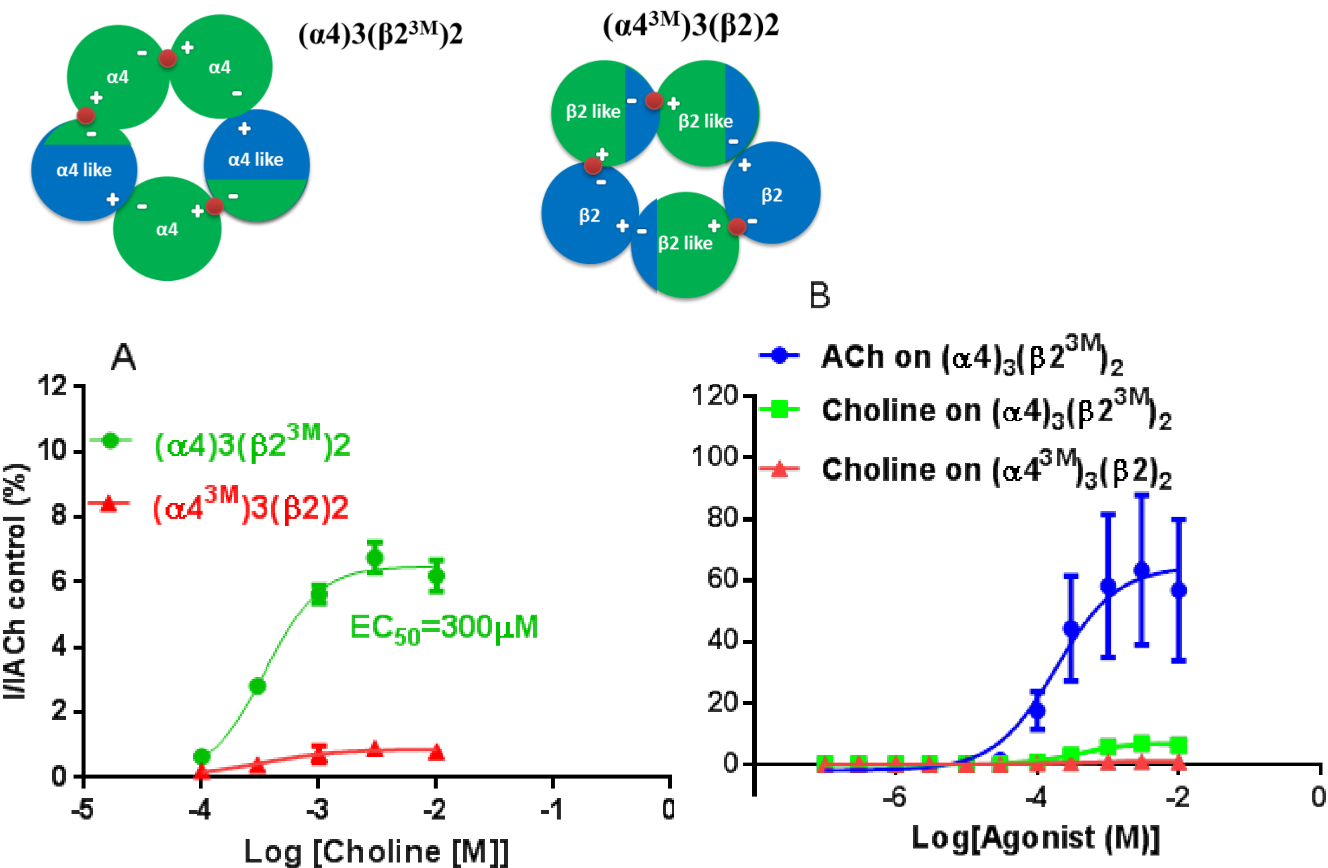

Figure 19. Concentration-response curve for choline and ACh on α4^{3M})3(β2)2 mutants receptors

A: Concentration-response relationship for choline on (α4)3(β2^{3M})2 (green) and (α4^{3M})3(β2)2 (red) nAChRs expressed in *Xenopus laevis* oocyte. B: ACh concentration response curve on (α4)3(β2^{3M})2 (blue) is compared to concentration-response relationship for choline on (α4)3(β2^{3M})2 (green) and (α4^{3M})3(β2)2 (red). Data analysis was performed as in Figure 15 and regression results are summarised in Table 7 on page 71.

These results resemble the efficacy and potency of choline on wild types (α4)3(β2)2. In contrast, choline had no effect on (α4^{3M})3(β2)2 receptors indicating that an α4-α4 site is required for choline activity and choline efficacy on mutants did not increase as a result of the presence of additional α4α4 like binding sites. These effects, are also observed on current traces (Figure 20 A and B).

