Immunotherapy approach to combat nicotine addiction

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IMMUNOTHERAPY APPROACH TO COMBAT NICOTINE ADDICTION

Submitted by Ghazaleh Pashmi

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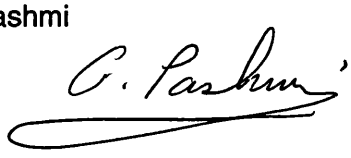

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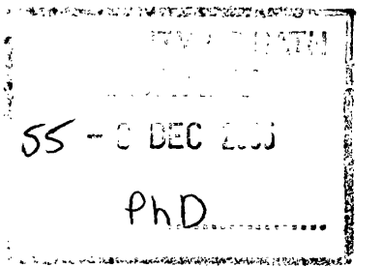
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Summary

Smoking is now recognized as the single largest avoidable cause of premature death and disability in Britain and probably the greatest avoidable threat to public health worldwide. There are several therapies available to combat nicotine addiction ranging from psychological therapy to pharmacological interventions such as Nicotine Replacement Therapy. However, success rates for these therapies individually and mixture of therapies together, are still low and can be improved. A new strategy in helping quit rates is immunotherapy. This research project has focused on targeting cotinine, the major metabolite of nicotine, to produce a vaccine as a cessation method.

The effect of cotinine on nicotine-evoked dopamine release was first examined using 96-well plate assay in chapter 2. Cotinine was shown to decrease nicotine - evoked dopamine release, probably by desensitising the nAChRs. $\alpha6\beta2^*$, $\alpha4\beta2$ receptor subtypes were implicated, using competitive antagonists. Trans-4-thiol cotinine was produced as a viable derivative and conjugated to ovalbumin in the appendix and chapter 3. Vaccination of rats generated anti-cotinine antibodies, although mid-point titres were low. Improvements were made in chapter 4 which increased the mid-point antibody titres. The improvements included change of carrier molecule to Tenatus Toxoid, allowing for 15 derivative attachments per carrier molecule, and change of adjuvant. The best concentration of conjugate to be used in vaccination was determined to be 5 μg which produced specific antibodies towards cotinine. Blood nicotine and cotinine concentrations after chronic nicotine treatment showed vaccination resulted in the retention of cotinine in the blood, presumably reducing the concentration reaching the brain, in chapters 4 and 5. Similar results were also obtained after acute nicotine treatment in chapter 5. The effect of vaccination on nicotine - evoked dopamine release was studied in chapters 4 and 5; an increase in nicotine-evoked dopamine release was observed in vaccinated animals. This suggests the retention of cotinine in the blood and the consequent reduction of antagonism of the actions of nicotine by cotinine, allowed nicotine to have a larger effect. Nicotine-induced locomotor activity was not affected by vaccination, however future work is needed to give conclusive results.

These results have provided preliminary proof of concept for this immunotherapy approach. Future *in vivo* experiments will elucidate the actions of this vaccine on addiction mechanisms and facilitate the development of this approach as a therapy to help people overcome nicotine addiction.

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Communications

Pashmi G., Parks S., Wonnacotte S. (2003) **Examination of (S)-(-)-Cotinine, the major metabolite of nicotine, on dopamine release in the rat striatum, using a 96-well plate assay.** British Neuroscience Association. Abstract, Vol 17, P29.05.

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Abbreviations

Å	angstrom
α Bgt	α-bungarotoxin
αCTMII	α-cototoxin MII
AChBP	acetylcholine binding protein
AChR	acetylcholine receptor
ANOVA	analysis of variance
B_{max}	maximal concentration of binding sites
BSA	bovine serum albumin
°C	degrees Celsius
CNS	central nervous system
cotcooh	N-alkyl pyridyly cotinine
cotsh	Trans-4-thiol cotinine
cpm	counts per minute
CPP	conditioned place preference
DA	dopamine
DHβE	dihydro-β-erythroidine
DNA	deoxyribonucleic acid
EC_{50}	agonist concentration which evokes a half-maximal response
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
g	gram
GABA	γ-aminobutyric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethane sulfonic acid]
h	hour
HRP	horseradish peroxidase
5-HT	5 Hydroxytryptamine
Hz	hertz
Ig	immunoglobulin
J	coupling constant
Kda	kilodaltons
Kg	kilogram
l	liter
λ	wavelength
μ	micro

mg	milligram
ml	milliliter
mM	milimolar
M	molar
mAb	monoclonal antibody
mAChR	muscarinic acetylcholine receptor
μCi	microciverts
μm	micrometer
μM	micromolar
mec	mecamylamine
mGluR	metabotropic glutamate receptor
min	minute
MW	molecular weight
M/z	mass spectra
nm	nanometer
NMDA	N-methyl-D-aspartate
NHS	National Health Service
niccooh	N-alkyl pyridyl nicotine
NMR	nuclear magnetic resonance
NRT	nicotine replacement therapy
NAChR	nicotinic acetylcholine receptor
OD	optical density
PB	phosphate buffer
PBS	phosphate buffer saline
PEI	polyethyleneimine
PMSF	phenylmethylsulfonylfluoride
s	seconds
SD	sodium dodecylsulphate
SD	sprague dawley
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel
TLC	thin layer chromatography
TT	tetanus toxoid
UV	ultraviolet
V	volts
v_{max}	infra-red spectra
VTA	ventral tegmental area

The nomenclature for all nicotinic acetylcholine receptor subunits described in this thesis are based on the NC-IUPHAR subcommittee recommended definitions published in 1999 (Lukas *et al* 1999).

"The rat is an animal which killed in sufficient numbers under carefully controlled conditions, will produce a PhD thesis"

Journal of Irreproducible Results

Chapter 1

Introduction

1.1 Overview of smoking

1.1.1 History of tobacco

In 1962, the Royal College of Physicians published its first report on the effects of smoking on health, drawing attention to the strong relationship between cigarette smoking and lung cancer. The report concluded that this association was probably causal, that smoking may also cause other diseases including chronic bronchitis and coronary heart disease, and that smokers may be addicted to nicotine. In the years since that report was published, the true scale of the harm caused by smoking has become apparent. Smoking is now recognized as the single largest avoidable cause of premature death and disability in Britain and probably the greatest avoidable threat to public health worldwide.

The tobacco plant, *nicotiana tabacum*, is native to the American continent. It is believed to have begun growing around 6000 BC and the American Indians started to use tobacco as early as 1st Century BC for medicinal and ceremonial purposes. Christopher Columbus was given tobacco, among other gifts, by American Indians in October 1492. Tobacco seeds and leaves were then brought back to Europe for the first time. However, tobacco smoking remained almost exclusively a male habit, at least in public, until the 19th Century. Although in 1571 a Spanish doctor, Nicholas Mondardes, claimed tobacco could cure 36 health problems, including toothache and cancer, in 1604 King James I produced a damning report entitled 'Counterblast to Tobacco' in which he said that smoking is a;

"Custome loathsome to the eye, hateful to the nose, harmful to the brain, [and] dangerous to the lungs."

However by mid-1660s trade in tobacco between America and Europe had become a major business and by 1770 England's role as a major tobacco trade centre between America and Europe had been established. In 1761 Dr John Hill performed possibly the first clinical study of tobacco's effects, and noted that snuff users were vulnerable to cancers of the nose. In 1826 the pure form of nicotine was isolated. The invention of manufactured cigarettes transformed tobacco smoking into a truly mass habit when the first cigarette factory opened in England in 1856. It was still unacceptable for women to smoke until the suffragette movement in the 1920s, when a significant number of women started smoking; by the 1940s the British tobacco smoking epidemic was in full force (Kiernan 1991).

1.1.2 Trends in smoking prevalence in Britain

Published data released by the Office for National Statistics shows smoking prevalence is on the decline and in 2004 was at 26% for men and 24% for women. However, inspection of age-specific rates in adults reveals that smoking prevalence has in fact been stable or increasing in recent years amongst most of the younger age groups, particularly in women. The rate at which new smokers are joining the prevalent smoking population has therefore been increasing for some years, making it likely that unless cessation rates begin to increase, the overall prevalence of smoking in the UK will soon begin to rise. Therefore better methods for effective smoking cessation are needed, hence the incentive for the research described in this thesis.

1.1.3 Risk factors and determinants of cigarette smoking

Risk factors and determinants of cigarette smoking come in several categories. The effect of gender on the likelihood of being a smoker is changing from being predominantly a male habit to becoming equal between the genders and may be overtaken by the female population (Freeth, Office for National Statistics 1998). Age is a major determinant of smoking behaviour with 28% of boys and 33% of girls of 15 being regular smokers. Among adults, the 20-24 age group show the greatest smoking prevalence, thereafter decreases progressively with age (Department of Health 1998). Socio-economic status is also a significant determinant of smoking behaviour. In 1996 the lowest consumers of tobacco were the professionals at 12% and the highest were the semi-skilled manual occupation groups at 39% (Department of Health 1998). However other measures of relative poverty or deprivation, including housing tenure, crowding, living in rented accommodation, being divorced or separated, unemployment, low educational achievement, and in women, single parent status, are also independently associated with an increased risk of smoking amongst adults (Royal College of Physicians 1992). Data for NHS Regional Office areas of England reveal that highest prevalence is in the North West Region at 30% and lowest in the South and West Regions at 25% therefore region of residence is also a determining factor (Department of Health 1998). Risk factors for smoking in children aged 11-15 in England include low educational achievement, living with parents who smoke and having siblings who smoke. Additional potential risks include low socio-economic status and having friends and teachers who smoke (Royal College of Physicians 1992).

1.1.4 Morbidity and mortality caused by smoking

The cost of smoking to health is vast. A report by Callum for Health Education Authority (1998) said, smoking is responsible for approximately one in every five

deaths in Britain which translates to 2300 people killed by smoking every week. When looking at individual causes of deaths, smoking caused 84% of all lung cancer and chronic obstructive lung disease deaths, 17% of ischaemic heart disease and 10% of strokes. Other diseases related to smoking include various forms of cancer such as kidney, bladder, stomach, pancreas and myeloid leukaemia. Other circulatory diseases include aortic aneurysm, myocardial degeneration and atherosclerosis. Diseases in the digestive system such as ulcers are also causes of death related to smoking. Overall, approximately one in every two smokers will die prematurely as a result of their smoking. However, the total loss to the community also depends on how premature their deaths are. Based on the distribution of deaths from smoking by age and mortality risks in never-smokers, it is estimated that in 1997 cigarette smoking accounted for the loss of 205000 years of life under age 65 and 551000 years of life under age 75 (Callum 1998).

1.1.5 The costs of smoking

There is also the monetary cost of smoking to society. These can be divided into two groups (Parrott *et al* 1998):

- The cost of the harmful effects of passive smoke exposure in non-smokers
- The costs imposed by smokers on the wider community, through the use of scarce health service resources or by lower productivity in the workplace. The total estimated cost was £1.5 billion for England in 1997.

1.2 Acetylcholine Receptors (AChR)

Addiction is a complex issue with many different factors contributing to the causes and effects of the condition. Ultimately, drug addiction begins with molecular interactions that alter the activity and metabolism of the neurons that are sensitive to that drug. Over time, this alters the properties of individual neurons and circuits, which leads to complex behaviours such as dependence, tolerance, sensitisation, and craving (Mansvelder *et al* 2002). Nicotine is the principle addictive component of cigarettes, which interacts with specific membrane receptors in the nervous system known as neuronal nicotinic acetylcholine receptors (nAChRs).

1.2.1 Classes of Acetylcholine Receptors

There are two classes of AChRs, the metabotropic muscarinic receptors and the ionotropic nicotinic receptors. In 1914, Sir Henry Dale, observed that the action of the esters of choline could be mimicked by some tissues by an alkaloid from *Nicotiania tabacum* (nicotine) and in other tissues by another alkaloid from *Amanita muscaria*

(muscarine). In doing so, Dale proved that the same substance (now known to be acetylcholine) could mediate distinctive responses in tissues through different molecules, therefore defining the existence of different receptor subtypes. However, the characterization of the nAChR became possible after the discovery that the electric organs (electroplax) of the sea dwelling rays of the *Torpedo* species contained huge amounts of nAChRs that led to the purification of milligrams of the protein (Changeux & Edelstein 1998). Research since then has propelled the nAChR group to be one of the most studied receptor groups. Both classes of receptor are present throughout the brain, with the smaller portion lying in the nicotinic group. From this section I will talk about the more relevant nicotinic acetylcholine receptors.

1.2.1.1 Nicotinic Acetylcholine Receptors

nAChRs respond to the neurotransmitter acetylcholine and have been implicated in a variety of physiological processes, including mediation of muscle contraction, neuronal development, neuroprotection, autonomic transmission and cognitive function (Lindstrom *et al* 1996, Role & Berg 1996, Changeux *et al* 1998, Dajas-Bailador *et al* 2000). The nAChRs are ligand gated ion channels from a supergene family containing the inhibitory $GABA_A$, GABAc and glycine receptors and the excitatory $5HT_3$ receptors (Ortells & Lunt 1995). The nAChR family is divided into two groups: neuronal nAChRs and muscle nAChRs.

1.2.1.2 nAChRs located at the neuromuscular junction

The nAChR located at the neuromuscular junction has a subunit composition of $(\alpha1)_2\beta_1\delta/\varepsilon\gamma$ (Ortells & Lunt 1995). These can form two different subtypes; the $(\alpha1)_2\beta_1\delta\gamma$ are found before innervation (developmentally) or after nerve damage, and the adult $(\alpha1)_2\beta\delta\varepsilon$ are found over the muscle surface with the same composition as in *Torpedo electroplax*. After innervation, nAChRs are concentrated at the endplate, with the ε subunit replacing γ in adult muscle, altering both single channel conductance and channel kinetics (Mishina *et al* 1986, Camacho *et al* 1993). Due to the lack of subtype diversity, accessibility and straightforward functional role, synthesis and structure, the function of the muscle-type nAChR has been characterized in relative detail compared to the neuronal nAChR group. The neuromuscular junction is a remarkably robust structure, which rarely fails, however myasthenia gravis is one of the very few disorders that specifically affect it (Drachman 1981, Graus & De Baets 1993). This disease affects about 1 in 2000 individuals, who show muscle weakness and increased fatigability resulting from a failure of neuromuscular transmission. This is caused by

the reduction of the amplitude of the endplate potential so that it often fails to reach threshold. Functionally, this results in the inability of muscles to produce sustained contractions, of which the characteristic drooping eyelids is a sign. In myasthenic muscle the number of receptors per endplate is on average, only about one-third of normal. An immunological explanation of the disappearance of receptors from the neuromuscular junction emerged in 1974, when the presence of antibody directed against the AChR protein was discovered in the serum of myasthenic patients (Drachman 1994). Myasthenia gravis can therefore be classified as an autoimmune disease.

1.2.1.3 Neuronal nAChR

The heterologous neuronal nAChRs are thought to contain 2 'α' subunits and 3 'non-α' subunits; the latter comprise of β and α5 subunits (Cooper *et al* 1991). These receptors show a high affinity for nicotine (Clark 1985). The 12 types of neuronally expressed nAChR subunits, α2-10 and β2-4, can potentially give rise to thousands of nAChR subtypes. However, this is not thought to be the case, as it is now known that the assembly of the nAChR follows certain combinatorial rules (Elgoyhen *et al* 2001, Galzi & Changeux 1995, Sargent 1993). When heterologously expressed the α7, α8 and α9 nAChR subunits can produce homopentamers (Elgoyhen *et al* 1994, Gotti *et al* 1994). These receptors show a high affinity for α-bungarotoxin (Schoepfer *et al* 1990, Elgoyhen *et al* 2001). The α2-6 and α10 nAChR subunits only form complexes with β subunits or other α subunits (Elgoyhen *et al* 2001, Le Novere *et al* 1996, Ramirez-Latorre *et al* 1996, Vernallis *et al* 1993). The pentameric structure of nAChRs is shown in figure 1.1.

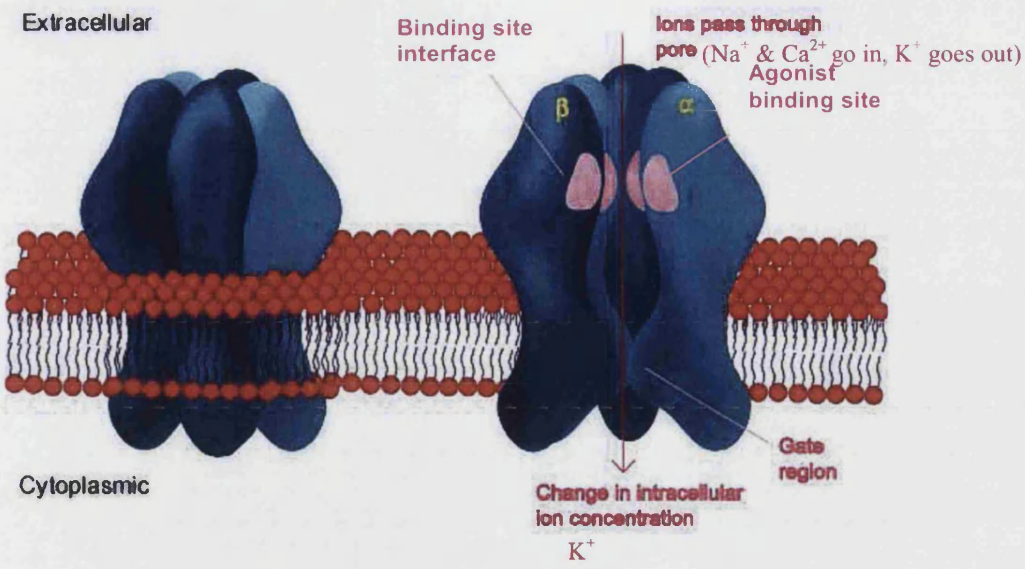


Figure 1.1: Pentameric structure of the neuronal nAChR

The five subunits are arranged around a central channel in a pseudosymmetric manner. The pentamer consists of 2 α subunits and 3 non-α subunits. There is a large extracellular domain containing two acetylcholine binding sites that are mainly confined to the α subunits, with some contribution at the interfaces from neighbouring subunits. (picture by Dr Amy Bradley)

The subunits contain two major domains, the N-terminal extracellular domain, which contains the agonist-binding site, and the transmembrane domain that contains the ion channel. The most recent model shows flattened subunits, rather than rod-like, which are arranged around the pore like the blades of a pinwheel with a clockwise torsion (LeNovere *et al* 1999).

The large extracellular N-terminal domain contains putative glycosylation sites, a disulphide-linked cysteine-loop between residues homologous 128 and 142 of all α or β subunits, which contains 13 amino acids, and the interface for agonist binding (Corringer *et al* 2000). It has been suggested that the cysteine-loop is involved in the assembly of the receptor from component subunits; in particular a conformational change in the cysteine-loop of the α subunit enables the association of a second subunit (Green & Wanamaker 1997).

The N-terminal region is glycosylated with up to 4KDa of carbohydrate polymers. There are four membrane-spanning regions (M1-M4) with a large cytoplasmic loop between M3 and M4 and a short C extracellular carboxyl terminus. The transmembrane

domains contain both β sheets and α helices. The second transmembrane segment, TM2 is orientated to line the channel producing a wide region at the extracellular surface narrowing towards the cytoplasmic face. High magnification imaging confirmed an α-helical conformation on TM2 (Revah *et al* 1990, Unwin 1995). The other 3 transmembrane domains contain α helical regions but also contain a large degree of β strand conformation (Unwin *et al* 2003).

1.2.1.4 The acetylcholine binding protein (AChBP)

Although electron microscopy has resolved the structure (9.6 Å), high resolution detail achieved by crystallographic methods has not been possible. However, this is circumvented for the ACh binding site by the recent discovery of the acetylcholine binding protein (AChBP) from the mollusc *lymnaea stagnalis.* The AChBP is produced by glial cells and is excreted into the extracellular space where it binds to free ACh, effectively lowering the concentration of ACh at synapses (Smit *et al* 2001). The AChBP is a homopentameric soluble protein, which appears to be analogous to the extracellular portion of the nAChR, with highest homology to α7-9 homopentamers. The cysteine-loop is present although the 13 amino acids between cysteines are not conserved. The high-resolution crystal structure demonstrated that the pentamer is 80 Å in diameter, 62 Å in height with an 18 Å channel through the centre. The subunits form an immunoglobulin-like fold and are arranged in a pin-wheel conformation (Brejc *et al* 2001).

The crystal structure confirmed the positions of the residues in the binding pocket, consisting of 3 loops (A, B, and C) contributed by the α subunit and 3 loops (D, E and F) from the neighbouring subunit. The contribution of loop E appears to be responsible for difference in agonist affinities between β2* and β4* nAChRs (Changeux & Edelstein 2001, Le Novere *et al* 1999).

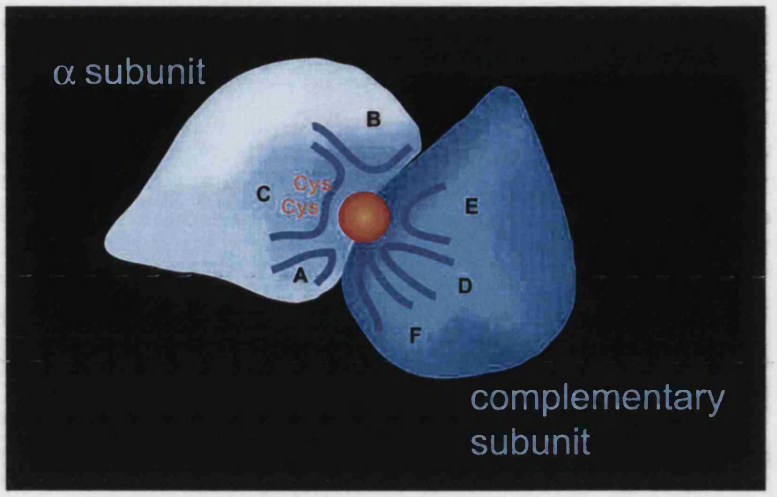


Figure 1.2: Representation of the ligand-binding site viewed from the extracellular face of the protein.

The binding site is formed at the interface of the α subunit and the complementary subunit, γ or δ in muscle receptors, α or β in neuronal receptors. Acetylcholine (orange orb) binds between 6 loops, 3 contributed from the α subunit, A, B and C, and 3 from the complementary subunit, D, E, and F. Loop C contains vicinal cysteines 192 and 193, (Cys), the majority of the other residues on the 6 loops are aromatic amino acids (Adapted from Changeux & Edelstein 2001, Le Novere et al 2001). (Picture by Dr Amy Bradly).

1.2.1.5 Sequential model or concerted allosteric model?

It was first thought that the nAChR opened and closed in a manner similar to that of an enzyme, binding of an agonist causing the receptor to open, called the 'sequential model'. However, in 1965 Monod, Wyman & Changeux proposed the 'concerted allosteric model'. In this model the nAChR spontaneously isomerises between discrete states via a series of allosteric transitions, where binding of the ligand is at a site distinct from the site of activity i.e. the ion channel (for reviews see Corringer *et al* 2000, Changeux & Edelstein 2001).

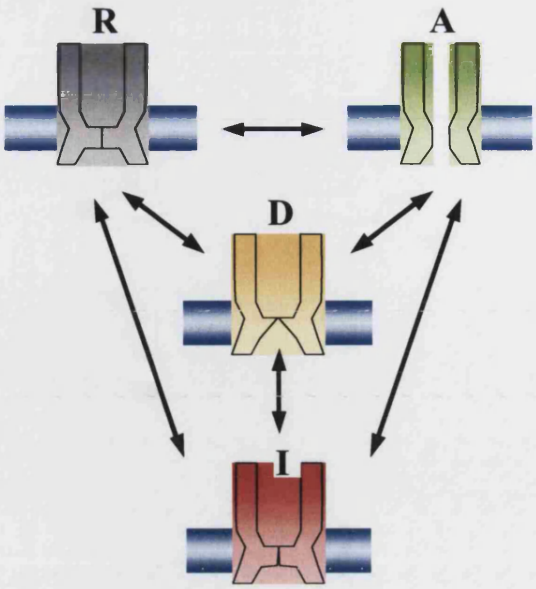


Figure 1.3: Diagram representing the minimal four state model of allosteric transitions of the nAChR.

From rapid mixing experiments performed on Torpedo membranes rich in nAChR, a model with a minimum of four states was developed. R is the low-affinity resting state that occurs in the absence of agonist, A represents the active receptor state, and D and I are desensitised states. For the Torpedo nAChR, the timescales of some of the transitions are as follows: R to A = μs to ms, towards D = ms to 100 ms, towards I = up to a minute.

Figure 1.3 shows that upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state which conducts the cations sodium, potassium and calcium. In the active (open) conformation, the nAChR binds agonists with low affinity. The continued presence of agonist leads to ion channel closure and receptor desensitisation. Although the receptor shows higher affinity for agonist binding in this condition, it is refractory to activation. A multiplicity of desensitised states is proposed to exist. The rates of desensitisation and recovery differ between subtypes with α7 displaying very rapid desensitisation. Prolonged agonist exposure may produce an inactivation state, from which recovery is very slow. Transitions between resting, open and desensitisation states are reversible and different ligands may stabilise different receptor states: agonists initially stabilise the activated state whereas competitive antagonists preferentially stabilise the closed state, either the resting or desensitised configuration (Sharples & Wonnacott 2001 Tocris report). So from a physiological point of view, a pulse of acetylcholine into the

synaptic cleft would stabilise the active state before proceeding to stabilise the higher affinity desensitised and inactive states. However, a low concentration of acetylcholine would match the high affinity of the inactive and desensitised states thus shifting the equilibrium in that direction. In this way acetylcholine could act as both an activator or inhibitor depending on the concentration present at the nAChR. This is important on the actions of nicotine in addiction and smoking.

1.3 Pharmacology and pharmacokinetics of nicotine

1.3.1 Chemistry of nicotine in tobacco smoke

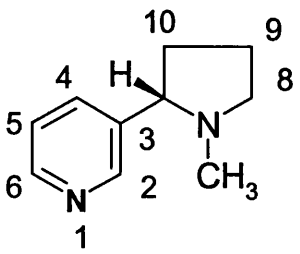


Figure 1.4: Chemical structure of nicotine molecule. *Nicotine is a water and lipid soluble drug, which in free base form, is readily absorbed via respiratory tissues, skin and gastrointestinal tract.*

Nicotine is a tertiary amine which exists in two sterioisomers; (S)-nicotine is the active isomer which binds to nACh receptors and is found in tobacco. Small quantities of (R)-nicotine are found in cigarette smoke after racemisation during smoking, which acts as a weak agonist on nAChRs.

A concise and accurate description of cigarette smoke was found in the Royal College of Physicians report 1997 which says: Cigarette smoke is composed of volatile and particulate phases. 500g gaseous compounds including nitrogen, carbon monoxide, carbon dioxide, ammonia, hydrogen cyanide and benzene have been identified in the volatile phase which accounts for about 95% of the weight of cigarette smoke; the other 5% consists of particulates. There are about 4000 different compounds in the particulate phase, of which the major one is nicotine. Other alkaloids include nornicotine, anatabine and anabasine. The particulate matter without its alkaloid and water content is called tar. Many carcinogens including polynuclear aromatic hydrocarbons, N-nitrosamines and aromatic amines, have been identified in cigarette tar.

1.3.2 Absorption of nicotine from tobacco products

When a cigarette is smoked, small droplets of tar containing nicotine are inhaled and deposited in the small airways and alveoli. As nicotine is a weak acid, at pH 5.5 (same as smoke from cigarettes) it is mostly ionized and does not freely cross cell membranes. This is why it is not absorbed through the buccal mucosa. In contrast, nicotine delivered through pipes and cigars, which have an alkaline pH of 8.5, is mostly unionized and well absorbed from the mouth (Russell *et al* 1981, Russell *et al* 1986). Once cigarette smoke reaches the small airways and alveoli, it is buffered to physiological pH and rapidly absorbed into the pulmonary alveolar capillary and venous circulation leading to the systemic arterial blood. This allows for rapid distribution around the body, only taking 10-19 seconds to reach the brain (Russell *et al* 1981). At this time the arterial blood perfusing the brain contains levels of nicotine which exceed venous levels by a factor of 2-6 fold allowing for rapid absorption into the brain. The nicotine levels in plasma and brain decline rapidly due to distribution to peripheral tissues, excretion and elimination.

The plasma nicotine levels of smokers who smoke multiple cigarettes during the day follow a pattern of oscillations between peak and trough. As nicotine has a half-life of only 4 hours, nicotine accumulates over 6-8 hours reaching levels in the plasma ranging from 20-40ng/ml (Russell 1990). This then falls progressively during the night. Different people seem to have a different tolerance to the levels of nicotine in their blood which makes the smoker regulate their intake of nicotine by self-titration, from a cigarette to achieve and maintain a desired level of nicotine. Thus circumventing the negative physiological effects caused by high nicotine levels, whilst preventing symptoms of withdrawal (review by Russell 1990). This can be done in a number of ways including changing the puff volume or number of puffs (Hasenfratz *et al* 1993). The rapid absorption of nicotine from cigarettes and the high arterial levels reaching the brain enhance the behavioural reinforcement from smoking. When the levels of nicotine fall, the nAChRs are allowed to resensitise between cigarettes. Tolerance to the toxic effects of nicotine such as nausea, rapidly develop and persist. Nicotine has a number of toxic or adverse effects, some of which are potentially relevant in disease pathogenesis. These include acute systemic effects such as headache and dizziness, local toxic effects such as mouth ulcers and chronic systemic effects such as cardiovascular disease.

1.3.3 Nicotine metabolism

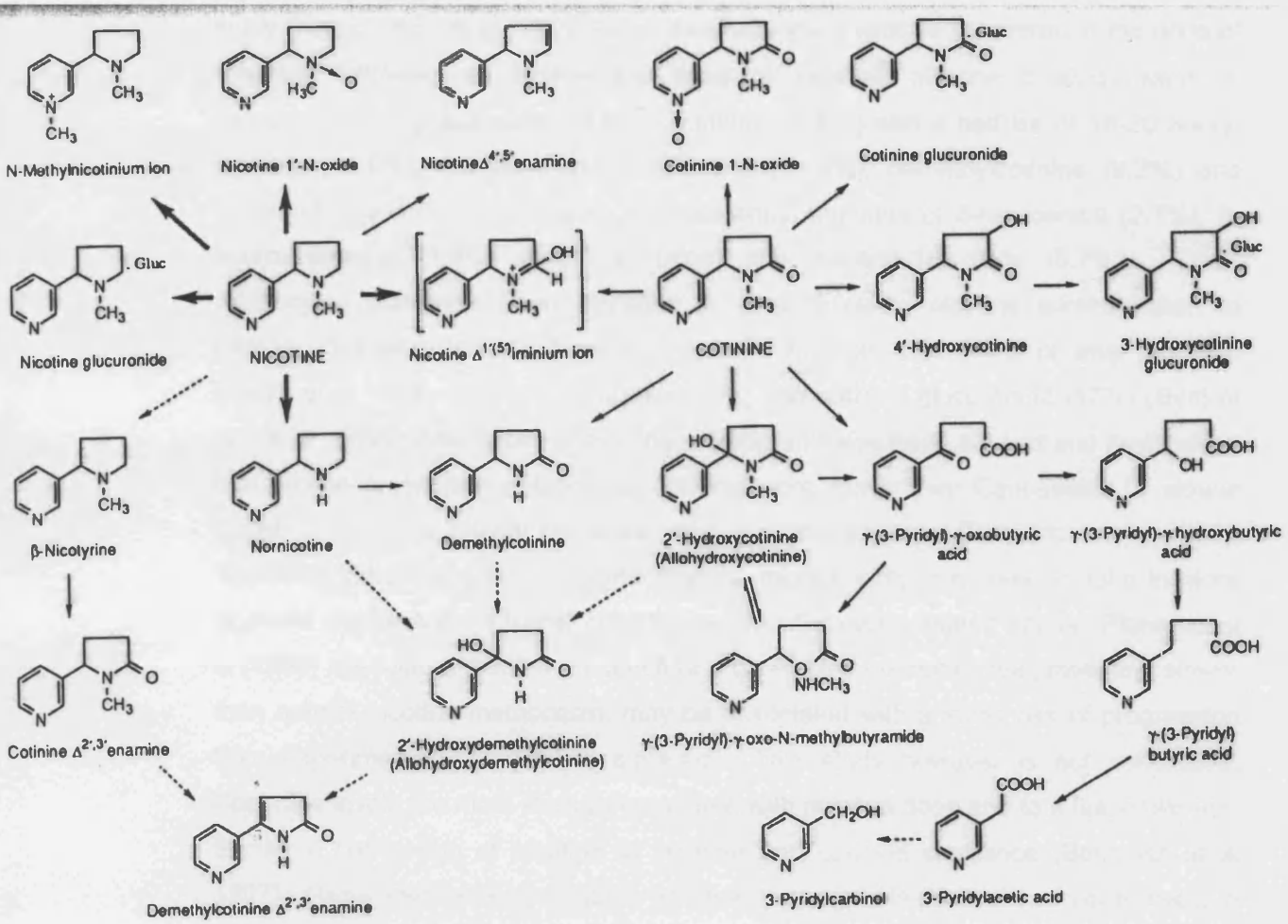


Figure 1.5: The metabolic pathway of nicotine in mammals.

Solid arrows show established pathways, broken arrows indicate unconfirmed reactions (Kyerematen & Vesell 1991).

Following administration, nicotine is extensively metabolised in the liver but also to a small extent in the lung and brain. Nicotine undergoes extensive hepatic biotransformation via a number of phase I and II metabolic pathways (figure 1.5) (Kyerematen *et al* 1991). It is established that regular smokers metabolise nicotine more slowly than non-smokers. Several cytochrome P450 enzymes and flavin mono-oxygenase have been reported to play a role in nicotine metabolism. However, CYP2A6 has been shown to be the main enzyme involved, especially in converting nicotine to cotinine via the route shown in figure 1.5. The principle metabolite of

nicotine is cotinine which has a much longer half-life (16-21h) than nicotine (4h), with average cotinine levels 15 times higher than those of nicotine (Benowitz 1996). In one study (Kyerematen *et al* 1990) the six most abundant species recovered in the urine of smokers following an intravenous dose of racemic nicotine (190μg) were 3-hydroxycotinine glucuronide (16.6%), cotinine (15.6%) with a half life of 16-20 hours, nicotine (14.9%), demethylcotinine enamine (11.3%), demethylcotinine (9.3%) and cotinine-N-oxide (7.4%). There were also small amounts of 3-norcotinine (2.7%), 3-hydroxycotinine (1.6%) (found in urine) and nicotine-1-N-oxide (0.7%). Other metabolites that have been identified in urine following nicotine administration to human volunteers include 4-oxo-4-(3-pyridyl) butanoic acid (14% of total nicotine) (Hecht *et al* 1999), nicotine glucuronide (3%) and cotinine glucuronide (17%) (Byrd *et al* 1992). Ethnic differences in nicotine metabolism have been studied and these show that African-Americans metabolised cotinine more slowly than Caucasians by slower oxidation to trans-3'-hydroxycotinine and N-glucuronidation (Benowitz *et al* 1999). Therefore, smokers who metabolise nicotine more rapidly may need to take in more cigarette smoke and are therefore at higher risk of smoking-related illness. Pianezza *et al* (1998) speculated that the presence of a CYP2A6*v1 mutant allele, reflecting slower than normal nicotine metabolism, may be associated with a lower risk of progression from experimental to addictive smoking. This study however is not conclusive. Continine levels are most strongly correlated with nicotine dose and to a lesser extent , fractional conversion of nicotine to cotinine and cotinine clearance (Benowitz *et al* 1997). Renal clearance of nicotine, which in turn controls partly the nicotine levels in plasma, depends on urine pH, being higher in acidic urine and lower in alkaline urine, and accounts for 2-35% of total nicotine clearance (Benowitz *et al* 1985). Other factors as mentioned before which influence nicotine levels in plasma and brain are distribution to peripheral tissues, excretion and elimination.

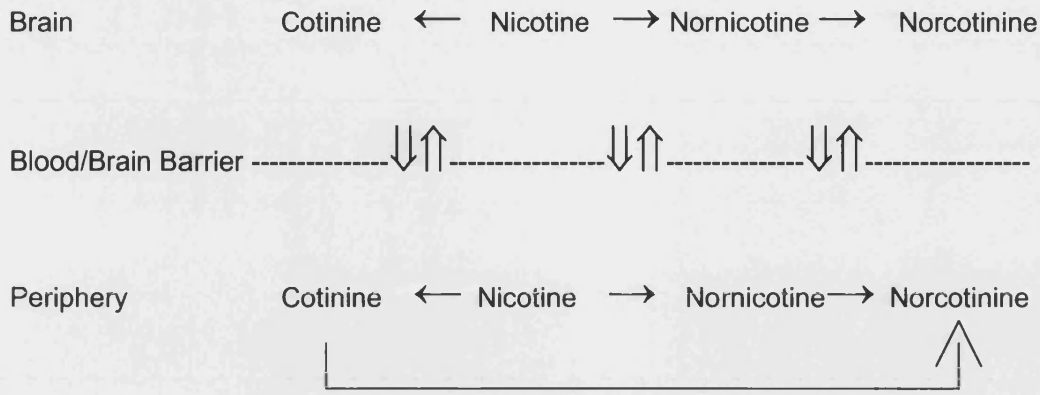


Figure 1.6: The origin of CNS metabolites of nicotine after peripheral nicotine administration (Crooks & Dwoskin 1997).

Figure 1.6 shows the current understanding of the origin of nicotine metabolites present in brain after peripheral nicotine administration. Little is known about the metabolism of nicotine in human brain. Pharmacokinetic studies have determined the amount of nicotine in brain after various routes of administration in several animal species (Nordberg *et al* 1989, Plowchalk *et al* 1992). Nicotine, cotinine, nornicotine, norcotinine and two minor unidentified metabolites appear in brain after a single dose of nicotine (Crooks *et al* 1995, 1997, Ghosheh *et al* 1999). Brain half-lives for cotinine, nornicotine and norcotinine were 6, 3 and 4 times longer, respectively, than the half-life of 52 minutes determined for nicotine, demonstrating that these biotransformation products have longer brain residence times compared with nicotine. Considering the chronicity of nicotine exposure during tobacco smoking and the relatively longer brain residence time of the metabolites, the possibility exists that after repeated peripheral nicotine administration, the metabolites of nicotine may accumulate in brain and reach pharmacologically significant concentrations (Ghosheh *et al* 1999).

1.4 Nicotine addiction

Addiction is a complex behavioural phenomenon with causes and effects that range from molecular mechanisms to social interactions. Ultimately, the process of addiction begins with molecular interactions that alter the activity and metabolism of the neurons that are sensitive to that drug. Over time, this alters the properties of individual neurons and circuits, which leads to complex behaviours such as dependence, tolerance, sensitisation and craving (Koob *et al* 1997, Nestler & Aghanjanian 1997). Various neural pathways and transmitter systems have emerged in research as compelling candidates for the processing of the psychoactive and addictive properties of nicotine.

1.4.1 Neuronal pathways associated with nicotine addiction

The ventral tegmental area (VTA) is located in the midbrain and has been shown to be involved in the rewarding and motivational effects of many addictive drugs, including cocaine (Philips *et al* 2003), and nicotine (Laviolette & Van der Kooy 2003, Corrigall *et al* 2000, Nisell *et al* 1994). The principle excitatory inputs to the VTA DA neurons are glutamatergic projections from the prefrontal cortex (kalivas *et al* 1989, Johnson *et al* 1992, Sesack & Pickel 1992, Taber *et al* 1995, Carr & Sesack 2000). The principle inhibitory inputs to VTA neurons are GABAergic including local interneurons and projections from Nacc and the ventral pallidum (Kalivas *et al* 1993). Cholinergic projections to the VTA come from brain stem nuclei, the pedunculopontine and tegmental nucleus (PPTg) and the lateral dorsal tegemental nucleus (LDTg). Ultrastructural analysis has shown that cholinergic boutons within the VTA contact postsynaptic structures with low levels of dopamine transporter expression (Garzon *et al* 1999). Numerous other neurotransmitters and neuromodulators influence the activity of the VTA including serotinine, norepinephrine and endogenous opiods (Tzschentke 2001), however I will only focus on GABA and glutamate inputs into the VTA DA neurons. DA release from VTA projections is caused by the balance of excitatory and inhibitory inputs and the intrinsic activity of the DA neurons. Nicotinic receptors of various subtypes are expressed by DA and GABA neurons and by the axon terminals of glutamatergic inputs and play an important physiological role in the modification of synaptic transmission within the VTA (figure 1.8).

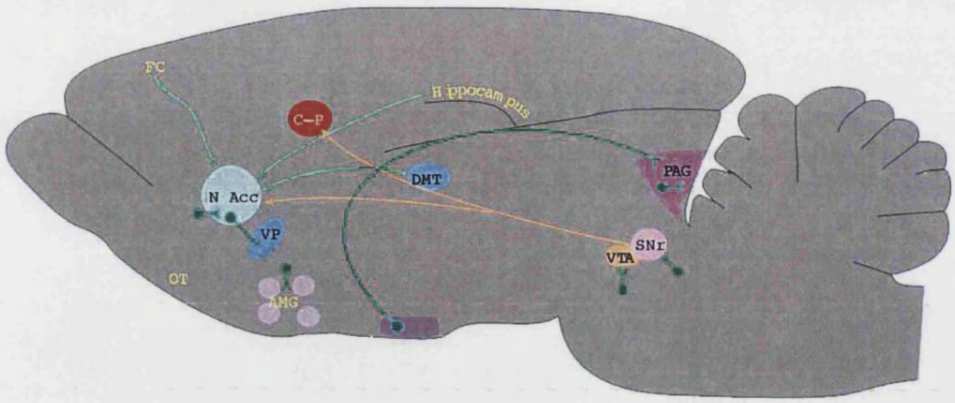


Figure 1.7: Sagittal section through the rat brain depicting the anatomical pathways implicated in the reinforcing effects of drugs of abuse. *Projections of the mesolimbic dopaminergic system to the nucleus accumbens (NAcc) are in orange, and are thought to be critical for psychomotor stimulation reward. They originate in the ventral tegmental area (VTA) and project to the NAcc, olfactory tubercle (OT) and ventral striatal domains of the caudate putamen (CP). Green pathways indicate opioid peptide containing neurones that may be involved in opiate and ethanol reward. $GABA_A$ receptor brain areas are shown in blue, and are thought to mediate some of the sedative/hypnotic rewards of ethanol. VP, ventral pallidum; SNr, substantia nigra pars reticulata; DMT, dorsal medial thalamus; PAG, periaqueductal gray; FC, frontal cortex; AMG, amygdala (modified from Koob 1999).*

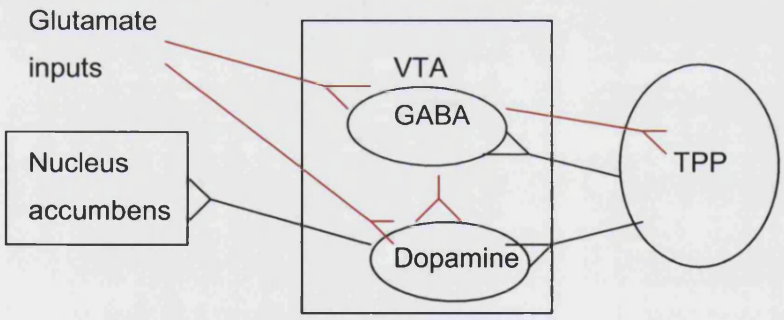


Figure 1.8: Schematic showing the DA and GABA neuronal populations within the VTA. *GABA neurons send descending projections to the TPP and provide inhibitory input to DA neurons. Both neuronal populations are activated by nicotine and receive excitatory glutamatergic inputs, which can regulate the relative activity of DA and GABA in the VTA (Picture taken from Laviolette & van der Kooy 2004).*

Dopamine neurons of the VTA express the α2-α7 and β2–β4 subunits (Charpantier *et al* 1998, Klink *et al* 2001). From these subunits, at least three pharmacologically distinct subtypes can be formed including the homomeric α7, which is preferentially

localized in the VTA in the midbrain, adjacent to the substantia nigra (Woolterton *et al* 2003). Most nAChRs of the GABA neurons in the VTA contain α4 and β2 subunits, which are blocked by mecamylamine and DHβE (Mansvelder *et al* 2002) and less than 25% of these neurons express the α3, α5, α6 and β4 subunits (Klink *et al* 2001).

Many addictive drugs including nicotine cause an increase in dopamine levels in the nucleus accumbens (NAcc) at the same concentrations that are achieved in serum during self-administration in rodents and humans (Nisell *et al* 1994, Imperato *et al* 1986, Stolerman & Jarvis 1995, Dani & Heinemann 1996, Picciotto *et al* 1998, Dani & De Biasi 2001). Within physiological range of plasma nicotine concentrations that are obtained by smokers (~0.5μM), nicotine potently activates DA neurons of the VTA, which is followed by desensitisation of nAChRs after continued exposure to nicotine (Pidoplichko *et al* 1997). This indicates that the acute excitatory actions of nicotine on DA neurons might signal its reinforcing reward effect, the long-lasting desensitisation of VTA nAChRs might represent a cellular basis of nicotine tolerance. This may explain reports from heavy smokers, that they enjoy most the first cigarette of the day (at a time when nAChRs would not be in a state of prolonged desensitisation). The Nacc levels have been shown to be important in reward by VTA lesion studies and microperfusion of the Nacc with DA receptor antagonists, mecamylamine and DHβE, both of which result in reduced self-administration of nicotine (Nisell *et al* 1994, Balfour 1991, Corrigall *et al* 1992, 1994, Louis & Clarke 1998). Therefore, while systemic administration of nicotine affects nAChRs in many brain regions such as Nacc, hippocampus and cortex, it is the nAChRs in the VTA that mediate the rewarding effects of nicotine. Although some drugs of abuse alter the activity of the VTA neurons to enhance DA release, preferentially in mesoaccumbens but not the nigrostriatal system (Imperato *et al* 1986, Benwell & Balfour 1997).

1.4.2 Functional properties of nAChRs in nicotine addiction

As nicotine influences neuronal activity, synaptic communication and behaviour through its effects on nicotinic receptors, it is important to discuss the functional properties of these receptors. There is great diversity in the sensitivity of the different receptor subtypes to nicotine and different affinities lead to different channel activation and subsequent desensitisation in the continued presence of the drug. Nicotinic receptor upregulation is where the receptor sensitivity and binding levels increase following nicotine pre-exposure, but this is not associated with changes in mRNA and is thought to reflect increased assembly (Olale *et al* 1997, Wang *et al* 1998).

Physiologically relevant nicotine concentrations have been shown to upregulate α4β2 – containing receptors (Buisson & Bertrand 2001). Upregulation has also been shown with higher nicotine concentrations in some cells (Rogers & Wonnacott 1997, Molinari *et al* 1998). There are some reports of an increase in ligand binding following nicotine pre-exposure (Rowell & Wonnacott 1990, Buisson & Bertrand 2001), while others have found a decrease in function (Marks *et al* 1993). These differences may be a result of different assays being used. However nicotine self-administration may enhance nAChR responses as pre-exposure to nicotine has been shown to sensitise animals to its locomotor and self-administration effects (Shoaib *et al* 1997). It is still not clear whether nicotine self-administration induces receptor upregulation *in vivo*, although high [^{3}H] nicotine binding in brain tissue from post-mortem smokers have been reported (Court *et al* 2000, Patterson & Nordberg 2000).

1.4.3 Excitation of nAChRs in nicotine addiction

In brain slice recordings from VTA DA neurons, glutamatergic transmission onto these neurons is enhanced by low concentrations of nicotine which is unaffected by TTX, which blocks action potential firing, suggesting that nAChRs mediating this effect are situated locally in the VTA, on the presynaptic glutamatergic terminals (Mansvelder & McGehee 2000). These nAChRs are sensitive to MLA, which is a selective inhibitor of α7 subunits (Alkondon & Albuquerque 1993). These subunits are well situated for modulating synaptic transmission as they have a high calcium permeability, and this calcium flux occurs at resting membrane potential when activated by agonists (McGehee & Role 1995).

In a cholinergic synapse, the Ach concentration rises within milliseconds to millimolar concentrations (Kuffler & Yoshikami 1975). However the nicotine concentration profile of a smoker's blood during cigarette smoking is very different and shows blood nicotine levels reaching 300-500nM several minutes after the initiation of smoking and concentrations close to 250nM are sustained for 10 minutes or more (Henningfield *et al* 1993, Gourlay & Benowitz 1997). The high affinity nAChRs including α4β2 and α3β2 subtypes have measurable activity at these concentrations on VTA DA neurons (Picciotto *et al* 1998) but with nicotine concentrations of 100-500nM, the somatic nAChRs desensitise within minutes (Dani *et al* 2000). However, *in vivo* studies show that a single systemic injection of nicotine enhances DA release in the Nacc for more than an hour (Di Chiara 2000). Therefore other mechanisms following activation of nAChRs must induce the long-term potentiation of the excitatory glutamatergic input,

and nicotine-induced depression of GABAergic transmission (Mansvelder & McGehee 2000).

Nicotine receptors are present on both the presynaptic glutamatergic terminals and DA neurons. When nicotine arrives in the VTA, it stimulates both directly, which can mimic the paired electrical stimulation of pre- and postsynaptic partners. Presynaptic α7 nAChRs provide rapid means to increase intracellular calcium presynaptically as these channels will gate the resting membrane potential (Seguela *et al* 1993), and simultaneous activation of the nAChRs on postsynaptic DA neurons will increase the likelihood of NMDA receptor activation due to depolarisation and relief of magnesium block. In mutant mice lacking the β2 containing subunit, there is no long-term activation of the DA system by nicotine, and the mice do not self-administer (Picciotto *et al* 1998). This suggests that without β2 containing nAChRs, nicotine does not depolarise the DA neuron enough to relieve magnesium block of NMDA receptors (Mansvelder & McGehee 2002).

1.4.4 Inhibitory control of nAChRs in nicotine addiction

In addition to excitatory inputs, GABAergic inputs into the VTA DA neurons provide inhibitory control. When nicotine reaches the VTA, nAChRs expressed by GABA neurons are activated and cause an increase in the firing rate of these neurons (Yin & French 2000). These are most likely to be of the non-α7 type that contain α4 and β2 subunits. When nicotine is applied to these receptors there is a transient increase in inhibitory inputs to DA neurons in the VTA. This effect would likely offset some of the excitatory effects of nicotine during the time of enhanced GABA transmission. These nAChRs on the GABA neurons desensitise rapidly causing a decrease of GABA neuron activity and the inhibitory input to the DA neurons diminish resulting in an increase in their action potential firing. The nAChRs on the GABA neurons recover very slowly from desensitisation. In the first 15 minutes after nicotine is present, GABA neurons do not respond at all to the next nicotine application and this takes approximately 1 hour to reach normal levels of nicotine sensitivity (Mansvelder *et al* 2002). The recovery of endogenous cholinergic transmission would require a similar time course. During this time DA neurons are therefore more active.

Although α7 receptors on the glutamatergic terminals also desensitise rapidly, the low nicotine concentrations associated with tobacco use induce much less desensitisation of these receptors. A 10 minute exposure to 250nM nicotine completely desensitises

the nAChRs on GABA neurons, however the enhancement of glutamatergic transmission does not show significant desensitisation. Thus excitatory inputs to VTA DA neurons are enhanced by nicotine while inhibitory GABAergic inputs are depressed (Mansvelder *et al* 2002). In addition, if DA neuron is depolarised sufficiently, the enhancement of glutamatergic transmission can induce long-term potentiation of these inputs.

1.4.5 Does a reduction in VTA GABAergic transmission actually contribute to nicotine addiction?

Rats and mice will readily self-administer $GABA_A$ receptor antagonists when they are locally infused into the VTA (David *et al* 1997), which also increases DA levels in the Nacc (Ikemoto *et al* 1997). Therefore it is likely that the reduction in GABAergic transmission following nAChR desensitisation in the VTA contributes to the reinforcing effects of nicotine. β2 subunits which mediate the effect of nicotine on GABA neurons have been shown to be necessary for the maintenance of nicotine self-administration in knock-out mice (Picciotto *et al* 1998, Grottick *et al* 2000). These mutant mice also showed a strong reduction in nicotine induced conditioned taste aversion indicating that this receptor is also implicated in the aversive effects of nicotine. I will talk about the aversive effects of nicotine later. Pre-treatment with specific antagonists of D1 and D2 DA receptor subtypes strongly attenuated nicotine self-administration (Corrigall *et al* 1991). Lesions of the mesolimbic DA system caused by 6-hydroxydopamine, a molecule that selectively destroys DA neurons, also attenuated nicotine self-administration (Corrigall *et al* 1992). Also, microinfusion of DHβE directly into the VTA attenuated nicotine self-administration but not the reinforcing effects of food or cocaine, implicating that the mesolimbic DA projections from the VTA are a crucial mediator of the reinforcing effects of nicotine (Corrigall *et al* 1994).

1.4.6 Role of Nacc shell vs core in nicotine addiction

Another issue to consider is the fact that the Nacc is a heterogeneous structure, which is composed of a shell that lies ventral and medial to the accumbal core. These two principle subdivisions of the accumbens are anatomically distinct, have different projection fields and are thought to subserve different functions (Heimer *et al* 1991, Zahm & Brog 1992). Subsequent studies showed that the acute injections of nicotine stimulate DA overflow in the medial shell of the accumbens, whereas they have no significant effect on extracellular levels of DA in he accumbal core (Benwell & Balfour 1992, Cadoni & Di Chiara 2000, Iyaniwura *et al* 2001). However, if rats are treated

repetitively with daily injections of nicotine, the DA response in the medial shell tends to diminish, significantly so in the study reported by Cadoni & Di Chiara (2000), whereas nicotine pre-treatment results in a regionally selective sensitisation of the response in the accumbal core (Benwell & Balfour 1992, Cadoni & Di Chiara 2000, Iyaniwura *et al* 2001).

The core of the accumbens sends projections to areas of the brain concerned with the control of motor activity (Zahm & Brog 1992). Initially therefore, it was assumed that the sensitised DA response to nicotine observed in the core of the accumbens of nicotine pre-treated rats, mediated the sensitised locomotor responses to the drug also observed in rats pre-treated with nicotine prior to the test day (Benwell & Balfour 1992, Cadoni & Di Chiara 2000). However some studies have revealed a dissociation between the sensitised increase in DA overflow in the accumbal core and the increase in locomotor activity evoked by nicotine in nicotine pre-treated rats. The sensitised DA response to the drug is inhibited by the prior administration of an antagonist of NMDA glutamatergic receptors, whereas the sensitised locomotor response is unaffected by these drugs (Shoaib *et al* 1994, Balfour *et al* 1996, Benwell *et al* 1996).

The microdialysis probes which are used in many studies to investigate the effects of drugs of dependence on extracellular DA in the NAcc do not directly measure the changes in DA release into the synaptic cleft, but detect changes in the DA concentration in the interstitial space between the cells. The increase in DA overflow, evoked by systemic injections of nicotine, appears to be mediated predominantly by receptors located on or close to the cell bodies of the VTA that influence impulse flow to the terminal field (Balfour *et al* 1998, 2000). Nicotine injections increase the proportion of midbrain DA neurons that exhibit burst firing (Nisell *et al* 1996), and there is evidence that increased burst firing of these neurons evokes a substantial increase in the DA concentration in the extracellular space sampled by a dialysis probe (Gonon 1997, Nissbrandt *et al* 1994). This led to the conclusion that the increases in extracellular DA found in both the accumbal medial shell and core of nicotine treated rats reflect the increase in burst firing of dopaminergic neurons evoked by the drug (Balfour *et al* 2000).

1.4.7 Hedonic properties on behaviour by nicotine

Microdialysis studies have shown that the presentation of natural rewards, such as palatable food or sucrose increase DA overflow in the medial shell when compared with the core in the NAcc. Whereas the presentation of a stimulus such as the odour

of food selectively stimulates DA overflow in the accumbal core (Hajnal & Norgren 2002, Bassareo & Di Chiara 1999). It also elicits sensitisation of the DA response to the core if the animals are subsequently permitted access to the food. One hypothesis by Balfour (2004), to explain the data derived from studies with both natural rewards and drugs of dependence, is that the major role of the DA projections to the medial shell is to confer hedonic properties on behaviours, rather than their outcomes, that are paired with increased extracellular DA in this subdivision of the structure. Therefore the behaviours themselves acquire reinforcing properties, which are related to the magnitude of the increase in extracellular DA in the medial shell. This increases the probability that behavioural responses that deliver a pleasurable reward will be repeated and learned efficiently. Psychostimulant drugs of dependence such as nicotine, evoke sustained and substantial increases in DA overflow in the accumbal medial shell, by acting directly on the neurons that project to the subdivision of the accumbens. Therefore behaviours and stimuli associated with delivery of the drug acquire powerful reinforcing characteristics that may be disproportionate to, or indeed independent of, the rewarding properties of the drugs per se.

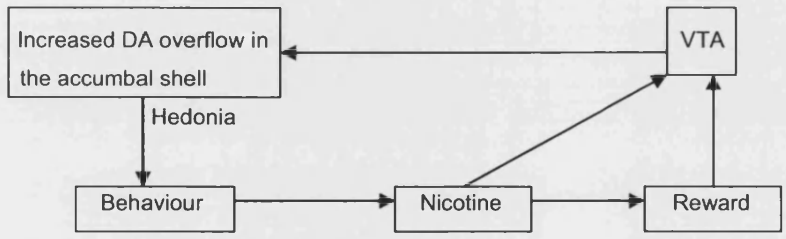


Figure 1.9: The role of increased dopamine overflow in the accumbal shell on responding to a reward.

Proposed circuitry by which a reward such as nicotine increases DA overflow in the acumbal medial shell. This increased DA overflow is hypothesised to confer hedonic characteristics on behaviour such as lever-pressing response that results in presentation of reward. Nicotine is illustrated to greatly enhance the hedonia associated with the behaviour by directly increasing extracellular DA in the accumbal medial shell. (Diagram and hypothesis from Balfour (review) 2004).

Some tests have shown that responding reinforced by injections of psychostimulant or the presentation of food are also associated with increased DA overflow in the accumbal core (Ito *et al* 2000, Bassareo & Di Chiara 1999). Balfour (1994) suggested that increased DA overflow in the core of the accumbens may also promote and maintain compulsive drug-seeking behaviour in animals in which the behaviour is

reinforced by delivery of the primary reward. This may suggest that sensitisation of this response plays a pivotal role in the transition to dependence. The sensitised increase in DA overflow in the accumbal core enhances the probability of compulsive drug-seeking behaviour in response to drug-associated stimuli, whereas the increase in DA overflow in the medial shell serves to amplify these effects on behaviour by enhancing the hedonic value of the behaviour itself. These two effects serve to make drug-seeking behaviour that results in delivery of drug both a powerfully reinforced and compulsive behaviour.

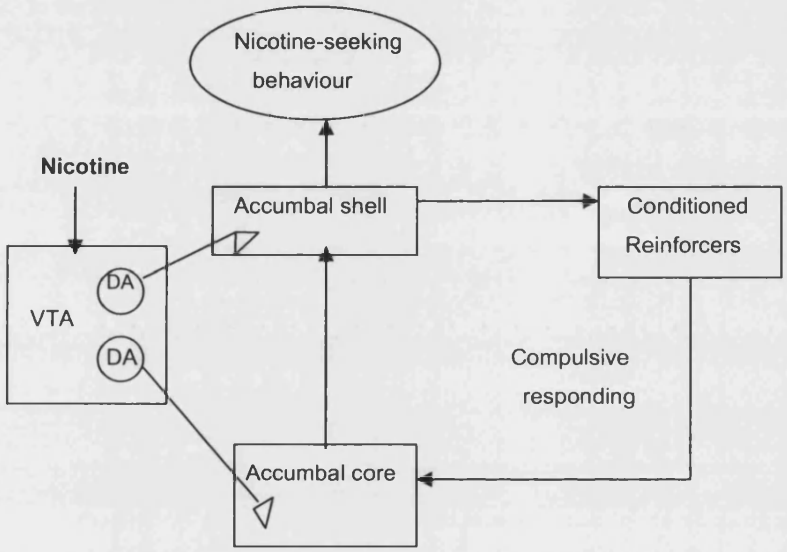


Figure 1.10: The putative role of hedonia and conditioned reinforcers in nicotine-seeking behaviour.

The figure summarises the mechanisms that have been proposed to explain how increased DA overflow in the medial shell and core of the NAcc, evoked by an injection of nicotine, play complementary roles in the expression of nicotine-seeking behaviour. The hypothesis suggests that in both subdivisions of the accumbens, extracellular DA serves to promote or amplify the signals that project from or through the structure. Stimulation of the projections to the medial shell of the accumbens enhances the hedonia value of the behaviour itself and of sensory and environmental stimuli associated with the delivery of nicotine. Stimulation of the projections to the accumbal core promotes the effects of conditioned reinforcers or stimuli on nicotine-seeking behaviour. These conditioned responses can be amplified further by stimulation of the DA projections to medial shell through which neurones from the core project. (Diagram and hypothesis from Balfour (review) 2004).

1.4.8 Aversive effects of nicotine on nicotine addiction

Nicotine however does not have powerful "euphoriant" properties and in some circumstances has potent aversive effects. Nicotine can produce powerful anxiogenic effects systemically and centrally (File *et al* 2000) through activation of nAChRs that contain β2, α4 and α7 subunits (Tucci *et al* 2003). The noxious effects felt by people include nausea, dizziness and coughs on their initial experience with tobacco (Kozlowski & Hartford 1976), however tolerance develops to these aversive effects with repeated exposure (Shoaib & Stolerman 1995). The precise neurological mechanism behind this is still unknown but it does indicate that chronic nicotine exposure might induce a functional alteration in the neuronal systems that mediate the aversive and/or rewarding effects of nicotine. A study using the conditioned place preference model (CPP) reported that both rewarding and aversive effects could be measured using the same test after microinfusions of nicotine itself onto the VTA (Laviolette & van der Kooy 2003). They showed a dose-dependant, biphasic curve for the motivational effects of nicotine in the CNS; whereas a lower nicotine concentration in the VTA produced an aversive effect, higher concentrations produced potent rewarding effects. With DA receptor blockade systemically or directly in the Nacc there was no attenuation of the rewarding effects of nicotine in chronically treated animals, however when the acute aversive effects of nicotine are blocked by interfering with mesolimbic DA receptor signalling, the rewarding effects of nicotine are potentiated, presumably by the removal of an aversive signal (Laviolette & van der Kooy 2003).

A view proposed by Berridge & Robinson (1998, 2001) states that after repeated drug exposure, sensitisation of the DA system, which signals the 'craving' for the drug, leads to compulsive drug seeking and use. Repeated nicotine exposure has been shown to sensitise DA pathways (Benwell & Balfour 1992) and increase DA receptor expression in the projection areas of the VTA DA system (Le Foll *et al* 2003) and increase the number of nAChR subtypes in the VTA (Ryan & Loiacono 2001). A related possibility is that plastic processes that increase DA responsiveness to nicotine within the VTA might represent an aberrant form of drug-induced associative learning. Interestingly, exposing humans to imagery of stimuli associated with smoking activates neuronal regions that are linked to drug-induced DA sensitisation processes such as the prefrontal cortex and amygdala (Brody *et al* 2002). Blockade of this DA-mediated incentive learning signal in chronically treated animals does reduce nicotine self-administration (Corrigall *et al* 1992, 1994, Picciotto *et al* 1998, 2002). However this does not explain why DA-mediated transmission seems to carry a specific aversive signal in the acute phase of nicotine exposure and reinforcing signal after chronic

nicotine exposure. An explanation by Laviolette & van der Kooy (review 2004) suggests that dysregulation of DA-mediated signalling during nicotine dependence and withdrawal after chronic exposure might be responsible for the aversive effects of nicotine withdrawal, which motivate the smoker to seek nicotine. Whereas, activation of nAChRs of DA neurons can signal an aversive effect in the early phases of nicotine exposure, the eventual desensitisation of these receptors, which takes place after the desensitisation of the nAChRs in VTA GABA neurons might account for the tolerance to the aversive properties of nicotine over time.

1.4.9 Prolonged or habitual smoking

As mentioned before, the neuronal nAChRs which mediate the effects of nicotine on mesolimbic DA neurons are desensitised by sustained exposure to the drug at concentrations significantly lower than those found in the plasma of rats that have self-administered nicotine for 1hr (Benwell *et al* 1995, Pidoplichko *et al* 1997, Shoaib & Stolerman 1999). This implies that as the concentration of nicotine in the brain reaches a level that results in desensitisation of the receptors, subsequent injections of the drug may not result in further stimulation of the DA-secreting neurons (Balfour *et al* 2000). In many self-administration studies the animals are trained and tested in discrete daily trials of 1 or 2 hours. For the remainder of the day, the animals experience enforced abstinence when the blood nicotine concentration will fall to a low level that is unlikely to maintain the nicotinic receptors in a desensitised state. The microdialysis results predict that at the beginning of each session of self-administration the initial injections of nicotine will stimulate DA overflow in the medial shell, and the DA concentrations in the extra-cellular space will remain elevated for 1 hour or so afterwards. As a result, for most if not all of the trial, the animals will be responding under conditions of raised extra-cellular DA. The hypothesis predicts that during this period the behaviour response that delivers drug will be reinforced thereby facilitating the acquisition and maintenance of responding for nicotine.

If inhaled nicotine exerts a similar sustained effect on DA overflow in the medial shell as that evoked in rats that receive daily injections of the drug, the microdialysis results suggest that cigarette smoking will increase DA overflow in smokers with low initial blood nicotine concentrations. Therefore the behaviour associated with delivery of the drug – cigarette smoking – and sensory cues associated with its delivery will acquire hedonic properties that may be disproportionate to the rewarding properties of the drug itself. However, it is also likely that many smokers, allowed to smoke ad libitum, will accumulate concentrations of nicotine in the brain that cause prolonged desensitisation

of the neuronal nAChRs, which mediate the response (Benowitz *et al* 1990, Balfour *et al* 2000). If this happens, smoking may not be associated with increased DA overflow in the medial shell of the accumbens and it becomes necessary to consider the reasons why habitual smokers continue to smoke under these circumstances.

One explanation could be that these smokers seek to maintain the brain nicotine concentration at a level that prevents withdrawal, therefore avoiding the aversive abstinence syndrome. This conclusion is supported by results where rats rendered nicotine-dependent by the constant infusion of the drug, show that nicotine administration alleviates both somatic and other behavioural signs evoked by its abrupt withdrawal (Malin *et al* 1992). However, the evidence from NRT experiments in abstinent smokers is less impressive as withdrawal symptoms are not completely alleviated (Foulds *et al* 1998, West 1998).

An alternative explanation may be that tobacco smoke is likely to be a rich source of sensory stimuli in the mouth and bronchi that can acquire the properties of conditioned reinforcers when paired with the delivery of nicotine from the smoke. These sensory cues play a fundamental role in regulating smoke intake and the craving to smoke (Rose *et al* 1993). These stimuli could also serve as conditioned reinforcers that maintain responding during periods when the receptors that mediate the effects of nicotine on DA overflow are desensitised and smoking may not result in increased DA overflow in the medial shell or core of the accumbens. This is supported by evidence that show non-nicotinic sensory-motor factors play a critical role in mediating the immediate responses to cigarette smoke that reinforce the habit (Rose *et al* 2000). Thus by smoking heavily the smoker can maintain the blood nicotine concentration at a level that prevents withdrawal while continuing to derive some positive reinforcement form the conditioned reinforcers present in smoke (Balfour *et al* 2000).

Due to the complexity of addiction and a large number of pathways involved, which are not fully understood, it is very difficult for a smoker to quit. It is often said that quitting is one the hardest things a person can do. Quitters relapse due to, in some cases severe withdrawal syndromes, and ex-smokers have reported to experience some craving even after 10 years. Although there are some strategies to aid smokers to quit (explained in section 1.5), there is definate room for improvement to allow for higher quit rates over longer periods of time. The immunotherapy approach is a new candidate to tackle this issue.

1.5 Nicotine Addiction Therapies

Nicotine addiction can be tackled in two ways via non-pharmacological approaches:

- Wide-reach approach.
- Intensive intervention.

These two approaches can work effectively together and on their own (Royal College of Physicians report 1997).

1.5.1 Wide-reach approach

This consists of interventions which reach the population on a large scale.

1) *Comprehensive community-orientated interventions* include posters, mass-media campaigns, telephone and self-help interventions, 'quit and win' contests etc. However, these have not been proven to be effective.
2) *Self-help interventions* such as leaflets and books have been marginally effective especially when they also include information about pharmacological interventions and are tailored to the individual smoker and their addiction rating. These methods have also been shown to be more effective when combined with other more intensive therapies.
3) *Telephone help-lines* have been found to elicit a large number of calls when well advertised. Although there is no research as yet showing their efficacy, they are a useful tool for referring callers to locally available face-to-face treatments.
4) *Brief advice by physicians or health professionals* has been shown to be effective especially with light smokers, although the efficacy is low. This however is difficult to initiate and maintain within the patient-doctor relationship.

1.5.2 Intensive interventions

1) *Behavioural interventions* are often offered in conjunction with pharmacological interventions over a series of weekly sessions. Both individual and group meetings have been shown to be effective. The large Lung Health Study (Anthonisen *et al* 1994) provides a persuasive confirmation of the effectiveness of intensive support combined with NRT, achieving a one-year abstinence rate of 35% versus 9% with usual care for hospitalised patients. There are two forms of behavioural interventions. One category is for relapse intervention which is a major problem causing the fall in success rates of 50-60% at one month to 20-30% one year later. This has been shown to be effective even for heavy smokers for a period of several weeks but there is no evidence yet of its longer efficacy. Social support is the second category which is believed to be

very important especially in group treatments. The 'buddying' system has shown a significant short-term effect in the first stages of abstinence. More work is needed on designing and evaluating social support treatments on a larger scale.

2) *Hypnosis and acupuncture* are very popular with smokers. The Cochrane group has reviewed 9 studies of hypnosis and 16 trials of acupuncture and concluded that evidence of specific efficacy is lacking. However, some people can be helped by numerous different unproven procedures, via non-specific and placebo effects.

1.5.3 Pharmacological approach

These include nicotine replacement therapy and non-nicotine medications.

1.5.3.1 Nicotine Replacement Therapy (NRT)

Acute nicotine withdrawal symptoms are the main short-term difficulty smokers face during a quite attempt. NRT works by breaking the quitting process into two phases. In the first phase smokers learn to cope with not smoking and the absence of the rapid boli of nicotine, whilst protected from the worst withdrawal effects by moderate levels of nicotine provided by NRT. The second phase is the gradual withdrawal of nicotine until none is used. NRT may make early relapse less rewarding therefore less likely to trigger a full-scale relapse. Another possible mechanism could be deconditioning because the link between pharmacological reinforcement and smoking behaviour may weaken during abstinence accompanied by NRT. The provision of a coping mechanism during the use of NRT can also play a crucial role in success rates.

1.5.3.1.1 Nicotine chewing gum

Nicotine gum has been subject to several reviews (Tang *et al* 1994, Silagy *et al* 1994). There is little doubt that nicotine gum is an effective NRT treatment, although the magnitude of the effect versus placebo is low with some trials reporting only a marginal or non-significant increase in quit rate compared to placebo. It is likely that during gum usage a substantial amount of nicotine is ingested, leading to extensive first pass metabolism and formation of metabolites.

There are 2 doses of nicotine gum available at 2mg and 4mg. Nicotine enters the blood stream via the buccal mucosa and the plateau blood nicotine level is reached after about 30 minutes. Only about 0.9mg nicotine from one piece of 2mg gum and 1.2mg from 4mg gum reaches the bloodstream. Smokers are recommended to chew

each piece for 30 minutes and chew one piece per hour not exceeding 15 gums per day.

1.5.3.1.2 Nicotine transdermal patch

There are 3 doses of patch available depending on the number of cigarettes smoked and time to first cigarette, and is aimed at reducing nicotine dosage over time. The highest dose delivers 1mg of nicotine per hour. 16 hour patches are available to avoid sleep-time nicotine dosing, however 24 hour patches are useful for preventing urges to smoke on waking. Nicotine absorption is slow, taking hours to reach plateau, however the simple instructions and ease of use makes the patch the most used NRT product (Tonnesen *et al* 1999, Hajek *et al* 1999).

1.5.3.1.3 Nicotine nasal spray

This is a bottle of nicotine solution, which is sprayed into a nostril by an air pump plunger via a nozzle. It provides the most rapid nicotine absorption rate among NRT products as it reaches plateau in just 10 minutes. One spray delivers 0.5mg of nicotine absorbed through the nasal mucosa. Smokers are recommended to use one spray in each nostril hourly, up to 16 times a day. Despite its initial irritant effects it is most effective for heavy smokers who seem to get used to it quickly (Sutherland *et al* 1992).

1.5.3.1.4 Nicotine inhaler

This consists of a plastic holder and cartridges containing a polythene plug with nicotine. A puff from the inhaler delivers nicotine vapour into the mouth and throat where it is absorbed. 20 puffs from an inhaler delivers the same amount of nicotine as one cigarette. Smokers are advised to use 6-12 cartridges per day, each for 3 puffing sessions. The inhaler is designed to replace the behavioural aspects of smoking without the harmful effects of cigarettes, however this is not a widely used device (Hajek *et al* 1999).

1.5.3.1.5 Nicotine sublingual tablet

These are designed to be held under the tongue until they dissolve within 20-30 minutes. They have a similar absorption and dose delivery profile to the gum and inhaler although they are also not widely used and their effectiveness has not been thoroughly studied.

The persistent presence of nicotine in the body during cigarette smoking is thought to be one of the contributing factors to the addictive nature of tobacco use. Conversely

the low levels of nicotine provided by NRT products attempt to relieve some of the withdrawal symptoms people suffer when they quit smoking (Lee *et al* 1993).

1.5.3.2 Non-nicotine medications

The main non-nicotine medication, which has shown success and is now widely used is buproprion. Although other medications such as lobeline, naltrexone and mecamylamine have been tested, they have not shown adequate results to boost them onto the market on a large scale.

1.5.3.2.1 Buproprion (Zyban)

This drug was originally produced as an antidepressant, but patients found they could quickly quit smoking after taking buproprion. This drug has dual action; it is a dopamine re-uptake inhibitor and, as rat studies have shown, during withdrawal dopamine levels drop, therefore this drug can maintain a more stable level of dopamine. It is also a nAchR antagonist, which reduces the effects of nicotine, therefore smokers do not get the desired effects of a cigarette (Slemmer *et al* 2000, Hemby *et al* 1997). It is given one week before cessation for 7-12 weeks. Its efficacy does not appear to be due to its antidepressant effects as the drug works equally well in smokers with and without a past history of depression. It has shown similar quit rates to NRT (Hughes *et al* 1999).

Keeping in mind the various nicotine cessation strategies and their efficacies explained above, they have only proved to have limited success. There still remains a need to enhance and develop therapies which can be more successful. Immunotherapy is a new strategy being studied which may prove to show more success in the battle against nicotine addiction.

1.6 Immunotherapy

1.6.1 Vaccines under investigation

A new strategy which has grown in popularity in the last few years, has been to target the drug rather than the brain through vaccination. This project is committed to the study of an immunotherapy approach to combat nicotine addiction, which will be explained in more detail later. Bonese *et al* (1974) were the first people to investigate the possibility of using drug-specific antibodies to reduce the effects of drugs of abuse, namely heroin. They actively immunized one monkey after it had been trained to self-administer both heroin and cocaine. They showed that immunization reduced the self-administration of heroin but not cocaine. Later Killian *et al* (1978) went on to transfuse

two non-immunized monkeys with morphine-specific antiserum from immunized monkeys. In this study, altered patterns of heroin self-administration lasted more than 3 weeks, when the effect of immunization reduced in parallel with antibody titres. Later, research was conducted for the treatment of cocaine addiction by Carrera *et al* (1995). They have produced a catalytic antibody to bind to the cocaine molecule in the blood before it reaches the brain and it is deactivated by a simple cleavage reaction that produces two molecules. The antibody then releases the molecules and is ready to bind another molecule. This therefore eliminates the addictive effects of cocaine. Results in vaccinated rats that were previously addicted to cocaine have shown that they ignore the drug. This concept is still under investigation.

The idea behind vaccination of rats against nicotine is to elicit the production of nicotine-specific antibodies which can bind and sequester nicotine in serum and extracellular fluid, reduce nicotine distribution to brain, and reduce many of nicotine's physiological and behavioural effects. Some practical features of vaccination such as long-lasting effects and avoiding the need for daily medication, may prove attractive. Potentially, a positive aspect of immunotherapy is that it does not affect neurotransmitters or receptors and should not have many of the adverse effects associated with other treatments.

The amount of nicotine reaching the brain, and the rate at which it does so, are important determinants of the initiation and maintenance of smoking (Benowitz 1996). Within limits, higher doses of nicotine are more rewarding than lower doses, and more rapid delivery of nicotine to the brain is more rewarding than slower delivery (smokers report cigarettes are more rewarding than a nicotine patch). Therefore the rate and extent of nicotine distribution to the brain provide potential therapeutic targets in which therapy is directed at the drug rather than the brain. In theory, nicotine vaccines should bind to nicotine and the antibody-nicotine complex would be too large to cross the blood-brain barrier, so that administered nicotine bound to the antibody is excluded from the brain. Therefore, vaccination can potentially alter the amount of nicotine reaching the brain. Vaccination may also reduce the rate at which nicotine enters the brain, although the mechanism by which this happens is unclear.

In 1997, Pentel group produced the first nicotine vaccine for which a nicotine derivative was conjugated to KLH (Heida *et al* 1997). After immunising rats with the nicotine vaccine (3 x 25μg over 5 weeks), antibody titres rose to over 10,000. Competition ELISA assays demonstrated that increasing concentrations of nicotine and nicotine

derivative increased the percent inhibition of antibody binding in a concentration-dependent manner, thus demonstrating their ability to bind to the antibody. The nicotine metabolites, nicotine-N-oxide and cotinine, did not appreciably bind to the antibody with concentrations up to 10^{-2} mol/L. Anti-nicotine antibodies did not recognise the structurally dissimilar compounds propranolol and acetylcholine. The nicotine binding capacity of anti-nicotine antibodies after active immunisation (1.3 μmol/L) exceeded the venous plasma levels of nicotine (up to 0.26 μmol/L) and was nearly equal to the arterial plasma levels reported in humans after smoking one or two cigarettes (Benowitz *et al* 1983, Henningfield *et al* 1993). Fourty minutes after intravenous administration of nicotine 0.03 mg/kg, there were 4 to 6 fold greater concentrations of nicotine in the plasma of immunised animals but there were no differences in brain levels of nicotine. However, when nicotine levels were examined at a more clinically relevant time point (3 minutes after intravenous nicotine administration), brain nicotine levels were reduced by 36%, while plasma concentrations raised 3 to 6 fold (Heida *et al* 1999). The pharmacokinetics are similar even after five repeated doses of nicotine (Keyler *et al* 1999), or after long-term exposure to nicotine before and during immunisation (Heida *et al* 2000).

To show the effectiveness of anti-nicotine antibodies for altering the behavioural effects of nicotine, nicotine polyclonal antibodies (50-150mg) were passively administered to rats (Pentel *et al* 2000). Antibody treatment dose-dependently attenuated nicotine-induced increases in systolic blood pressure and completely prevented nicotine-induced increases in locomotor activity measured 25 hours later. In nicotine-dependent rats, passive administration of 150mg of nicotine antibodies also prevented nicotine reversal of abstinence signs (Malin 2001). Malin *et al* (2002) also showed that passive immunization against nicotine attenuated nicotine discrimination. Rats were trained in a two lever operant chamber to press different levers after a nicotine or saline injection. After passive immunization, the immunized rats performed a significantly lower percentage of their lever presses on the nicotine lever compared to the non-immunized rats. In 2003 they published a paper showing that vaccination reduces nicotinic distribution to the brain not only by sequestering nicotine in serum but also by redirecting tissue distribution disproportionately away from the brain, such that nicotine concentrations are reduced to a greater extent in the brain than in other tissue (Satoskar *et al* 2003). They also showed that maternal vaccination against nicotine reduced nicotine distribution to foetal brain in rats (Keyler et al 2003).

To test the effects of the nicotine vaccine, nicotine-induced seizures were examined in rats. Immunisation reduced the incidence of seizures induced by a high (2mg/kg) dose of nicotine (Tuncok *et al* 2001). The nicotine vaccine was even more effective in preventing seizures if rats were first pre-exposed to nicotine (1mg/kg/day for 6 days) before high dose nicotine challenge. This vaccine was tested in humans in early 2002 by Nabi Biopharmaceuticals under the trade name Nic VAX.^{TM1}. This vaccine is a nicotine conjugate vaccine, conjugated to a carrier protein, recombinant exoprotein A (rEPA). In the phase I trials, 20 healthy non-smoker adults were randomly assigned to receive either an intramuscular injection of 200μg of the vaccine or placebo. Blood samples showed that a single dose of the vaccine resulted in a rapid immune response (within 7 days of vaccination) and generated substantial amounts of nicotine specific antibodies that were maintained or continued to increase through 60 days after vaccination. Adverse effects included mild to moderate local reactions to vaccination that were temporary and required no therapeutic intervention (Nabi Pharmaceuticals 2002).

At least three other nicotine vaccines are currently under development. Xenova Group plc began phase I clinical testing with TA-NIC^{TM} (a nicotine derivative coupled to rec cholera toxin B) in September 2001. The vaccine's safety, tolerability and immunogenicity were investigated in 60 Belgian volunteers, and preliminary results in both smokers and non-smokers have shown the vaccine to be safe and well tolerated both systemically and locally. The vaccine was administered by intramuscular injection and investigated at two different dose levels in a variety of dose regimes. The vaccine generated a specific anti-nicotine response, which is especially important in preventing nicotine from reaching the brain (Xenova.co.uk 2002). Preclinical studies of this vaccine produced high titres of nicotine-specific antibodies in mice and altered the pharmacokinetic distribution of a nicotine challenge (Kasaian 1998). Specifically one minute after an intravenous injection, nicotine levels in the brain decreased while plasma levels increased. Therefore, the antibodies may have the capacity to block the psychoactive effects of nicotine.

De Villiers *et al* (2002) have developed a nicotine-KLH conjugate vaccine which produced antibodies that recognised nicotine and the minor metabolite nornicotine, but not the major metabolites cotinine or nicotine-N-oxide. Immunisation in rats showed a reduction in the outflow of dopamine in the NAcc shell. They have also shown that immunization against nicotine alters the distribution but not half-life of nicotine (de

Villiers *et al* 2004). They also showed that vaccinated rats do not re-instate nicotine self-administration behaviour when exposed to nicotine (Lindblom *et al* 2002).

Whether vaccination will prove clinically useful is still unclear and will hinge upon a variety of pharmacokinetic and behavioural factors. In rats, pharmacokinetic effects of vaccination are greatest in those with the highest titres of antibodies in serum. Therefore it will be important to achieve sustained high antibody titres in humans in order for the vaccine to be effective. A key question regarding the use of vaccination is whether smokers will attempt to compensate for reduced nicotine effects by increasing their smoking, either deliberately or unintentionally. Since vaccination efficacy depends upon both the concentration of nicotine-specific antibodies in serum and the magnitude of the nicotine dose, compensation may be possible.

In a clinical setting, vaccination against nicotine is not likely to duplicate or replace existing medication, as it has no effect on the severity of tobacco withdrawal. The main effect of vaccination will be to blunt the rewarding effects of nicotine. Therefore it may be useful to use vaccination in conjunction with other therapies. Vaccination may be better suited to relapse prevention, in which the goal is to block the priming effect of a few puffs or a few cigarettes, than to smoking cessation where the antibody will be presented with large cumulative daily doses of nicotine. Vaccination can be achieved even in the presence of nicotine so that smokers could be vaccinated while they are still smoking, in preparation for a quit attempt (Heida *et al* 2000). However, this is already done in smoking cessation services using NRT while cutting down on nicotine consumption with a view to quit. More research needs to be done to study the effectiveness of this approach. Another possible role for vaccination is primary prevention of smoking among high-risk teens but this will require not only efficacy but considerable confidence in vaccine safety.