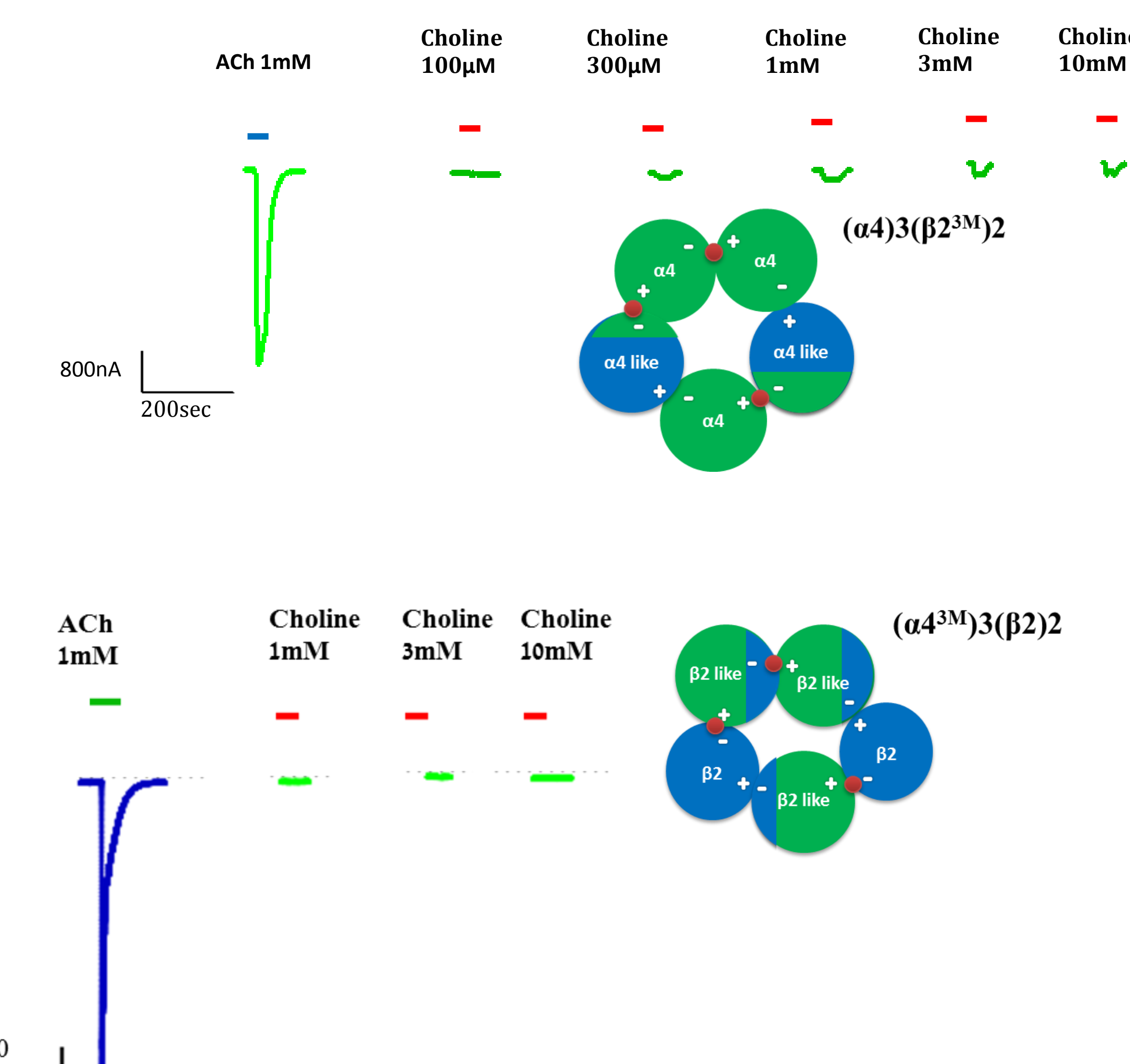

Figure 20. Sample current traces for an oocyte expressing the (α4)3(β2)2 mutants. Currents shown in A result from (α4)3(β2^{3M})2 and those in B are from (α4^{3M})3(β2)2. The first trace results from ACh 1mM (control) application followed by current traces of increasing concentration of choline. A 10 min. waiting period was included between each application. The holding potential was always -60mV.

The results show that choline does not interact with receptors that lack α4α4 binding site and may explain why it only interact with the native (α4)3(β2)2 nAChRs, which theoretically means that this compound would have potentiating activity with other agonists able to bind at α4β2 binding sites of the same receptor. To investigate this hypothesis further, investigation on whether choline could potentiate the effect of ACh was explored by testing various concentrations of choline in the presence of a fixed concentration of ACh on the native (α4)3(β2)2 nAChRs, The results of these experiments are presented in the next section.

3.6 Effect of Choline and ACh Co-application on (α4)3(β2)2 nAChRs

In order to evaluate the effects of choline on ACh responses on (α4)3(β2)2 nAChRs, increasing concentrations of choline were mixed with a fixed concentration of 1μM ACh. In a subsequent experiment the effects at different concentrations (1 μM, 10 μM and 100 μM) of ACh were investigated, and data were plotted on a graph to construct concentration-response curves shown in Figure 21.