The immunotherapy approach for nicotine addiction is starting to get some attention. More research into the actions of the vaccine in relation to nicotine addiction may prove very interesting. For example, does the nicotine vaccine have any effect on the addiction pathway? If so, how? Does the decrease in nicotine in the initial stages of smoking reduce the aversive effects of nicotine? With lower nicotine concentrations in the brain, does tolerance to these aversive effects take place or take longer to occur? If so, will addiction only develop in persistent smokers? Is addiction to nicotine produced in the smoker with lower levels of nicotine in the brain, as there is less reward associated with the hedonic properties instigated by smoking? With already

established smokers going through withdrawal due to a lower level of nicotine in the brain, will other products such as NRT relieve withdrawal? As mentioned before one major reason for relapse is the severity of withdrawal symptoms. Perhaps intensive counselling will play a larger role in the treatment of nicotine addiction alongside vaccination and new relapse prevention methods need to be investigated. With more research and a better understanding of its therapeutic use in both animals and humans vaccination may become a viable nicotine cessation therapy.

1.6.2 Why target cotinine in an immunotherapy strategy?

Cotinine (Figure 1.5) is the most extensively studied nicotine metabolite. Until recently it has been generally accepted that cotinine is devoid of pharmacological activity (Benowitz 1986, Benowitz *et al* 1983). However, some more recent reports have suggested that cotinine (or its metabolites) may have some physiologic activity. Details of a small clinical study to assess the effect of cotinine in the treatment of cigarette smoking have been published as a conference abstract (Liberto 1995). The authors conclude that the preliminary data showed that cotinine had efficacy as an aid to quitting cigarette smoking. Another publication (Keenan *et al* 1994) describes a two-way crossover study to assess the effects of oral cotinine and placebo on various withdrawal symptoms in abstinent smokers. The results showed that for a number of subjective withdrawal symptoms (ie restlessness, tension/anxiety) the placebo was more effective than cotinine in relieving these symptoms. Somewhat surprisingly this study has been recently used to support a US patent application concerning the use of cotinine as a smoking cessation aid.

A case study (Benowitz *et al* 1987) concerning nicotine poisoning following massive cutaneous exposure to nicotine provides further evidence that cotinine or other nicotine metabolites may have antagonistic activity. The patient presented with nausea and recurrent vomiting. Blood levels of nicotine measured between 5 and 12 hours following exposure to nicotine remained fairly constant, whereas cotinine levels increased from around 400 ng/ml to 800 ng/ml within the same period, however nausea was resolved after around 8 hours following exposure to nicotine. It is possible that the rise in cotinine concentration (or other metabolite) could have been responsible for the abolition of poisoning symptoms.

The development of tolerance to the acute effects of nicotine is well documented (Porchet *et al* 1987), although the reasons for this remain unclear. One group (Porchet *et al* 1988) has produced a pharmacokinetic/ pharmacodynamic model to explain the

development of tolerance to the acute effects of nicotine. The model developed utilized a hypothetical "metabolite" antagonist of nicotine which may be cotinine. A reasonable fit was obtained between the observed and predicted data using this approach.

Studies conducted *in vivo* in rats and mice have shown that the number of nicotine receptors in the brain increases after chronic administration of nicotine and induction of tolerance (Marks *et al* 1983). In this paper the authors postulated that nicotine or a nicotine metabolite could act directly as an antagonist.

The effects of oral nicotine and subcutaneously administered nicotine have been studied in rats (Balfour 1980). Oral nicotine was not an adequate substitute for rats made behaviourally dependent upon nicotine injections in a shock avoidance test. Furthermore in rats treated with oral nicotine in the drinking water (5.4mg/kg/day) there were no changes in plasma corticosterone levels. However this treatment did block the increase in plasma corticosterone seen in response to a subcutaneous injection of nicotine. Pre-treatment with cotinine was without effect on the changes in corticosone levels seen with nicotine injections suggesting that cotinine was not acting as an antagonist in this case, however this observation does not rule out putative antagonistic activity of other metabolites.

Other recent work has demonstrated that cotinine can antagonize the effects of nicotine, for example one study (Vainio *et al* 1998) demonstrated that cotinine inhibits nicotine-induced noradrenaline release *in vitro*. The authors speculated that cotinine may sustain the addictive changes or relieve the abstinence symptoms in smokers by antagonizing the effects of nicotine. They have also shown that cotinine and nicotine inhibit each other's calcium responses in bovine chromaffin cells, and cotinine may also be desensitising the nicotinic cholinergic receptors, possibly by acting as a low-affinity agonist at these receptors. (Vainio *et al* 2000). Tests done on the nucleus accumbens show that cotinine blocks nicotine induced dopamine release (Sziraki *et al* 1999). This can be explained by the findings that cotinine acts as an agonist to evoke dopamine release and perhaps causes desensitisation of the nAChRs in the striatum and therefore in practice inhibit nicotine induced dopamine release (Dwoskin *et al* 1999).

As it has been demonstrated here, there is conflicting evidence about the actions of cotinine. More evidence shows that cotinine seems to be antagonizing the overall effects of nicotine possibly through desensitisation of the nAChRs. With this idea in

mind cotinine was used as a primary target for the immunotherapy approach in nicotine cessation.

1.6.3 Aims of the immunotherapy approach targeting cotinine

The aims of this project are to target cotinine, the major metabolite of nicotine and raise antibodies to eliminate it from the blood before it reaches the brain, and proceed on to pharmacological tests to find out what effects this has on the brain in relation to nicotine addiction.

The rational behind this project is; once a cigarette has been smoked by a vaccinated smoker, nicotine will be metabolised to cotinine in the blood. Cotinine will then be retained in the blood and eliminated before it reaches the brain via the cotinine antibodies. This will in turn stop the antagonistic actions of cotinine (through initial agonism causing desensitisation of nicotinic AchRs) on nicotine in the brain. Therefore nicotine will have a larger desired effect on the smoker, which in turn will encourage the smoker to consume fewer cigarettes. This is better for the health of the smoker and also with the aid of other therapies such as NRT, the smoker may have a better chance of successfully quitting.

The experimental aims of this project are:

- As there is conflicting evidence in the literature regarding the effects of cotinine on nicotine addiction, this will be studied further using dopamine release experiments in the striatum.
- The first step in the production of the vaccine will be to produce a derivative of cotinine with the feasibility to attach to a carrier molecule. This derivative-carrier complex will need to have 10-15 derivatives and expose the cotinine molecule to allow for specific recognition by the immune system.
- The derivative-carrier complex will then need to be incorporated with adjuvent and injected into rats and the immune response tested.
- The injection regime needs to be optimised to produce the maximal response.
- Appropriate ELISA methods need to be developed to test the immune response and its specificity to cotinine.
- Blood nicotine and cotinine levels will then need to be measured to examine whether cotinine is retained in the blood.
- Dopamine release in the striatum will be measured to examine whether vaccination has an effect.

- The effect of vaccination will also be examined on nicotine induced locomotor activity as a test to see whether vaccination has an effect on the behaviour of animals.

This hypothesis is of course not without flaw. There are some factors that may affect the outcome of this immunotherapy approach, which will need further examination beyond the scope of this project. For example, metabolism of nicotine and cotinine may be altered by vaccination. This has not been shown to be the case in a limited study by de Villiers *et al* (2004), however it is an important issue to consider and examine. If cotinine is taken up by antibodies in plasma, it may increase rate of shift of the metabolism of nicotine into cotinine, thereby increasing the amount of cotinine in the blood which may mean that less nicotine is reaching the brain. This may suggest that administration of nicotine by NRT and the proposed increase in its effect due to the eradication of the antagonism caused by cotinine, as hypothesised in this study, may have a reduced effect. Another question to address is whether enough cotinine will be kept away from the brain to cause a functional increase in the actions of nicotine in the brain. Also, will this increase in effect of nicotine in the brain mean less demand for nicotine intake by the smoker in a practical situation?

There are many aspects to nicotine addiction as explained in this chapter, and the effect of a cotinine vaccine in this hypothesis is only tackling the effects of smoking on the mesolimbic dopimanergic pathway related to reward in a limited manner. Questions which will need further investigation following this project include;

- Will the decrease in cotinine in the brain have an effect on excitatory inputs into the VTA via glutamatergic transmission from the prefrontal cortex?
- Will the inhibitory inputs into the VTA via GABAergic neurons have an effect on the addiction pathways?
- Will the decrease in cotinine and the subsequent larger effect of nicotine show different characteristics on the dopamine released in the Nacc shell and core and therefore on the hedonic properties of nicotine?
- The aversive effects of nicotine may also be altered with the decrease of cotinine and the subsequent increase of nicotine effect in the brain.

As with the nicotine vaccines under investigation, other aspects of addiction such as withdrawal and behavioural support also need to be addressed alongside vaccination to allow for a robust therapy package for nicotine addiction.

The rational behind the immunotherapy approach targeting cotinine has been explained. In the final section of this introduction I will talk more about the immune responses in the body, characteristics of antibodies and what is involved in designing the right vaccine fit for the purpose of this immunotherapy approach against nicotine addiction.

1.6.4 Overview of immunology

To give an overview of the immunology involved in this project I have used a variety of text books (Immunology by Goldsby *et al*, Biology by Campbell, Antibodies; a laboratory manual by Harlow *et al*).

The body has two defence systems against unwelcome intruders. One system is non-specific and consists of two lines of defence; the first line of defence is external and consists of epithelial tissue that cover and line the body via skin and mucous membranes. The second line is internal and is triggered by chemical signals and uses antimicrobial proteins and phaygocytic cells that attack any invaders that penetrate the body's outer barriers. The second system, which is specific, is the immune system, which comes into play simultaneously with the second line of non-specific defence. This includes the production of specific defensive proteins called antibodies as well as several different types of cells that are derived from white blood cells called lymphocytes. The four key features characterize the immune system are specificity, diversity, memory and self/nonself recognition.

1.6.4.1 Passive Immunity

Immunity elicited in one animal can be transferred to another, providing passive immunity, by injecting it with serum from the first. Passive immunization does not activate the immune system, it generates no memory response and the protection provided is transient. Passive immunization can be acquired through several processes:

1. Natural maternal antibodies transferred from mother to foetus.
2. Immune globulins which are antibody-containing solution derived from human blood, obtained by cold ethanol fractionation of large pools of plasma, available in intramuscular and intravenous preparations.
3. Humanized monoclonal antibody.
4. Antitoxins which are antibodies derived from the serum of animals that have been stimulated with specific antigens.

1.6.4.2 Active Immunity

The goal of active immunization is to elicit protective immunity and immunological memory. When active immunization is successful, a subsequent exposure to the agent elicits a heightened immune response that successfully eliminates the antigen. Active immunity can be achieved by natural infection with a microorganism or by artificial administration of a vaccine.

1.6.4.3 Humoral and cell-mediated responses

The immune system can mount two different types of responses to antigens: a humoral response and a cell-mediated response. Humoral immunity results in the production of antibodies, which are secreted by B lymphocytes and circulate as soluble proteins in blood plasma and lymph. Cell-mediated response depends on the direct action of cells (T lymphocytes) rather than antibodies.

Lymphocytes originate from pluripotent stem cells in the bone marrow or in the foetus, mainly in the liver. Lymphocytes that then differentiate into T cells are those that migrate to the thymus, and B cells remain in the bone marrow to continue their maturation. Mature B cells and T cells are most concentrated in lymph nodes, the spleen and other lymphatic organs, where they are most likely to encounter antigens. Both have specific antigen receptors on their plasma membranes. The antigen receptors on B cells are membrane-bound antibody molecules specific for a certain antigen. The antigen receptors on T cells are different than in antibodies but do recognize antigens as specifically as antibodies. Any lymphocytes with receptors for molecules present in the body are destroyed or are rendered non-functional during maturation, leaving only lymphocytes that are reactive against foreign molecules. This gives rise to self-tolerance. The self molecules tolerated by an individual's immune system are a collection of molecules encoded by a family of genes called the major histocompatibility complex (MHC). Two main classes of MHC molecules mark cells as self. Class I MHC molecules are located on all nucleated cells. Class II MHC molecules are restricted to a few specialized cell types such as macrophages, B cells and activated T cells.

Each antigen, by binding to specific receptors, selectively activates a tiny fraction of cells from the body's diverse pool of lymphcotyes; this relatively small number of selected cells gives rise to a clone of millions of effector cells, all dedicated to

eliminating the specific antigen that stimulated the humoral or cell-mediated response. This reaction upon the first exposure to an antigen constitutes the primary immune response. Between initial exposure to an antigen and maximum production of effector cells, there is a lag time of 5 to 10 days.

When antigens bind to specific receptors on the surface of a lymphocyte, the lymphocyte is activated to divide and differentiate, giving rise to a population of effector cells, which defend the body in an immune response. B cells differentiate to plasma cells, which secrete antibodies that help eliminate antigens. T cell effector cells include cytotoxic T cells (T_C), which kill infected cells and cancer cells, and Helper T cells (T_H) which secrete protein factors called cytokines to regulate neighbouring cells. Cytokines help B cells and T cells therefore act in both the humoral and cell-mediated responses.

The secondary immune response occurs if the body is exposed to the same antigen at some later time. This response is faster than in the primary immune response and only takes 3-5 days and lasts longer (peaks after 14 days after the second exposure) than the primary immune response. The immunological memory is based on long-lived memory cells, which are produced along with the relatively short-lived effector cells of the primary immune response, however they are not active at this time. These memory cells proliferate rapidly when exposed again to the same antigen. This gives rise to a new clone of memory cells as well as new effector cells.

In most cases, the selective activation of a B cell to form a clone of plasma cells and memory cells is a two-step process. One step as mentioned before is the binding of antigen to specific receptors on the surface of the B cell. The other step involves macrophages and helper T cells. After a macrophage engulfs pathogens by phagocytosis, fragments of the partially digested antigen molecules are bound by class II MHC molecules. The two molecules are transported to the plasma membrane and displayed on the surface of the macrophage (antigen-presenting cell). A helper T cell's specific receptors recognize this self/nonself combination of MHC and a particular antigen fragment. This activates the T cell, which proliferates and forms a clone of helper T cells. The helper T cells then secrete cytokines, which selectively stimulate B cells that have already encountered the particular antigen. After the binding of antigen to a B cell, the cell takes in a few of these foreign molecules by endocytosis. The B cell then displays antigen fragments bound to class II MHC markers on the cell surface. The helper T cell's receptor recognizes and binds this antigen-MHC complex.

The antigens that evoke the response described above are known as T-dependent antigens. Most antigens are T-dependent. T-independent antigens trigger the humoral response without involvement of macrophages and T cells. However, the humoral response is generally much weaker and no memory cells are generated. These will not be discussed further as they are not involved in the immune response generated in this project. Once B cells are activated by T-dependent antigens, a clone of plasma cells are produced and each of these effector cells secretes as many as 2000 antibodies per second for the 4-5 day lifetime of the cells.

1.6.4.4 Antibodies

Antibodies constitute a class of proteins called immunoglobulins (Igs). Every antibody has at least two identical sites that bind to the epitope. A typical antibody has four polypeptide chains joined to form a Y-shaped molecule: two identical light chains and two identical heavy chains. Both heavy and light chains have constant regions which are responsible for the antibody's effector function. At the tips of the Y-shaped molecule's two arms are the variable regions, which function as the antigen-binding sites. The antigen-binding site is responsible for an antibody's recognition function of epitopes. There are five types of constant regions which determine the five major classes of mammalian immunoglobulins;

IgM - which are the first circulating antibodies to appear in response to an initial exposure to an antigen and decline in concentration rapidly. They consist of five Y-shaped monomers arranged in a pentamer structure.
IgG - which are the most abundant of the circulating antibodies. They readily cross the walls of blood vessels and enter tissue fluids and trigger the action of the complement system. These are the immunoglobulins most expected to be present in this project.
IgA - which are produced by cells in mucous membranes to prevent attachment of viruses and bacteria to epithelial surfaces.
IgD - which don't activate the complement system and are mostly found on the surface of B cells, probably functioning as an antigen receptor.
IgE - which are slightly larger than IgG molecules and are present in blood in small concentrations. They cause mast cells and basophils to release histamine and other chemicals which cause an allergic reaction.

Antibodies do not destroy an antigenic invader directly. The composition of the antigen-antibody complex causes several effector mechanisms. These include neutralization, agglutination of particulate antigens, precipitation of soluble antigens

which forms immobile precipitates that are captured by phagocytes, and activation of the complement system.

1.6.4.5 Immunogenicity

Immunogenicity is the ability to induce a humoral and / or cell mediated immune response:

B cells + antigen = effector B cells + memory B cells

T cells + antigen = effector T cells + memory T cells

Antigenicity however is the ability to combine specifically with the final products of the above responses ie: antibodies and / or cell - surface receptors. Some small molecules called haptens are antigenic but incapable, by themselves, of inducing a specific immune response. Proteins are the most potent immunogens, and are used by immunologists as immunogens in most experimental studies of humoral immunity and cell-mediated immunity. These proteins include ovalbumin (44 KDa) and tetanus toxoid (150KDa).

Immunogenicity is determined in part by several properties of the immunogen. In order to elicit an immune response, a molecule must be recognized as nonself by the biological system. Also, the greater the phylogenic distance between two species, the greater the structural disparity between them. There is a correlation between size of a macromolecule and its immunogenicity. The most active immunogens tend to have a MW of over 100KDa, and the least active are below 5-10KDa. Chemical complexity also contributes to immunogenicity for example all four levels of protein organization (primary, secondary, tertiary and quaternary) contribute to its immunogenicity. Large, insoluble macromolecules are generally more immunogenic than small, soluble ones because the larger molecules are more readily phagocytosed and processed.

1.6.4.6 Vaccine design

Several factors must be considered when designing a successful vaccine. The development of an immune response does not necessarily mean that a state of protective immunity is achieved. What is critical is which branch of the immune system, the humoral or the cell-mediated branch, is activated. A second factor is the development of immunological memory. For example, a vaccine that induces a protective primary response may fail to induce the formation of memory cells, leaving the host unprotected after the primary response to the vaccine subsides. For a compound to elicit a primary antibody response and a strong secondary response, it

must contain an epitope that can bind to the cell-surface antibody of a virgin B cell and it must promote cell-to-cell communication between B cells and helper T cells. The development of both humoural and cell-mediated responses requires interaction of T cells with an antigen that has been processed and presented together with major histocompatibility complex (MHC) molecules. Binding of the antigen to the surface antibody molecule of a virgin B cell determines the specificity of the resulting antibodies as the antigen-binding site on the surface antibody molecule will be identical to the binding site on the secreted antibodies.

Even if a macromolecule has the properties that contribute to immunogenicity, its ability to induce an immune response will depend on certain properties of the biological system that the antigen encounters. Some of these properties include immunogen dosage and route of administration and an optimal mixture of the two can induce a peak immune response in a given animal. An insufficient dose fails to activate enough lymphocytes or induce a state of immunological unresponsiveness or tolerance. Conversely, an excessive high dose can also induce tolerance. A single dose of most experimental immunogens will not induce a strong response; rather repeated administration or boosters, over a period of weeks is usually required. Boosters increase the clonal proliferation of antigen - specific T cells or B cells and thus increase the lymphocyte populations specific for the immunogen. The immunogens are usually administered by a variety of routes such as intravenous, intradermal, intramuscular, intraperitoneal and subcutaneous. The administration route strongly influences which immune organ and cell populations will be involved in the response. In intravenous injections, the antigen is carried first to the spleen, whereas antigens administered subcutaneously move first to local lymph nodes. This affects the subsequent immune response.

Adjuvants are substances that when mixed with an antigen and injected enhance the immunogenicity of that antigen. They are used to boost the immune response when an antigen has low immunogenicity or when only small amounts of antigen are available. Precisely how adjuvants increase the immune response is not known. Alum (aluminum potassium sulfate) appears to prolong antigen persistence, increase local inflammation by inducing granuloma formation and maybe enhance co-stimulatory signals. When an antigen is mixed with alum, the salt precipitates the antigen. This results in a slower release of antigen from the injection site, so that the effective time of exposure to the antigen increases from a few days without adjuvant to several weeks with adjuvant. The alum precipitate also increases the size of the antigen, increasing the likelihood of

phagocytosis. When alum stimulates the local inflammatory response, it attracts both phagocytes and lymphocytes. This infiltration of cells often results in formation of a dense macrophage-rich mass of cells called a granuloma. Because the macrophages in a granuloma are activated, this mechanism also enhances T_H cell activation.
Chemical coupling of a hapten to a large protein (carrier) yields an immunogenic hapten-carrier conjugate. When this is injected, animals produce antibodies specific for 1) the hapten, 2) unaltered epitopes on the carrier protein, and 3) new epitopes formed by combined parts of both the hapten and carrier. As mentioned before, a hapten cannot function as an immunogenic epitope. But when multiple molecules of a single hapten are coupled to a carrier protein, the hapten becomes accessible to the immune system and can function as an immunogen.

The theories presented in this section regarding the immune system and vaccine design were all taken into account when making the derivatives (in the appendix) and vaccines in chapter 3 and their further optimisation in chapters 4 and 5.

Chapter 2

Dopamine Release

2.0 Effects of nicotine and cotinine on dopamine release using 96-well plate assay.

2.1 Introduction

As discussed in the Introduction section 1.6.2, cotinine has been shown to have various effects in relation to nicotine addiction and its actions on nAChRs. The aim of this chapter is to investigate the effect of cotinine on dopamine release in the striatum in comparison and in conjunction with nicotine. A high throughput method using a 96-well plate assay developed by Puttfarcken *et al* (2000) was used in this study with variations. This is a static release system carried out in 96-well filter plates, with a support membrane to separate tissue slices from the bathing medium (containing the released transmitter) that can be removed by vacuum filtration. The pharmacological data presented for nicotine-evoked [^{3}H]-dopamine release (Puttfarcken *et al* 2000), are comparable to results obtained from conventional superfusion systems, therefore this method is useful for rapid determination of the effects of agonists and antagonists on [^{3}H]-dopamine release. The advantages of using minces as described in section 2.3.3 are its speed and ease of preparation.

Previous studies have estimated that approximately 85% of mouse striatal nAChRs are $\alpha4\beta2$ on synaptosomes on DA terminals (Marks *et al* 1998, Whiteaker *et al* 2000). Sharples *et al* (2000) and Puttfarcken *et al* (2000) have suggested the involvement of $\alpha4\beta2$ nAChRs in [^{3}H]-dopamine release in rats. The role of other nAChR subtypes in dopamine release has been suggested by the finding that αCTMII–sensitive nAChRs account for 40% of nicotine-evoked dopamine release in mouse striatum (Grady *et al* 2002, Kulak *et al* 1997, Kaiser *et al* 1998), suggesting the involvement of $\alpha3$ and/or $\alpha6$-containing nAChRs (Champtiaux *et al* 2002). Kaiser & Wonnacott (2000) showed that antagonism of αCTMII was additive with that of $\alpha7$-selective antagonists. They concluded that $\alpha7$ nAChRs on striatal glutamate terminals elicit glutamate release, which in turn acts at ionotropic glutamate receptors on dopamine terminals to stimulate dopamine release.

Several antagonists were used to study which nAChRs are involved in nicotine and cotinine -evoked [^{3}H]-dopamine release in this assay. The antagonists used were:

1) Mecamylamine which is a non-selective nAChR antagonist.

2) α-Conotoxin MII was originally defined as a potent antagonist at α3β2 nAChRs expressed in *Xenopus oocytes*, with inhibition of responses occurring in the subnanomolar range (Cartier *et al* 1996). Since its isolation from the venom of the fish-hunting cone snail *Conus magus*, it is now synthesized commercially and has been used in several functional studies in native systems. Using α-Conotoxin MII in the studies examining dopamine release from rodent striatal dopaminergic neurons, has led to the identification of two distinct populations of nAChRs (Kulak *et al* 1997, Kaiser *et al* 1998). This toxin partially blocks [^{3}H]-dopamine release from rat striatal synaptosomes stimulated with nicotine (Kulak *et al* 1997) or anatoxin-a (Kaiser *et al* 1998). This was interpreted as evidence for the heterogeneity of presynaptic nAChRs on dopamine terminals, with one population containing an α3β2 interface. Although recent reports have suggested that the population may contain an α6β2 inerface (Champtiaux *et al* 2002). Therefore, the selectivity of α-Conotoxin MII may be more complicated than originally thought.

3) α-bungarotoxin is isolated from the venom of the snake *Bungarus multicinctus* and is a potent and selective pseudoirreversible antagonist at α7 nAChRs (Davies *et al* 1999). This toxin has been used to inhibit α7 nAChRs in a wide variety of functional studies, ranging from superfusion (Kaiser & Wonnacott 2000) to electrophysiology (Gray *et al* 1996). The disadvantage of its use in functional studies is the long pre-incubation time needed (1h) when using nanomolar concentrations, which may adversely affect some tissue preparations. However, this problem may be overcome by increasing its concentration with a shorter pre-incubation period.

4) Dihydro-β-erythroidine (DHβE) was derived from seeds of the *Erythrina spp.*. It has been shown to inhibit β2 nAChR subunits in low concentrations (Crooks *et al* 1995). Many pharmacological studies have used this compound as a selective antagonist at α4β2*nAChRs (Alkondon & Albuquerque 1993). However, its selectivity at this subtype is questionable, as it has been shown to inhibit other subtypes of heteromeric nAChRs expressed in *Xenopus oocytes* with a concentration range of 0.19 to 1.3 μM (Harvey *et al* 1996).

The experiments presented in this chapter were conducted by myself and two undergraduate project students, Sarah Parks and Christine Robinson, under my immediate supervision.

2.2 Materials

2.2.1 Animals

Rats used were male Sprague Dawley rats with a weight range of 250-300 grams. Four were housed per cage, in a smoke-free environment with food and water available *ad libitum*.

2.2.2 Drugs and Chemicals

Cotinine, (-)-nicotine, dihydro-β-erythroidine (DHβE), mecamylamine, nomifensine and pargyline were purchased from Sigma Chemical Company Ltd., Poole, Dorset, UK. Alpha-bugarotoxin (α-bungarotoxin) was purchased from Molecular Probes, Eugene, UK. Alpha-conotoxin MII (αCTMII) was purchased from Tocris, Bristol, UK. [7,8-^{3}H]-dopamine (41-45 Ci/mol) was purchased from Amersham International, Amersham, Bucks., UK. Microscint^{TM} 40 (liquid scintillation cocktail) and Optiplate^{TM}-96 (opaque, top-count specific, 96-well plates) were purchased from Packard Bioscience Company, Groningen, Netherlands. Millipore Multiscreen^{TM} BV clear plates were purchased from Millipore Corporation, Bedford, UK.

All other chemicals used were of analytical grade or higher and were bought from standard commercial sources.

2.3 Method

2.3.1 Preparation

Buffer 1: Krebs-bicarbonate (188mM NsCl, 2.4mM KCl, 2.4mM$CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO4, 25mM NaHCO3, pH 7.4). This was gassed with 95% O_2/ 5% CO_2 for 1 hour. Pargyline (10μM), glucose (0.9 g) and ascorbic acid (0.09 g) were added (pH 7.4). This buffer is used in the first stage of the assay.

Buffer 2: Nomifensine (50nM) was added to buffer 1. Nomifensine is a dopamine re-uptake inhibitor and is used to prevent [^{3}H]-dopamine being taken up into the tissue once it has been released by the tissue to allow for measurement. Buffer 2 was used in the preparation of the drugs used in the assay, as well as incubating the filter plates for 30 minutes at 37°C prior to the experiment. The drugs to be used in the assay were prepared prior to the experiment and transferred into nunc 96-well plates.

Sucrose-HEPES (0.32M sucrose, 5mM HEPES) was used to hold brain tissue prior to striatal mince preparation.

2.3.2 Dissection of the striatum

Four striata (a striatum from the left and right hemisphere of the brain) from two rats were used per experiment. The rats were killed by cervical dislocation and decapitation (schedule 1 method).

2.3.3 Preparation of striatal minces

The striata were sliced using a McIllwain tissue chopper to create 250μm x 250μm tissues minces. This was done by chopping the tissue 3 times, each time rotated by 60°. The minces were then triturated in 5ml buffer 1 and incubated for 4 minutes at 37°C. The minces were then washed 3 times in 5ml buffer 1 over 15 minutes at 37 °C.

2.3.4 Pre-loading of tissue with [^{3}H]-dopamine

[^{3}H]-dopamine (50nM) was added to the 5ml suspended minces and incubated at 37°C for 30 minutes, with regular agitation. The minces were then washed 5 times in buffer 2 over 30 minutes at 37°C and re-suspended in 10ml buffer 2.

2.3.5 Stimulation of tissue

The buffer soaking the filter plate was removed using vacuum filtration and the striatal minces were distributed evenly over the 96-well filter plate (100μl per well). This was then vacuum filtered, leaving the minces on the filter. The drugs used for pre-incubation were added (70μl per well) and incubated for the required duration (the standard time was 5 minutes unless otherwise stated), at 37°C. 8 wells were used per drug condition.

The solution was vacuum filtered into an opaque Optiphase plate (basal step). The drugs used for stimulation of tissue (70μl per well) were added and incubated for the required duration (the standard time was 5 minutes unless otherwise stated), at 37 °C. The solution was vacuum filtered into another opaque Optiphase plate (release step).

The filter plate was frozen at –20°C overnight.

2.3.6 Measurement of radioactivity

Scintillation fluid was added (170μl per well) to both Optiphase plates and radioactivity was measured using Microbeta machine.

After the filter plate has been frozen overnight the filters were taken out of each well and placed in individual scintillation vials. Scintillation fluid (4ml) was added to each vial. Radioactivity remaining in the tissue was measured using a scintillation counter over 1 minute.

2.3.7 Data analysis

The ratio of the [^{3}H]-dopamine released in the release step to the basal step was calculated which gives the degree of drug-evoked release. This was then compared to the total amount of radioactivity contained within the tissue and the final release was calculated using the equation below:

% Release = Stimulated release / (Stimulated release + Tissue) x 100

Previously efficiencies of the microbeta (34.5%), and scintillation counter (53.6%) were calculated and these were taken into account when calculating release.

Curve fitting for the nicotine and cotinine dose response experiments were carried out using the Hill Equation below:

$$f = t / (1+(K/X)^*n) + s$$

where:

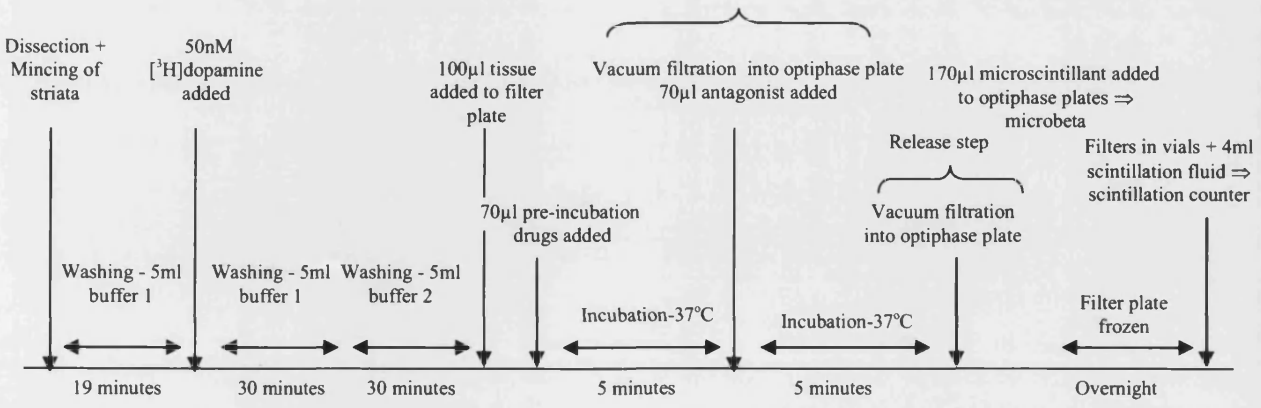
K = EC_{50} value (agonist concentration which evokes a half-maximal response)

s = minimum agonist concentration

t = maximum agonist concentration

n = Hill number (n = 1)

2.3.8 Time-line Summary



2.4 Results

2.4.1 Dose response for nicotine and cotinine evoked [^{3}H]-dopamine release

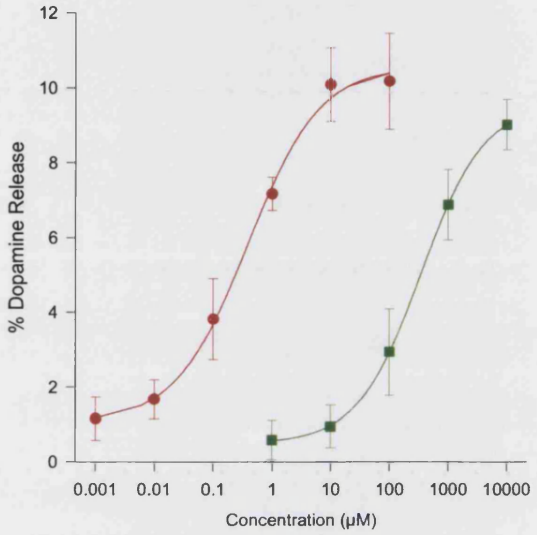


Figure 2.1: Dose response curves for nicotine and cotinine evoked [^{3}H]-dopamine release.

Striatal minces were pre-loaded with [^{3}H]-dopamine and buffer 2 only was used to pre-incubate the tissues (5 minutes). In the basal stage nicotine (1nM - 100μM) or cotinine (1μM – 10mM) were used to stimulate the tissue (5 minutes). Nicotine-evoked (—) and cotinine-evoked (—) dopamine release were calculated. 8 wells per drug treatment were used in each experiment and this was repeated in 3 experiments (n=3).

[^{3}H]-dopamine release evoked by nicotine or cotinine ranged between 0.6% and 10.2% of the total amount of [^{3}H]-dopamine at the start of the release stage. The EC_{50} value was calculated which denotes the drug concentration that evokes 50% of the maximum [^{3}H]-dopamine released. Nicotine (EC_{50} = 381nM) requires a thousand fold lower concentration than cotinine (EC_{50} = 342μM) to evoke the same %[^{3}H]-dopamine release. This is in accordance with the observation that cotinine has a thousand fold lower affinity for nAChR than nicotine (Quick & Lester 2002).

GCMS analysis of the cotinine used in these experiments shows 0.0022% impurity of nicotine present. This is insufficient to account for the shift in potency.

2.4.2 Characterization of nicotine and cotinine - evoked [^{3}H]-dopamine release

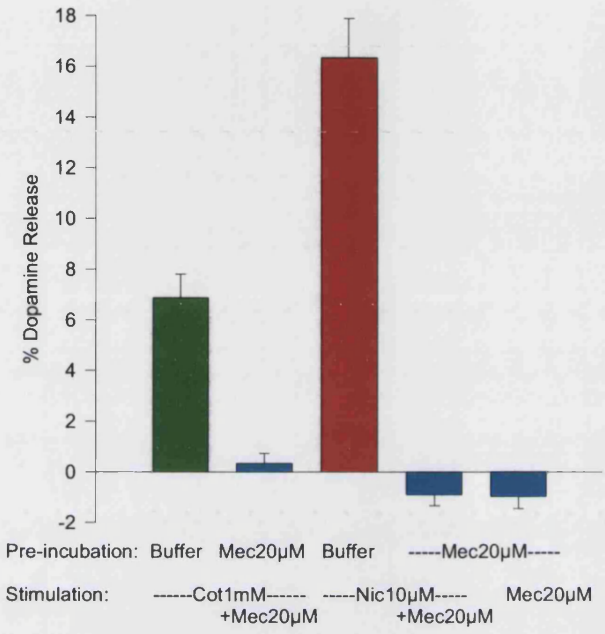


Figure2.2: Effect of mecamylamine on nicotine and cotinine evoked [^{3}H]-dopamine release.

Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 (5 minutes) and stimulation with cotinine (1mM) ■ and nicotine (10μM) ■ (5 minutes). Incubation with mecamylamine (20μM) ■ ,with both cotinine and nicotine-evoked [^{3}H]-dopamine release was blocked. 8 wells per drug treatment were used in each experiment and this was repeated in 3 experiments (n=3).

Figure 2.2 shows both nicotine and cotinine – evoked [^{3}H]-dopamine release were blocked by the non-selective nicotinic antagonist mecamylamine. This suggests both nicotine and cotinine have an agonist effect on [^{3}H]-dopamine release via nAChRs.

2.4.3 Effect of cotinine on nicotine - evoked [^{3}H]-dopamine release

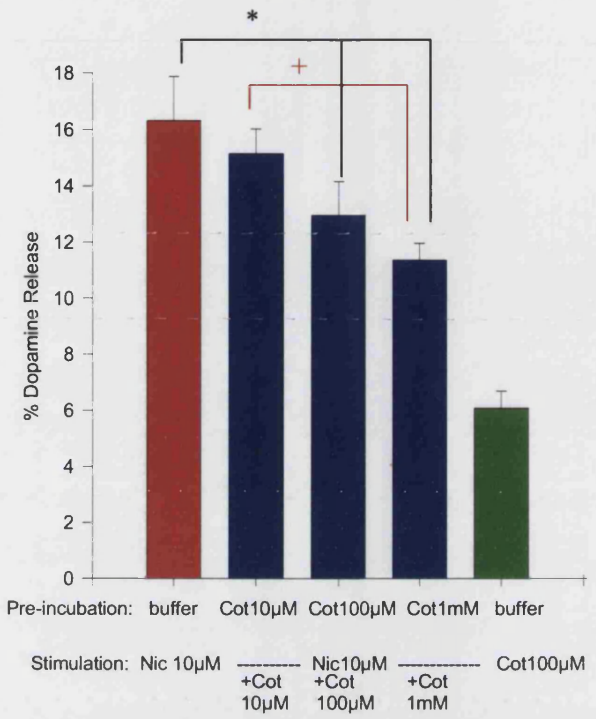


Figure 2.3: Effect of cotinine on nicotine – evoked [^{3}H]-dopamine.

*Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 (5 minutes) and stimulation with nicotine (10μM) ■ and cotinine (100μM) ■ (5 minutes) showed [^{3}H]-dopamine release similar to that seen in figure 2.2. When pre-incubated with cotinine (10μM - 1mM) and stimulated with both nicotine (10μM) and cotinine (10μM –1mM) ■ nicotine evoked [^{3}H]-dopamine release decreased with increasing cotinine concentrations. 8 wells per drug treatment were used in each experiment and this was repeated in 3 experiments (n=3). * = significantly different ($p<0.05$), using one way analysis of variance (ANOVA) test with Dunnet post hoc – multiple comparisons versus control group (10μM nicotine alone). + = significantly different ($p<0.05$), using one way analysis of variance (ANOVA) test with Turkey post hoc – all pair-wise multiple comparison.*

Figure 2.3 shows pre-exposure to cotinine produces a significant decrease in nicotine (10μM) – evoked [^{3}H]-dopamine release at 100μM and 1mM concentrations. This suggests that cotinine is antagonising the actions of nicotine. As both nicotine and cotinine – evoked [^{3}H]-dopamine release were blocked by the non-selective nicotinic antagonist mecamylamine (figure 2.2), the most likely explanation is that prior exposure

to low concentrations of cotinine can desensitise the nAChRs. This is further discussed in the discussion (section 2.5).

2.4.4 Which nAChR subunits are involved in nicotine and cotinine - [^{3}H]-dopamine release?

2.4.4.1 Dihydro-β-erythtoidine

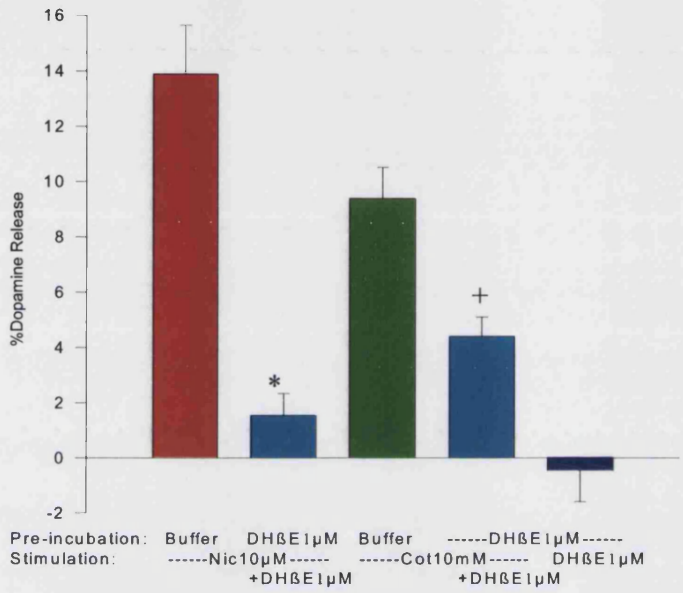


Figure 2.4: The effect of dihydro-β-erythtoidine (DHβE) on nicotine and cotinine – evoked [^{3}H]-dopamine release.

*Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 (5 minutes) and stimulation with nicotine (10μM) ■ and cotinine (10mM) ■ (5 minutes) showed [^{3}H]-dopamine release similar to that seen in figure 2.2. When pre-incubated with DHβE (1μM) and stimulated with both nicotine (10μM) and DHβE (1μM) ■ nicotine-evoked [^{3}H]-dopamine release decreased significantly. When pre-incubated with DHβE (1μM) and stimulated with both cotinine (10mM) and DHβE (1μM) ■ nicotine-evoked [^{3}H]-dopamine release also decreased significantly. Pre-incubation and stimulation with DHβE (1μM) alone ■ showed no [^{3}H]-dopamine release. 8 wells per drug treatment were used in each experiment and this was repeated in 3 experiments (n=3). * = Significantly different from nicotine alone ($p<0.05$) using Student's unpaired t-Test. + = Significantly different from cotinine alone, $p<0.05$ using Student's unpaired t-Test.*

DHβE is a β_2* nAChR antagonist (Harvey *et al* 1996, Alkondon & Albuqurque 1993), and was used in this experiment to determine whether the β_2* subunit is involved in nicotine and cotinine - evoked dopamine release. Figure 2.4 shows β_2* is one of the receptors involved in nAChR evoked [^{3}H]dopamine release from dopaminergic terminals. Nicotine shows a 89% reduction in nicotine evoked [^{3}H]dopamine release in the presence of DHβE compared to a 54% reduction for cotinine in the presence of DHβE, however it does not produce complete antagonism as seen by mecamylamine in figure 2.2. The antagonism shown by DHβE is greater against nicotine than cotinine. This may be due to the high concentration of nicotine used, which may be causing a desensitisation of the receptors during pre-incubation in this assay and therefore adding to the reduction of the dopamine response by the actions of the antagonist DHβE.

2.4.4.2 αConotoxin MII

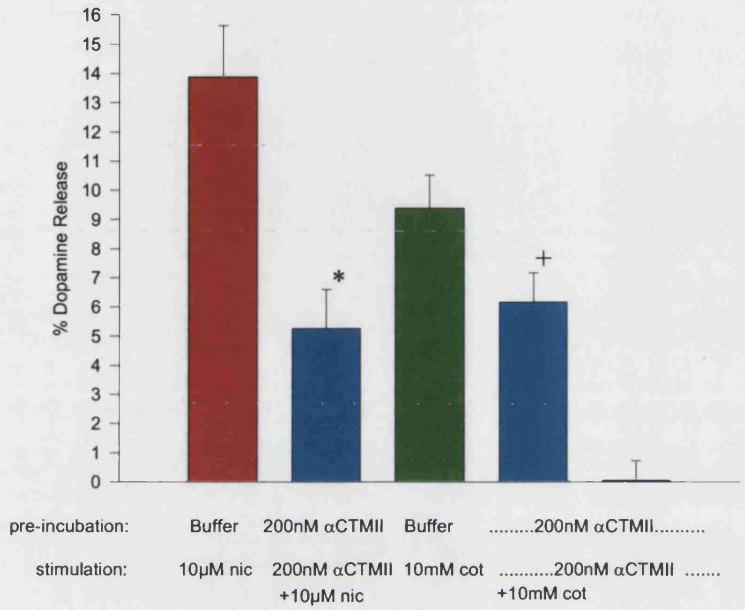


Figure 2.5: The effect of αConotoxin MII (αCTMII) on nicotine and cotinine – evoked [^{3}H]-dopamine release using 96-well plate assay.

*Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 (5 minutes) and stimulation with nicotine (10μM) and cotinine (10mM) (5 minutes) showed [^{3}H]-dopamine release similar to that seen in figure 2.2. When pre-incubated with αCTMII (200nM) and stimulated with both nicotine (10μM) and αCTMII (200nM) , nicotine-evoked [^{3}H]-dopamine release decreased significantly. When pre-incubated with αCTMII (200nM) and stimulated with both cotinine (10mM) and CTMII (200nM) , nicotine-evoked [^{3}H]-dopamine release also decreased significantly. Pre-incubation and stimulation with αCTMII (200nM) alone showed no [^{3}H]-dopamine release. 8 wells per drug treatment were used in each experiment and this was repeated in 4 experiments (n=4). * = Significantly different from nicotine alone ($p<0.05$) using Student's unpaired t-Test. + = Significantly different from cotinine alone, $p<0.05$ using Student's unpaired t-Test.*

αCTMII has been shown to be an $\alpha_3/\alpha_6\beta_2$* nAChR antagonist (Cartier *et al* 1996, Harvey *et al* 1997, Kaiser *et al* 1998), and was used in this experiment to determine whether the $\alpha_3/\alpha_6\beta_2$* subunits are involved in nicotine and cotinine - evoked dopamine release. Figure 2.5 shows $\alpha_3/\alpha_6\beta_2$* is one of the receptors involved in nAChR evoked [^{3}H]dopamine release from dopaminergic terminals. Nicotine shows a 62% reduction in

nicotine evoked [^{3}H]dopamine release in the presence of αCTMII compared to a 34% reduction for cotinine in the presence ofαCTMII, however it does not produce complete antagonism as seen by mecamylamine in figure 2.2.

2.4.4.3 α-Bungarotoxin

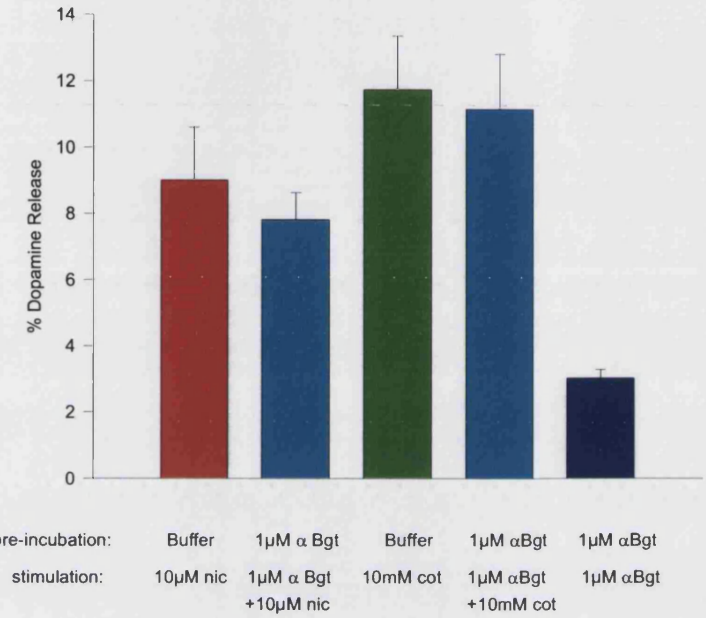


Figure 2.6: The effect of α-Bungarotoxin (αBgt) on nicotine and cotinine – evoked [^{3}H]-dopamine release using 96-well plate assay.

Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 (5 minutes) and stimulation with nicotine (10μM) ■ *and cotinine (10mM)* ■ *(5 minutes) showed [^{3}H]-dopamine release similar to that seen in figure 2.2. When pre-incubated with αBgt (1μM) and stimulated with both nicotine (10μM) and αBgt (1μM)* ■ *, nicotine-evoked [^{3}H]-dopamine release did not show a significant change. When pre-incubated with αBgt (1μM) and stimulated with both cotinine (10mM) and αBgt (1μM)* ■ *nicotine-evoked [^{3}H]-dopamine release also showed no significant change. Pre-incubation and stimulation with αBgt (1μM) alone* ■ *showed very little [^{3}H]-dopamine release. 8 wells per drug treatment were used in each experiment and this was repeated in 3 experiments (n=3).*

αBgt has been shown to be an α7 nAChR antagonist (Davies *et al* 1999), and was used in this experiment to determine whether the α7 subunits are involved in nicotine and cotinine - evoked dopamine release. However, under these conditions (figure 2.6), it does not show any significant effect. This may suggest α7 is not involved in

nicotine and cotinine - evoked [^{3}H]dopamine release in the striatum contrary to Kaiser & Wonnacott (2000). However these results may be due to αBgt's slow binding kinetics (Sharples & Wonnacott 2000, Davies *et al* 1999), and the short pre-incubation time of this assay, which may have been insufficient to allow αBgt to elicit an effect.

2.4.4.4 Optimisation of assay

This experiment was conducted as part of the optimisation of the assay before all experiments with αCTMII in this chapter were conducted. αCTMII is known to adhere to the surface of the wells during incubation. Addition of BSA in the buffer was predicted to reduce adhesion.

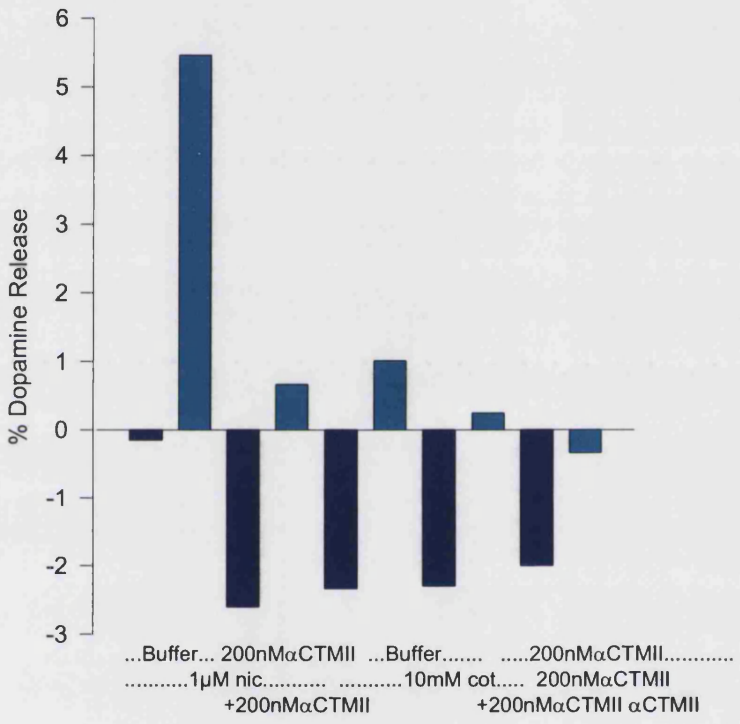


Figure 2.7: Effect of assay buffer with and without BSA (0.01%) on nicotine and cotinine – evoked [^{3}H]-dopamine release using 96-well plate assay.

Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with buffer 2 or 200nM αCTMII (5 minutes) and stimulation with nicotine (1μM) or cotinine (10mM) (5 minutes) showed BSA reduces the nicotine and cotinine – evoked [^{3}H]-dopamine release. BSA also increased the ability of αCTMII to block nicotine and cotinine – evoked [^{3}H]-dopamine release. However, αCTMII still blocked nicotine and cotinine – evoked [^{3}H]-dopamine release effectively without the addition of BSA. 8 wells per drug treatment were used in this experiment and this was only done once to acquire the effect of BSA.

As BSA was shown to reduce the effects of nicotine and cotinine – evoked [^{3}H]-dopamine release and αCTMII was shown to still be effective without BSA, it was concluded that BSA was not necessary for use in assays with αCTMII.

2.5 Discussion

2.5.1 Pharmacology

Nicotine has been shown in this study to evoke dopamine release in rat striatal minces in a dose dependent manner (figure 2.1). This is in accordance with previous published data by Dwoskin *et al* (1999), as well as in our laboratory; dopamine release in the striatum has been observed, in response to a local application of nicotine via the microdialyisis probe (Marshall *et al* 1997). The dose response curve for cotinine (figure 2.1) follows the same sigmoidal pattern as seen for nicotine (figure 2.1, Dwoskin *et al* 1999), in which the curve is shifted to the right. The EC_{50} values show that 1000-fold higher concentration of cotinine is required to elicit the same dopamine release as produced by nicotine. This is in accordance with the report that cotinine has a 1000-fold lower affinity for nicotine binding sites on nAChRs, than nicotine (Sharples & Wonnacott 2001, Abood *et al* 1981).

Both nicotine and cotinine - evoked dopamine release were shown to be inhibited by mecamylamine (figure 2.2), which is a non-selective nAChR antagonist (Dwoskin & Crooks 2001), suggesting that both drugs evoke dopamine release via nAChRs.

To find out which nAChRs are involved in the actions of nicotine and cotinine –evoked dopamine release, in this assay, 3 competitive antagonists which work on a variety of nAChRs were used. Competitive antagonists interact reversibly with the nAChR at or close to the agonist binding site, stabilising the receptor in a conformation with the channel closed and preventing access for agonists. This inhibition is surmountable with increasing agonist concentration.

DHβE is a purely competitive antagonist for neuronal nAChRs. At low concentrations, it has been shown to inhibit nAChR subtypes containing β2 (see section 2.1, Crooks *et al* 1995). In this assay, DHβE has shown a significant inhibition of the nicotine and cotinine–evoked dopamine release (figure 2.5), suggesting that β2 subunits are involved in this mechanism, which is in accordance with literature (Zoli et *al* 2002, Sharples *et al* 2000).

α-Conotoxin MII has previously been reported to act on α6β2 * subunits (see section 2.1, Zoli *et al* 2002, Sharples *et al* 2000). α-Conotoxin MII was shown to inhibit both nicotine and cotinine – evoked dopamine release in this assay (figure 2.5), suggesting involvement of the α6*β2* subunits in both the actions of nicotine and cotinine.

A slight decrease in nicotine and cotinine - evoked dopamine release is seen when α-bungarotoxin is present in the pre-incubation and stimulation stages (figure 2.6). This however, is not significant. This may suggest α7 subunits are not involved in this mechanism, however, as seen in previous publications (see section 2.1), α7 has been shown to be present in the striatum and has been shown to act on α7 subunits on glutamate terminals which are indirectly involved in nicotine – evoked dopamine release within the striatum (Kaiser & Wonnacott 2000). Also α-Bungarotoxin has been reported to act on α7 nAChR subunits by McGehee & Role (1995). There is however evidence that shows α7 nAChRs do not appear to mediate [^{3}H]dopamine release from striatal synaptosomes as the α7-selective antagonists α-bungarotoxin and α-conotoxin MII are without effect (Rapier *et al* 1990, Grady *et al* 1992, Kulak *et al* 1997). However, it has previously been noted that 50nM methyllcaconitine, a potent α7-selective antagonist, produced a partial inhibition of anatoxin-a-evoked [^{3}H]dopamine release from striatal synaptosomes (Kaiser & Wonnacott 2000), in agreement with the earlier observation of Clarke & Reuben (1996). Therefore, the lack of dopamine release inhibition by this antagonist, in this assay, may be explained by its slow association binding kinetics. Typically a pre-incubation of one hour with the toxin would be necessary to achieve a complete blockade using a low concentration of α-bgt (10nM) (Sharples & Wonnacott 2001, Davies *et al* 1999). A higher concentration may be needed to evoke a response in this assay, α7 components may only appear at 100 μM of nicotine (Wonnacott *el at* 2000). Relating this information to this assay, pre-incubation of 5 minutes of a 1μM concentration of α-bgt may not be sufficient to elicit an inhibition on nicotine and cotinine – evoked dopamine release.

Nicotine–evoked dopamine release was decreased when striatal minces were pre-incubated and stimulated with cotinine in a dose-dependent manner (figure 2.3). This suggests that cotinine is acting as an antagonist towards the actions of nicotine. However as figures 2.1. and 2.2 suggest, cotinine can also act as an agonist on nAChRs. As previously published, pre-treatment of synaptosomes with nanomolar concentrations of nicotine has shown a decrease in the ability of acute nicotine stimulation to evoke dopamine release (Marks *et al* 1993). This behaviour has been

attributed to nAChR desensitisation. This is when prolonged stimulation results in receptors entering a temporary inactive state (See section 1.2.1.5). This desensitisation theory can be attributed to exposure to cotinine causing desensitisation in this assay.

2.5.2 Implications of cotinine vaccine

This desensitisation theory is important in the hypothesis of the vaccine project (see section 1.8 & 1.9). As discussed in the introduction, the effects of cotinine are contradictory in the literature. The results in this chapter have been able to add to the theory of cotinine acting as an agonist on nAChRs. However, by desensitisation these receptors, it produces a decrease in nicotine - evoked dopamine release. If a cotinine specific vaccine can retain cotinine in the blood and therefore reduce its concentration in the brain, then nicotine will be able to produce a larger effect, resulting in reducing the need for nicotine from cigarettes.

Chapter 3

Trial 1

3.0 Production and verification of conjugates – Trial 1

3.1 Introduction

The aims of this trial were to conjugate the derivatives summarized in the appendix section 7.4 onto the carrier molecule ovalbumin, to allow for increased immunogenicity (see section 1.6.4 & 7.1). The conjugates were then formulated with adjuvant to increase the immune response, and injected into rats to examine the immune response. Blood nicotine and cotinine concentrations were also determined to demonstrate effect of vaccination after chronic nicotine treatment with osmotic minipump.

3.2 Materials

All chemicals were obtained from standard commercial suppliers and all equipment used is present at the University of Bath.

Buffers: Phosphate buffer; 100mM Na_2HPO_4, 100mM NaH_2PO_4.

Phosphate buffer saline (PBS); 150mM NaCl, 100mM Na_2HPO_4, 100mM NaH_2PO_4.

3.3 Conjugation of *N*-alkyl pyridyl cotinine (cotcooh) and *N*-alkyl pyridyl nicotine (niccooh) to ovalbumin

3.3.1 Methods

Conjugation reactions for both *N*-alkyl pyridyl cotinine and *N*-alkyl pyridyl nicotine are performed simultaneously and with the same protocol except for areas stated in the text.

The derivative (4mg) was dissolved in 4 drops of water and stirred (pH blow 6). Ten-fold molar excess 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (27.4mg for niccooh, 26.2mg for cotcooh) over derivative was added and stirred at room temperature for 5 minutes. Eighty-fold molar excess derivative was added to the derivative (8mg ovalbumin in 2ml phosphate buffer pH 7.4) and stirred overnight at room temperature. The solution was then dialysed against PBS(pH 7.4) at 4°C for 8 hours with 2 changes of PBS.

The chemical reaction between the derivatives and ovalbumin is shown in figure 3.1.

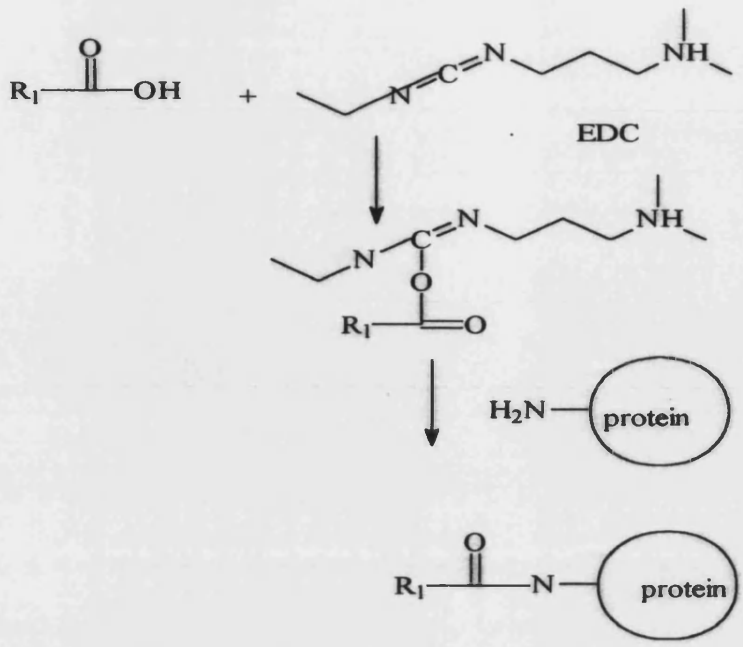


Figure 3.1: Conjugation of derivative containing a carboxyl group (N-alkyl pyridyl cotinine and N-alkyl pyridyl nicotine) with a carrier protein (ovalbumin).

The first stage of the reaction is the attachment of EDC to the carboxyl group of the derivative, producing an active site (R1 represents the remaining structure of the nicotine or cotinine derivatives). In the second stage of the reaction, the amine groups present in ovalbumin then react with the active site of the derivative to produce a carbodiiamide bond resulting in an ovalbumin-nicotine or ovalbumin-cotinine conjugate. There can be up to 15 derivative attachments per ovalbumin molecule.

3.3.2 Results

3.3.2.1 Conjugation was estimated photometrically

The conjugated product was tested by spectrophotometry using phosphate buffer as blank and 4mg/ml ovalbumin in phosphate buffer as control. The product was measured at 260nm and 280nm, and the ratio calculated. This was done to show a general picture of whether any conjugation had occurred.

Chemicals tested	λ260nm	λ280nm	Ratio
Ovalbumin alone	1.458	2.302	0.633
N-alkyl pyridyl cotinine conjugate	3.478	3.245	1.07
N-alkyl pyridyl nicotine conjugate	3.347	3.175	1.05

Table 3.1: Spectrophotometry results showing mean absorbance measured at λ260nm and λ280nm and the calculated ratio.

The OD260 values (figure 3.1) have increased significantly for both conjugates compared to that of ovalbumin alone. This is due to the addition of the pyridyl ring structures present in the derivatives, to ovalbumin, which are detected at the 260nm range. This has in turn increased the ratio. The increase in the OD280 values compared to ovalbumin alone, may be caused by the presence of the pyridyl ring structure which is also detected at this wavelength due to the overlap in the spectra. The results show the derivatives have been conjugated onto the carrier molecule although this test alone is inconclusive.

3.3.2.2 Conjugation was estimated by SDS-PAGE electrophoresis

To qualitatively determine the conjugation of the derivatives to ovalbumin, electrophoresis gels were run, stained with Coomasie blue, or used in Western Blotting for both conjugates. SDS-PAGE electrophoresis is used for separation of proteins by apparent molecular weight using an electric current. In the Coomasie blue staining technique, the separated proteins are dyed to allow for recognition. In the western blotting technique, separation is followed by electrophoretic transfer onto a suitable membrane, where the proteins are immobilized. This enables the membranes to be probed with specific antibodies, and subsequent secondary antibodies, to reveal the presence of individual proteins.

The gels were run under standard conditions as described in literature (Harlow, Lane, Antibodies - a laboratory manual, by Cold Spring Harbor Laboratory 1998).

Recipes for the SDS gels used were as follows:

SDS Gel 7.5%	Stacking gel
4.95 ml distilled H_2O	2.5 ml distilled H_2O
2.5 ml 1.5M Tris pH 8.8	1 ml 0.5M Tris pH 6.8
100 µl 10% SDS	10% AMPS
10% AMPS	5 µl TEMED
2.55 ml Acrylamide / Bisacrylamide	0.4 ml Acrylamide / Bisacrylamide

(Acrylamide/Bisacrylamide 30% stock 'Protogel'; National Diagnostics)
5 µl TEMED

Gels were cast in the Bio-Rad mini-Protean II SDS gel system between clean glass plates and 1mm spacers. Once the 7.5% SDS gel had set, stacking gel was applied with a 10 sample well comb and allowed to set.

Sample preparations already made up in Laemmli buffer (1:1, 20µl), (Laemmli buffer; 2%SDS, 100mM Dithiothreitol (DDT), 6mM Tris pH 6.8, 0.01% bromophenol blue), were thawed and 20µl were then loaded onto the gel along with BioRat marker (5µl). Ovalbumin in phosphate buffer (4mg/ml) was used as control. This was run for 10 minutes at 80V and 200V for 45 minutes in a Bio-Rad mini-protean II system with 80ml of 5X electrode buffer (0.12M Tris, 1.2M glycine 0.5% SDS, diluted in distilled H_2O to give 400ml).

3.3.2.3 Electrophoresis gel was stained with Coomasie Blue

One gel from section 3.3.2.2 was stained with Coomasie blue for 4 hours using method described in literature (Harlow, Lane, Antibodies - a laboratory manual, by Cold Spring Harbor Laboratory 1998).

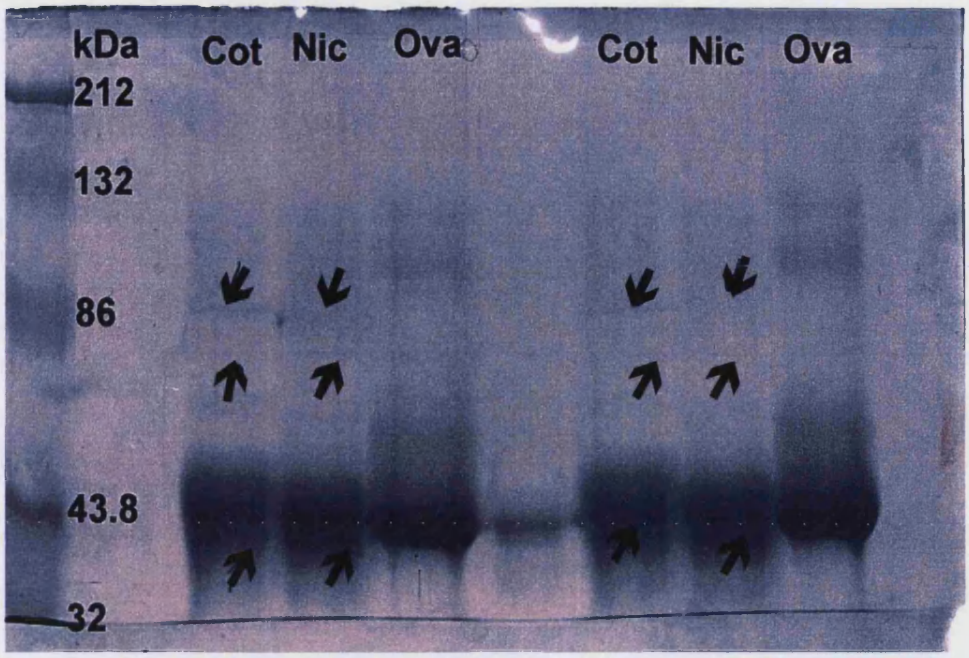


Figure 3.2: SDS-PAGE gel stained with Coomasie blue showing N-alkyl pyridyl cotinine and N-alkyl pyridyl nicotine conjugates, compared with untreated ovalbumin.

Samples (20μl) of N-alkyl pyridyl cotinine-ovalbumin conjugate (Cot) and N-alkyl pyridyl nicotine-ovalbumin conjugate (Nic) and ovalbumin alone (Oval) were subjected to SDS-Page as described in section 3.3.2.2. Molecular weight standards (left hand well) were run in parallel. Gels were stained with Coomasie blue for 4 hours. Same samples are shown in duplicate on the gel.

The staining (figure 3.2) suggests that conjugation has occurred as there is a slight rise in the bands at 40-50 KDa compared to the ovalbumin alone wells. This is accounted for as ovlabumin has a MW of 46 KDa and the rise in the band is caused by the addition of several derivatives onto the ovalbumin molecule. There are several lines between the 80-100kDa regions, which are not present in the ovalbumin only wells. This may have been caused by the conjugation of two ovalbumin molecules together, as the MW is approximately double that of one ovalbumin molecule. This gel is not very accurate as there is an over loading of the wells with conjugate and ovalbumin. This was a preliminary experiment and due to constraints of time, it was not repeated to get better band definition. It was used purely as a quick observation to decide whether to move onto immunisation.

3.3.2.4 Western Blotting was conducted on electrophoresis gel

Western blotting was conducted using method described in literature on one gel from section 3.3.2.2 (Harlow, Lane, Antibodies - a laboratory manual, by Cold Spring Harbor Laboratory 1998), with the following changes.

The gel was blotted using Sigma semi-dry blotting transfer equipment onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) previously soaked in transfer buffer (0.05M Tris, 0.05M glycine, 0.0375% SDS, 20% methanol), at 80 mA for 40 minutes. The methanol prevents the gel swelling and keeps proteins adsorbed to the nitrocellulose membrane.

After blotting, the non-specific binding sites were blocked in 4% non-fat milk powder (Bio-Rad Laboratories Inc) / PBS for 1 hour on a shaker at room temperature. The membranes were probed with primary antibodies (sheep anti-cotinine antibody 4μl in 20ml blotto;1% Marvel / PBS + 0.05% Tween-20) on a shaker at 4°C overnight. The blots were washed 3 times for 5 minutes with PBS then incubated on a shaker at room temperature for 1 hour with HRP conjugated secondary antibodies (anti-sheep horse raddish peroxidase, 4μl in 20ml blotto), diluted to manufacturers' recommended concentration. Secondary antibodies were removed and blots were washed a further 3 times with PBS. Electro-chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech AB, Uppsala Sweden) was prepared according to instructions, (1:1 solution 1 and 2, to give 7 ml/blot), and applied to blots for 1 minute. Excess ECL reagent was removed and the membranes were then wrapped in plastic film and secured in a film holder, blots were then exposed to photographic film to visualise, typically exposure times ranged from 30 seconds to 6 minutes.

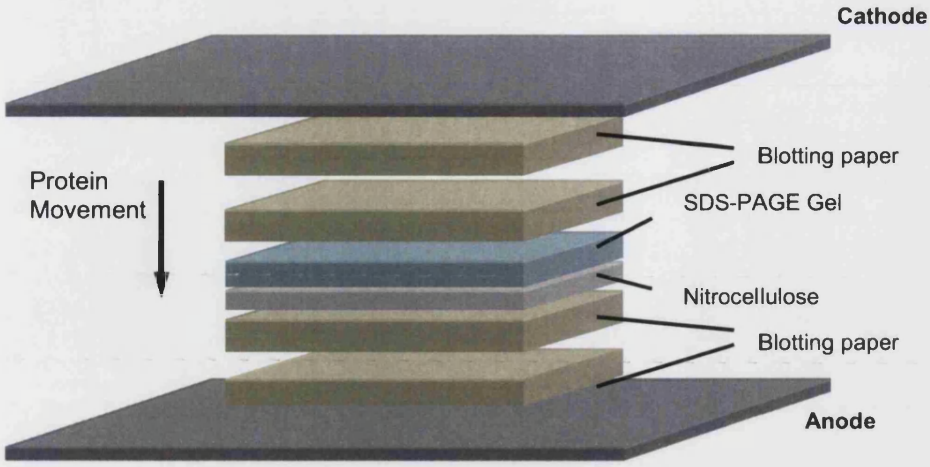


Figure 3.3: Diagram of semi-dry blotting apparatus. *(Picture by Dr Amy Bradly)*
Blotting paper soaked in transfer buffer sandwiches the nitrocellulose membrane and the SDS gel containing the elecrophoretically separated proteins. A current is applied through the electrodes causing the proteins to migrate out of the gel matrix onto the nitrocellulose membrane.

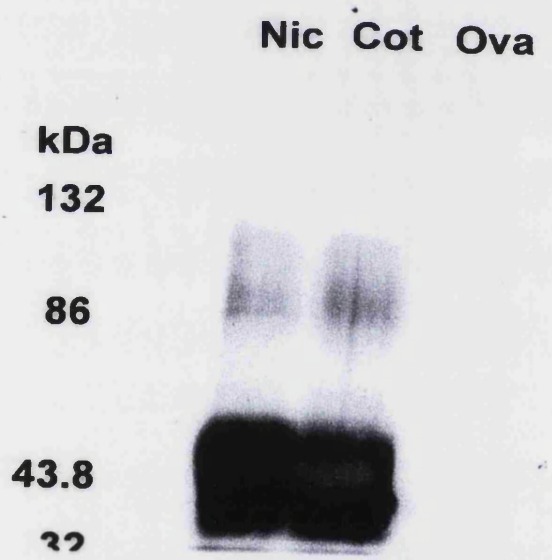


Figure 3.4: Western blot of N-alkyl pyridyl cotinine and N-alkyl pyridyl nicotine conjugates, compared with untreated ovalbumin.
Samples (20μl) of N-alkyl pyridyl cotinine (Cot) and N-alkyl pyridyl nicotine (Nic) conjugates and ovalbumin alone (Ova) were subjected to SDS-Page and western blot using sheep anti-cotinine antibodies as primary antibody, as described in section 3.3.2.4. Molecular Weight standards (left hand well) were run in parallel. Same samples are shown in duplicate on the gel.

The results (figure 3.4) show that sheep anti-cotinine antibody has recognized the nicotine and cotinine conjugates but not the ovalbumin alone. This shows conjugation has occurred, although the sheep anti-cotinine antibody can not distinguish between the nicotine and cotinine conjugates. This is not cause for alarm at this stage as commercial antibodies may produce such results. The bands are in similar regions as presented in the Coomasie blue gel in figure 3.2, however better results can be obtained with less loading of the wells with conjugate to obtain better band definition. Due to time constraints this blott was not repeated.

3.4 Conjugation of trans-4-thiol cotinine (cotsh) to ovalbumin

3.4.1 Conjugation methods

Before the conjugation reaction could be performed, trans-4-thiol cotinine was tested to find out whether oxidation of the sulfhydryl group had occurred, despite the derivative being stored under nitrogen. To do this and also to quantify the conjugated product at the end of the experiment, Ellmans' Reagent (5,5'-dithiobis-(2-nitrobenzoic acid) was used, which reacts with the sulfhydryl groups on the derivative, to produce a chromophore with maximum absorbance at 412nm. This assay will estimate the total numbers of peptide sulfhydryls present before and after conjugation.

Imject Maleimide Activated Immunogen Conjugation Kit was used to perform the conjugation. This experiment was conducted in 3 stages.

1. Conjugation buffer (200μl) provided in the conjugation kit was added to all wells in a microwell plate. Hapten solutions (10μl) were added in duplicate wells. The hapten solutions were cysteine standards used for the standard curve (a range from 3.0mM, 2.5mM, 2.0mM, 1.5mM, 1.25mM, 1.0mM, 0.75mM, 0.5mM, 0.25mM in conjugation buffer). The derivative (3.6mg/ml) was dissolved in conjugation buffer. 10μl was added in separate duplicate wells. Conjugation buffer (10μl) was added in separate duplicate wells as control. Ellmans' Reagent (20μl, 1mg/ml reagent in conjugation buffer) was then added to all wells. The plate was incubated for 15 minutes at room temperature and absorbance was measured at 412nm using a microwell plate reader.
2. Pre-activated ovalbumin molecule (0.6mg/ml) provided in the conjugation kit was dissolved in deionised water and the derivative (20 μl) made in stage 1 was added. The solution was incubated at room temperature for 2 hours. This was then dialysed against PBS at 4°C for 8 hours with 2 changes of PBS.
3. The Ellmans' test was conducted in the same manner as in stage 1, using 20μl of the conjugated solution produced in stage 2.

3.4.2 Conjugation was estimated photometrically - results

Standard curves showing absorbance at 412nm for cysteine solutions over a range of concentrations were drawn for both stages 1 (figure 3.5) and 3 (figure 3.6) of the Ellmans' test.

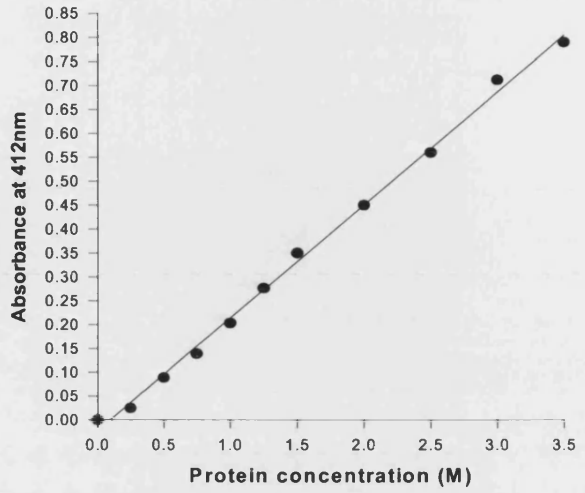


Figure 3.5: Standard curve showing absorbance at 412nm for cysteine solutions over a range of concentrations from stage 1 of the Ellmans' test.

The sulfhydryl group absorbance reading before conjugation was 0.784 nm, giving a 3.39M concentration of sulfhydryl groups present.

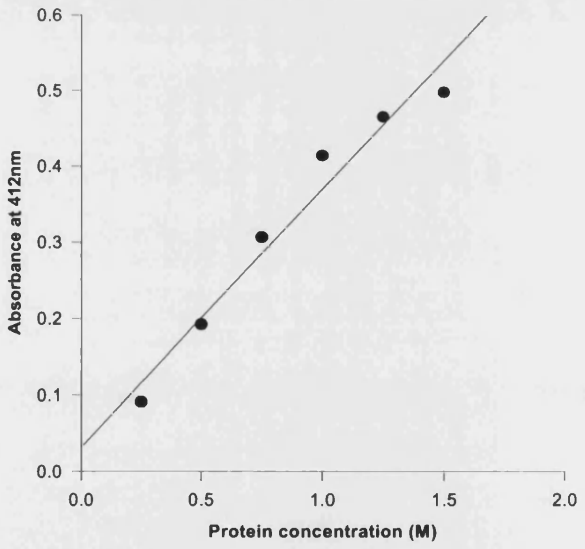


Figure 3.6: Standard curve showing absorbance at 412nm for cysteine solutions over a range of concentrations from stage 3 of the Ellman's test.

The activated ovalbumin conjugated to trans-4-thiol cotinine produced an absorbance reading of 0.003 nm, giving a 0.008M concentration of sulfhydryl groups present after conjugation.

The standard curves allowed for the calculation of the concentration of derivative before and after conjugation to ovalbumin (table 3.2).

	Mean absorbance at 412nm (nm)	Sulfhydryl derivative Concentration (M)
Before conjugation	0.784	3.39
After conjugation	0.003	0.008

Table 3.2: Spectrophotometry results showing mean absorbance measured at λ412nm and concentration of sulfydryl derivative, present before and after conjugation to ovalbumin.

The concentration of derivative was 3.39M before conjugation, which decreased to 0.008M after conjugation to ovalbumin. This is because the free sulfhydryl derivatives which are recognized in the Ellmans' test, were no longer present due to reaction with the ovalbumin molecule as shown in figure 3.7. However, these results alone are inconclusive.

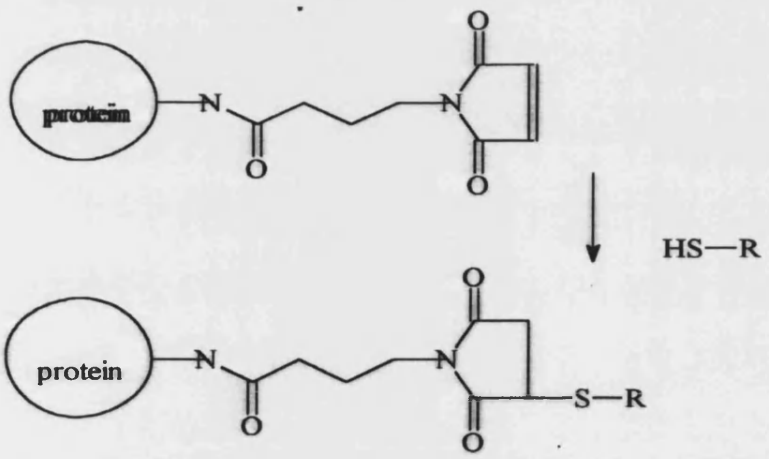


Figure 3.7: Conjugation of derivative containing a sulfhydryl group (Trans-4-thiol cotinine) with a carrier protein (ovalbumin).

Activated ovalbumin from Imject Maleimide Activated Immunogen Conjugation Kit contains the active site (R1 represents the remaining structure of the nicotine or cotinine derivatives), which reacts with the sulfhydryl group on the derivative producing a maleimide bond, resulting in an ovalbumin-cotinine conjugate.

3.4.3 Conjugation was estimated by SDS-PAGE electrophoresis and coomasie blue staining

To qualitatively determine the conjugation of the derivative to ovalbumin, electrophoresis gels were run, and stained with Coomasie blue, using the same protocol as for the *N*-alkyl pyridyl cotinine and *N*-alkyl pyridyl nicotine molecules (see Section 3.3.2.2 & 3.3.2.3).

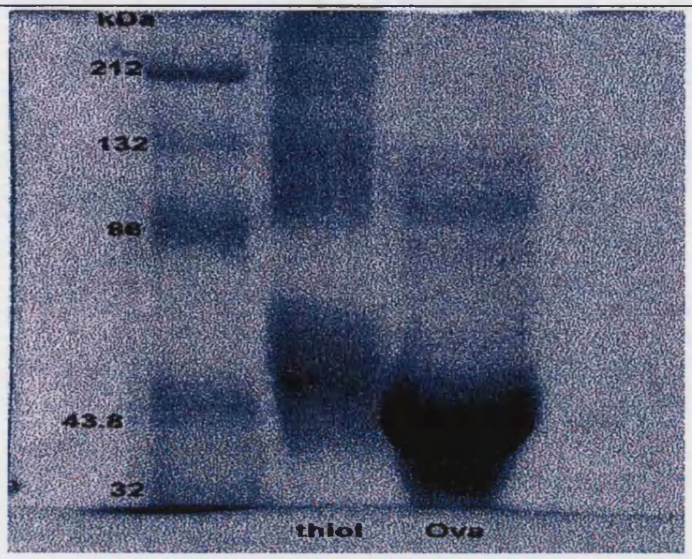


Figure 3.8: SDS-PAGE gel stained with Coomasie blue showing trans-4-thiol cotinine conjugate, compared with untreated ovalbumin.

Sample (20μl) of trans-4-thiol cotinine conjugate (thiol) and ovalbumin alone (Ova) were subjected to SDS-Page as described in section 3.3.2.2. Molecular Weight standards (left hand well) were run in parallel. Gels were stained with Coomasie for 4 hours.

The staining (Figure 3.8) shows that conjugation has occurred as there is a definite rise in the band in the thiol column, meaning an increase in the molecular weight of the conjugated molecules compared to the ovalbumin alone caused by the attachment of several derivatives. This band is broad, perhaps due to varying numbers of sulfhydryl groups attached to the ovalbumin molecule or due to the overloading of the wells with the conjugate as seen in the ovalbumin well. Due to time constraints this experiment was not repeated. There are also bands present around the 80-100Kda MW as seen in figure 3.2 perhaps due to the conjugation of two ovalbumin molecules together.

3.4.4 Western Blotting was conducted on electrophoresis gel

To qualitatively determine the conjugation of the derivative to ovalbumin, electrophoresis gels were run, and western blotting conducted with the same protocol as for the *N*-alkyl pyridyl cotinine and *N*-alkyl pyridyl nicotine molecules (see Section 3.3.2.2 & 3.3.2.4).