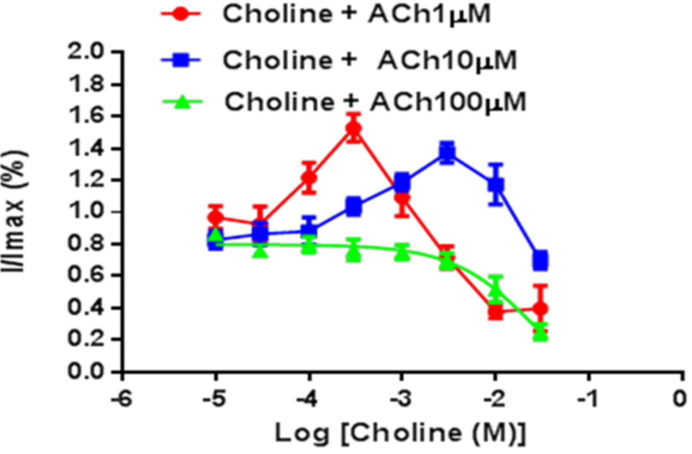

Figure 21. Concentration-response relationships for ACh and choline co-application on wild type (α4)3(β2)2 nAChRs expressed in Xenopus laevis oocytes.
The cartoon next to the curve illustrates wild type (α4)3(β2)2 receptor.

The concentration-response curves as shown in Figure 21 (above) indicate that choline has a dual effect on (α4)3(β2)2 when it is co-applied with ACh. Low concentrations of choline result in an apparent potentiating effect with 1 μM and 10 μM ACh, but these effects are reversed (inhibited) by choline in concentrations of 1mM or higher. The observed potentiation effects are not high enough to conclude that they are a result of synergism of ACh and choline effects, and it cannot be concluded whether they are additive effects as potentiation did not appear at all concentrations. No potentiation was observed when choline was co-applied with 100 μM ACh, but inhibition occurred with an IC_{50} value of 26mM (Figure 19C). This indicates that much higher concentration of choline are required to inhibit responses evoked by higher levels of ACh. These effects are clearly shown on individual concentration-response curve as well as the traces of current induced by each concentration of ACh co-applied with choline (Figure 22A-C).

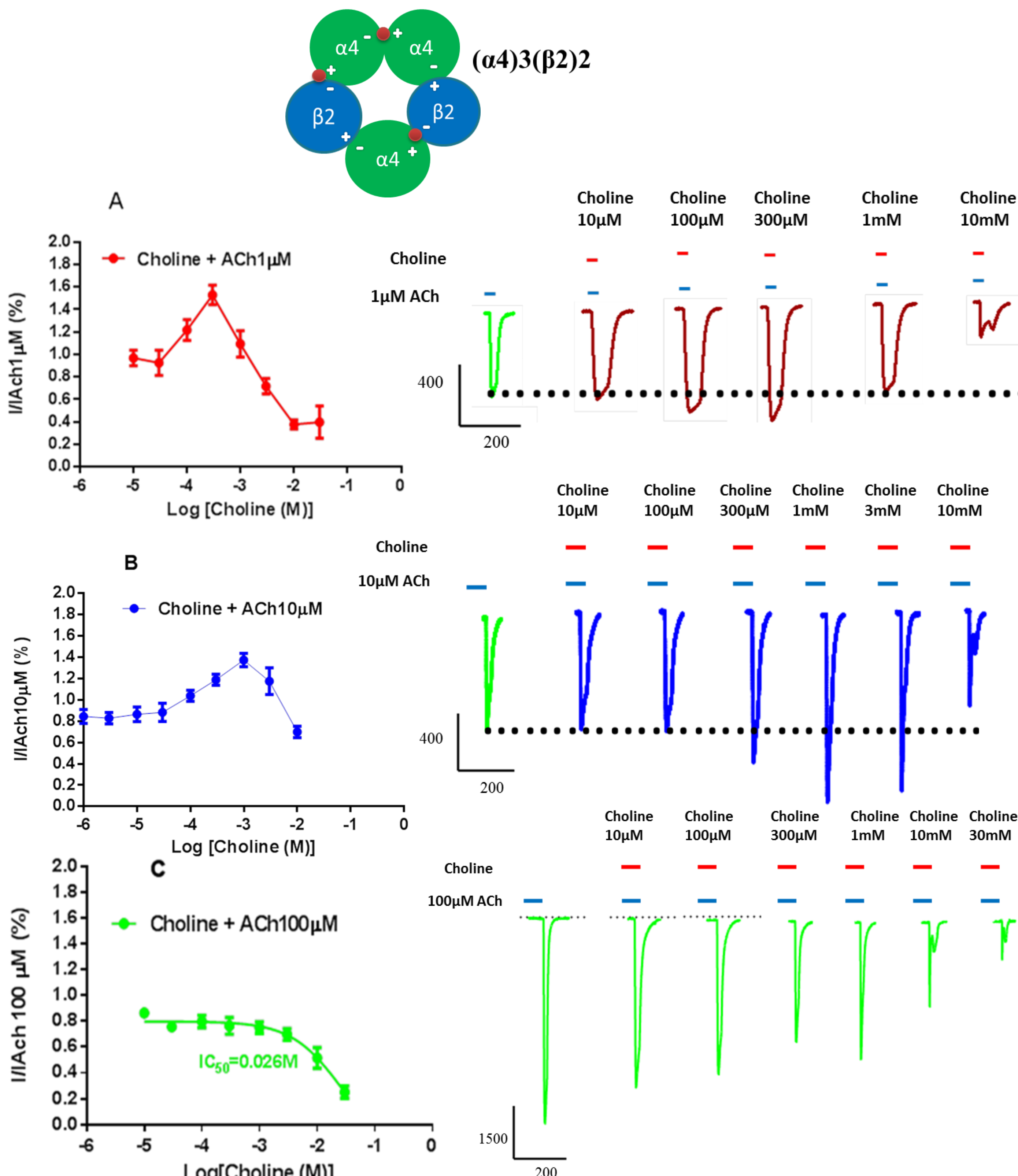

Figure 22. Concentration-response relationships for ACh and choline co-application on (α4)3(β2)2 nAChRs expressed in *Xenopus laevis* oocytes.
Increasing concentrations of choline were applied with three different fixed concentrations of ACh 1μM (A), 10 μM (B) and 100 μM (C). Peak current amplitudes were normalised to the fixed saturating concentrations of 1μM, 10 μM and 100μM ACh respectively. Regression results are shown in Table7 on page 71. Raw data of induced current are also shown (right) and the dotted line indicates the level of the maximum current induced by the control.