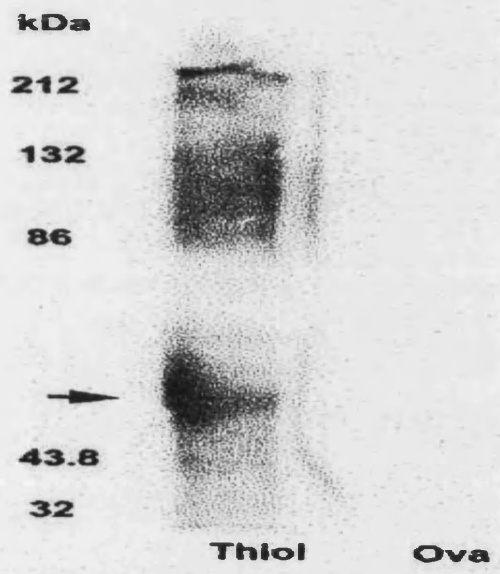


Figure 3.9: Western blot of trans-4-thiol cotinine conjugate, compared with untreated ovalbumin.

Samples (20μl) of which trans-4-thiol cotinine conjugate (Thiol) and ovalbumin alone (Ova) were subjected to SDS-Page and western blot using sheep anti-cotinine antibodies as primary antibody, as described in section 3.3.2.4. Molecular Weight standards (left hand well) were run in parallel.

The western blot (Figure 3.9) shows that the sheep anti-cotinine antibody has recognized the cotinine conjugate but not the ovalbumin alone. This shows conjugation has occurred. Again the band seen is broad which can be improved with less loading of the wells. This was not done due to time constraints.

3.5 Formulation of vaccines

The N-alkyl pyridyl cotinine, N-alkyl pyridyl nicotine and trans-4-thiol derivatives conjugated to ovalbumin were formulated for vaccination into rats. Ovalbumin alone was used as control conjugate. Rats injected with saline alone were also tested as control. All vaccines were made in the same manner. Alum (10mg/ml) from GlaxoSmithKline was used and diluted to 4mg/ml in PBS. Alum was added to

conjugate drop wise while vortexing over 1min. This was left for 30 minutes at room temperature, in which time vortexing every 10 minutes for 1 minute each time. An experiment plan is shown in more detail in table 3.3.

3.6.1 Injection regime

The injection regime used was recommended by Dr Pascal Mettens from GlaxoSmithkline and taken from Heida *et al* (2000) with variations (Also see section 1.7). The same regime was applied for all animals. Injections were given subcutaneously. Following immunization, minipumps were used to study the effect of chronic nicotine administration for a 14-day period.

Injection regime for 34 rats (This is explained further in table 3.3):

Day 1	Bleed & first injection
Day 21	Bleed & second injection
Day 35	Bleed & first booster
Day 43	Bleed & minipump insertion
Day 57	Kill & brain dissection & blood collection

The rats used varied in weight between 220g and 290g at the start of the experiment. This was the closest range of weight possible with the rats available. The rats were 6-7 weeks old at the beginning of the experiment, which is the age in which the parental antibodies are no longer able to cause any effect on the production of antibodies against this vaccine.

It was noted that no difference was seen in rat behaviour or locomoter activity after injections and minipump insertion.

3.7 Surgical Procedure

All surgical procedures that are described in this thesis conform to UK Home Office Regulations and were performed under project licence number PPL 30/2112 .

3.7.1 Materials & Animals

Male Sprague-Dawley (SD) rats, weighing 220-290g from the University of Bath breeding colony were used. (-)nicotine base was obtained from Sigma Chemicals Co. (Poole, Dorset, UK). Alzet™ osmotic minipumps (Model 2001) were purchased from Charles River Laboratories (UK). Wound clips were from Scientific Laboratory Supplies (Nottingham, UK). Other miscellaneous surgical equipment and chemicals were obtained from standard sources.

3.7.2 Methods

3.7.2.1 Blood taken for antibody titre determination

The rats were injected subcutaneous with corresponding vaccines and bleeding was done via tail lateral vein, under general anaesthetic (Isoflurane). Approximately 200µl of blood was taken at the time of each bleed. This was heated at 37°C for 30 minutes, centrifuged at 4°C, 1500G, for 30 minutes, serum taken off and centrifuged again at 4°C, 1500G, for 30minutes. The final serum was taken off, 0.01% sodium azide added and frozen at -70°C.

3.7.2.2 Chronic nicotine treatment of rats by 14-day osmotic minipumps

The rats previously immunized with cotinine and control vaccine were chronically treated with (-)nicotine delivered via osmotic minipumps for 14 days, 4mg (-)nicotine base/kg/day. Minipumps were loaded with either nicotine base in saline or saline as control, with the pH adjusted to 7.4 with conc. HCl. The concentration of nicotine in the pump was calculated according to the following equation:

$$\frac{\text{4mg/kg/day x estimated weight of rat on day 7}}{\text{24hrs x Pumping Rate of Minipump/hr (in ml)}}$$

The answer gives the concentration of free base nicotine to be used in mg/ml.
Pumping rate of the minipumps used was 0.00049 ml/hour. The nicotine was inserted into the minipump and % efficiency of filling the pump was calculated by:

$$\frac{\text{Weight of pump after filling – weight of pump before filling}}{\text{maximum volume of minipump available (229µl) x 100.}}$$

All minipumps were within 97-100% filled. All nicotine solutions were made in PBS pH 7.4, and filtered with 0.2µl filter. For control rats, minipumps filled with only PBS were used.
Animals were placed under isoflourane anaesthesia (3% isoflourane:1.4l/min O_2), and minipumps implanted dorsally, 2-3cm behind the shoulder blades. This area was shaved with electric clippers and swabbed with 70% ethanol / 0.5% hibitane. A 1.5cm incision was made and blunt dissection performed to create a subcutaneous skin pocket large enough to accommodate the minipump. The minipump was swabbed with isopropanol and inserted, with the delivery portal facing towards the tail of the animal.

Antibiotic powder (Cicatrin™) was puffed into the wound and the incision closed using 2-3 wound clips. The minipumps start releasing nicotine after 4 hours of being in the rat therefore the time was noted and rats were sacrificed 14 days after this time. When the rats were sacrificed the minipump was taken out and checked to see if the membrane inside had collapsed therefore nicotine delivered, and the delivery tube not blocked. All minipumps used were shown to have worked at the end of the experiment. Behaviour of the animal was monitored repeatedly throughout the 14 days. At the end of the 14 days the animals were sacrificed using a schedule 1 method. Trunk blood was taken for serum nicotine and cotinine concentration analysis (section 3.9) and antibody titre analysis (section 3.8).

3.7.2.3 Blood taken for nicotine and cotinine concentration determination

This was done after the rats are killed and trunk blood was collected in ependorf tubes containing 10µl of 20 units/ml heparin. Blood was kept on ice and centrifuged at 2500 rpm for 15 minutes. The serum was removed, and re-centrifuged in the same manner. The final sera were frozen at -20°C and sent to ABS Laboratories for analysis. ABS Laboratories determined the nicotine and cotinine concentrations in the serum by Gas Chromatography.

No. of rats	Vaccine type	Amount of conjugate	Amount of alum	Minipump treatment
3	Saline	Saline - 150µl	-	Nicotine
3	Saline	Saline - 150µl	-	Saline
5	Ovalbumin	120µg in 30µl	120µl-4mg/ml	Nicotine
5	Ovalbumin	120µg in 30µl	120µl-4mg/ml	Saline
3	*N*-alkyl pyridyl nicotine	120µg in 30µl	120µl-4mg/ml	Nicotine
3	*N*-alkyl pyridyl nicotine	120µg in 30µl	120µl-4mg/ml	Saline
3	*N*-alkyl pyridyl cotinine	120µg in 30µl	120µl-4mg/ml	Nicotine
3	*N*-alkyl pyridyl cotinine	120µg in 30µl	120µl-4mg/ml	Saline
3	Trans-4-thiol cotinine	120µg in 30µl	120µl-4mg/ml	Nicotine
3	Trans-4-thiol cotinine	120µg in 30µl	120µl-4mg/ml	Saline

Table 3.3: Experiment plan showing number of SD rats used in each treatment group, amount of conjugate and adjuvant used and osmotic minipump treatment.

Each injection is made up of conjugate, saline and alum as described in this table. The injection regime is shown in section 3.6.1. There is no difference in composition of the solution administered in the injections or boosters. The difference is only in name relating to when in the injection regime, vaccination is conducted.

3.8 Enzyme-Linked Immunosorbent Assay (ELISA)

3.8.1 Materials

Maxisorp 96-well plates (Nunclon), were used for ELISA antibody titre determination.
Carbonate buffer - 1 tablet in 100ml deionised water.
Saturation buffer – PBS,Tween 20 (0.1%), Decomplemented Newborn Calf Serum (4%), BSA(1%).

3.8.2 Methods & Results

3.8.2.1 Measurement of antibody titres towards ovalbumin in sera from rats injected with ovalbumin

This experiment was done to determine the amount of antibodies produced towards ovalbumin, in control animals, which were injected with only ovalbumin.

Methods

Maxisorp 96 well plates were coated with ovalbumin (10μg/ml in carbonate buffer, 50μl), and left overnight at 4°C. Plates were emptied and saturated with saturation buffer plus caseine (1%) (100μl, pH 7.4) for 1 hour at 37°C. Sera from ovalbumin-injected rats were serially diluted (1:2 times each time) starting from 1:200 dilutions in saturation buffer.

50μl of each dilution was added to the wells and incubated at 37°C for 1 hour. Plates were washed 3 times with PBS-Tween (0.1%). Biotinylated anti-rat Ig from sheep was prepared (1:3000) in saturation buffer and added to wells (50μl). This was incubated for 1 hour 15 minutes at 37°C. Plates were washed 3 times with PBS-Tween (0.1%) and incubated with streptavidine-biotinylated HRP (1:1000 in saturation buffer, 50μl), for 30 minutes at 37°C. Plates were washed 5 times with PBS-Tween (0.1%)and incubated with single component TMB peroxidase (50μl) for 10 minutes in the dark. Sulphuric acid was then added (0.4 N, 50μl) and absorbance measured using a plate reader at 420nm and 620nm and the difference taken away to give final absorbance.

Results

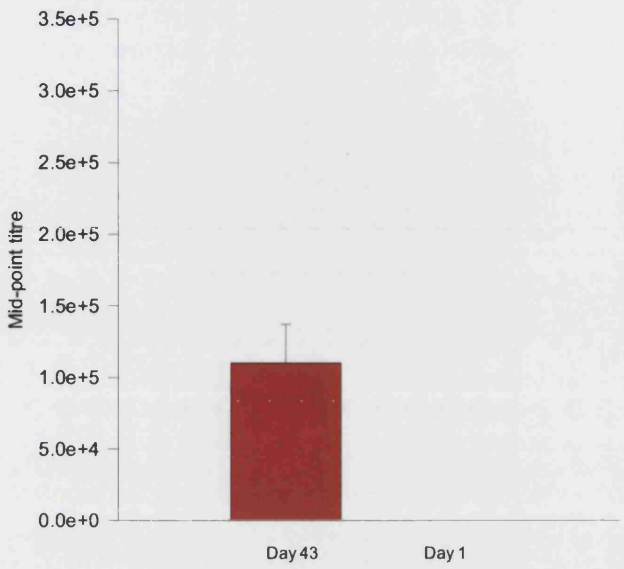


Figure 3.10: Mid-point antibody titres from rats vaccinated with ovalbumin alone.
The titres were measured in sera from rats on days 1 before injection and day 43 after injection with ovalbumin alone, and tested by ELISA using ovalbumin-coated wells. There are 10 rats in each group.

The results (figure 3.10) are expressed as midpoint titres using SoftMaxPro software. No titres were produced for rats on day 1 as expected. These results show mean antibody titres of 110082±27175 from 10 rats injected with control vaccine containing ovalbumin only, from sera taken on days 43. The titres obtained against ovalbumin are high as expected, and shows that ovalbumin alone is producing a good immune response. There is a large standard error which is normal in animal studies and can be associated to inter-animal variations. Sera from day 1 show no antibody titres against ovalbumin as expected. Only sera from days 1 and 43 were tested to keep costs down. This shows the immune response after all injections were made.

3.8.2.2 Measurement of titres towards conjugates in sera from rats injected with each conjugate

This was done in four assays to determine the best ELISA method for obtaining accurate antibody titres. The first method was to coat the 96-well plates with the conjugate, the second method was to determine whether the titres obtained were from

the effects of ovalbumin or from the conjugates, ie. Are antibodies raised towards the target conjugate? The third method was to coat the 96-well plates with derivative only, and the fourth method was a sandwich ELISA.

3.8.2.2.1 Conjugate coated 96-well plates

Method

The method is the same as in section 3.8.2.1 with the following variations:

Maxisorp 96 well plates were coated with conjugates ie. Derivatives attached to ovalbumin (10μg/ml in carbonate buffer, 50μl), and left overnight at 4°C. Plates were also coated with ovalbumin in the same way as mentioned in section 3.8.2.1. Serum from rats injected with the three conjugates, were serially diluted (1:2 times each time) starting from 1:200 dilutions in saturation buffer. 50μl of each dilution was added to the wells and incubated at 37°C for 2 hour.

Results

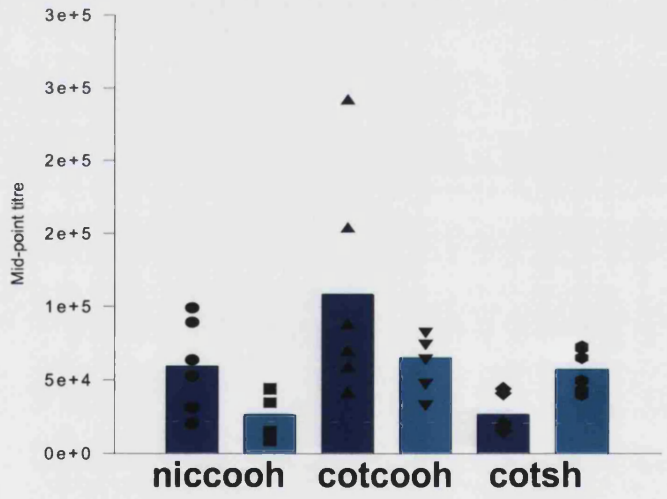


Figure 3.11: Mid-point antibody titres from rats vaccinated with N-alkyl pyridyl nicotine (niccooh), N-alkyl pyridyl cotinine (cotcooh) and trans-4-thiol cotinine (cotsh).

The titres were measured in sera from rats on day 43 after injection with the three vaccines and tested by ELISA using ovalbumin-coated wells ■ and corresponding conjugate coated wells ■. There were 6 rats in each group with the bars illustrating mean titre and symbols representing each rat.

The results show (figure 3.11) all sera demonstrate antibodies raised against ovalbumin on plates coated with ovalbumin. When sera were tested on plates coated

with the corresponding conjugate, all plates again demonstrated titres. These titres are largely due to antibodies raised against ovalbumin. However, for N-alkyl pyridyl nicotine and N-alkyl pyridyl cotinine sera, there is a fall in titres tested on conjugate coated plates compared to the corresponding ovalbumin coated plates. This may be because all antibodies raised against ovalbumin alone or conjugate are not detected in this assay. It is not possible to distinguish whether the titres shown are towards ovalbumin alone or from the conjugate. The point to note here is the increase in titres in the trans-4-thiol cotinine injected sera tested on the conjugate coated plates compared to ovalbumin coated plates. This demonstrates that antibodies have been raised against the conjugate as well as ovalbumin alone, however this assumption is not conclusively proven in this experiment and is in need of further investigation. This effect of antibodies raised against ovalbumin is tested further in section 3.8.2.2.2.

3.8.2.2.2 Effect of ovalbumin on measurement of antibody titres in plates coated with conjugate.

This experiment was conducted to examine the effect of antibodies raised against ovalbumin, on the antibody titres produced against the conjugates (see section 1.7.6). This was done by binding ovalbumin antibodies in the sera to ovalbumin coated plates, removing the remaining serum from those wells and placing it in conjugate coated plates. The ELISA was then conducted to find out whether this one fold dilution of ovalbumin antibodies had any effect on the titres shown for the conjugate.

Methods

Maxisorp 96 well plates were coated with ovalbumin (10μg/ml in carbonate buffer, 50μl), and left overnight at 4°C. Plates were emptied and saturated with saturation buffer plus caseine (1%) (100μl, pH 7.4) for 1 hour at 37°C. Serum from rats injected with the three conjugates, were serially diluted (1:2 times each time) starting form 1:20 dilutions in saturation buffer. 50μl of each dilution was added to the wells and incubated at 37°C for 2 hour. The serum was then taken out of the wells and placed on new plates coated with derivatives conjugated to ovalbumin [96 well plates were coated with conjugates (10μg/ml in carbonate buffer, 50μl), and left overnight at 4°C. All plates were emptied and saturated with saturation buffer plus caseine (1%) (100μl, pH 7.4) for 1 hour at 37°C]. These new plates were then incubated for 1 hour at 37°C and washed 3 times with PBS Tween-20 (0.1%). Biotinylated anti-rat Ig from sheep was prepared (1:3000) in saturation buffer and added to the wells (50μl). This was incubated for 1 hour 15 minutes at 37°C. Plates were washed 3 times with PBS Tween

(0.1%) and incubated with streptavidine-biotinylated HRP (1:1000 in saturation buffer, 50μl), for 30 minutes at 37°C. Plates were washed 5 times with PBS Tween-20 (0.1%)and incubated with single component TMB peroxidase (50μl) for 10 minutes in the dark. Sulphuric acid was then added (0.4 N, 50μl) and absorbance measured by a plate reader at 450nm and 620nm and the difference taken away to give final absorbance.

Results

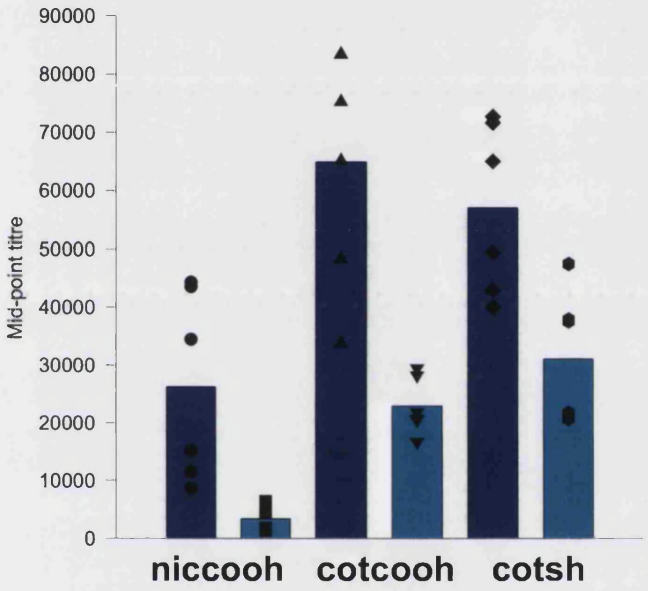


Figure 3.12: Mid-point antibody titres from rats vaccinated with N-alkyl pyridyl nicotine (niccooh), N-alkyl pyridyl cotinine (cotcooh) and trans-4-thiol cotinine (cotsh).

The titres were measured in sera from rats on day 43 after injection with the three vaccines and tested by ELISA using corresponding conjugate coated wells ■ and corresponding conjugate coated wells with one fold ovalbumin dilution ■ There were 6 rats in each group with the bars illustrating mean titre and symbols representing each rat.

The results (figure 3.12) show a marked decrease in titres with the one fold dilution for all sera tested on plates coated with their corresponding conjugates, compared to ELISA with no ovalbumin dilution. This suggests some of the ovalbumin antibodies have been taken out of the sera at the first plate stage, however a large amount may still remain which may be eliminated if more dilutions were done. This method does

not give accurate antibody titres generated towards the conjugates. Therefore, a more accurate ELISA method was investigated in section 3.8.2.2.3.

3.8.2.2.3 Derivative coated 96-well plates

This experiment was conducted using only derivatives, therefore eliminating coating of ovalbumin onto ELISA wells. The antibody titres obtained will therefore be for the derivative only and not ovalbumin.

Methods

The method is the same as in section 3.8.2.1 with the following variations. Maxisorp 96 well plates were coated with the three derivatives N-alkyl pyridyl nicotine (niccooh), N-alkyl pyridyl cotinine (cotcooh) and trans-4-thiol cotinine (cotsh), (1μg/ml in carbonate buffer, 50μl) and left overnight at 4°C. Sera from rats injected with the three conjugates were serially diluted (1:2 times each time) starting from 1:20 dilutions in saturation buffer. 50μl of each dilution was added to the corresponding wells and incubated at 37°C for 2 hour.

Results

These results (figure 3.13) show that there were no antibody titres measured from sera injected with N-alkyl pyridyl nicotine or N-alkyl pyridyl cotinine conjugates tested on plates coated with corresponding derivatives. This suggests that no antibodies specifically recognizing the N-alkyl pyridyl nicotine and N-alkyl pyridyl cotinine derivative sections of the conjugates were produced. Antibodies towards and trans-4-thiol cotinine were produced as shown in figures 3.13, 3.14, 3.15. Titres were produced as early as day 21 and reached their maximum on day 43. There was a reduction in titres after minipump treatment on day 57, although this was not significant. This reduction is an encouraging sign as it may be due to an antibody-conjugate complex being produced, therefore reducing the amount of antibodies being detected, as the antibody-conjugate complex is not likely to be detected by ELISA. These titres however are very low and are not sufficient for further neurochemical studies.

Figure 3.14 demonstrates the antibody titre curve on days 21, 35, 43 and 57, for a sample rat shown in figure 3.13. This illustrates both the changes in antibodies over time and the success of the cotinine derivative in stimulating the production of antibodies which recognise cotinine.

Figure 3.15 demonstrates the mid-point antibody titres for the same sample rat as in figure 3.13, on a linear scale on days 21, 35, 43 and 57. This illustrates the changes in antibodies with time and treatment.

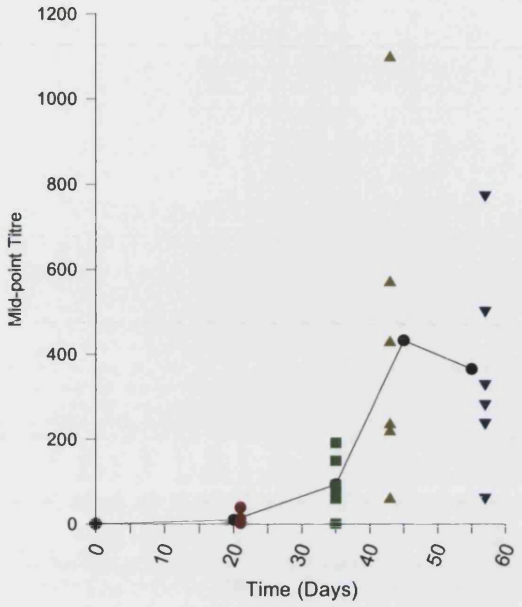


Figure 3.13: Mid-point antibody titres from rats vaccinated with trans-4-thiol cotinine.
The titres were measured for sera taken on days 21, 35, 43 and 57. The sera were tested by ELISA using trans-4-thiol cotinine derivative-coated wells. There were 6 rats in each group with the line illustrating mean titre, and symbols representing each rat.

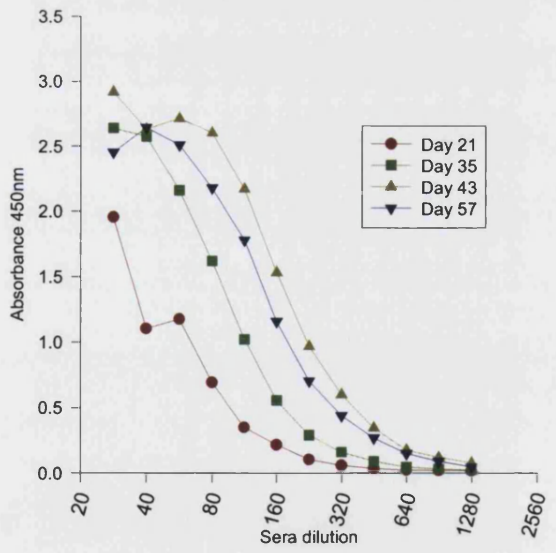


Figure 3.14: Antibody titre curve for one rat vaccinated with trans-4-thiol cotinine.
The titres were measured for sera taken on days 21, 35, 43 and 57. The sera were tested by ELISA using trans-4-thiol cotinine derivative-coated wells.

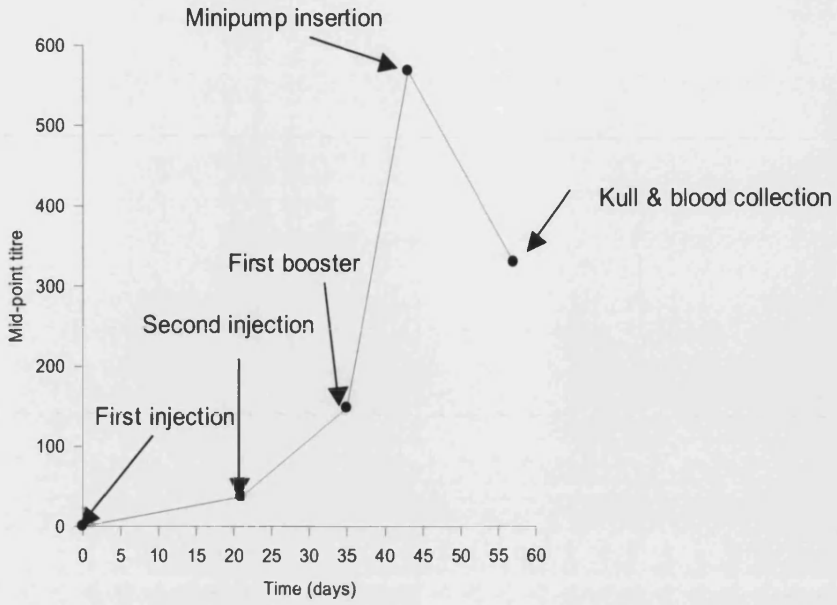


Figure 3.15: Mid-point antibody titre from one rat vaccinated with trans-4-thiol cotinine. *The mid-point titres were measured for sera taken on days 21, 35, 43 and 57. The sera was tested by ELISA using trans-4-thiol cotinine derivative-coated wells.*

3.8.2.2.4 Sandwich ELISA

This experiment was done to determine whether a sandwich ELISA would give more conclusive antibody titres.

Method

96 well plates were coated with commercial anti-cotinine IgG (10μg/ml in carbonate buffer, 50μl), and left overnight at 4°C. Plates were emptied and saturated with saturation buffer plus caseine (1%) (100μl, PH 7.4) for 1 hour at 37°C. The three different derivatives (1μg/ml in carbonate buffer, 50μl) were added to the plates and incubated for 1 hour at 37°C. The plates were washed 3 times with PBS Tween-20 (0.1%). Sera from rats injected with one of the three conjugates were serially diluted (1:2 times each time) starting from 1:20 dilutions in saturation buffer. 50μl of each dilution was added to the wells and incubated at 37°C for 1 hour. Plates were washed 3 times with PBS Tween-20 (0.1%). Biotinylated anti-rat Ig from sheep was prepared (1:3000) in saturation buffer and added to the wells (50μl). This was incubated for 1 hour 15 minutes at 37°C. Plates were washed 3 times with PBS-Tween (0.1%) and incubated with streptavidine-biotinylated HRP (1:1000 in saturation buffer, 50μl), for 30 minutes at 37°C. Plates were washed 5 times with PBS Tween (0.1%) and incubated

with single component TMB peroxidase (50μl) for 10 minutes in the dark. Sulphuric acid was then added (0.4 N, 50μl) and absorbance measured by a plate reader at 450nm and 620nm and the difference taken away to give final absorbance.

Results

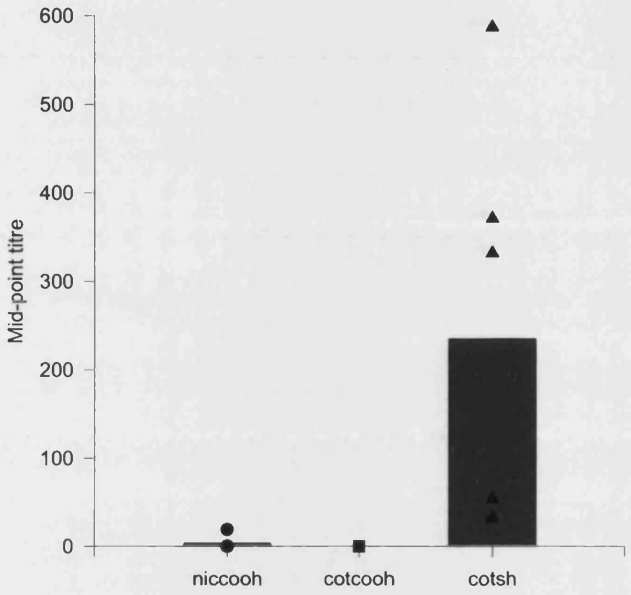


Figure 3.16: Mid-point antibody titres from rats vaccinated with N-alkyl pyridyl nicotine (niccooh), N-alkyl pyridyl cotinine (cotcooh) and trans-4-thiol cotinine (cotsh).
The titres were measured in sera from rats on day 43 after injection with the three vaccines and tested by sandwich ELISA. There were 6 rats in each group with the bars illustrating mean titre and symbols representing each rat.

These results (figure 3.16) show no antibody titres for N-alkyl pyridyl nicotine (niccooh) and N-alkyl pyridyl cotinine (cotcooh) conjugates. There are antibodies raised against the trans-4-thiol cotinine (cotsh) conjugate, hence confirming results in figure 3.13.
Figure 3.17 illustrates a correlation between mid-point titres illustrated in figure 3.15 for rats injected with trans-4-thiol vaccine on day 43 and sera tested on ELISA coated with derivative, and the same sera tested by sandwich ELISA. The sandwich ELISA shows a lower average mid-point titre than the derivative coated ELISA, suggesting that the derivative coated ELISA method gives the most accurate results. Therefore it was used in all antibody titre determination experiments in the rest of the project.

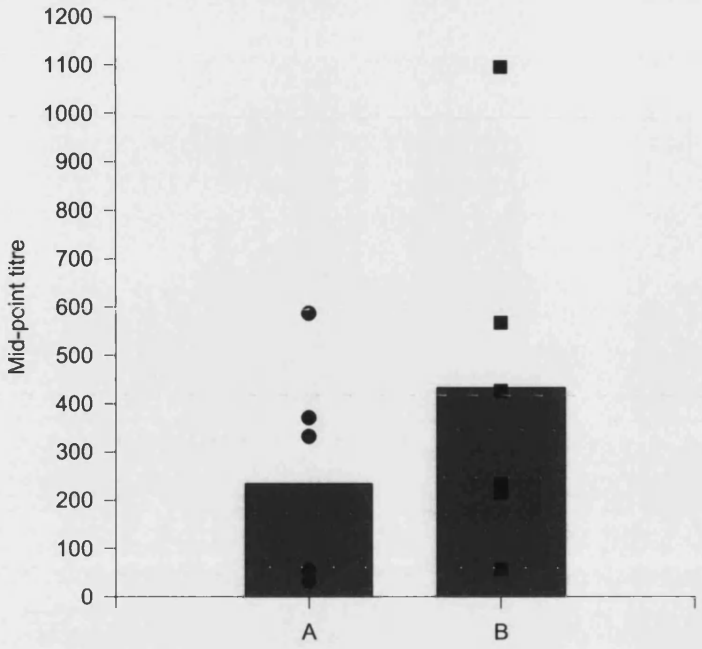


Figure 3.17: Mid-point antibody titres from rats vaccinated trans-4-thiol cotinine (cotsh). *The titres were measured in sera from rats on day 43 after injection with trans-4-thiol cotinine and tested by sandwich ELISA (A) and derivative coated ELISA (B). There were 6 rats in each group with the bars illustrating mean titre and symbols representing each rat.*

Control

Sera from a group of rats with saline injections were also tested as control, on both ovalbumin coated and derivative coated plates. The methods were the same as described in sections 3.8.2.2.3 and 3.8.2.1. The results showed no production of antibodies, as expected, towards ovalbumin or the derivatives.

3.9 Results of rat serum analysis for nicotine and cotinine concentrations in rats treated with nicotine by osmotic minipump.

The aim of this experiment was to measure the concentration of nicotine and cotinine in rat serum treated with nicotine minipump with the different vaccines. Dopamine release experiments were planned for this set of vaccinated rats, however due to the very low antibody titres obtained, these experiments were deemed too expensive to conduct.

Vaccine Type	[Nicotine] ng/ml	[Cotinine] ng/ml
Saline	90.1±17.2 (n=3)	461.4±67.4 (n=3)
Ovalbumin	98.1±12.9 (n=5)	461.0±55.0 (n=5)
N-alkyl pyridyl nicotine	92.4±12.4 (n=3)	443.1±64.0 (n=3)
N-alkyl pyridyl cotinine	77.6±26.4 (n=3)	433.0±15.6 (n=3)
Trans-4-thiol cotinine	67.9±31.5 (n=3)	573.3±21.5 (n=3)

Table 3.4: Nicotine and cotinine concentrations in sera from rats immunized with 4 different vaccines and treated with nicotine by osmotic minipump.

The results show (table 3.4), rats receiving saline had negligible levels of serum nicotine (mean 1.0 ± 0.2) and cotinine (mean 15.8 ± 4.8) in all vaccine types.

Nicotine treatment produced levels of nicotine and cotinine in the expected range in non- vaccinated and control vaccinated rats. (Sanderson *et al* 1993).

The cotinine concentration for the trans-4-thiol cotinine vaccine showed an increase compared to the saline and ovalbumin only control vaccines, (this was not significant using Student's unpaired t-Test). However, the slight increase is an encouraging sign as it may be due to an antibody-cotinine complex being produced, therefore cotinine (metabolised after chronic nicotine treatment) is retained in the blood probably by the precipitation mechanism of antibodies as described in section 1.7.4).

The cotinine concentration in the blood does not vary significantly in the presence of the *N*-alkyl pyridyl nicotine and *N*-alkyl pyridyl cotinine vaccines, compared to ovalbumin alone or saline vaccine. This may be because high antibody titres were not produced in response to these derivatives. Another reason may be that the antibodies did not recognise nicotine or cotinine respectively from those conjugates.

In contrast to cotinine, nicotine concentrations showed some variation between groups of conjugates. For the *N*-alkyl pyridyl nicotine vaccine, the nicotine concentration is the same as both control vaccines. This may also be because high antibody titres were not produced in response to the derivative, or another reason may be that the antibodies did not recognise nicotine.

The *N*-alkyl pyridyl cotinine and trans-4-thiol cotinine vaccines do show a slight fall in nicotine concentration in the blood (not significant using Student's unpaired t-Test). However, this may be due to inter-animal variation or it may be a consequence of the vaccine. If it is a real effect, this could mean antibodies have been produced in high enough titres and they recognise nicotine. Alternatively, antibodies may have recognised cotinine, retained it in the blood and therefore increased the rate of metabolism of nicotine to cotinine. Further study is needed to allow for conclusive answers.

3.10 Discussion

3.10.1 Conjugation

The 3 derivatives discussed in the appendix (section 7.4) were taken forward for conjugation to the carrier protein ovalbumin. The qualitative analysis conducted by Coomasie blue staining and western blot of the *N*-alkyl pyridyl nicotine, *N*-alkyl pyridyl cotinine and trans-4-thiol cotinine conjugates (section 3.3.2.3, 3.3.2.4, 3.4.3, 3.4.4) showed that conjugation had occurred. However, quantitative analysis is needed to determine how many molecules of the derivatives are attached to each ovalbumin molecule. This is important as up to 14 derivative attachments per carrier molecule are needed to produce a viable immune response (see section 1.6.4). HPLC work was conducted for quantitative analysis, however due to incomplete hydrolysis of the conjugate, I was not able to get accurate results. As this initial trial was performed as a learning experience, more quantitative analysis was left until trial 2.

3.10.2 Immunisation

The conjugates were taken forward for injection into animals to determine whether any immune response was produced. The four different ELISA methods tested showed that the derivative coated plate method identified the highest mid-point titres (section 3.8.2.2.3). This assay was developed for use in the next trial.

The *N*-alkyl pyridyl nicotine and *N*-alkyl pyridyl cotinine conjugates showed very low antibody titres. This may have been due to insufficient conjugation of derivatives onto the carrier molecule, therefore insufficient activation of the humoral and cell-mediated responses (as discussed in section 1.6.4). However, trans-4-thiol cotinine conjugate vaccine did induce an immune response and gave some encouraging titres. The antibodies identified were probably IgG immunoglobulins as these are the most abundant antibodies in the blood (see section 1.6.4). This may be due to a more robust conjugation of derivative to carrier molecule by maleimide bonds. Mid-point titres of approximately 10,000 are needed to produce a sufficient antibody response to allow for studies on the effects of the vaccine in the animal in relation to nicotine addiction as shown by Heida *et al* (1997). However, titres obtained in this trial were low and not sufficient to conduct neurochemical studies.

3.10.3 Effects of nicotine

Heida *et al* (2000) showed an increase in blood nicotine levels after nicotine vaccination, caused by the binding and accumulation of nicotine to the antibodies in serum. There was a trend in this trial of an increase in cotinine levels in the sera (discussed in section 3.9), however the results were not significant and this was repeated in the next trial to allow for better interpretation of the actions of the vaccine. Trans-4-thiol cotinine derivative was taken forward to the next trial, where modifications were introduced to increase the immune response.

3.10.4 Conclusions

This first trial allowed for the right conjugate (trans-4-thiol cotinine) to be taken forward to the next trial and lessons were learnt on possible methods of improving the antibody titres which are described in full in trial 2.

Chapter 4

Trial 2

4. Optimisation of injection regime and neurochemical study –Trial 2

4.1 Introduction

Several changes were made to trial 1 to enhance the immune response. As mentioned in section 3.10, trans-4-thiol cotinine was the only conjugate taken forward for this trial. A new conjugation method was used to allow for the attachment of this derivative to a new carrier protein, tetanus toxoid (150KDa). This was done to enhance the number of derivative attachments on to the carrier molecule. Three concentrations of conjugate were injected into rats to determine which dose elicited the best immune response. The adjuvant was changed from Alum to GlaxoSmithKline adjuvant AS2V to increase the immune response.

4.2 Materials

All chemicals were obtained from standard commercial suppliers and all equipment used was present at the University of Bath.

Buffers: Phosphate buffer; 100mM Na_2HPO_4, 100mM NaH_2PO_4.

Phosphate buffer saline (PBS); 150mM NaCl, 100mM Na_2HPO_4, 100mM NaH_2PO_4.

4.3. Conjugation of Trans-4-thiol cotinine (cotsh) to Tetanus Toxoid (TT)

4.3.1 Activation of Tetanus Toxoid

The first step was to activate the lysine groups in the TT molecules (14-16 lysines have previously been activated using TT by GSK team), to enable the presentation of an active site for conjugation with sylfhydryl group in trans-4-thiol cotinine.

TT (5mg/ml in phosphate buffer pH 7) was stirred at room temperature, with SGMBS (5 μmol) for 2 hours, Final volume 2ml). This was then purified by FPLC (PD-10 desalting column – Amersham Pharmacia Biotech). Phosphate buffer (pH 6.8) was used to elute the activated TT at a flow rate of 40ml/hr. 15 fractions of 4 ml each were taken and absorbance was measured at 280nm. The fractions showing the desired TT in the profile reading on basis of increased UV absorption were pooled. The Lowry assay was conducted to determine the protein concentration.

4.3.2 Protein determination by Lowry Assay

Protein was determined using the Lowry assay (Lowry *et al* 1959). A Bovine Serum Albumin (BSA) standard curve was constructed using triplicate samples of BSA

dissolved in PBS (pH 6.8) over a concentration range of 10μg/ml – 200 μg/ml. TT samples were diluted 1:10, 1:20 and 1:40 in PBS (pH 6.8) in triplicate. Each sample (200 μl of BSA standards and TT samples) was placed in LP4 tubes and 10% SDS (200 μl) and assay reagent (1% $CuSO_4$, 2% Na/K, 2% $NaCO_3$/NAOH 0.1N, 1ml) were added. The tubes were vortexed and incubated for 10 minutes at room temperature. Folin's Reagent (100μl) was added to each tube, vortexed and incubated for 30 minutes at room temperature. Protein was determined by measurement of optical density at 750nm versus blank (assay buffer plus Folin's Reagent) using Helios Gamma spectrophotometer.

4.3.3 Determination of the number of activated sites on TT by Ellmans' Assay

This was done to determine the number of activated sites on TT. Samples (100μl) of phosphate buffer (blank – 100 mM, pH 6.8), activated TT from section 4.3.1 and unmodified TT were assayed in triplicate. 2-mercaptoethanol (1mM, pH 8, 50μl) was added to all samples and incubated for 30 minutes (37°C). Ellmans' Reagent (100mM, pH 8, 100μl) and PB (100mM, pH 8, 1ml) were added to all samples and incubated for 15 minutes at room temperature. Optical density (OD) was measured at 412nm using a Helios Gamma spectrophotometer and the number of activated sites calculated as below:

(Blank OD – modified TT OD) / (Molar extinction coefficient of TNB (reported to be $14150M^{-1}cm^{-1}$) x concentration of sulfhydryl solution*

Concentration of sulfhydryl solution * = protein concentration from Lowry Assay x 1000 / MW of TT x final volume

The final number of activated sites is calculated by taking away the number of activated sites on the unmodified TT (<1), from the number of activated sites on the activated TT.

Results: The number of activated sites on TT was calculated to be 15.