Similar choline effects to those observed in our results have been found in previous studies [133, 135]. In fact, both potentiating and inhibiting effects of choline on α4β4 and α3β4 nAChRs and choline's inhibiting effects on α4β2 nAChRs were reported (Table 6). Using the two-microelectrode voltage clamp technique, it was shown that choline concentrations from 10 μM to 10mM potentiated responses evoked by 1 μM ACh on α4β4 receptors expressed in *Xenopus* oocytes, but ACh responses were inhibited by choline concentration higher than 10mM on the same receptors[135].

In the same study, it was demonstrated that responses evoked by ACh 300 μM on α4β4 were inhibited by choline with an IC_{50} of 0.87mM [135], which is 3 times higher than the IC_{50} required for inhibition of ACh 100 μM responses on (α4)3(β2)2 in the present study. Although the above study was done on α4β4 nAChRs subtype, our results were generated using similar experimental methods and come to the same conclusion; namely, that choline has a dual activity and modulates the response of ACh on (α4)3(β2)2 nAChRs. In addition both α4β2 and α4β4 nAChRs presented small differences in agonist affinities for a number of ligands tested; for example only 2 fold affinity differences for ACh [142].

Table 6. Comparison of our Results with Previous Findings

nAChR subtype	Choline effects		
	Agonize	**Potentiation**	**Inhibtion**
Rat α4β2 expressed in *Xenopus* oocytes (This Study)	**Weak partial agonist at (α4)3(β2)2, Imax: 4% of ACh Imax EC_{50}=0.4 mM**	**Concentrations of choline 10 µM to 300 µM potentiated responses evoked by 1µM and 10 µM ACh**	**Concentrations of choline >1mM inhibited responses evoked by 1µM or 10 µM ACh**
Rat α7 [80]	Selective full agonist, EC_{50} = 1.6 mM		
Bovine α7 [134]	A weak full agonist, EC_{50} = 0.43 mM.		
Rat α3β4 from hippocampal and dorsal striatal slices [133]		ACh Potentiation by choline concentration < 3 µM	ACh Inhibition by choline >3 µM, IC_{50} of 15 µM
Rat α4β4 expressed in *Xenopus* oocytes [135]		Choline enhanced responses evoked by 1µM ACh	300 µM ACh was inhibited by choline, IC_{50} = 0.87mM
Rat α4β2 from hippocampal and dorsal striatal slices [133]			100 µM ACh was inhibited by 200 µM to 10 mM of choline, IC_{50}=372 µM

Note. Choline behaved as an agonist or inhibitor on some nAChRs, and choline potentiated or inhibited ACh evoked responses on other subtypes.

A regulatory activity of choline on α3β4 and α4β2 nAChR-mediated glutamate excitatory postsynaptic currents (EPSCs) from neurons of rat hippocampal and dorsal striatal slices using whole-cell patch-clamp recordings was also demonstrated by Alkondon et al. [133]. In their study, concentrations of choline lower than 3 µM did enhance the magnitude of ACh-induced N-methyl-D-aspartate (NMDA) EPSCs, but higher concentration of choline inhibited NMDA EPSCs in CA1 stratum radiatum (SR) interneurons of rat hippocampal slices with an IC_{50} of 15 µM. Interestingly, 100 µM ACh on α4β2 nAChRs in CA1 SR interneuronswere inhibited by choline with an IC_{50} of 372 µM. This inhibition potency is 65 times higher than the inhibition potency observed in our results for 10 µM to 30 mM choline with 100 µM ACh on (α4)3(β2)2 expressed in *Xenopus* oocytes.

The changes in responses evoked by 1 µM or 10 µM ACh on (α4)3(β2)2 appear to be significant with 300 µM choline. This observation led to the question of what could happen if choline is pre-incubated with receptors expressed in *Xenopus* oocytes followed by application of increasing concentrations of ACh. It was hypothesised that the prior incubation of choline may result in important potentiation of ACh response. This pre-incubation experiment was then designed and the findings are discussed in the following section.