4.3.4 Coupling of modified TT to trans-4-thiol cotinine (cotsh)

The concentration of trans-4-thiol cotinine needed was calculated by multiplying the concentration of modified TT from section 4.3.1, by molecular weight of trans-4-thiol cotinine. This is then added drop-wise to the solution of activated TT, and incubated for 1 hour at room temperature while stirring (TT-cotsh). To prepare the control, a

sample of modified TT from section 4.3.1 was added to cysteine (4mg/ml in PBS 150mM, pH 6.8) and incubated for 1 hour at room temperature (TT-cysteine). The TT-cotsh sample was dialysed for 2 hours using Slide A-lyzer discs (3500MWCO - Pierce) against phosphate buffer (2mM, pH 6.8) with 3 buffer changes. Cysteine (4mg/ml in phosphate buffer 100mM, pH 6.8) was then added to quench the unattached sites present on TT-cotsh, and incubated for 30 minutes at room temperature while stirring. TT-cotsh and TT-cysteine preparations were then dialysed overnight (4°C) using dialysis membrane (12-14KDa – Medicell International), against phosphate buffer (2mM, pH 6.8) with 3 buffer changes. Aliquots (100µl) of TT-cysteine and TT-cotsh before and after quenching were set-aside for Lowry Assay. The volumes of the TT-cysteine and TT-cotsh were then measured and these samples were filtered using a millex filter (0.22µm) in a sterile environment. The volume after filtration was also measured (needed for calculations using Lowry and Ellman's tests as described in the following paragraph).

Lowry Assay was conducted on TT-cysteine and TT-cotsh after filtration samples (as described in section 4.3.2). Ellmans' test was also conducted on the following samples; TT-cotsh before quenching, TT-cotsh before and after filtration, TT-cysteine before and after filtration (as described in section 4.3.3), to determine the number of activated sites still remaining. These sites are not detected when trans-4-thiol cotinine or cysteine are attached, therefore the number of attachments per TT molecule can be derived. In the trans-4-thiol cotinine samples, to find out whether these attachments are trans-4-thiol cotinine or cysteine from the quenching step, a comparison of number of attachments was made between the samples of before and after quenching.

Results: In the TT-cotsh sample, 15 attachments of trans-4-thiol cotinine were made per TT molecule. This is in accordance with number of attachments acquired by GSK who in their own work have shown to illicit a high immune response (literature not available to public).

4.4 Formulation of vaccines

The adjuvant was changed from Alum to GlaxoSmithkline adjuvant AS2V to increase the immune response. AS2V has been shown by GSK to illicit a high immune response (literature not available to public).

All vaccines were made in the same manner. The correct amount of TT-cotsh conjugate (1μg, 5μg, 25μg) and TT-cysteine conjugate (25μg) used as control vaccine, were made up in PBS (200mM) and mixed with adjuvant in a 1:1 ratio. Adjuvant was added to conjugate drop wise while vortexing over 10 seconds. This was incubated for 10 minutes at room temperature while stirring. The vaccine was vortexed before injection.

4.5. Injection regime

The injection regime used was recommended by Dr Pascal Mettens from GlaxoSmithkline and taken from Heida *et al* (2000) with variations. This was changed from trial 1 to study the effect of more boosters on the amount of antibodies produced. Due to the limitation of animal numbers, the second booster was only conducted on the 1μg and 25μg TT-cotsh vaccine groups. The minipumps were used to deliver a constant rate of nicotine applied to the rats over a 7-day period. This is different in comparison to the 14-day minipump treatment of trial 1. This change was made to reduce the number of days of the animal treatment, however the other protocols related to the minimpup procedure were the same (section 3.7.2.2).

Day 1	Bleed & first injection
Day 21	Bleed & second injection
Day 35	Bleed & third injection
Day 49	Bleed
Day 78	Bleed & first booster
Day 98	Bleed & 7 day osmotic minipump insertion
Day 105	Kill – bleed & brain dissection for dopamine release assay – 5μg/ml vaccine group and control
Day 184	Bleed & second booster for 1μg and 25μg vaccine groups
Day 205	Bleed
Day 226	Bleed

Rats were 6-7 weeks old at the beginning of the experiment, which is the age in which the parental antibodies are no longer able to cause any effect on the production of antibodies against this vaccine. The rats used varied in weight between 220g and 290g at the start of the experiment. This was the closest range of weights possible with the rats available. There were 10 rats per vaccine group. In the 5μg/ml vaccine group which were treated with osmotic minipumps, 5 rats were treated with saline and 5 with nicotine (4mg/ml/kg/day).

It was noted that no difference was seen in rat behaviour or locomoter activity after injections and minipump insertion.
All further procedures undertaken are the same as described in section 3.6 and 3.7.

4.6 Enzyme-Linked Immunosorbent Assay (ELISA)

The protocol described in section 3.8.2.2.3 - Derivative coated 96-well plate ELISA, was used with variations, to determine antibody titres using TT-cotsh and TT-cysteine vaccinated rat sera. The plates were coated with trans-4-thiol cotinine (1μg/ml in carbonate buffer, 50μl) and the serum was serially diluted (1:2 times each time) starting from 1:100 dilutions in saturation buffer.

An ELISA was conducted using TT-coated maxisorp 96 well plates using the same protocol as section 3.8.2.2.3, to determine antibody titres produced against TT. The results were too high to calculate the mid-point titres. As the aim of this project was to study the effect of the antibodies specifically raised against cotinine, as well as the high cost of TT, this experiment was not optimised to determine titres against TT.

4.6.1 Immune response for trans-4-thiol cotinine – Tetanus Toxoid conjugate - 1μg

Antibodies towards trans-4-thiol cotinine were produced as shown in figure 4.1. Titres were produced as early as day 21 but were too low to measure mid-point titres, therefore only those from day 35 onwards were used. Average titres reached their maximum on day 105 and decreased for the remaining injection period. The titres obtained are high and within a reported range (Heida *et al* 2000).

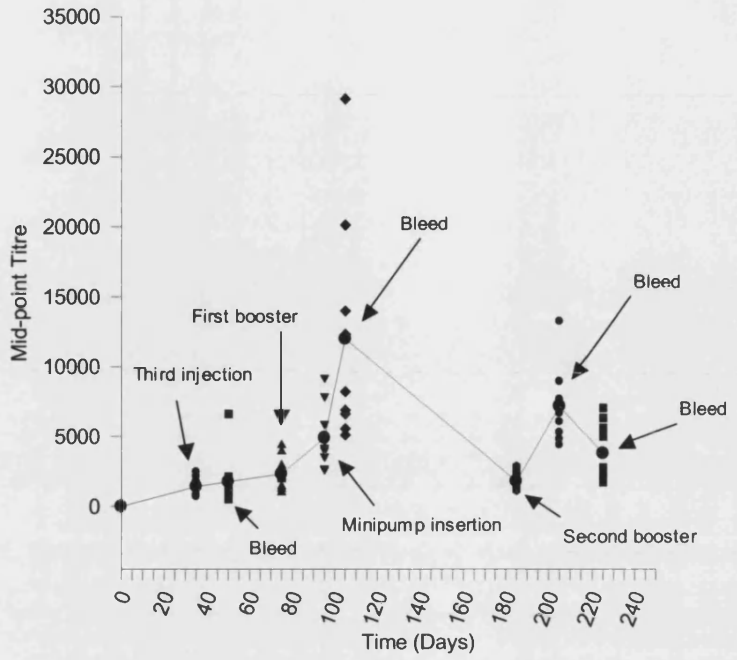


Figure 4.1: Mid-point antibody titres from rats vaccinated with 1μg Tetanus Toxoid - Trans-4-thiol cotinine.

The titres were obtained from sera taken on days 35, 49, 78, 98, 105, 184, 205 and 226. The sera were tested by ELISA, using trans-4-thiol cotinine derivative coated wells. n = 10 for each group with the line illustrating mean mid-point titre, and symbols representing mid-point titre for each rat.

4.6.2 Immune response for trans-4-thiol cotinine – Tetanus Toxoid conjugate - 5μg

Antibodies towards trans-4-thiol cotinine were produced as shown in figure 4.2. Titres were produces as early as day 21 but were too low to measure mid-point titres accurately, therefore only those from day 35 onwards were used. Average titres reached their maximum on day 105. The titres obtained are high and within a reported range (Heida *et al* 2000).

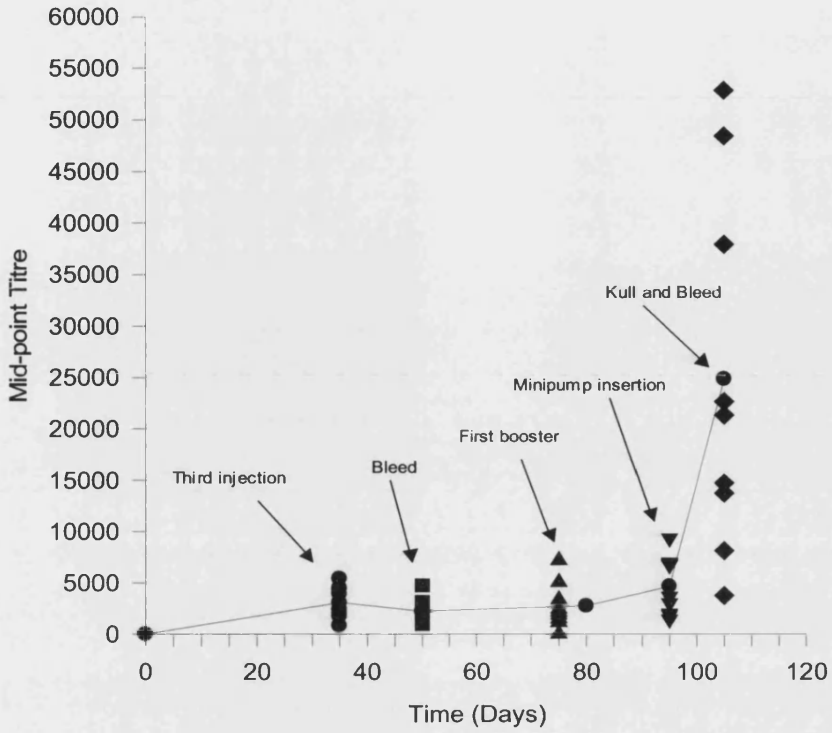


Figure 4.2: Mid-point antibody titres from rats vaccinated with 5μg Tetanus Toxoid - Trans-4-thiol cotinine.

The titres were obtained from sera taken on days 35, 49, 78, 98, 105. The sera were tested by ELISA, using trans-4-thiol cotinine derivative coated wells. n = 10 for each group with the line illustrating mean mid-point titre, and symbols representing mid-point titre for each rat.

4.6.3 Immune response for trans-4-thiol cotinine – Tetanus Toxoid conjugate - 25μg

Antibodies towards trans-4-thiol cotinine were produced as shown in figure 4.3. Titres were again produced as early as day 21 but were too low to measure mid-point titres accurately, therefore only those from day 35 onwards were used. Average titres reached their maximum on day 105. The titres obtained are high and within a reported range (Heida *et al* 2000).

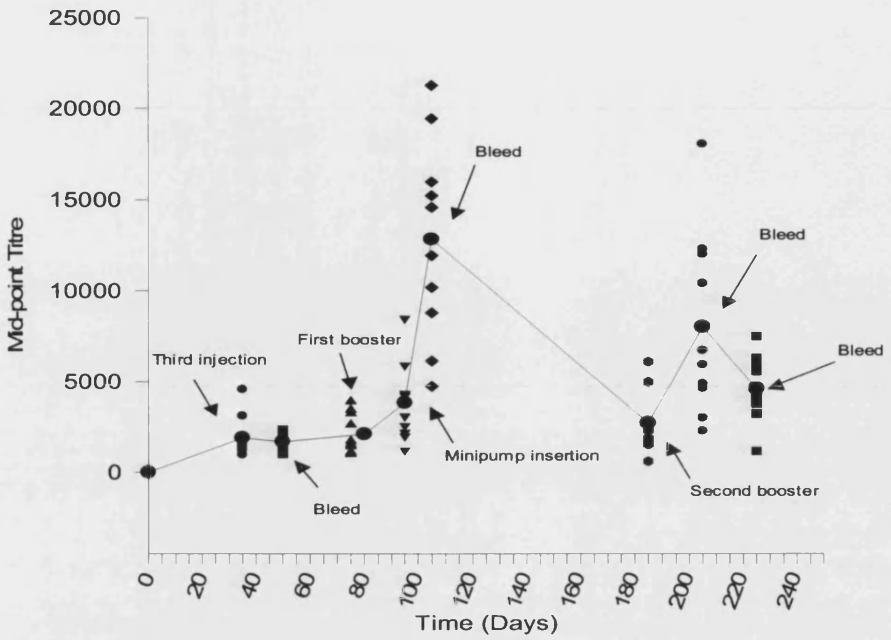


Figure 4.3: Mid-point antibody titres from rats vaccinated with 25μg Tetanus Toxoid - Trans-4-thiol cotinine.

The titres were obtained from sera taken on days 35, 49, 78, 98, 105, 184, 205 and 226. The sera were tested by ELISA, using trans-4-thiol cotinine derivative coated wells. n = 10 for each group with the line illustrating mean mid-point titre, and symbols representing mid-point titre for each rat.

4.6.4 Summary of immune response for Trans-4-thiol cotinine – Tetanus Toxoid conjugate – 1, 5 and 25μg

A summary of the dose response experiment to determine which vaccine gave the highest mid-point titre antibody production is illustrated in figure 4.4. In each case, the highest mid-point titre was observed on day 105. Comparison of these values (figure 4.4) shows that the 5μg TT-cotsh conjugate produced the highest mid-point titre.

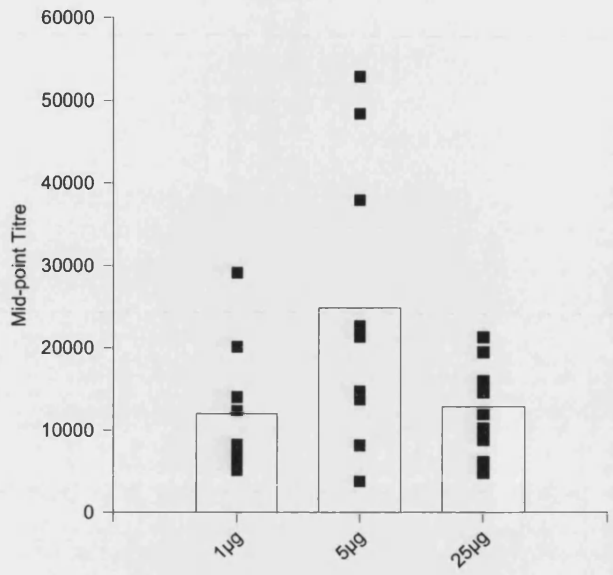


Figure 4.4: Mid-point antibody titres from rats vaccinated with 1, 5 and 25µg Tetanus Toxoid – trans-4-thiol cotinine.

Comparison of the mid-point titres obtained on day 105. The sera were tested by ELISA using trans-4-thiol cotinine derivative coated wells. n = 10 in each group with the bars illustrating mean mid-point titre, and symbols representing mid-point titres for each rat.

4.6.5 Competitive ELISA to determine the specificity of antibodies produced

4.6.5.1 Optimisation of derivative coating concentration for ELISA plate

The first step in determining the specificity of antibodies present in sera was to optimise the competitive ELISA method. This was done to determine the optimal concentration of trans-4-thiol cotinine derivative for coating the ELISA plates. To do this, a variety of concentrations of trans-4-thiol cotinine (0.001-1µg/ml) were tested, using the normal ELISA method as described in section 3.8.2.2.3, with a sample serum from a rat vaccinated with trans-4-thiol cotinine 5µg conjugate on day 98 (figure 4.5).

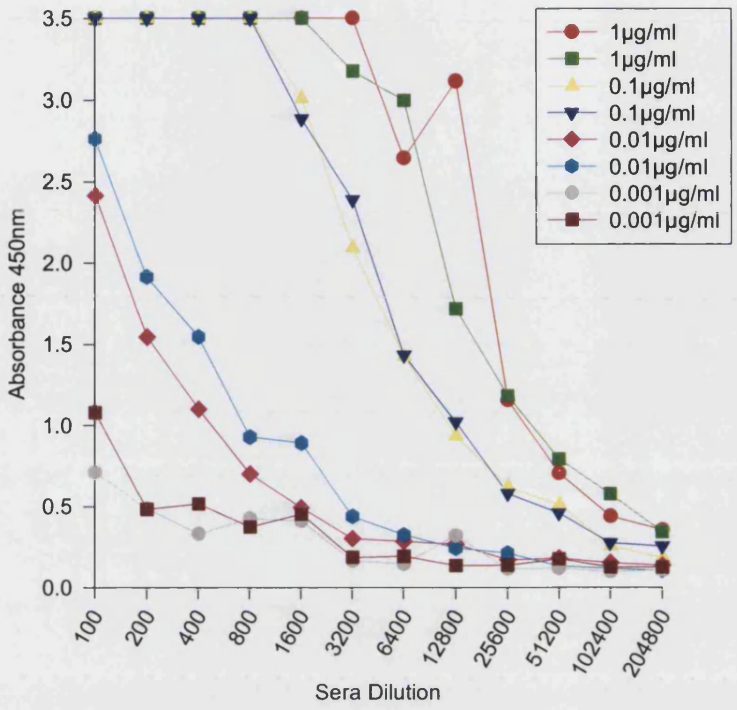


Figure 4.5: Antibody titres from serum from a sample rat using ELISA plate coated with varying concentrations (0.001-1µg/ml) of the derivative cotsh.

The serum from a rat vaccinated with 5µg TT-cotsh on day 98 was used. Each concentration of derivative coating was done in duplicate shown as two curves per concentration.

The most suitable coating concentration is the lowest needed concentration to give a full antibody titre curve. From Figure 4.5 it can be determined that this concentration lies between 0.1-0.01µg/ml.

Therefore another ELISA was conducted within this range with a more concise gradient. The concentrations used were 0.1, 0.05, 0.025 and 0.01µg/ml (figure 4.6).

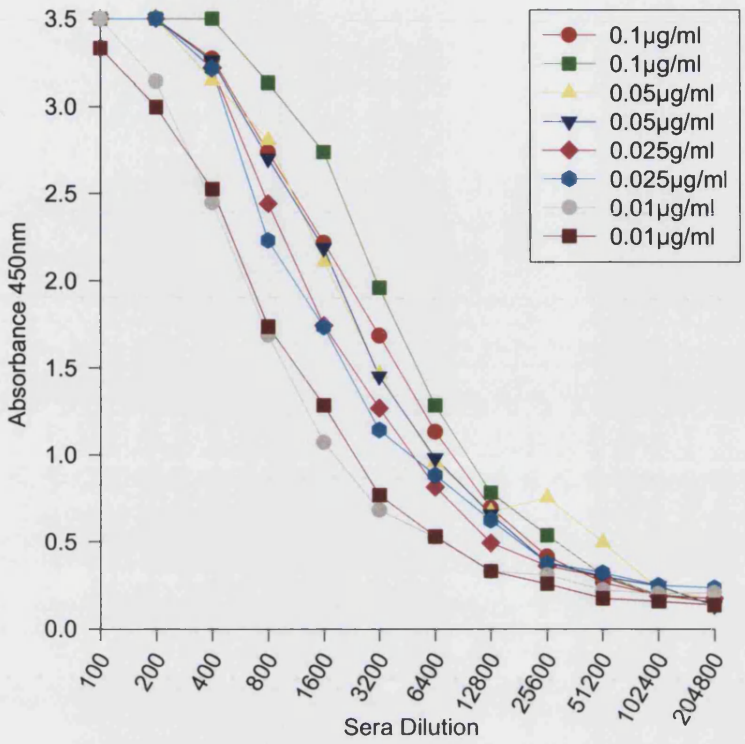


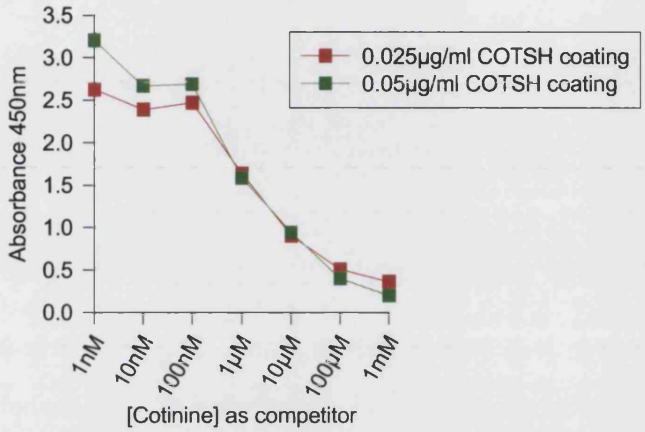
Figure 4.6: Antibody titre from serum from a sample rat using ELISA plate coated with varying concentrations (0.1-0.01µg/ml) of the derivative cotsh.

The serum from a rat vaccinated with 5µg TT-cotsh on day 98 was used. Each concentration of derivative coating was done in duplicate shown as two curves per concentration

From Figure 4.6 it can be seen that the most suitable concentrations are 0.05 µg/ml and 0.025 µg/ml. However, it is difficult to distinguish which concentration is most suitable of the two as they show a similar profile.

Therefore a competitive ELISA was conducted using ELISA plates coated with both concentrations of derivative to distinguish which concentration gave the best titre readings (figure 4.7). Nicotine and cotinine were used as competitor drugs (1mM-1nM). The method for competitive ELISA is described in section 4.6.5.2.

A



B

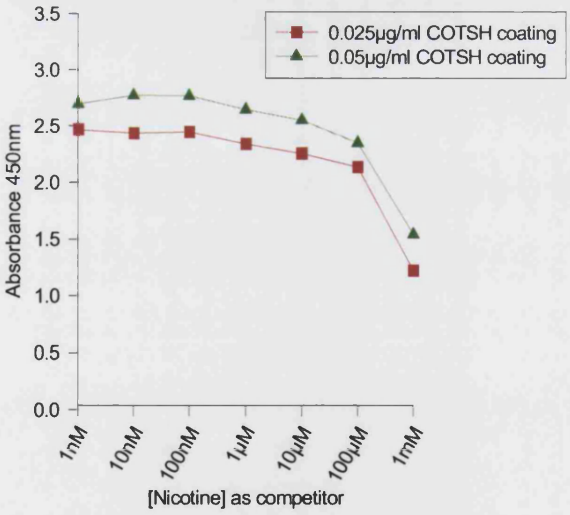


Figure 4.7: Antibody titres from serum from a sample rat using a competitive ELISA. The serum from a rat vaccinated with 5µg TT-cotsh on day 98 was used. The ELISA plate was coated with two concentrations (0.05 and 0.025µg/ml) of the derivative trans-4-thiol cotinine.

Cotinine (A) and nicotine (B) were used as competitor drugs (1mM-1nM). The method for competitive ELISA is described in section 4.6.5.2.

Figure 4.7 shows clear competition of antibody detection by cotinine compared to a lack of effect by nicotine, except at high concentrations which are not present in blood after chronic nicotine treatment (figure 3.4). This is probably caused by antibody-cotinine complexes being formed, which are not detectable by ELISA.

The ELISA plates coated with 0.05 μg/ml trans-4-thiol cotinine show a slightly higher titre. A larger n number would be needed to give a conclusive result, however due to time constraints, 0.05 μg/ml trans-4-thiol cotinine derivative was chosen for all competitive ELISA experiments.

4.6.5.2 Competitive ELISA

4.6.5.2.1 Methods

The competitive ELISA was done on sera from rats injected with 5μg TT-cotsh vaccine on day 98. The other sera were not tested due to cost and time constraints, however this will give a good indication on the specificity of the antibodies raised against the vaccine. The control vaccine was not tested, as any antibodies raised against TT-cysteine are not likely to affect the actions of cotinine, as they are not able to recognize cotinine.

The highest sera dilutions from the linear part of the curve obtained from previous ELISAs were incubated with 7 ten-fold dilutions of competitors (1mM – 1nM). The competitors used were nicotine, cotinine, nornicotine, norcotinine, trans-3-hydroxycotinine, nicotine-1-oxide and cotinine-N-oxide (purchased by GSK). These are all metabolites of nicotine (see chapter 1, section 1.3.1.2). Sera and competitors were incubated at 4°C overnight. A normal ELISA was then done (as described in section 3.8.2.2.3) with the following variations; after the 0.05μg/ml trans-4-thiol cotinine coated plates were incubated with casein and washed as normal, 100μl of the sera-competitor solution were added in duplicate. There was no serial dilution of the serum as one specific dilution was taken at the beginning of the experiment. This was incubated for 2 hours at 37°C. The ELISA was then conducted as described in section 3.8.2.2.3.

4.6.5.2.2 Results

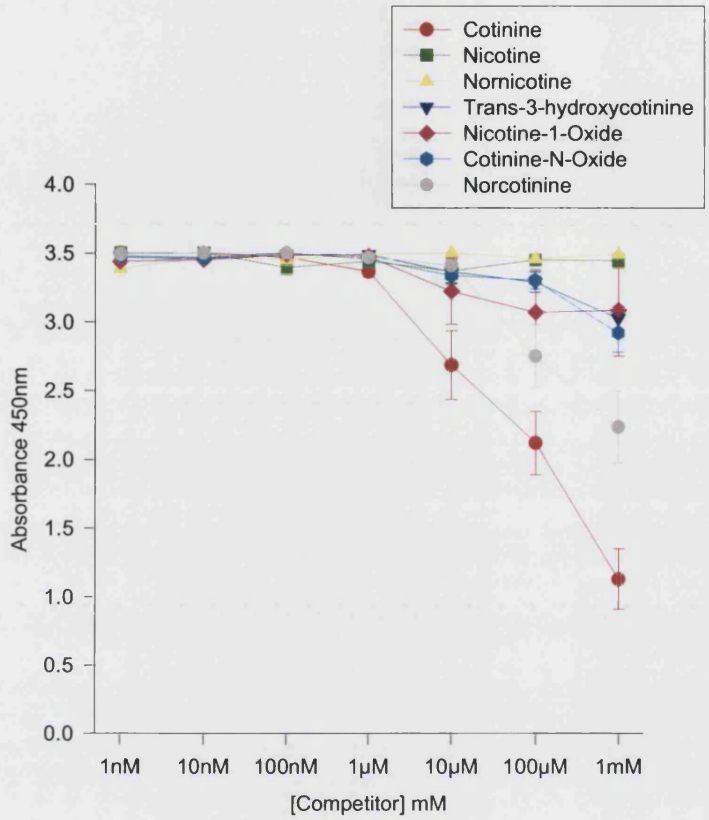


Figure 4.8: Competitive ELISA to determine specificity of antibodies in sera in relation to nicotine and some of its metabolites.

The sera used are from 5µg TT-cotsh vaccinated rats on day 98. The sera were tested by using 0.05µg/ml trans-4-thiol cotinine derivative coated wells. n = 10 rats for each competitor.

The competitive ELISA (figure 4.8) illustrates that the antibodies obtained from vaccination with 5µg TT-cotsh vaccine show specificity for cotinine over all metabolites tested. Norcotinine does show slight competition with the vaccine, however this is not an important finding. Although norcotinine has been shown to be present and metabolised in rat brain, there is no literature to suggest it has any pharmacological activity (Crooks & Dwoskin 1997, Ghosheh *et al* 2001).

There seems to be a difference in inhibition between figures 4.7 and 4.8. In figure 4.7 the inhibition seems to start occurring with 100µM competitor, whereas in figure 4.8, the curve seems to start occurring with 1µM competitor. The point to note is that in

figure 4.7, the highest absorbance reached is 2.5 and the curve seems to start going down at 2.0. At this absorbance of 2.0, figure 4.8 shows inhibition at the same concentration (100μM). I suggest that the problem lies with why the highest absorption levels of 3.5 are not reached. This maybe because the serial dilution in figure 4.7 was only begun at 1:20, and not 1:100 as in figure 4.8. This may mean that more antibody are present in the sera and therefore more inhibitor needed to have the same effect. This is of course only a hypothesis and needs further investigation. Experimental error could also account for this difference and as figure 4.7 is n=1, more experiments are needed to clarify this point.

4.7 Results of rat serum analysis for nicotine and cotinine concentrations in rats treated with nicotine osmotic minipump.

The aim of this experiment was to determine the effect of vaccination on rat serum nicotine and cotinine concentrations. Rats vaccinated with TT-cotsh (5μg) and TT-cysteine (25μg) were chronically treated with nicotine or saline by osmotic minipump, on days 98-105. The same procedures for bleeding, osmotic minipump preparation, surgical procedures and sera cotinine and nicotine concentration determination were used, as described in section 3.6 and 3.7. Cotinine and nicotine concentration measurements were done by gas chromatography at ABS laboratories. In short, this technique separates out the components within the sera and the concentration of cotinine and nicotine are detected. The cotinine levels detected will include the antibody-bound cotinine as separation experiments such as hydrolysis are not conducted previous to gas chromatography.

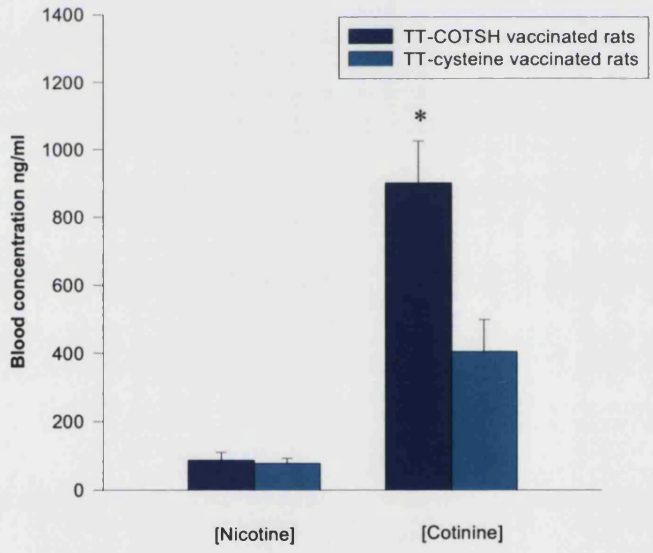


Figure 4.9: Nicotine and cotinine concentration in serum from rats immunized with 5μg TT-cotsh vaccine and TT-cysteine (control), and treated with 7-day nicotine osmotic minipump.

*Nicotine was administered at a rate of 4mg/kg/day. Sera were taken on day 105. n = 5 rats per minipump treatment group. * = Significantly different from corresponding control TT-cysteine vaccine ($p < 0.05$ $p = 0.0129$) using student's unpaired t-test.*

Rats receiving saline had negligible levels of serum nicotine (mean 1.0 ± 0.5ng/ml) and cotinine (mean 3.75 ± 0.5ng/ml) in both vaccine groups.

Nicotine treatment produced levels of nicotine (77.3 ± 15.2) and cotinine (403.4 ± 95.1) in the expected range for the nicotine dose used, in control vaccinated rats (figure 4.9) (Sanderson *et al* 1993).

The cotinine concentration for the TT-cotsh vaccine sera (901 ± 124) increased significantly compared to the control vaccine (403.4 ± 95.1), when treated with nicotine (figure 4.9). This result is as expected, possibly due to the cotinine specific antibodies binding to cotinine in the blood producing an antibody-conjugate complex, and therefore retaining cotinine in the blood (Heida *et al* 1997).

4.8 Effect of vaccination on nicotine-evoked dopamine release from striatal minces

The rats vaccinated with 5μg TT-cotsh were treated with 7-day nicotine or saline osmotic minipumps on day 98-105. The two striata from each animal were dissected and the effect of this nicotine treatment on nicotine–evoked dopamine release was measured.

The same procedures for osmotic minipump preparation and surgical procedures were used as described in section 3.6 and 3.7. The 96-well plate [^{3}H]-dopamine release assay described in sections 2.2 and 2.3 was used.

There was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in both TT-cotsh and TT-cysteine vaccinated rats (figure 4.10). However in this experiment there is no significant change in nicotine – evoked [^{3}H]-dopamine release between TT-cotsh and TT-cysteine vaccinated rats ($p=0.059$). The statistics show that significance in this case is close and with further experiments and an increase in the number of rats in the experiment, significance may be reached. This is further investigated in the next trial. Nicotine - evoked [^{3}H]-dopamine release was unaffected by vaccination in saline treated rats.

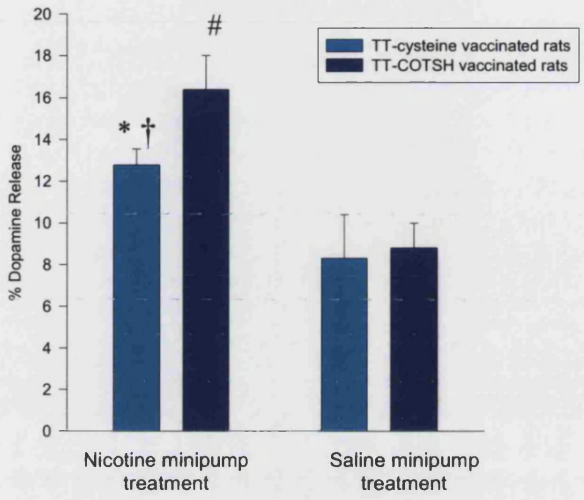


Figure 4.10: Effect of chronic nicotine treatment on nicotine – evoked [^{3}H]-dopamine release using 96-well plate assay.

Rats were chronically treated with nicotine or saline osmotic minipump for 7 days. Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with nicotine (100μM, 5 minutes) and stimulation with nicotine (100μM, 5 minutes) produced [^{3}H]-dopamine release. 8 replicates were used for each condition and this was done for 4-6 rats per group.

** = Significantly different from control TT-cysteine vaccinated rats treated with saline minipump ($p<0.05$) using Student's unpaired t-Test.*

***# =** Significantly different from TT-cotsh vaccinated rats treated with saline minipump ($p<0.05$) using Student's unpaired t-Test.*

† = Not quite significant from corresponding TT-cotsh vaccinated rats treated with nicotine minipump ($p=0.059$) using Student's unpaired t-Test.

4.9 Discussion

4.9.1 Have the improvements from trial 1 been effective?

The change in carrier molecule from Ovalbumin to Tetanus toxoid was shown to be a successful strategy as the desired number of attachments of derivative (15 cotsh per TT molecule), were made and this was successfully determined using Lowry and Ellmans' tests. The more cotinine epitopes present on the conjugate vaccine, the higher the recognition of cotinine by the immune response, therefore increasing the amount of antibodies produced. There is a correlation between size of a macromolecule and its immunogenicity as discussed in the introduction (section

1.6.4.5), therefore increasing the size of the antigen by adding a larger carrier molecule (TT) compared to ovalbumin in trial 1 has improved the immune response. The change in adjuvant from Alum to GSK AS2V was also a successful strategy as in conjunction with the increase in derivative attachments and change of carrier molecule, the immune response was increased dramatically (average mid-point titres >1:10000) to desired levels where it has been shown to produce adequate drug-specific binding affinity (Hieda *et al* 1997). This was done in a vaccine against nicotine where titres at this level have been shown to elicit binding of a substantial fraction of a single dose of nicotine in serum.

The successful optimisation of the competitive ELISA method allowed for the determination of the specificity of the antibodies to detect cotinine alone. As shown in figure 4.8, nicotine and the other metabolites tested were not recognized by the cotinine antibodies present in the sera. The metabolites used in this trial were chosen as they were easily obtained and may contribute to the actions of nicotine in relation to nicotine addiction. For example nornicotine has been shown to increase dopamine release in a calcium-dependent manner from superfused rat striatal slices (Dwoskin *et al* 1993). It is important to obtain an immune response towards the cotinine molecule only, as the metabolites of nicotine have a similar structure to cotinine (as seen in figure 1.5), and the identification of the oxygen molecule on the pyrolydine ring in cotinine, by the immune cells is paramount in enabling a specific response. The specificity of the antibody therefore enables us to determine only the effect of the reduction of cotinine to the brain and not other mechanisms that may contribute to nicotine addiction. In conclusion the results show a proof of concept for the initial aims of this project: cotinine specific antibody titres are produced in the desired quantity due to the production of a viable cotinine vaccine.

The 5 μg TT-cotsh conjugate produced the highest mid-point titres in comparison to the 1 and 25 μg TT-cotsh conjugates (figure 4.4). An insufficient dose fails to activate enough lymphocytes or induce a state immunologic unresponsiveness or tolerance. This could explain the reduced immune response for the 1 μg conjugate. Conversely, an excessive high dose can also induce tolerance, which could explain the reduced immune response in the 25 μg conjugate. In each conjugate concentration, the highest mid-point titre was observed on day 105. The 1 and 25 μg vaccines did show a fall in antibody titres on day 184 which then increased by day 205 but did not reach the maximum titre previously achieved, and progressively fell once again by day 226. This

suggests that one booster alone does not produce a long lasting effect in this immune response but the second booster activates the memory response. This is probably caused by the long-lived memory cells, which are produced along with the relatively short-lived effector cells of the primary immune response, after the second booster. These memory cells proliferate rapidly when exposed again to the same antigen, giving rise to a new clone of memory cells as well as new effector cells, demonstrated here by the increase of antibody titres after the second booster. The fall in antibody titres is probably caused by the actions of the immune cells, by getting rid of this foreign molecule (explained further in section 1.6.4.3).
This pattern needs to be further investigated to determine improvements in the vaccination regime to produce a high and long lasting immune response.

4.9.2 Effects of nicotine

The significant increase in blood cotinine, but not nicotine, concentrations after chronic nicotine treatment, seen in TT-cotsh vaccinated rats in comparison to control vaccinated rats suggests an antibody-cotinine complex formation in sera. Therefore cotinine is retained in the blood probably by the precipitation mechanism of antibodies as described in section 1.6.4.4). This is an important finding, suggesting that the specific antibodies produced towards cotinine are acting on free cotinine in the blood. This is similar to results obtained in the production of a nicotine vaccine (Hieda *et al* 1997), where nicotine concentrations were increased in sera after nicotine treatment. In this project, I was not able to determine specific binding concentrations for cotinine to the antibody due to practical constraints but this is a crucial piece of information to allow for complete characterization of the immune response.

There was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in both TT-cotsh and TT-cysteine vaccinated rats (figure 4.10). A search of the literature reveals that few reports exist regarding the effect of chronic nicotine treatment on dopamine release *in vitro*, with examination of dopamine release *in vivo* regarded as a more suitable system (DiChiaria 2000). The analysis of dopamine release from rat striatal slices found an increase in dopamine release after treatment with nicotine by daily injection (Yu & Wecker 1994). In contrast, Marks *et al* (1993) showed that dopamine release from mouse brain synaptosomes declined after chronic nicotine infusion suggesting desensitisation / inactivation of nAChRs. Similar results have also been obtained in our laboratory.

The dopamine release assay was conducted to determine whether vaccination causes any change in the reported increase in dopamine release after chronic nicotine treatment. In TT-cotsh vaccinated animals, following chronic nicotine treatment, there was a trend towards increased nicotine-evoked dopamine release, compared with the response in tissue form rats receiving the control vaccine. However this failed to reach statistical significance, suggesting vaccination does not have an effect on dopamine release in the striatum, using this assay. However, increasing the n number of animals, and / or the titres achieved during the nicotine treatment may enhance this difference. This is discussed further in trial 3.

Chapter 5

Trial 3

5.0 Neurochemical and Behavioural studies to evaluate the effect of vaccination – Trial 3

5.1 Introduction

This final trial was conducted to assess the effect of vaccination against cotinine on a variety of parameters. These parameters included the study of mid-point titres, nicotine and cotinine concentrations after chronic and acute nicotine treatment, dopamine release in the striatum, and locomotor activity. The locomotor activity tests are a new addition in this trial and a short review of the literature as well as methodologies used are explained in section 5.7, followed by results for the effects of acute and chronic nicotine treatment on locomotor activity and a discussion of the results obtained. The vaccine used in this trial was prepared in the same manner as trial 2 using the 5μg TT-cotsh conjugate and 25μg TT-cystiene conjugate as control (section 4.3 and 4.4). However the number of trans-4-thiol cotinine attachments to TT was 11 per TT molecule compared to 15 per TT molecule in trial 2. This change in the vaccine may cause an effect on the immune response however, it could not be improved due to time and financial limitations.

5.2 Injection regimes

There were two groups of rats used in this study. One group (regime 1) was used to study the effect on nicotine-evoked dopamine release and blood cotinine and nicotine concentrations after chronic nicotine treatment using 7-day osmotic minipumps. The injection regime was kept as similar to the injection regime in trial 2 as possible allowing for differences in access to the animals at the time. The second group (regime 2) was used to study locomotor activity and blood nicotine and cotinine concentrations after acute nicotine injection. The injection regime was changed in this group to examine any differences that may occur in the immune response with a different injection regime (allowing 4 weeks between the second injection and first booster). This was also done for convenience, to allow for experiments in both injection regimes to be conducted in the same period of time.

Regime 1

Day 1 – First injection

Day 21 – Second injection

Day 35 – Third injection & bleed

Day 84 – First Booster & bleed

Day 91 – Minipump insertion & bleed

Day 98 – kill - dopamine release assay, blood taken for [nicotine & cotinine] analysis & ELISA

Regime 2

Day 1 - First injection

Day 21 - Second injection

Day 35 - Third injection & bleed

Day 95 - First Booster & bleed

Day 116 - Bleed

Day 119 - Start of Locomotor activity experiment

Day 143 - End of locomotor activity experiment

Day 150 - Bleed

Day 170 - Second Booster

Day 184 - Acute nicotine injection, 8 hours later rats were killed - blood taken for [nicotine & cotinine] analysis

The rats used varied in weight between 220g and 290g at the start of the experiment. This was the closest range of weight possible with the rats available. The rats were 6-7 weeks old at the beginning of the experiment which is the age in which the parental antibodies are no longer able to cause any effect on the production of antibodies against this vaccine. There were 10 rats per vaccine group in regime 1 and 14 rats per vaccine group in regime 2. In regime 1 vaccine group which were treated with osmotic minipumps, 5 rats were treated with saline and 5 with nicotine (4mg/kg/day).

It was noted that no difference was seen in rat behaviour or locomoter activity after injections and minipump insertion.

All further procedures undertaken are the same as described in section 3.6 and 3.7.

In regime 2 vaccine group, which were studied for locomotor activity, 7 rats were injected with saline, and 7 rats were injection with nicotine (0.4 mg/kg). Further details are described in section 5.8.

5.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The protocol described in section 3.8.2.2.3 – derivative-coated 96-well plate ELISA was used with variations, to determine antibody titres using TT-cotsh and TT-cysteine vaccinated rat sera. The plates were coated with trans-4-thiol cotinine (1μg/ml in carbonate buffer, 50μl) and the serum was serially diluted (1:2 times each time) starting from 1:100 dilutions in saturation buffer.