3.7 Effect of ACh with Choline Pre-incubation on (α4)3(β2)2 nAChRs

Choline effects on responses evoked by ACh on (α4)3(β2)2 nAChRs were evaluated by three minutes incubation of oocytes with 300μM choline —at which concentration a maximum potentiation was observed when choline is coapplied with 1 μM ACh— and then followed by ACh application. Subsequent experimentation was conducted for increasing concentrations of ACh, and the concentration response relationship was constructed (Figure 20). Incubation of oocytes with choline resulted in a dramatic decrease of ACh efficacy and potency as compared to responses of ACh on oocytes without choline pre-incubation. The maximum response of ACh on (α4)3(β2)2 receptors without choline pre-incubation fell from 120.5% to 52.4% when ACh was applied after choline pre-incubation, which is a decrease of nearly 70%. ACh potency (EC_{50}) also steeply declined from 0.19mM (pEC_{50}±SE = 3.71±0.14) to 2.3mM (pEC_{50}±SE = 2.64±0.97), which is around 10 times less potent. It was also found that the potency of ACh on (α4)3(β2)2 nAChRs incubated with choline was six times less than the potency of choline on the same receptors. However, ACh efficacy on receptors incubated with choline remained higher (13 times) than the efficacy of choline.

From these results, it is believed that prior incubation of choline with (α4)3(β2)2 receptors has limited the full response of ACh. As opposed to choline and ACh co-application, these two ligands are not applied at the same time. In fact, we believe that choline binds the receptors in the first instance and the later application of ACh interacts with remaining unoccupied receptors thus a downward shift of the maximal concentration-response curve occurs (Figure 23).

Inhibition of ACh responses by choline preincubation was also observed on α7 nAChRs in a previous study. Using patch-clamp experiments on rat α7 from neurons of hypothalamic tuberomammillary slices, 2 to 5 minutes preincubation of 80 μM choline resulted in more than 60% efficacy reduction of 200 μM ACh [116].

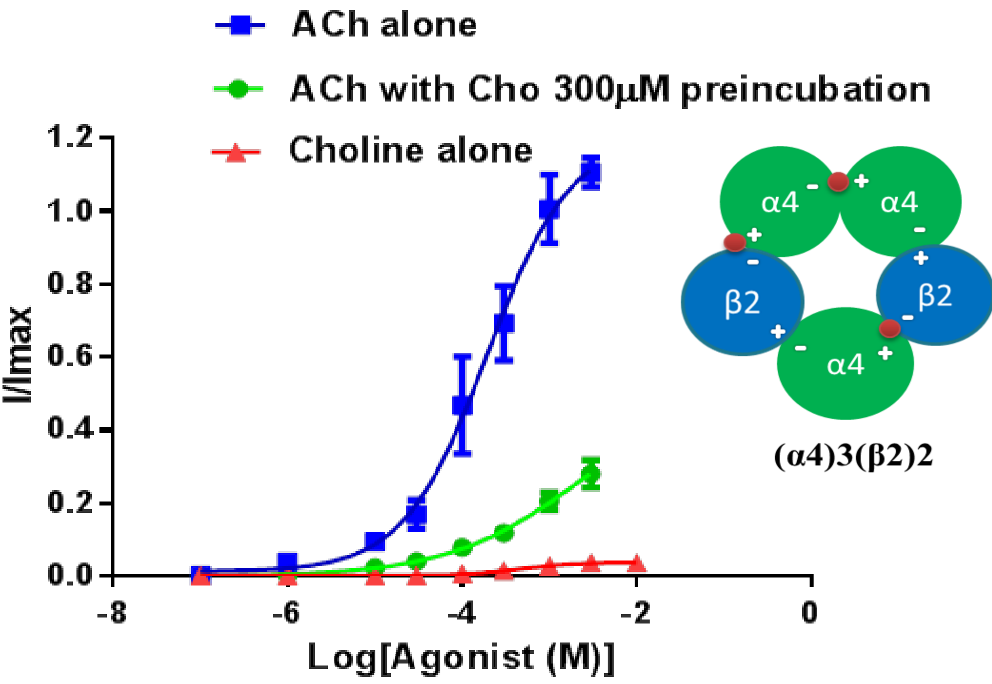

Figure 23. Concentration-response relationship for ACh and choline on (α4)3(β2)2 nAChRs compared to curve for ACh recorded after pre-incubation with 300 μM choline.
The cartoon next to the curve is an illustration of (α4)3(β2)2 receptors. Electrophysiology experiments and data analysis were performed as described in the legend to Figure. 15. Table 7 on page 71 shows regression results.

In this experiment, as shown on a sample of current traces (Figure 24), constant choline concentration of 300 μM was first applied and was run for 3 minutes (red line) before being stopped to let ACh solution (blue line) on the oocyte until the maximum response was achieved. A zoom-in on the 1mM ACh current trace (Figure 24) shows that the response induced by 1mM ACh (610.9nA) on cells pre-incubated with 300 μM choline is half of the maximum current induced by ACh (control) without choline pre-incubation (1271.88nA).