An ELISA was also conducted in this trial using TT-coated maxisorp 96 well plates using the same protocol as section 3.8.2.2.3, to determine antibody titres produced

against TT. The results were too high to calculate the mid-point titres (as explained further in section 4.6).

5.3.1 Immune response for trans-thiol cotinine – Tetanus Toxoid conjugate (5μg) from injection regime 1

Antibodies towards trans-4-thiol cotinine were produced as shown in figure 5.1. Titres were produced as early as day 21 but were too low to measure mid-point titres, therefore only those from day 35 onwards were used. Average mid-point titres reached a maximum on day 91 and remained at this level until day 98. The titres obtained are high, however they are lower than the titres obtained in trial 2 (figure 4.4). This may have been caused by the lower number of derivative attachments (11cotsh per TT) on the carrier molecule compared to the number of attachments in trial 2 (15 cotsh per TT) as explained in section 5.1. Another reason for the fall in immune response may be the efficacy of the adjuvant used. GSK have shown that the efficacy of AS2V adjuvant was decreased after three months from production, and in this trial AS2V was made one month before injection. This could not be avoided due to time constraints as AS2V was produced at GSK.

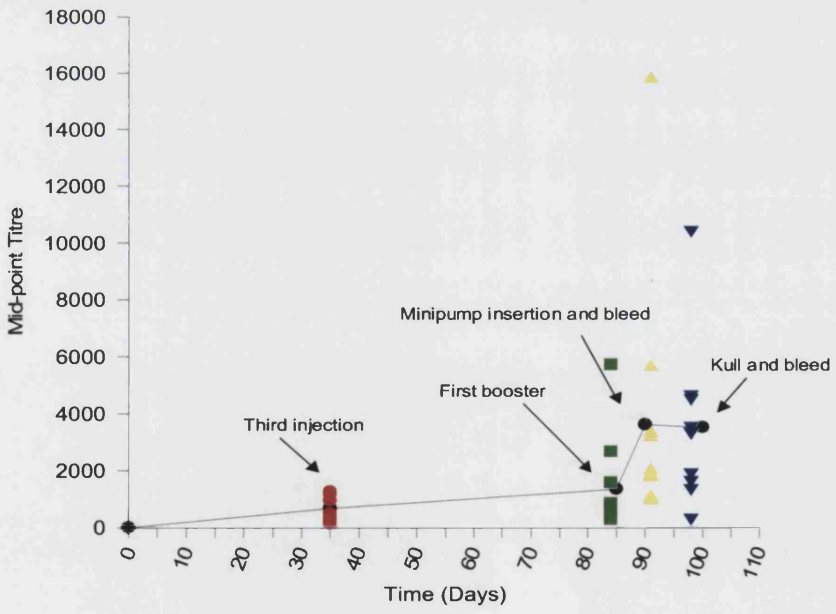


Figure 5.1: Mid-point antibody titres from rats vaccinated with 5μg TT-cotsh.

The titres were obtained from sera taken on days 35, 84, 91. The sera was tested by ELISA using trans-4-thiol cotinine derivative coated wells. n = 10 rats for each group with the bars illustrating mean mid-point titre, and symbols representing mid-point titre for each rat.

5.3.2 Immune response for TT–cotsh conjugate (5μg) from injection regime 2

Antibodies towards trans-4-thiol cotinine were measured as shown in figure 5.2. Titres were produced as early as day 21 but were again, too low to measure mid-point titres, therefore only those from day 35 onwards were used. Average mid-point titres reached their maximum on day 116 and remained at this level until day 150. The titres obtained are high, however the average titres are lower than the titres obtained in trial 2. Individual rat titres do reach those obtained in trial 2 in the 1:10000 region. The decreased in average titres may have, been caused by the lower number of derivative attachments on the carrier molecule Tetanus Toxoid, or the possible lower efficacy of AS2V adjuvant explained further in section 3.3.1. Antibody titres were not able to be obtained on day 184, 14 days after the second booster, as it was not possible to take blood from these animals before or after the acute nicotine injection on that day.

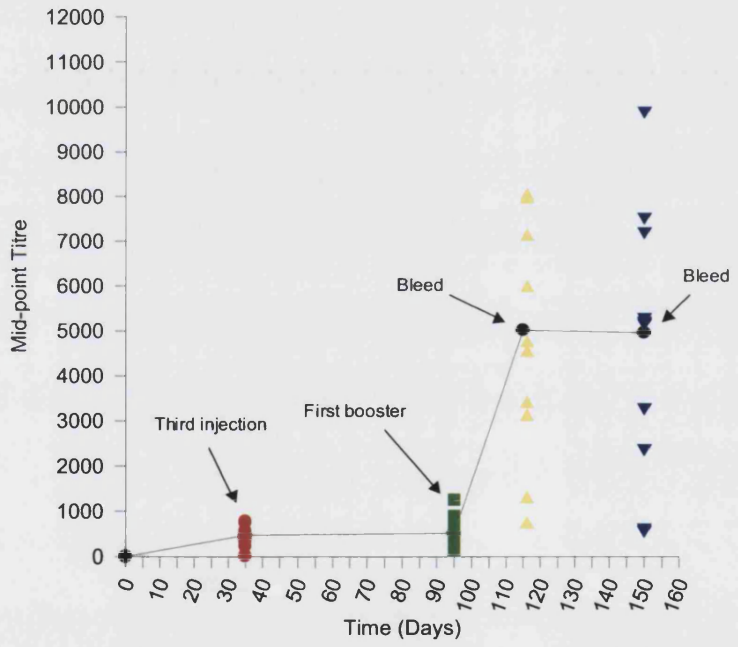


Figure 5.2: Mid-point antibody titres from rats vaccinated with 5μg TT-cotsh.

The titres were obtained from sera taken on days 35, 95, 116, 150. The sera were tested by ELISA using trans-4-thiol cotinine derivative coated wells. n = 10 rats for each group with the bars illustrating mean mid-point titre, and symbols representing mid-point titre for each rat.

5.4 Results of rat serum analysis for nicotine and cotinine concentrations in rats chronically treated with nicotine by osmotic minipump.

Rats vaccinated with TT-cotsh (5μg) and TT-cysteine (25μg) were chronically treated with nicotine (4mg/kg/day) or saline by osmotic minipump, on days 91-98. The same procedures for bleeding, osmotic minipump preparation, surgical procedures and sera cotinine and nicotine concentration determination were used, as described in section 3.6 and 3.7.

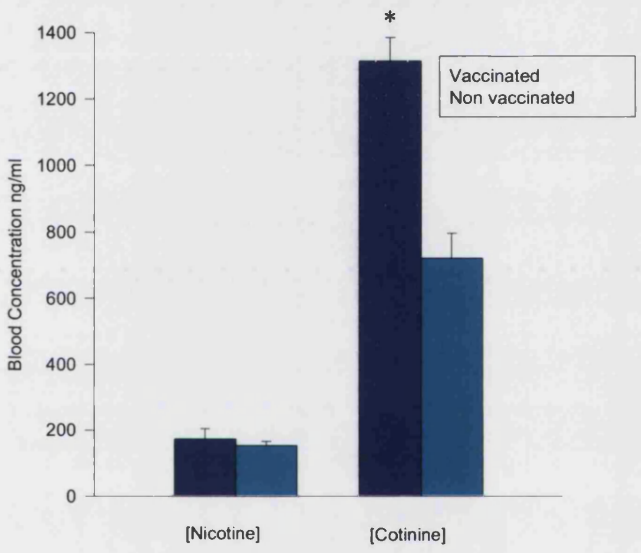


Figure 5.3: Nicotine and cotinine concentration in serum from rats immunized with 5μg TT-cotsh vaccine and TT-cysteine (control), and treated with 7-day nicotine osmotic minipump in regime 1.

Nicotine was administered at a rate of 4mg/kg/day. Sera were taken on day 98. n = 5 rats per minipump treatment group.

***** = *Significantly different from corresponding rats vaccinated with TT-cysteine (control) (p <0.05 p = 0.0129) using student's unpaired t-test.*

Rats receiving saline had negligible levels of serum nicotine (mean 2.0 ± 0.226ng/ml) and cotinine (mean 6.35 ± 1.627ng/ml) in both vaccine groups.

Nicotine treatment produced levels of nicotine (152.6 ± 13.6) and cotinine (720.6 ± 75.8) in the expected range for the nicotine dose used, in control vaccinated rats (figure 5.3) (Sanderson *et al* 1993). The cotinine concentration for the TT-cotsh vaccine sera (1312.4 ± 70.93) increased significantly compared to the control vaccine (720.6 ± 75.8),

when treated with nicotine (figure 5.3). This result is as expected, possibly due to the cotinine specific antibodies binding to cotinine in the blood producing an antibody-conjugate complex, and therefore retaining cotinine in the blood (Heida *et al* 1997).

5.5 Results of rat serum analysis for nicotine and cotinine concentrations in rats, 8 hours after nicotine injection.

Rats vaccinated with TT-cotsh (5μg) and TT-cysteine (25μg) from regime 2, were acutely treated with nicotine (0.4mg/kg) or saline by subcutaneous injection, on day 184. Eight hours after injection, the rats were killed and blood was taken for nicotine and cotinine concentration determination (see section 3.7 for procedures). There were 7 rats per injection group. This experiment was done to show if vaccination causes an effect on acute nicotine treatment.

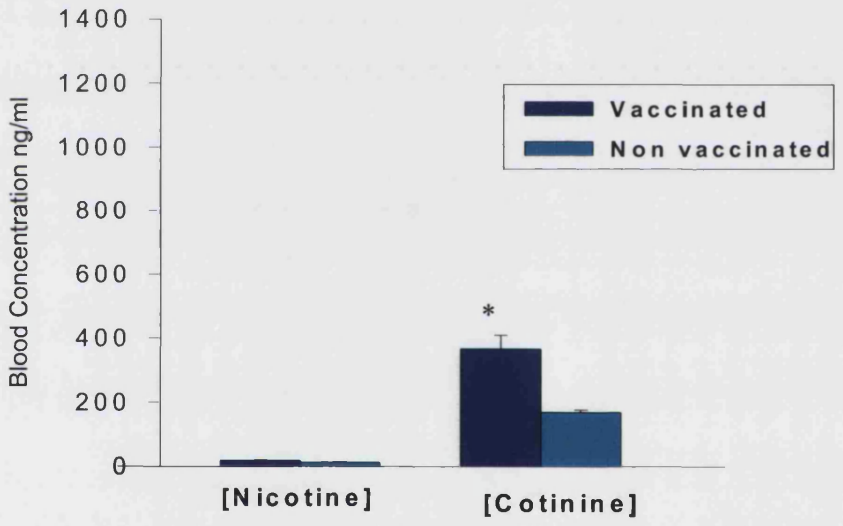


Figure 5.4: Nicotine and cotinine concentration in serum from rats immunized with 5μg TT-cotsh vaccine and TT-cysteine (control), and treated with nicotine injection (0.4mg/kg) in regime 2.

Acute nicotine treatment (0.4mg/kg) was administered by subcutaneous injection on day 184, and sera were taken eight hours after injection, with 7 rats per nicotine or saline treatment group. ******* *= Significantly different from corresponding rats vaccinated with TT-cysteine (control) vaccine ($p < 0.05$ $p = 0.0129$) using student's unpaired t-test.*

Rats receiving saline had negligible levels of serum nicotine (mean 2.0 ± 0.226ng/ml) and cotinine (mean 6.35 ± 1.627ng/ml) in both vaccine groups.

Nicotine treatment produced levels of nicotine (12.3 ± 1.9) and cotinine (167.2 ± 8.5) in the expected range for the nicotine dose used, in control vaccinated rats (Sanderson *et al* 1993).

The cotinine concentration for the TT-cotsh vaccine sera (365.2 ± 43.3) increased significantly compared to the control vaccine (167.2 ± 8.5), when treated with acute nicotine (0.4mg/kg) (figure 5.4). This result shows that vaccination was acting within eight hours after introduction of nicotine into the system, allowing for metabolism of nicotine to cotinine. The cotinine specific antibodies may then have bound to cotinine in the blood, in large amounts to allow the production of antibody-conjugate complexes, which retained cotinine in the blood in large enough quantities to produce a significant increase in cotinine concentration (Heida *et al* 1997).

5.6 Effect of vaccination on nicotine- evoked dopamine release in the striatum

The rats vaccinated with 5μg TT-cotsh were treated with 7-day nicotine or saline osmotic minipumps in regime 1 on days 31-38. The two striata from each animal were dissected and the effect of this nicotine treatment on nicotine-evoked dopamine release was measured.

The same procedures for osmotic minipump preparation and surgical procedures were used as described in section 3.6 and 3.7. The 96-well plate [^{3}H]-dopamine release assay described in sections 2.2 and 2.3 was used. The nicotine concentration used in these experiments was 10μM.

There was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in TT-cotsh vaccinated rats (figure 5.5). This is in accordance with results obtained in trial 2 (figure 4.10).

There was a significant decrease in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in TT-cysteine vaccinated rats (figure 5.5). This is in contradiction with results obtained in trial 2 (figure 4.10).

However in this experiment there is no significant change in nicotine – evoked [^{3}H]-dopamine release between TT-cotsh and TT-cysteine vaccinated rats ($p=0.058$). The statistics show that statistical significance in this case is close and with further experiments and an increase in the number of rats in the experiment, significance may be reached.

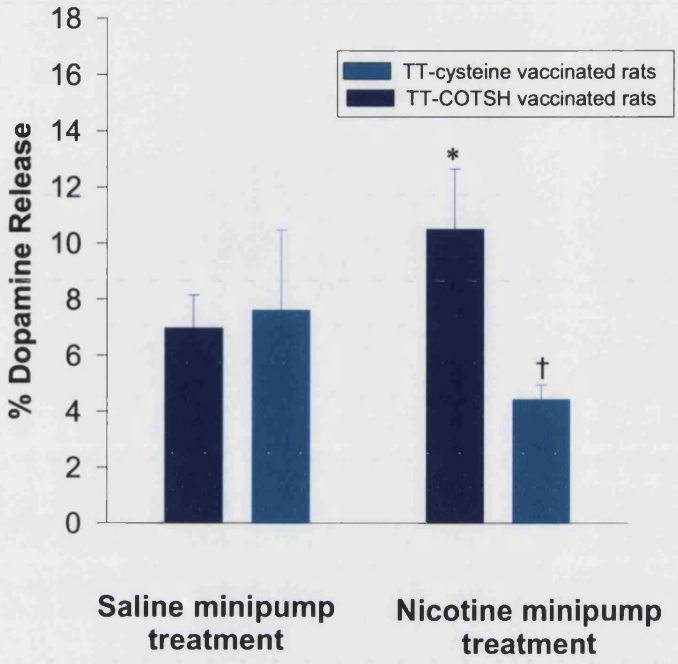


Figure 5.5: Effect of chronic nicotine treatment on nicotine – evoked [^{3}H]-dopamine release.

Striatal minces were pre-loaded with [^{3}H]-dopamine. Pre-incubation with nicotine (10μM, 5 minutes) and stimulation with nicotine (10μM, 5 minutes) showed [^{3}H]-dopamine release after chronic nicotine treatment by 7-day osmotic minipump. 8 wells per treatment were used in each experiment and this was done for 5 rats per group from regime 1.

† = Significantly different from control TT-cysteine vaccinated rats treated with saline minipump ($p<0.05$) using Student's unpaired t-Test.

** = Significantly different from corresponding TT-cotsh vaccinated rats treated with saline minipump ($p<0.05$) using Student's unpaired t-Test*

5.7 Effect of vaccination on nicotine – evoked locomotor activity

5.7.1 Introduction

Under a regime of daily exposure to nicotine, tolerance to the depressant effect on locomotor activity begins to develop after a single exposure, and is clearly present after a week (Shoaib *et al* 1994, Domino 2001). Sensitisation to the locomotor stimulation

produced by nicotine takes longer to develop, with a clear effect visible after 10 days or more (Shoaib & Stolerman 1992, Whiteaker *et al* 1995, Kelsey *et al* 2002).

Enhanced locomotor response to nicotine in animals pretreated with the drug prior to test day is associated with potentiation of its effects on dopamine secretion in the nucleus accumbens (Benwell & Balfour 1992). Pre-treatment with nicotine causes a regionally selective sensitisation of its stimulatory effects on the mesoaccumbens dopamine system, which has been implicated in the locomotor stimulant response to nicotine and its ability to reinforce self-administration . The sensitisation evoked by daily injections of nicotine is associated with a regionally selective down-regulation of the control of mesoaccumbens dopamine neurons by inhibitory autoreceptors and depends on co-stimulation of NMDA glutamatergic receptors. It is suggested that the sensitisation is related to enhanced burst firing of mesoaccumbens neurons, which results in an enhancement of dopamine release into the extracellular space between the cells where it acts upon putative extrasynaptic dopamine receptors. (Balfour *et al* 1997). There are some studies which suggest that up-regulation of the nicotinic acetylcholine receptors may be responsible, in part, for the sensitisation to nicotine (Shoaib *et al* 1997). This study was done to examine the effects of nicotine on locomotor activity in vaccinated vs non-vaccinated rats, as well as the effect of nicotine sensitisation on locomotor activity in vaccinated vs non-vaccinated rats. This study was carried out in the Department of Pharmacy and Pharmacology at the University of Bath, in conjunction with Dr Paul J Mitchell.

5.7.2. Methods

STUDY GROUPS

TT - COTSH vaccinated rats	-	Saline injections	(n=7)
	-	Nicotine injections	(n=7)
TT - Cysteine vaccinated rats	-	Saline injections	(n=7)
	-	Nicotine injections	(n=7)

Rats were placed in the locomotor activity chamber for 60 minutes prior to injection to habituate the rats to the new environment. Locomotor activity was monitored immediately upon introduction to the activity chamber and for 90 minutes after injection of nicotine or phosphate buffer (total time = 150 minutes) (Figure 5.6). Nicotine hydrogen tartrate in phosphate buffer (100mM, 0.4mg/kg, pH 7.4) or phosphate buffer as control (100mM, pH 7.4) was administered by subcutaneous injection.

The locomotor cages had 11 infrared beams in the surround. Breaking of the beams was registered on the computer, allowing for measurement of line crosses and repeated breaking of the beam. The number of line crosses was the measurement used to quantify locomotor activity. Cage crosses were measured at 5-minute epochs and 30-minute blocks were analysed. Rats were housed in cages of 4 for the duration of the trial, with unlimited access to food and water, in rooms where the temperature is maintained at 20 ± 1°C and humidity at 50 ± 10%. There was a regular light-dark cycle with lights on from 0.700 – 19.00 hours. All behavioural experiments were carried out during the light phase of the cycle with 1 rat per locomotor activity chamber.

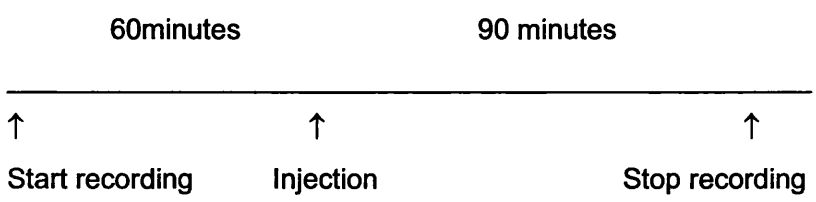


Figure 5.6: Time course of each locomotor activity recording.
Locomotor activity was monitored immediately upon the introduction of rats to the activity chamber and for 90 minutes after injection of nicotine or phosphate buffer (total time = 150 minutes).

	Day 1	Day 2	Day 3	Day 4
Group 1	Saline	Nicotine	Saline	Start Sensitisation
Group 2	Saline	Saline	Nicotine	Start Sensitisation

Table 5.1: Experiment plan demonstrating which treatments were administered on specific days prior to the sensitisation experiment.
Locomotor activity was measured in rats with saline injections on day 1, to determine base rate activity and habituate all rats to the experimental procedures. Locomotor activity measured on days 2 and 3 are to study the effect of acute nicotine treatment, allowing for all rats to be tested with both nicotine and saline to increase the number of animals in each group. Beginning on day 4, the effect of sensitisation on locomotor activity was measured for 21 days, in rats in the two groups with nicotine or saline treatment (7 days/week).

The dosing and recording was done as shown in table 5.2:

	Mon	Tues	Wed	Thurs	Fri	Sat	Sun
Dose	√	√	√	√	√	√	√
Recording	√	-	√	-	√	-	-

Table 5.2: Experiment plan for a sample week, showing dosing and recording regime.
All rats were treated with nicotine (0.4mg/kg) or phosphate buffer by subcutaneous injection everyday for three weeks, and locomotor activity was measured on Monday, Wednesday and Friday. There were 9 sessions in total.

5.7.3 Results

5.7.3.1 Acute nicotine treatment

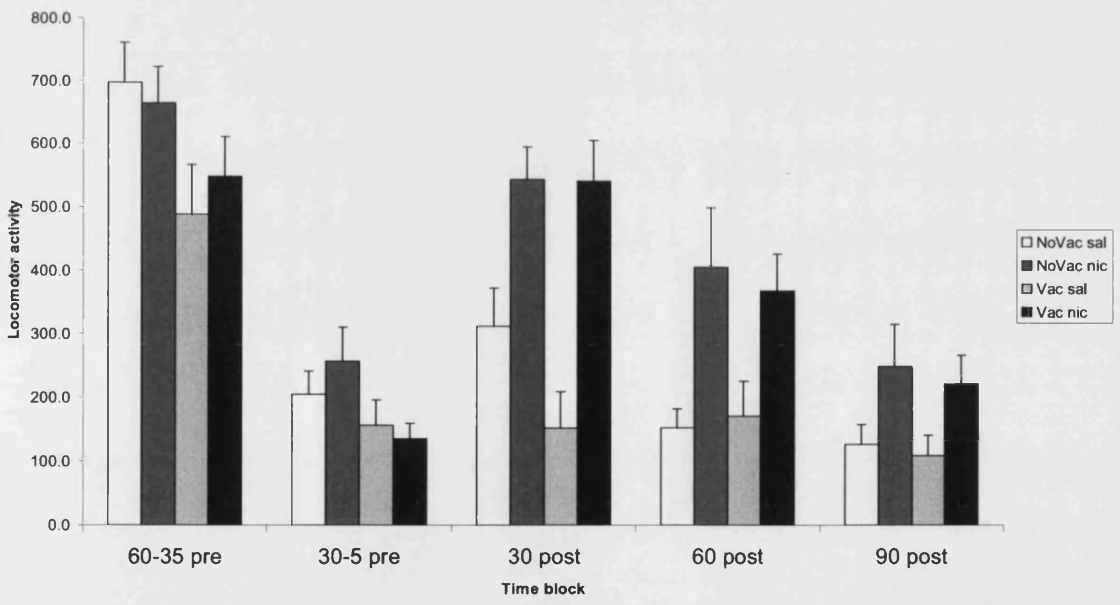


Figure 5.7: Effect of acute nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.
Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. n = 14

There is a high level of activity when animals are first placed in the locomotor chamber (60-35 pre) in all groups, which decreases in the next 30 minutes as the animals are habituated to the new environment. Acute nicotine induced hyperactivity is seen in vaccinated and non-vaccinated animals. The same hyperactivity is not seen in saline treated animals (Figure 5.7). Immunisation with cotinine vaccine shows no significant difference in nicotine induced locomotor activity compared to control vaccinated animals.

5.7.3.2 Chronic nicotine treatment produced sensitisation

The results are represented per half hour of locomotor activity measurement over the 9 sessions of tests as described in section 5.7.2, in the TT-cotsh and TT-cysteine vaccinated animals treated with nicotine or saline.

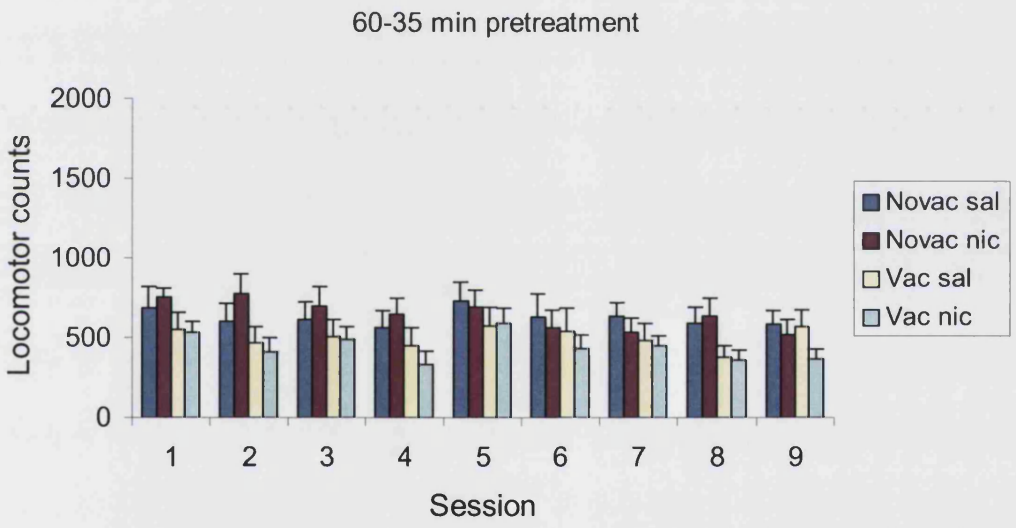


Figure 5.8: Effect of chronic nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.

Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. The graph represent locomotor activity measured in the 60-35 minute period prior to nicotine or saline treatment. n = 7

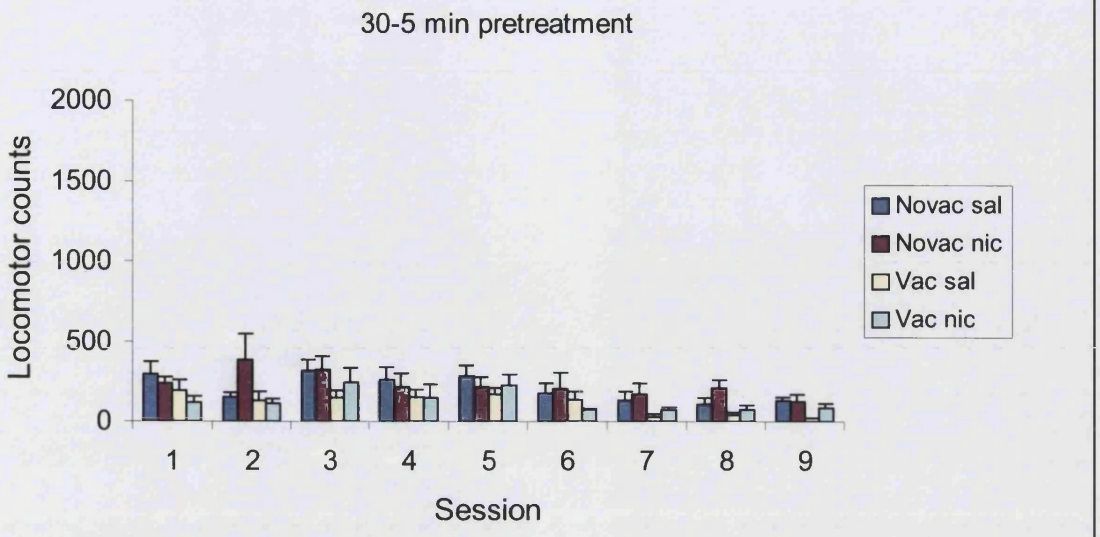


Figure 5.9: Effect of chronic nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.

Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. The graph represent locomotor activity measured in the 35-5 minute period prior to nicotine or saline treatment. n = 7

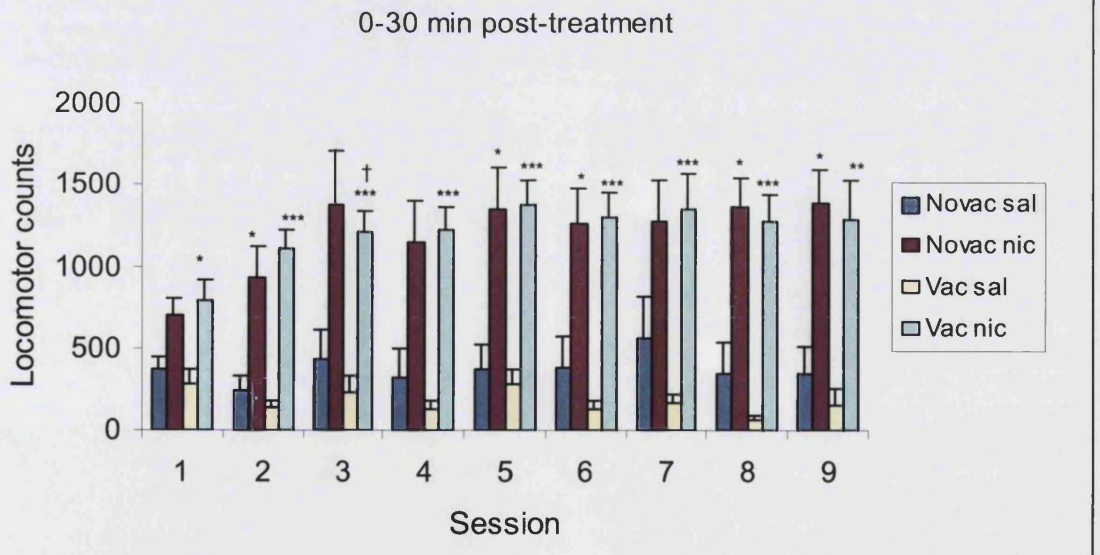


Figure 5.10: Effect of chronic nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.

Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. The graph represent locomotor activity measured in the 0-30 minute period after nicotine or saline treatment. n = 7

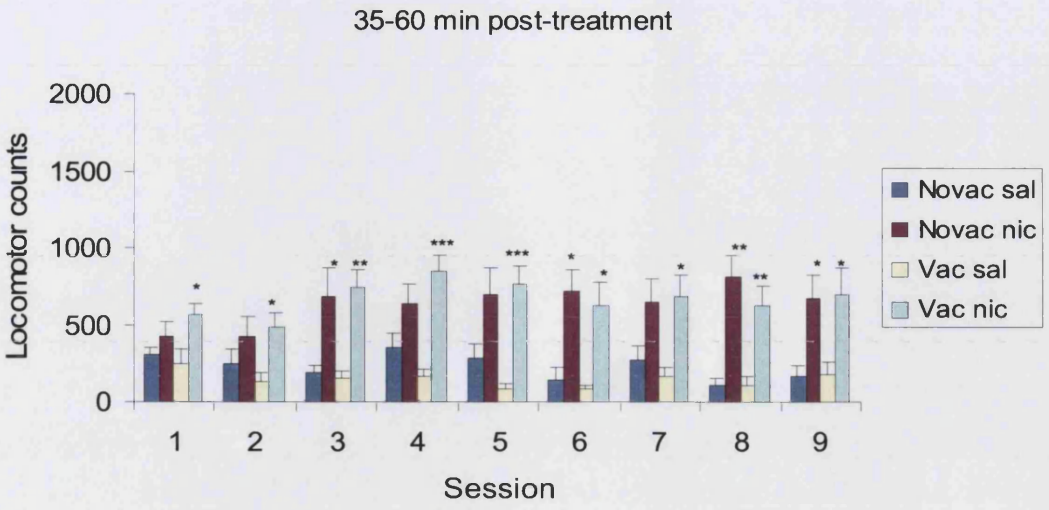


Figure 5.11: Effect of chronic nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.

Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. The graph represent locomotor activity measured in the 35-60 minute period after nicotine or saline treatment. n = 7

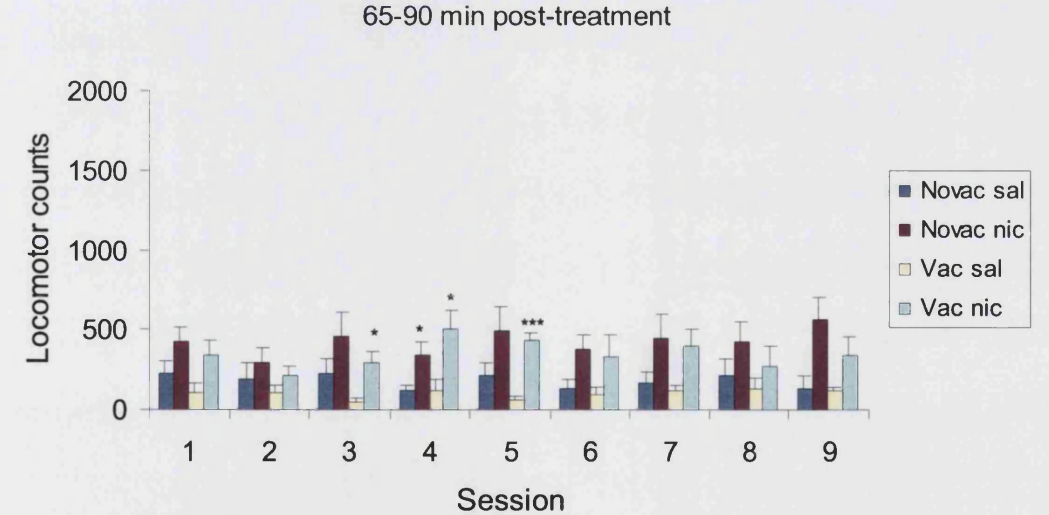


Figure 5.12: Effect of chronic nicotine treatment on locomotor activity in rats vaccinated with TT-cotsh and control TT-cysteine.

Rats vaccinated with TT-cotsh and TT-cysteine were treated with 4mg/kg nicotine or saline and locomotor activity was measured as described in section 5.7.2. The graph represent locomotor activity measured in the 65-90 minute period after nicotine or saline treatment. n = 7

The statistical analysis for figures 5.10, 5.11 and 5.12 are shown below. This was done to allow the reader to be able to compare the figures easily.

* *One-way ANOVA post hoc showed significance in comparison to respective saline group ($p<0.5$)*

** *One-way ANOVA post hoc showed significance in comparison to respective saline group ($p<0.01$)*

*** *One-way ANOVA post hoc showed significance in comparison to respective saline group ($p<0.001$)*

† *Repeated measures ANOVA post hoc $p < 0.05$ in comparison to session 1*

Figures 5.8 and 5.9 show a high level of activity when animals are first placed in the locomotor chamber (60-35 pre-treatment) in all groups, which decreases in the next 30 minutes as the animals are habituated to the new environment.

In the first 30 minutes after nicotine treatment (figure 5.10), nicotine induces significant hyperactivity in vaccinated and non-vaccinated animals. This does not occur in animals treated with saline. There is a period of development in the nicotine-induced increase of locomotor activity between sessions 1 to 3 for 30 minutes post dosing (figure 5.10). The increase in response to nicotine only reached significance in vaccinated animals in session 3, 30 minutes post treatment (figure 5.10) and is maintained mainly until 60 minutes post treatment (figure 5.11), however some hyperactivity is seen in session 5, 65-90 minutes post treatment (figure 5.12).

There is no evidence for differences in locomotor activity between vaccinated and non-vaccinated animals throughout the acute nicotine treatment. However, baseline activity in vaccinated animals is predominantly lower than in non-vaccinated animals, in the habituation sessions (60-5 minutes pre-treatment, figure 5.8, 5.9), and consistently lower in chronically treated animals (figure 5.10, 5.11, 5.12).

In summary, although nicotine – induced locomoter activity has been shown in this experiment, vaccination does not seem to cause an effect.

5.8 Discussion

5.8.1 Immunisation

The overall immune response in this trial was lower than in trial 2 for both injection regimes, possibly due to the lower number of derivative attachments to tetanus toxoid.

However mid-point titres from Regime 2 reached a higher level at the end of the experiment (day 150), compared to regime 1 (day 98), however this did include an extra booster of the vaccine (section 5.2). Further study is needed to examine both regimes more closely with the vaccine used in trial 2, to determine which injection regime activates the most robust immune response.

5.8.2 Effect of nicotine

The significant increase in blood cotinine but not nicotine concentrations after both acute and chronic nicotine treatment, seen in TT-cotsh vaccinated rats in comparison to control vaccinated rats, suggests antibody-cotinine complex formation in sera. Therefore cotinine is retained in the blood probably by the precipitation mechanism of antibodies as described in section 1.7.4. This is similar to results obtained in the production of a nicotine vaccine (Hieda *et al* 1997), where nicotine concentrations were increased in sera after nicotine treatment.

There was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in TT-cotsh vaccinated rats which is in accordance with results obtained in trial 2. However, nicotine has evoked a decrease in dopamine release compared to saline in non-vaccinated animals which is in accordance with Marks *et al* (1993). Therefore there seems to be a trend towards a larger difference in nicotine-evoked dopamine release and saline treatment in vaccinated animals compared to non-vaccinated animals. This decrease in dopamine release in non-vaccinated rats may be due to inactivation of nAChRs caused by nicotine treatment.

However in this experiment there is no significant change in nicotine – evoked [^{3}H]-dopamine release between TT-cotsh and TT-cysteine vaccinated rats ($p=0.058$). The statistics show that statistical significance in this case is close and with further experiments and an increase in the number of rats in the experiment, significance may be reached. These results are in accordance with the hypothesis of the cotinine vaccine project ie: cotinine is retained in blood causing a reduction in cotinine reaching the brain, allowing for nicotine to have a larger effect on dopamine release.

The nicotine induced locomoter activity was not affected by cotinine vaccination. This may be due to the low antibody titres during the locomotor activity experiments. Enough cotinine may not have been retained in the blood to have a significant effect on locomotor activity. Optimisation of the immune response and a repeat of these

locomotor experiments are necessary to determine whether the cotinine vaccine causes a behavioural change in rats. Passive immunisation of rats with nicotine vaccine (Pentel *et al* 2000) completely prevented nicotine-induced increases in locomotor activity measured 25 hours after nicotine injection, therefore reducing the amount of nicotine reaching the brain can cause an effect on locomotor activity. It would be interesting to find out if an increase in the actions of nicotine through vaccination against cotinine would cause an increase in locomotor activity.

Chapter 6

Discussion

6.1 Summary

The socio-economic consequences of nicotine addiction are vast as explained in the introduction. Many methods are adopted to treat nicotine addiction, including therapeutic intervention and counselling, the long term success rates have been limited and there continues to be a need for more effective treatments. A novel approach that has gained a significant degree of interest in recent years is the use of vaccines designed to raise antibodies against this drug of abuse and prevent its entry into the brain, therefore this concept would be targeting the drug and not the brain.

Cotinine was chosen as the target molecule for the vaccine. The hypothesis behind this target is that cotinine is produced in the body by the metabolism of nicotine. Specific antibodies to cotinine in the blood eliminate the compound before it reaches the brain. This will in turn stop the antagonistic actions of cotinine (through initial agonism causing desensitisation of nicotinic AchRs) on nicotine in the brain. Therefore nicotine will have a larger desired effect on the smoker, which in turn will encourage the smoker to consume fewer cigarettes. This is better for the health of the smoker and also with the aid of other therapies such as NRT, the smoker may have a better chance of successfully quitting.

The effects of cotinine and/on nicotine in the rat brain were first studied in chapter 2, as there is conflicting evidence in the literature regarding its mechanism of action (described in detail in the introduction). In the 96-well plate assay used to test the effects of various drugs on dopamine release in the striatum, nicotine and cotinine were shown to act as agonists on nAChRs in a dose-dependent manner, and cotinine showed a 1000-fold lower potency than nicotine, and probably caused desensitisation of the receptors when used in conjunction with nicotine. This conclusion was made as a decrease in nicotine-evoked dopamine release was seen when cotinine was used in conjunction with nicotine. This is in accordance with previous published data (Dwoskin *et al* 1999, Marshall *et al* 1997, Sharples & Wonnacott 2001, Abood *et al* 1981). This finding backs up the cotinine vaccine hypothesis. Removing or reducing the cotinine concentration in the brain by antibodies in the blood, will reduce the antagonism of cotinine on nicotine-evoked dopamine release as well as possibly affecting other mechanisms of nicotine addiction related to nAChRs. This will in turn increase the effects of nicotine on the addiction pathway allowing for the reduction of the need for nicotine by smokers. With other forms of nicotine cessation such as NRT, smokers will in this way be aided in their quit attempt.

The effects of cotinine on nAChRs in the striatum of rats were also studied to acquire a better understanding of the mechanism of action of cotinine. It was established that cotinine acts on nAChRs in the striatum, as mecamylamine which is a non-selective nAChR antagonist, was able to block dopamine release produced by cotinine stimulation. This is also in accordance with literature (Dwoskin & Crooks 2001).

To study which nAChRs are involved in this mechanism various antagonists were used and it was concluded that β2 and α6β2* subtypes are involved in cotinine-evoked dopamine release. This finding is also in accordance with literature (Zoli et *al* 2002, Sharples *et al* 2000). In this assay α7 was not shown to be involved in cotinine-evoked dopamine release, which is contradictory to published evidence (see section 2.1 and 2.5), however this may have been due to the limitations of the assay which is explained in full in section 2.5.

Three derivatives were made as described in the appendix, targeting both cotinine and nicotine. They were then conjugated to ovalbumin in chapter 3. It was however, not possible to conclusively determine whether conjugation had occurred and if so how many derivatives were attached per ovalbumin molecule. This was because of the lack of time and expertise at the beginning of the project. However, the conjugates were moved on to the immunisation stage to see the effect of the vaccine on the immune response. After immunization of SD rats with these vaccines, only trans-4-thiol cotinine produced measurable mid-point titres, however the titres were too low to be used to study the effects of the vaccine in neurochemical experiments. This may have been due to insufficient conjugation of derivatives onto the carrier molecule, therefore insufficient activation of the humoral and cell-mediated responses (as discussed in section 1.6.4).