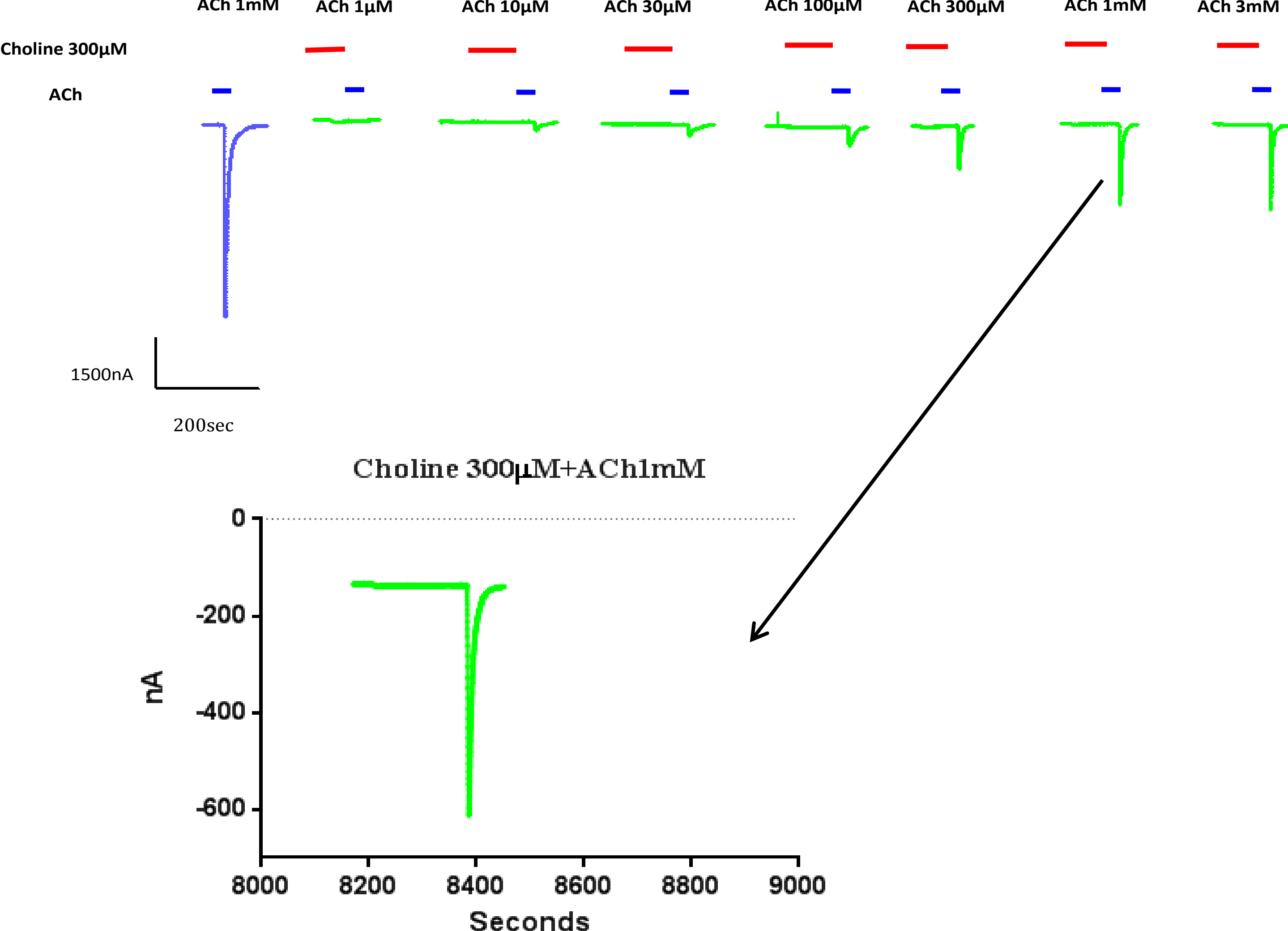

Figure 24. Sample current traces for ACh applied on an oocyte expressing the wild types (α4)3(β2)2 nAChRs pre-incubation with 300 µM choline.
The zoom-in on the 1mM ACh current trace is shown for close examination. Also note that 1mM ACh was used as a control and the holding potential was -60mV.

This experiment shows an apparent washout of prior-incubated choline because the more an increased concentration of ACh is applied on receptors, the higher are the observed current-evoked responses. This may be explained by displacement of choline on receptors, which are then occupied by ACh.

We believe that it would be of interest to maintain constant choline concentration along with application of increasing concentrations of ACh to evaluate the effects of constant choline concentration on ACh efficacy and potency. Nonetheless, results show that a receptor that has been exposed to choline becomes less sensitive and less responsive to ACh. The low response to ACh may be a result of either desensitisation of the receptors or competition binding between choline and ACh. These would have been distinguished by applying a much higher concentration of ACh on oocytes pre-incubated with choline. If the receptors were desensitised by choline pre-incubation, their response to ACh is decreased and the full maximum response cannot be achieved by increasing concentration of ACh. Contrary to receptors desensitisation, in the case of choline and ACh competition the ACh full maximum response will be reproduced if a much higher concentration of ACh is applied on oocytes pre-incubated with choline.

Table 7. Regression Results for Different Concentration-Response Relationships

	(α4)2(β2)3		(α4)3(β2)2		$(\alpha4^{3M})3(\beta2)2$		$(\alpha4)3(\beta2^{3M})2$	
	pEC_{50} [b]	Imax [c]	pEC_{50}	Imax	pEC_{50}	Imax	pEC_{50}	Imax
ACh	5.5±0.08	1.04±0.03	3.9±0.09	1.05±0.05	5.12±0.03	1.02±0.02	3.79±0.03	1.03±0.02
Choline	-	-	3.35±0.48	0.04±0.01	3.51±0.28	0.01±0.00	3.46±0.05	0.07±0.00
Choline (increasing concentration) + 1 µM ACh	-	-	4.06±0.73	1.11±0.06	-	-	-	-
Choline (increasing concentration) + 100 µM ACh	-	-	1.58±0.31	0.22±0.38	-	-	-	-
ACh (increasing concentration) + 300 µM Choline pre-incubation [d]	-	-	2.64±0.97	0.52±0.34	-	-	-	-

Note. In this table, ligands ACh and choline were applied separately, in co-application or with choline pre-incubation on wild type or mutant (α4)3(β2)2 and (α4)2(β2)3 nAChRs. The regression results are pEC_{50} defined as the negative logarithmic value of EC_{50} and Imax defined as the maximum response produced by a ligand.