The concentrations of nicotine and cotinine were studied in this initial trial after chronic nicotine treatment via osmotic minipump, however as anticipated, the vaccines did not produce significant changes in blood nicotine or cotinine levels. The other vaccines may not have been able to illicit an immune response due to the insufficient conjugation of derivatives on the carrier molecule. Heida *et al* (2000) showed an increase in blood nicotine levels after nicotine vaccination, caused by the binding and accumulation of nicotine to the antibodies in serum. There was a trend in this trial of an increase in cotinine levels in the sera (discussed in section 3.9), however the results were not significant and this was repeated in the next trial to allow for better

interpretation of the actions of the vaccine. Trans-4-thiol cotinine derivative was taken forward to the next trial, where modifications were introduced to increase the immune response. In trial 1 an ELISA assay was optimised to detect antibody titres and it was determined that coating the wells with the derivative produced the most accurate analysis of antibody titre, in comparison to coating with conjugate or using a sandwich ELISA. This assay was used for the rest of the project.

The trans-4-thiol cotinine vaccine was taken forward to trial 2 for optimisation. This was done by changing a variety of parameters:

- There is a correlation between size of a macromolecule and its immunogenicity as discussed in the introduction (section 1.6.4.5), therefore increasing the size of the antigen by adding a larger carrier molecule (TT) compared to ovalbumin in trial 1 improved the immune response.
- It was also determined that 5μg of the vaccine was the optimal concentration producing the highest mid-point titres. Therefore this concentration was used for the rest of the project.
- Conjunction with the increase in derivative attachments to the new carrier molecule, resulted in a dramatic increase in the immune response (average mid-point titres >1:10000) to desired levels where it has been shown to produce adequate drug-specific binding affinity (Hieda *et al* 1997). It was determined that 15 derivatives per tetanus toxoid molecule were attached which is in the desired region to illicit a high immune response.
- More boosters were also administered. The high levels of titres produced were probably caused by the long-lived memory cells, which are produced along with the relatively short-lived effector cells of the primary immune response, after the second booster. These memory cells proliferate rapidly when exposed again to the same antigen, giving rise to a new clone of memory cells as well as new effector cells, demonstrated here by the increase of antibody titres after the second booster.
- The change in adjuvant from Alum to GSK AS2V was also a successful strategy.
- High mid-point titres towards cotinine were obtained in this trial and after development of a competitive ELISA, the antibodies produced were shown to be specific to cotinine in comparison to nicotine and other nicotine metabolites which have been shown to have an effect on nicotine addiction. For example nornicotine has been shown to increase dopamine release in a calcium-

dependent manner from superfused rat striatal slices (Dwoskin *et al* 1993). The specificity of the antibody therefore enables us to determine only the effect of the reduction of cotinine to the brain and not other mechanisms that may contribute to nicotine addiction. The nicotine vaccines made by Pentel group (1997), De Villier *et al* (2002) or the Xenova vaccine have not shown specificity of antibody to their chosen drug to this degree.

This improved vaccine produced a positive result on blood cotinine concentrations after chronic nicotine treatment. It was shown that cotinine was retained in the blood.
This is similar to results obtained in the production of a nicotine vaccine (Hieda *et al* 1997), where nicotine concentrations were increased in sera after nicotine treatment.
This retention of cotinine in the blood probably causing a reduction in the concentration of cotinine in the brain, in accordance with the project hypothesis as explained earlier.

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In trial 3, two injection regimes were used to allow for dopamine release and locomotor activity studies. The vaccine used in this trial was the same as in trial 2, however only 11 derivatives per carrier molecule were shown to be conjugated. This may have been due to decomposition of the derivative before conjugation, as a long period had elapsed since the production of the derivatives at the beginning of the project. This contributed to less robust results in this trial. For example, Lower mid-point titres were obtained in trial 3 compared to trial 2. Another factor which may have contributed to this was the fall in efficacy of adjuvant used due to long period of storage after its production. However, cotinine was still retained in the blood after acute and chronic nicotine treatment as seen in trial 2.

There was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in both TT-cotsh and TT-cysteine vaccinated rats in trial 2. However in trial 3 there was a significant increase in nicotine - evoked [^{3}H]-dopamine release when rats were chronically treated with nicotine compared to saline, in TT-cotsh vaccinated rats (same as trial 2) but, nicotine evoked a decrease in dopamine release compared to saline in non-vaccinated animals which is in accordance with Marks *et al* (1993). Therefore there seems to be a trend towards a larger difference in nicotine-evoked dopamine release and saline treatment in vaccinated animals compared to non-vaccinated animals. This decrease in dopamine release in non-vaccinated rats may be due to inactivation of nAChRs caused by nicotine treatment.

The analysis of dopamine release from rat striatal slices found an increase in dopamine release after treatment with nicotine by daily injection (Yu & Wecker 1994). In contrast, Marks *et al* (1993) showed that dopamine release from mouse brain synaptosomes declined after chronic nicotine infusion suggesting desensitisation/inactivation of nAChRs. Similar results have also been obtained in our laboratory.

The nicotine evoked dopamine release experiments produced contradictory results between chapter 4 and 5. However, the vaccine did produce a trend in changing the effect of nicotine on dopamine release in the striatum, which may preliminarily suggest that the hypothesis of the vaccine can be plausible i.e.; cotinine is retained in the blood, reducing the amount reaching the brain, which in turn is imposing an effect on dopamine release in the brain. This pathway has been shown to be involved with reward in nicotine addiction, concluding that this vaccination has the potential of having an effect on nicotine addiction

Unfortunately the vaccine did not cause a significant effect on locomotor activity both with acute and chronic nicotine treatment. However, the low antibody titres were perhaps responsible for the lack of significance in the difference between locomotor activity in vaccinated and non-vaccinated rats. The cotinine concentration in the brain may not have been reduced to a large enough degree to produce a significant change in nicotine-induced locomotor sensitisation.

This project has given a proof of concept for the cotinine vaccine. It has shown that a vaccine specific for cotinine has been produced giving some preliminary results on the effect of immunization on biological and neurochemical parameters.

6.2 Future perspective

The cotinine vaccine project is at its preliminary stages. After obtaining specific antibodies towards cotinine, there are countless areas of examination of the immune response.

One route to take should be to obtain the concentration of antibodies in the blood after immunization and the binding affinity of the antibody for the antigen as these are important parameter to investigate. They may allow for the understanding of the actions of the antibodies produced against cotinine, and the effectiveness of the antibodies in retaining cotinine in the blood and for how long. Such experiments were conducted by Heida *et al* (1997) who demonstrated that the nicotine binding capacity of

anti-nicotine antibodies after active immunisation (1.3 μmol/L) exceeded the venous plasma levels of nicotine (up to 0.26 μmol/L) and was nearly equal to the arterial plasma levels reported in humans after smoking one or two cigarettes (Benowitz *et al* 1983, Henningfield *et al* 1993). Fourty minutes after intravenous administration of nicotine 0.03 mg/kg, there were 4 to 6 fold greater concentrations of nicotine in the plasma of immunised animals but there were no differences in brain levels of nicotine. However, when nicotine levels were examined at a more clinically relevant time point (3 minutes after intravenous nicotine administration), brain nicotine levels were reduced by 36%, while plasma concentrations raised 3 to 6 fold (Heida *et al* 1999). The pharmacokinetics were similar even after five repeated doses of nicotine (Keyler *et al* 1999), or after long-term exposure to nicotine before and during immunisation (Heida *et al* 2000). Preclinical studies of the Xenova TA-NIC™ (a nicotine derivative coupled to rec cholera toxin B) vaccine produced high titres of nicotine-specific antibodies in mice and altered the pharmacokinetic distribution of a nicotine challenge. Specifically one minute after an intravenous injection, nicotine levels in the brain decreased while plasma levels increased. They concluded that the antibodies may have the capacity to block the psychoactive effects of nicotine.

It would also be important to study what happens to the bound cotinine in the body. Does it cause an effect in other parts of the body? Measurement of antibody-cotinine complex as well as cotinine and nicotine in different organs between vaccinated and non-vaccinated animals is one way of achieving this. In 2003 Satoskar *et al* showed that vaccination against nicotine reduces nicotinic distribution to the brain not only by sequestering nicotine in serum but also by redirecting tissue distribution disproportionately away from the brain, such that nicotine concentrations are reduced to a greater extent in the brain than in other tissue. They also showed that maternal vaccination against nicotine reduced nicotine distribution to foetal brain in rats (Keyler *et al* 2003).

The De Villier vaccine also showed that immunization against nicotine altered the distribution but not half-life of nicotine (de Villiers *et al* 2004). Therefore studying the pharmacokinetics of nicotine and cotinine in cotinine vaccinated animals is also an important area to study to get a better understanding of its actions in the body. Will the half -life of cotinine remain the same? If so will the antibodies be able to retain and aid in the elimination of cotinine effectively as cotinine has a much longer half-life compared to nicotine.

Another area of investigation could be the characterization of the immunoglobulins activated by the vaccine as this would be useful to understand the immune response more thoroughly. It would also be important to study the time period in which the immune response is effective. Pentel *et al* (1997) used an injection regime over 5 weeks and similar time periods were used in the other nicotine vaccines. Would boosters be needed throughout the life of the "ex-smoker" to prevent relapse or would it only be an effective tool during cessation?

The animal species can be changed to find out effect of vaccination on other species as well as examining whether a better immune response can be obtained.

A large array of neurochemical and behavioural examinations can been conducted to study the effect of the cotinine vaccine on nicotine addiction. For example, locomotor activity tests must be conducted again with a higher immune response in the animals to find out the effect of vaccination against cotinine on nicotine-evoked sensitisation. Passive immunisation of rats with nicotine vaccine (Pentel *et al* 2000) showed attenuated nicotine-induced increases in systolic blood pressure and completely prevented nicotine-induced increases in locomotor activity measured 25 hours later.

Other behavioural experiments can also be conducted. For example in nicotine-dependent rats, passive administration with nicotine vaccine prevented nicotine reversal of abstinence signs (Malin 2001). These experiments were done using passive immunisation however, with active immunisation with the cotinine vaccine we can use the same behaviour experiment to study the effect of reduced cotinine on abstinence symptoms. Will these symptoms be reduced?

Self-administration experiments can be conducted to examine whether the cotinine vaccine does produce a reduction in nicotine intake *in vivo* in animals who have been previously "addicted" to nicotine. This would provide a good insight into the actions of the vaccine on the complex behavioural components of nicotine addiction. This was tested using passive immunization against nicotine which attenuated nicotine discrimination using a two lever operant chamber (Malin *et al* 2002). The De Villier vaccine also showed that vaccinated rats do not re-instate nicotine self-administration behaviour when exposed to nicotine (Lindblom *et al* 2002).

Microdialysis experiment would be a good tool to study the effect of vaccination on different neurotransmitter actions involved in nicotine addiction such as dopamine and

noradrenaline release in the areas of the brain which are involved in the addiction pathway including the NAcc core and shell. De Villiers *et al* (2002) showed a reduction in the outflow of dopamine in the NAcc shell with their nicotine vaccine. The questions below were raised in the introduction and microdialysis experiments are one tool to examine these questions:

- Will the decrease in cotinine in the brain have an effect on excitatory inputs into the VTA via glutamatergic transmission from the prefrontal cortex?
- Will the inhibitory inputs into the VTA via GABAergic neurons have an effect on the addiction pathways?
- Will the decrease in cotinine and the subsequent larger effect of nicotine show different characteristics on the dopamine released in the Nacc shell and core and therefore on the hedonic properties of nicotine?
- The aversive effects of nicotine may also be altered with the decrease of cotinine and the subsequent increase of nicotine effect in the brain.

Long term trials should also be set up to examine the effect of vaccination on the longer term parameters of nicotine addiction such as withdrawal and craving in animal models.

Of course, the effect of the cotinine vaccine would be most interestingly studied in humans to find out whether it provides a positive and effective tool to combat nicotine addiction, in the very complex physiological and psychological behaviours related to tobacco smoking.

Appendix Derivative Production

7.0 Production of nicotine and cotinine derivatives

7.1 Introduction

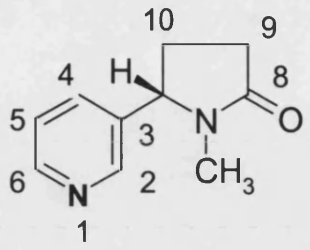


Figure 7.1: Chemical structure of Cotinine molecule. *Cotinine is the major metabolite of nicotine. It has a similar structure with added oxygen molecule to position 8 on the pyrolidine ring. It accounts for 70-78% of metabolised nicotine, with a half-life of 16-20 hours in humans.*

Immunogenicity (refer to section 1.6.4 in Introduction) is the ability of a molecule to induce an immune response and is determined by both the intrinsic chemical structure of a molecule and whether or not the host animal can recognize the compound. For a compound to elicit a primary antibody response and a strong secondary response, it must contain an epitope that can bind to the cell-surface antibody of a virgin B cell and it must promote cell-to-cell communication between B cells and helper T cells. When an immunogen is too small in size, as the cotinine hapten is in this project, the T helper cells are often not activated resulting in no response from the immune system. To remedy this problem, the immunogen must be conjugated to a carrier protein. The first step to making a vaccine for cotinine is the successful conjugation of cotinine onto an immunogenic carrier protein in order to induce an antibody response *in vivo*. To do this, the cotinine molecule (figure 7.1) must be derivatised so it can be easily linked to the carrier protein. This is the focus of the appendix.

One consideration in producing this cotinine vaccine is to be able to successfully obtain a patent for an anti-cotinine vaccine, and to do this the induced antibodies must predominantly recognize cotinine as opposed to nicotine. This would then avoid infringing the existing patents in the area of nicotine vaccines.

Cotinine itself could be linked to an immunogenic carrier protein via a number of different positions on its structure (positions 1, 5, 6 or 10) (figure 7.1). As the key differentiation factor from nicotine is the carbonyl group on the pyrolidine ring, the ideal linkage site would be on the pyridine ring at position 1, 5 or 6. Once conjugated via one of these sites, the pyrolidine ring would be presented to the body's immune system and therefore increase the chances of obtaining a cotinine specific antibody response.

Unfortunately it is not possible to predict on paper which linkage would yield the best immune response therefore work was done on several different linkage sites. The actual nature of the linker should be 4-6 carbons in length to adequately expose the cotinine hapten to the immune system. Ideally at the end of the linker there should be a sulfhydryl group which will enable the cotinine-linker conjugate to bind to the protein via a maleimide bond. This type of chemical linkage lends itself well to large-scale vaccine manufacture. However for initial studies there could be a carboxyl group at the end of the linkage which can then be linked to the protein via a carbodiiamide bond.
The production of the derivatised cotinine and nicotine molecules were undertaken by Dr. Sharn Ramaya and Professor Tim Gallagher at University of Bristol.

7.2 Materials

All commercially obtained reagents were purified and dried according to accepted procedures (Purification of Laboratory Chemicals, Perrin, Armarego & Perrin, 2nd Edition). Infrared spectra (ν_{max}) were recorded in the range of 3500-3100 cm^{-1} using a Perkin-Elmer spectrophotometer as a thin film on NaCl plates. Nuclear Magnetic Resonance (NMR) spectra were recorded at the specified field strength and in the solvent indicated using standard pulse techniques on a Joel GX 270, Lambda 300 or a Joel GX 400 spectrometer. All samples were dissolved in deuterochloroform using tetramethylsilane (TMS) as an internal reference. Chemical shifts are quoted in parts per million (ppm) downfield from TMS. Coupling constants (*J*) are quoted in Hz. Mass spectra (*m/z*) (electron ionisation (EI) or chemical ionisation (CI) were obtained using a Fisons/VG Analytical Autospec System at the University of Bristol. Flash column chromatography was carried out on silica gel (Merck 9385) or 60 H silica gel (Merck TLC). Thin layer chromatography (TLC) was carried out using Merck glass-backed Kieslegel 60 F_{254} plates. Chromophoric compounds were visualized by UV light (254 nm) and subsequently staining with alkaline potassium permanganate solution. Evaporation of solvents was carried out on a Buchi rotary evaporator. All chemicals were obtained from Aldrich.

7.3 Methods and Results

7.3.1 Synthesis of 6-Amino Nicotine

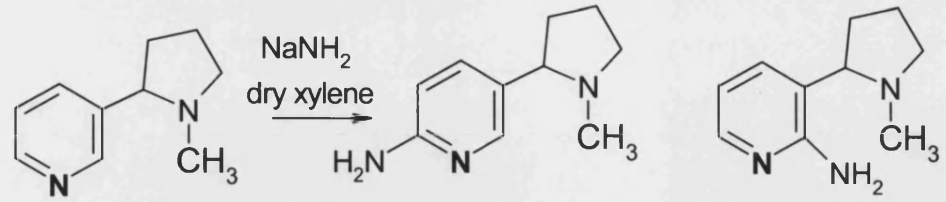


Figure 7.2: Synthetic reaction for generating 2- and 6-amino nicotine from nicotine.

This synthesis was carried out as a preliminary stage for the attachment of a viable linker on the 6 position of the nicotine pyridine ring.

This method (figure 7.2) was based on literature method of Casida *et al* (1999). Sodium amide (3.1g, 0.08 mol) was added portion wise, to a solution of (-)-nicotine (4.95cm^3,0.03 mol) in *p*-xylene (dry, distilled from P_2O_5, 12.5cm^3). The mixture was then heated at 130°C for 12 hours. The resulting brown mixture was cooled to room temperature, and concentrated hydrochloric acid was added carefully until pH1. The organic phase was extracted with ether (3x50cm^3), and the aqueous phase was made again alkaline (pH 14) with 20% aq NaOH and then extracted with ether (3x50cm^3). The combined extracts were dried (Na_2SO_4), filtered and concentrated *in vacuo* to give a brown slurry. Purification in order to separate this product from the corresponding 2-amino isomer was done by flash chromatography using 0-50% methanol / dichloromethane as eluant gave 6-amino nicotine (2.81g, 51%) as a brown oil.

δ_H (300 MHZ, $CDCl_3$) 7.97 (1 H, d, *J* 2.4, H-2), 7.49 (1 H, dd, *J* 2.4, 8.5, H-4), 6.52 (1 H, d, *J* 8.5, H-5), 4.47 (2 H, br s, NH_2), 3.22 (1 H, t d, *J* 1.9, 9.3), 2.93 (1H, d d, *J* 7.5, 9.3), 2.25-1.80 (5 H, m), 2.14 (3 H, s, CH_3).

7.3.2 Synthesis of 6-Amino Cotinine

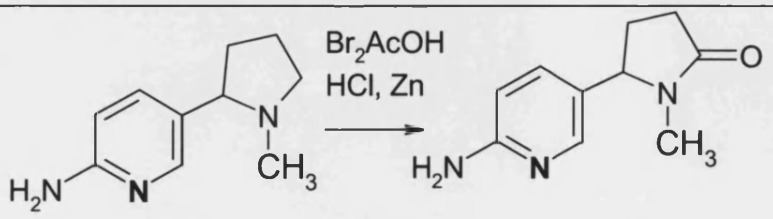


Figure 7.3: Synthetic reaction for generating 6-amino cotinine from 6-amino nicotine.

This synthesis was carried out as a preliminary stage for the attachment of a viable linker on the 6 position of the cotinine pyridine ring.

This method (figure 7.3) was based on literature method: Acheson *et al* (1979). Bromine (2.26 cm^3) in acetic acid (2.8 cm^3) and water (0.6 cm^3) were added over one hour to a stirred solution of 6-amino nicotine (1.13g, 6.38 mmol) in concentrated acetic acid (11.3 cm^3) and water (2.8 cm^3). After one hour at room temperature, water (45 cm^3) was added and the mixture stirred and heated to 85°C for 15 minutes, and then cooled to room temperature.

Concentrated acetic acid (4.5 cm^3), concentrated hydrochloric acid (2.3 cm^3) and water (1.1 cm^3) were added, followed by zinc dust (3.96g) which was added portion wise. The mixture was stirred at room temperature for 12 hours. After filtering, the solution was basified using aqueous ammonia 0.88. Extraction with chloroform (4 x 50 cm^3), drying the combined extracts over Na_2SO_4, filtering and removing the solvents *in vacuo* gave a yellow-brown oil. Recrystallisation of the crude product from benzene gave 6-amino cotinine as a pale yellow solid (416 mg, 34%).

δ_H (270 MHz, $CDCl_3$) 7.93 (1 H, d, *J* 2.2, H-2), 7.28 (1 H, d d, *J* 2.2, 8.5, H-4), 6.56 (1 H, d, *J* 8.5, H-5), 4.72 (2 H, br s, NH_2), 4.44-4.39 (1 H, m), 2.70-2.40 (3 H, m), 2.64 (3 H, s, CH_3), 1.94-1.78 (1 H, m).

Problems were encountered in purifying 6-amino nicotine, therefore attempts were made to oxidize the mixture of 2- and 6-aminonicotine directly to the target 6-aminocotinine which was isolated in 41% yield.

7.3.3 N(6)-(Alkyl) 6-Amino Cotinine

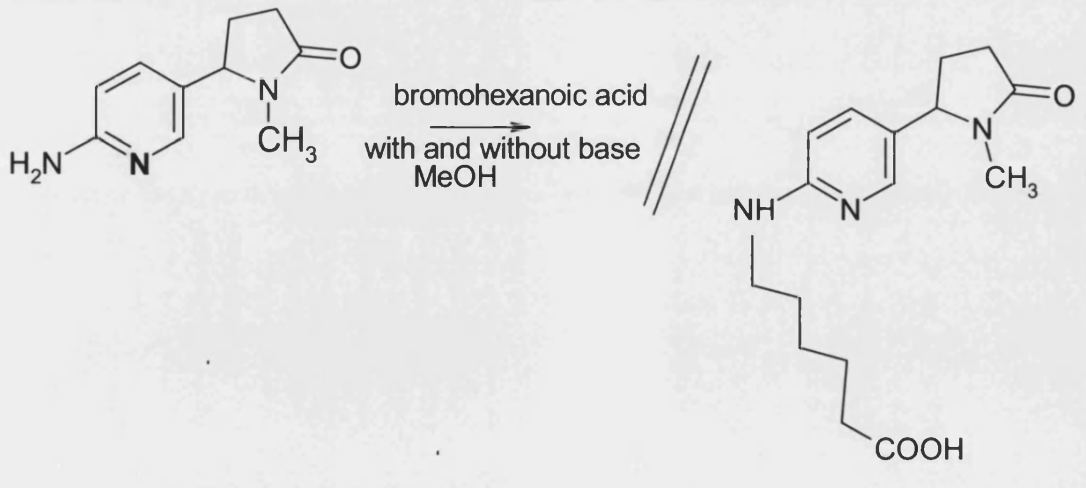


Figure 7.4: Synthetic reaction for addition of carboxyl group on 6-amino cotinine.

The aim of this experiment was to attach a carboxyl group at the end of a 6-carbon linker to produce a viable derivative.

The above reaction did not proceed using 6-bromohexanoic acid in Methanol, and only gave back starting material. Using 1 equivalent of K_2CO_3 also failed to give the desired product. Using 2 equivalents of K_2CO_3 also gave starting material, and then warming the reaction mixture to 40°C overnight also gave back starting material.

Similarly, we failed to isolate the *N*-alkylated product when 5-bromopentanoic acid was used, with and without added K_2CO_3 (1, 2 equivalents K_2CO_3 , and heating to 40°C overnight). Under all conditions only starting material was isolated.

Literature suggests that direct alkylation of 2-aminopyridine takes place at the pyridine nitrogen. However, there are reports that when $NaNH_2$ is used as base, the amino NH_2 undergoes alkylation.

Following some work done by Beresnevichyus (1992), on 2-amino pyridines, the following reaction was tried. The reaction involved the addition of acrylic acid to a refluxing solution of 6-aminocotinine in toluene. Unfortunately, when purifying using an ion exchange column (Amberlite resin), no carboxylic acid containing product was isolated. We cannot assume that this did not occur, only that it was not isolated.

The reaction (figure 7.5) was repeated, this time evaporating off the solvent and analysed by $_1$H NMR. To a boiling solution of 6-amino cotinine (100mg, 0.5 mmol) in toluene (1cm^3), acrylic acid (0.04 cm^3, 0.6mmol) was added and the boiling continued for another hour. During the heating an oil separates out. The reaction mixture was cooled to room temperature, and the solvent removed *in vacuo*. Water (5cm^3) and dichloromethane (5cm^3) was added, and the aqueous phase was washed with further portions of dichloromethane (3 x 5 cm^3). The organic extracts were combined and the solvent removed *in vacuo*. Purification by flash chromatography using 2% methanol/ dichloromethane as eluent, gave the 6-amino cotinine conjugate (12mg, 9%) as a colourless oil.

δ_H (270 MHz, $CDCl_3$) 8.66 (1 H, br s, NH), 8.36 (1 H, d, *J* 8.5, H-5), 8.21 (1 H, d, *J* 2.2, H-2), 8.21 (1 H, d d, *J* 2.2, 8.6, H-4), 6.51 (1 H, d d, *J* 1.5, 17.0, H-14a), 6.36 (1 H, d d, *J* 9.8, 17.0, H-13), 5.84 (1 H, d d, *J* 1.5, 9.8, H-14b), 4.54 (1 H, m), 2.70 (3 H, s, CH_3), 2.67-2.46 (3 H, m), 1.97-1.79 (1 H, m); δc (75MHz, $CDCl_3$) 175.4 (C-8), 163.7 (C-12), 151.5 (C-6), 146.2 (C-2), 136.6 (C-4), 132.5 (C-7), 130.8 (C-13), 128.9 (C-14), 114.5 (C-5), 61.9 (C-3), 30.0 (C-9), 28.3 (C-10), 28.2 (CH_3); *m/z* (EI $^+$) 245 [(M^+), 65%].

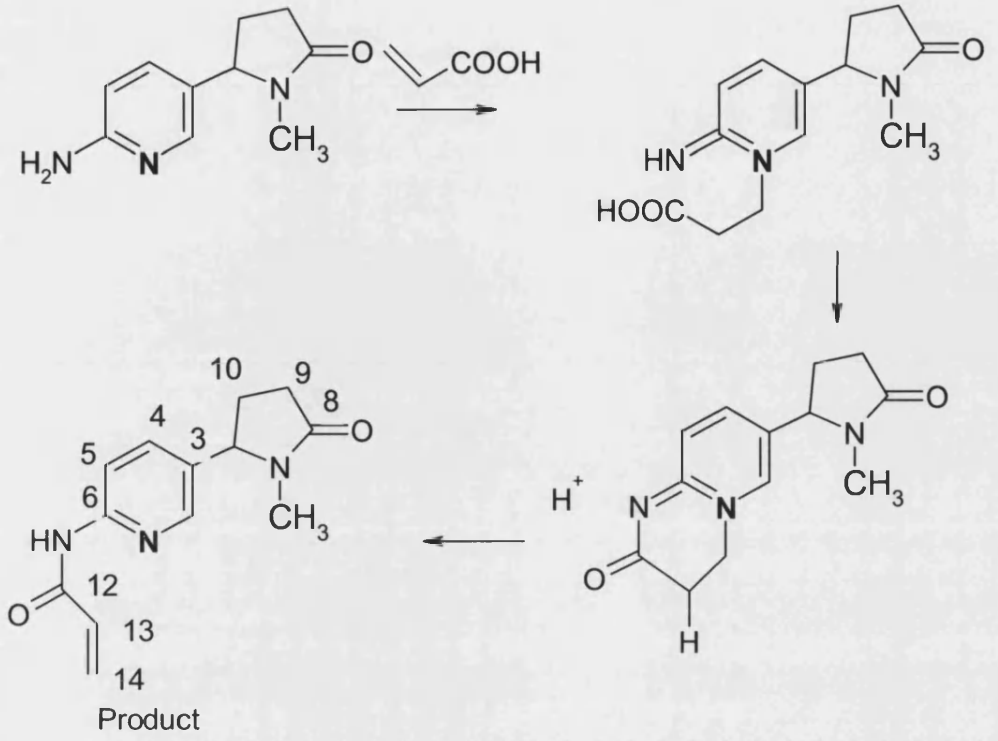


Figure 7.5: Synthetic reaction for the production of N(6)-(Alkyl)6-amino cotinine from 6-amino cotinine.

We speculated that this product is formed by initial *N*-alkylation on the pyridine nitrogen, followed by internal acylation, mediated by thermolysis. The structure of the product was clear from the NMR data, which showed the alkenyl signals clearly. The product represents an unusual and unexpected product that also offers potential in terms of the long term objectives of the project. However, it was not taken forward at this stage due to the difficulty of its production and low yield.

7.3.4 Preparation of N-alkyl pyridyl cotinine

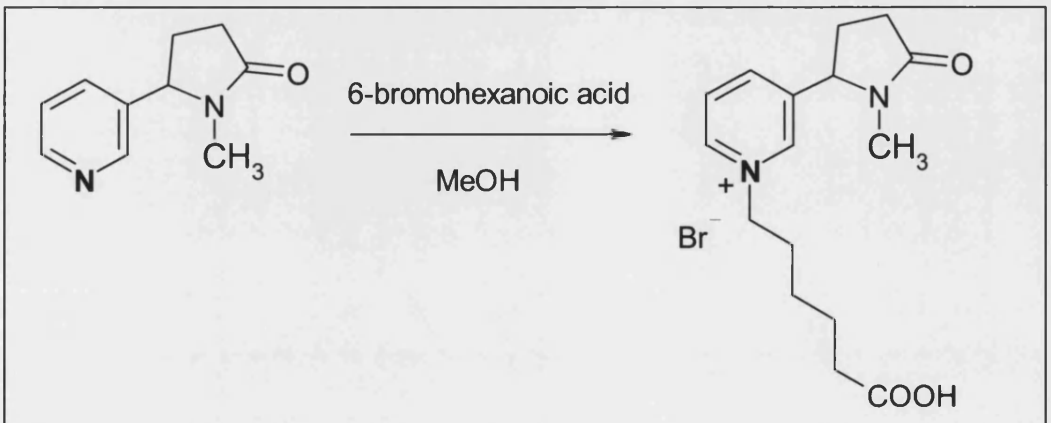


Figure 7.6: Synthetic production of N-alkyl pyridyl cotinine from cotinine.

The aim of this experiment was to attach a carboxyl group at the end of a 6-carbon linker on position 1 of the cotinine pyridine ring, to enable the cotinine-linker conjugate to bind to the carrier molecule via a carbodiiamide bond.

GSK supplied a procedure for alkylation on pyridine nitrogen of nicotine. In this case, it was possible to isolate the product using filtration. However, when the procedure was applied to cotinine, using 6-bromohexanoic acid as the alkylation agent, the product was not easily isolated by filtration. Attempts to precipitate the product directly using hexane, petrol 40-60°C, ethanol, ether and ethyl acetate failed to give a suitable solid, ether and EtOAc gave very fine suspension, which could not be filtered which is why this was not satisfactory. Therefore a different procedure was tried.

The reaction (figure 7.6) was conducted under nitrogen, and all glassware was pre-dried in an oven at 150°C for at least 12 hours prior to use. (-)-Cotinine (200mg, 1.1mmol) was dissolved in dry methanol (1cm^3, distilled from CaH_2) and cooled to O°C in an ice bath and 6-bromohexanoic acid (244mg, 1.25 mmol) in dry methanol was added drop wise. The solution was then allowed to stir at room temperature for 24 hours. The solvent was then removed *in vacuo*, and water (5cm^3) and CH_2Cl_2 (5cm^3) were then added to the residue. The aqueous phase was washed with CH_2Cl_2 (6 x 5 cm^3), until the aqueous phase only showed one baseline product by TLC in methanol. The water was removed (freeze drier) to give the *N*-alkylpyridyl cotinine conjugate (90mg, 27%) as a colourless oil.

[Found; (M^+), 291.1718. $C_{16}H_{23}O_3N_2$ requires (M^+) 291.1709]; δ_H (270 MHz, $CDCl_3$) 8.84 (2 H, d, *J* 6.3), 8.47 (1H, d, *J* 7.9), 8.09 (1 H, d d, *J* 6.3, 7.9), 5.03 (1 H, m), 4.62 (2 H, t, *J* 7.3), 2.72-2.54 (6 H, m), 2.36 (2 H, t, *J* 7.3), 2.09-1.88 (3 H, m), 1.68-1.56 (2H, m), 1.41-1.29 (2 H, m); *m/z* (EI^+) 291 [M^+, 3%], 176 [M – ((CH_2)$_5CO_2H$), 42%].

This yield of 27% could not be increased despite trying longer times (room temperature for 48 hours), warming to 40°C for 12 hours and increasing the number of equivalents of 6-bromohexanoic acid from 1 equivalent to 2 equivalents.

The corresponding reaction using 5-bromovaleric acid was also tried, but only 5% of the desired product was isolated. This is presumably due to the ease with which the pyridyl-*N*-alkylated product could cyclise. This type of reactivity might suggest that the product would not be a stable entity under the conditions required for the project.

The formation of a succinamate ester derivative of the *N*-alkyl pyridyl cotinine was then attempted. Unfortunately using both DCC and water soluble EDC as coupling agents, gave water soluble products that could not be separated. The sensitivity anticipated for the succinamate ester precluded further investigation. However, this product was

taken forward for conjugation to a carrier molecule and injection into rats, to find out whether it would raise sufficient specific antibodies.

7.3.5 Preparation of N-alkyl pyridyly nicotine

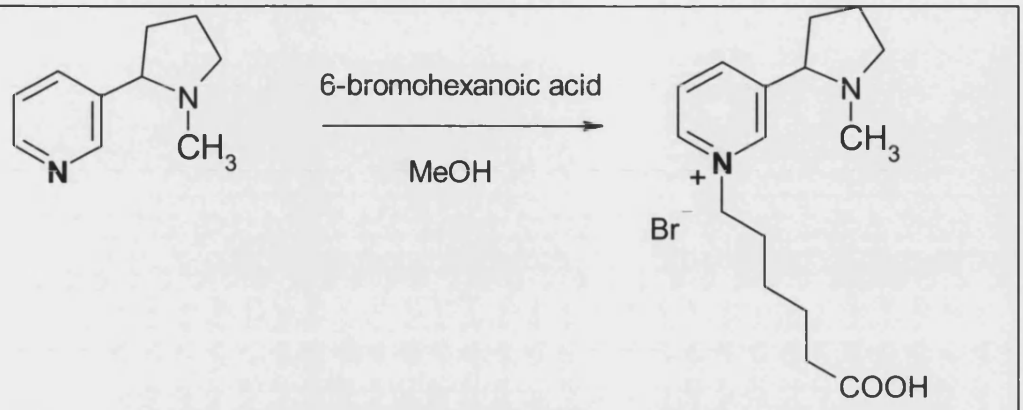


Figure 7.7: Synthetic production of N-alkyl pyridyl nicotine from nicotine.

The aim of this experiment was to attach a carboxyl group at the end of a 6-carbon linker on position 1 of the nicotine pyridine ring, to enable the nicotine-linker conjugate to bind to the carrier molecule via a carbodiiamide bond.

The reaction (figure 7.7) was conducted under nitrogen, and all glassware was pre-dried in an oven at 150°C for at least 12 hours prior to use. Nicotine (200mg, 1.2mmol) was dissolved in dry methanol (2cm^3, distilled from CaH_2) and cooled to O°C in an ice bath and 6-bromohexanoic acid (257mg, 1.32 mmol) in dry methanol was added drop wise. The solution was then allowed to stir at room temperature for 18 hours. The solvent was then removed in *vacuo*, and water (5cm^3) and CH_2Cl_2 (5cm^3) were then added to the residue. The aqueous phase was washed with CH_2Cl_2 (6 x 5 cm^3), until the aqueous phase only showed one baseline product by TLC in methanol. The water was removed (freeze drier) to give the *N*-alkylpyridyl nicotine conjugate (425mg, 27%) as a sticky red oil.

δ_H (270 MHz, $CDCl_3$) 8.96 (1H, br.s, H-2), 8.83 (1H, br.d, *J* 6.2 Hz H-6), (8.58 (1H, br.d, *J* 8.3Hz, H-4), 8.04 (1H, d d, *J* 6.2, 8.3 H-5).

N-alkylation of nicotine can be done via the pyridine or pyrrolidine nitrogen. The desired product is via alkylation of the pyridine ring. Alkylation of the pyrrolidine ring produces an undesired product and a mixture of dueterisomers. The NMR signals show this undesired product but could not be isolated. This did not occur in the corresponding cotinine derivative. This final product was taken forward for conjugation

to a carrier molecule and injection into rats, to find out whether it would raise sufficient specific antibodies.

7.3.6 Incorporation of a thiol moiety into racemic trans-4-cotinine carboxylic acid.

This experiment was conducted to attach a sulfhydryl group at the end of the linker on position 10 on the pyrolidine ring, to enable the cotinine-linker conjugate to bind to the carrier molecule via a maleimide bond.

The racemic trans-4-cotinine was first coupled using DCC as the coupling agent, but the desired product was not isolated. An alternative approach was investigated using the corresponding acid chloride. No good evidence was obtained to show that this reactive intermediate was either generated or stable under the conditions needed. It was also not possible to generate a mixed anhydride using isobutylchloroformate.

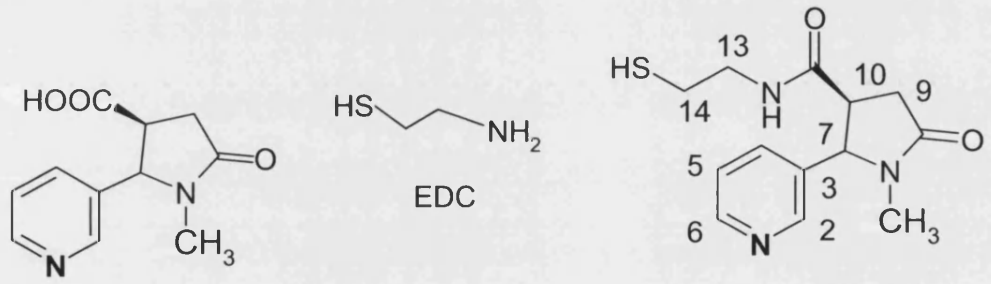


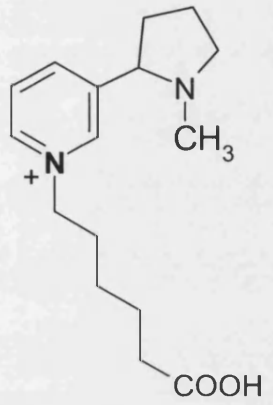
Figure 7.8: Synthetic production of trans-4-thiol cotinine from trans-4-cotinine carboxylic acid.

This reaction (figure 7.8) was conducted under nitrogen, and all glassware was pre-dried in an oven at 150°C for at least 12 hours prior to use. (±)-*trans*-4-Cotinine carboxylic acid (200mg, 0.9mmol), 2-aminoethanethiol.HCl (103mg, 0.9 mmol) and triethylamine (0.13cm^3, 1.8mmol) were dissolved in dry DMF (1.5 cm^3). The reaction mixture was cooled to 0°C in an ice bath, and EDCI.HCl (174mg, 0.9mmol) was then added to the above solution. The reaction mixture was warmed to room temperature and stirred for a further 12 hours. The solvent was removed *in vacuo*, and water (5 cm^3) and CH_2Cl_2 (5 cm^3) were added to the residue. The aqueous phase was washed with CH_2Cl_2 (4 x 5 cm^3). The combined organic extracts were dried over Na_2SO_4, filtered, and the solvent removed *in vacuo*. Purification by flash chromatography using 5% methanol/ dichloromethane as eluent, gave the product (136mg, 54% yield) as a colourless oil.

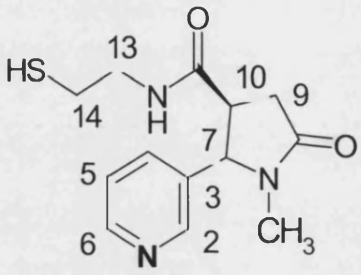
[Found; (M^+), 279.1045. $C_{13}H_{17}O_2N_3S$ requires (M^+) 279.1041]; δ_H (400MHz, $CDCl_3$) 8.58 (1 H, d d, *J* 4.9, H-4), 8.50 (1 H, d, *J* 2.0, H-2), 7.60 (1 H, d t, *J* 2.0, 7.8, H-6), 7.38 (1 H, d d, J 4.9, 7.8, H-5), 6.77 (1 H, br t, *J* 5.4, NH), 4.79 (1 H, d, *J* 6.8, H-7), 3.51-3.35 (2 H, m, H-9a, H-9b), 2.92-2.68 (5H, m, H-10, H-13a, H-13b, H-14a, H-14b), 2.64 (3 H, s, CH_3), 1.32 (1 H, t, *J* 8.3, SH); ν_{max} (thin film)/cm^{-1} 3500-3100 (br NH), 2543 (S-H), 1684 (C=O); *m/z* (EI^+) 279 [M^+, 65%]. The product is racemic.

7.4 Discussion

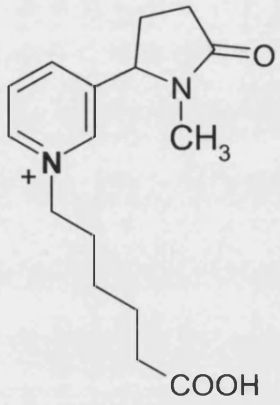
The three derivatives taken forward from these syntheses are:



N-alkyl pyridyl nicotine (niccooh) derivative



Trans-4-thiol cotinine (cotsh) derivative



N-alkyl pyridyl cotinine (cotcooh) derivative

These three derivatives were chosen as they were the most easily obtained and produced the largest yields. The *N*-alkyl pyridyl nicotine was taken forward to be tested for its immunological response, for a nicotine vaccine being considered by GSK. The trans-4-thiol cotinine derivative is probably the most viable derivative as it lends itself to conjugation to a carrier molecule via a maleimide bond, which is most suitable for large scale vaccine production. The linker is on position 10 of the pyrolidine ring, which allows for enough space for the epitope to expose the oxygen molecule on cotinine, which is the only difference between cotinine and nicotine. Therefore production of specific antibodies towards cotinine may be possible. *N*-alkyl pyridyl cotinine was taken forward as it can be conjugated to a carrier molecule via a carbodiiamide bond, which is suitable for this initial study. The linker is on position 1 of the pyridine ring, which also allows for the epitope to expose the oxygen on cotinine and production of specific antibodies towards cotinine may be possible.

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