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[b] pEC_{50}: Negative logarithmic value of EC_{50} (pEC_{50}= $-LogEC_{50}$)

[c] Imax: Maximum response produced by a ligand on the receptor

[d] Pre-incubation: pre-treatment of the receptor with a ligand

Chapter 4

Discussion

The study of choline and nAChRs is of great interest because of their crucial role in physiological functions. It has been reported that choline is required for early CNS development [113]. It has been demonstrated that choline has a prominent role in cognitive, memory and neuroprotective functions [130]. These functions are mainly modulated by nAChRs and a number of conditions, such as smoking addiction and diseases including dementia and AD, are associated with dysfunction of nAChRs and an increase in brain choline levels [26, 47, 115, 143]. The nAChRs are pentameric ion channels situated on post synaptic neurons in cholinergic synapses where they mediate the response of ACh. These neurons have an absolute requirement of choline to synthesise ACh, and choline is reproduced from degradation of ACh by AChE to terminate ACh signaling[3]. Therefore, choline and ACh coexist in the synaptic cleft during ACh neurotransmission. Choline and ACh cohabitation in the synaptic cleft, together with the fact that the two molecules are structurally similar, justify the study of their individual and possible combined effects on the nAChRs.

There are currently 12 different subunits (α4-α10 and β2-β4) that can be assembled in various combinations of five subunits to make an individual nAChR subtype, and each nAChR subtype is characterised by its pharmacological and physiological properties [2, 144]. Different compounds, including choline, have been tested on various nAChRs subtypes for the purpose of developing and designing new molecules that may translate into therapeutics.

The present study investigated the activity of choline on different stochiometries of rat α4β2 nAChRs, the most abundant and widely distributed nAChRs subtypes in rat as well as human brains. Choline effects were investigated on both wild and engineered α4β2 nAChRs expressed in *Xenopus laevis*.

In this study, α4β2 nAChRs were expressed by altering the ratio of α4 and β2 subunit mRNA injected into *X. laevis* oocytes. The obtained receptors successfully responded to ACh with potency consistent with previous work [59] and these predominantly consist of (α4)2(β2)3 containing nAChRs EC_{50}= 3.4 μM (pEC_{50}±SE=5.5±0.08) and (α4)3(β2)2 containing nAChRs EC_{50}= 100 μM (pEC_{50}±SE=5.5±0.08).

Our findings revealed a stoichiometry-dependent activity of choline on wild type α4β2 nAChRs. In fact, choline did not show any response on (α4)2(β2)3 stoichiometry in which α4-α4 binding site is not present. In contrast, choline activated (α4)3(β2)2 stoichiometry in which at least an α4-α4 binding site is present. At this receptor stoichiometry, choline showed a weak low affinity agonist effect with EC_{50}=400 μM (pEC_{50}±SE=3.35±0.48) and maximum efficacy four percent as compared to the control maximum response (ACh 1mM).

We could not find previous similar studies that have tested choline on individual stochiometries of α4β2 nAChRs. However, our results can be indirectly compared to other available published data on choline activity on nAChRs. One study, for example, has found that choline does not activate α4β2 nAChRs on hippocampal neurons [80], but the stoichiometric composition of α4β2 nAChRs was not known. Our results may partly explain why choline effects were not previously observed on α4β2 nAChRs on hippocampal neurons. One reason may be that, as opposed to

Xenopus oocytes, choline was tested on neuron preparations that possibly bear different receptor subtypes and may require a much higher concentration of choline to induce an activity. Another possible explanation may lie in the α4β2 stochiometry composition in the neuron preparations, since we observed no effects of choline on (α4)2(β2)3 receptors.

Our results also agree with other studies done in the past. For instance, an early study suggested that choline is a weak partial agonist of nAChRs receptors and has very low potency to account for the central nervous system effects [132]. More recent studies have suggested that choline behaves as a selective full agonist of α7 nAChRs and does activate and/or inhibit a number of other nAChRs. In fact, it has been demonstrated that choline is a full agonist of both rat and bovine α7 with EC_{50} of 1.6mM and 0.43 mM respectively [80, 134]. Moreover, it has been shown that choline has dual effects on α3β4 and α4β4. It was reported that choline is a partial agonist for α3β4 nAChRs on PC12 cells but is a voltage dependent blocker of α3β4 with an IC_{50} of 0.97mM [134].

In this study, we also tested choline on engineered α4β2 nAChRs, which allowed us to identify differences in choline sensitivity on α4α4 and α4β2 binding sites. In this case, we showed that choline activated receptors that contained one native α4α4 and two engineered α4α4 binding sites, (α4)3($\beta2^{3M}$)2 although the introduction of more α4α4 binding sites did not increase the maximum efficacy. In contrast, we did not observe effects on receptors that contained two native α4β2 and one engineered α4β2 binding sites, ($\alpha4^{3M}$)3(β2)2. These differences in choline sensitivity on engineered receptors may be explained by the structural composition and properties of α4α4 and α4β2 binding sites. In fact, three residues (His142, Gln150, and Thr152) on α4 and

three residues on β2 (Val136, Phe144, and Leu146) differentiate between α4α4 and α4β2 binding sites [59]. The residues on α4 bear a number of nitrogen and oxygen atoms able to make hydrogen bonds, thus the α4α4 binding site may prefer a hydrophilic molecule like choline. In contrast, the three residues on β2 tend to be more hydrophobic due to lipophilic residues facing the binding site, which may result in less favourable binding of choline. If these explanations are correct, they may also clarify why choline did not activate the wild type (α4)2(β2)3 receptors, which only contain two α4β2 binding sites.

In our investigation, choline and ACh co-application showed that, depending on concentration, choline potentiates or inhibits responses evoked by ACh on (α4)3(β2)2. The concentration of choline, ranging from 10 μM to 300 μM, potentiated responses evoked by 1 μM and 10μM ACh, but those responses were inhibited by choline concentrations above 1mM. In fact, in physiological conditions choline concentrations range between 10–20 μM [114, 115], and these are relatively similar to the concentrations that potentiated ACh evoked responses. Furthermore, in pathological conditions, choline levels can reach 100 μM, which is still in range of the concentrations of choline that showed potentiating effects of ACh responses. The fact that the effects we observed in *Xenopus* oocytes take place at concentrations that are physiologically relevant suggests an important physiological role for choline. This role is to maintain ACh-mediated neurotransmission in normal conditions by enhancing the low responses of ACh and reducing overstimulation due to high concentration of ACh. This supports the hypothesis that choline may have other physiological roles beyond that of being a precursor and metabolite of ACh.

Moreover, similar observations of choline regulating ACh activity on α4β4 [135] and α3β4 [133] have been reported (see Table 4).

We also showed that a prior application of choline on (α4)3(β2)2 nAChRs results in a dramatic reduction of ACh evoked responses. This particular observation may be explained by the structural properties of binding sites in the (α4)3(β2)2 receptors. Choline, which is more lipophilic than ACh, may more likely interact with the α4α4 binding site. It is also known that, in order to observe a full ACh response on (α4)3(β2)2, it is necessary for ACh to bind to both the α4α4 and α4β2 binding sites [59]. Consequently if choline is pre-incubated with the receptors, it will partly prevent ACh from binding on the α4α4 binding site, resulting in decreased efficacy.

Overall, for the first time choline effects have been demonstrated on each of the α4β2 stochiometries and we have proposed that choline has a selective activity on wild type receptor stochiometry bearing at least one α4α4 binding site. This has been also confirmed on mutant receptors. Moreover, our study revealed that choline (depending on concentration) enhances or inhibits ACh effects on (α4)3(β2)2 receptors. These results reinforce dual activity of choline on some nAChRs reported in previous studies.

Chapter 5

Conclusion

Nicotinic acetylcholine receptors in the brain constitute an important group of receptors in the cholinergic system and contribute to various CNS functions, including learning and memory, attention, and emotional processing. A large number of neuronal disorders such as Alzheimer's, Parkinson, schizophrenia and addiction are related to nAChRs dysfunction. The present project has focused on α4β2, the most abundant nAChRs subtype and one that is widely distributed in the mammalian brain. The main goal was to pharmacologically evaluate the effects of choline on α4β2 nAChRs expressed in *Xenopus laevis* oocytes using the two-electrode voltage clamp electrophysiology technique. This study, in contrast to previous research, has investigated the effects of choline on different stoichiometries of wild types α4β2 nAChRs, (α4)3(β2)2 and (α4)2(β2)3.

It was demonstrated that choline activates (α4)3(β2)2 stoichiometry, and no visible choline response on (α4)2(β2)3stochiometry, which has never been found before. Further investigation of choline effects on engineered α4β2 receptors has confirmed that choline preferably activates receptors containing at least one α4α4 interface, which is only present in the (α4)3(β2)2 stoichiometry. We recommend further binding and structural studies of choline on α4β2nAChRs to explore the reason behind selective stochiometry activation.

The potentiatiation and inhibition of ACh evoked responses on (α4)3(β2)2 receptors by, respectively, low and high concentrations of choline suggest that choline may

have a role in maintaining ACh neurotransmission in normal range. Our results provide additional support for the hypothesis that choline has a relevant physiological role other than that of simply being a precursor and metabolite of ACh

The prior incubation of (α4)3(β2)2 nAChRs with choline has enormous effects on ACh induced currents. There is a strong belief that choline competes with ACh on α4α4 binding site, which may consequently lead to decreased (α4)3(β2)2 sensitivity and responsiveness of ACh application. However, this needs to be investigated in further studies.

The findings from this study contribute to the understanding of the function of nAChRs, particularly α4β2 subtype. The observed effects of choline on α4β2 receptors could lead future investigation of nAChRs in the purpose of designing novel ligands that may translate into therapeutics.

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