1 Chapter 1 - Introduction

1.1 Alzheimer's disease overview

1.1.1 Prevalence, symptoms, diagnosis

Dementia is the term used to define a progressive decline in cognition which is serious enough to interfere with activities of everyday living. There are many causes of dementia, the causes differing in neuropathological features and in their clinical manifestations. Alzheimer's disease (AD) is the most common, representing at least 40% of dementia cases and possibly up to 80% (Nussbaum *et al.*, 2003), other causes of dementia including frontotemporal dementia, Lewy body dementia, cerebrovascular infarction and endocrine disorders (Karlawish *et al.*, 2003). AD is an insidious, irreversible form of dementia, which in its latter stages is extremely debilitating, necessitating full-time care for the sufferer and, ultimately, it is also fatal. It has been estimated that by the year 2025, 14 million people worldwide will suffer from AD (Dineley *et al.*, 2001) and in the U.S. it is the only one of the ten most common causes of death whose incidence is increasing (Minino *et al.*, 2006). The aetiology of the disease is complex and incompletely understood, involving many different neurochemical abnormalities.

1.1.2 Neuropathology of AD

The pathological hallmarks of AD are neuronal loss (particularly cholinergic neurons), extracellular accumulations of β-amyloid peptides (Aβ) as (senile)

neuritic plaques and accumulations of abnormal tau-protein as neurofibrillary tangles (NFTs). The clinical hallmarks of AD are progressive impairments in memory, judgement, orientation, decision making, language (Nussbaum *et al.*, 2003) and, it should be noted, one of the (earliest) impairments observed in AD patients involves decreased attentional function (Foldi *et al.*, 2002; Perry *et al.*, 1999). Many, though not all, AD sufferers also display affective disorders, particularly depression and/or aggression. The clinical diagnostic criteria of AD are based on impairments in memory, along with one or more of aphasia, apraxia, agnosia or disturbance in executive functioning (DSM-IV, 2000). These deficits must be sufficient to cause impairments in day-to-day living and represent a decline from former cognitive abilities.

Essentially however, the diagnosis of AD in the clinical setting is still one of exclusion, where all other possible causes of dementia must excluded. Definitive diagnosis of AD can only be made *post-mortem* (in light of *ante-mortem* assessments) upon the discovery of neuritic plaques and neurofibrillary tangles in the neocortex (The National Institute on Aging, 1997). Concurrent with the pathological changes, biochemical changes occur in the brains of AD patients, the most significant of which is the degeneration of cholinergic neurons.

1.1.3 β-Amyloid, NFTs

Several pre-dispositions for AD have been identified. Firstly, the over production of the amyloid precursor protein (APP). APP is causally related to AD due to its

conversion to Aβ peptides. These peptides can be between 40 and 43 amino acids in length ($A\beta_{40}$ - $A\beta_{43}$). Aβ peptides are formed when APP is proteolysed with the enzyme β-secretase, as opposed to an alternate protolytic pathway via α-secretase which results in end-products which cannot aggregate into plaques (Clark *et al.*, 2003). Aβ peptides (predominately $A\beta_{42}$ (Clark *et al.*, 2003)) congregate into amyloid plaques which are a hallmark of AD. They however are poorly correlated with the degree of cognitive deficit (Bennett *et al.*, 2006; Hyman, 2001). Prior to their transformation into plaques, the Aβ deposits can accumulate in neurons, often occluding the dendrites. This accumulation can thence lead to cell lysis and plaque formation (Wang *et al.*, 2002). Along with this, Aβ peptides damage neurons by inducing membrane lipid peroxidation, rendering them vulnerable to excitotoxicity (Mattson *et al.*, 1998). Interestingly, APP is also known to have trophic functions and neuroprotective qualities. It has the ability to modulate neuronal excitability and protect neurons from excitotoxic, metabolic and oxidative insults (Mattson *et al.*, 1998).

Found simultaneously with Aβ plaques in AD are neurofibrillary tangles (NFTs). As Aβ plaques are extracellular accumulations, NFTs are intracellular ones. The main fibrous component of NFTs are paired helical filaments (PHFs), which are formed by abnormal, hyperphosphorylated, tau proteins (Bramblett et al., 1993; Goedert, 1996; Hasegawa *et al.*, 1997).

Tau protein is abundantly found in the brain where it is normally concentrated in axons. It is a member of the microtubule-associated protein family and its primary function is to maintain microtubule stability (Law *et al.*, 2001). In the adult human brain, six isoforms of tau protein are found, ranging from 352 to 441 amino acids in length. All six isoforms have been found to contribute to PHFs.

Hyperphosphorylation of tau protein greatly reduces its ability to bind to microtubules. This leads to the destabilisation of microtubules, resulting in impaired cellular trafficking and neuronal death (Law et al., 2001; Goedert, 1996).

1.2 Cholinergic system in AD

1.2.1 Overview of cholinergic system, particularly corticopetal projections arising in the CBF

As mentioned above, alongside Aβ and NFT accumulations, the most significant change which occurs in the brain in AD is the degeneration of cholinergic neurons, specifically cholinergic afferent neurons projecting from the cholinergic basal forebrain (CBF) comprising: the medial septum (MS) the cholinergic efferents of which project mainly to the hippocampus; the vertical and horizontal limbs of the diagonal band of Broca (DBB) the cholinergic efferents of which project mainly to the olfactory bulbs, cingulate and occipital cortex, and the nucleus basalis magnocellularis (NBM; also referred to as the nucleus basalis of Meynert in humans) which projects via cholinergic efferents to the frontal and parietal cortices and amygdala (Waite *et al.*, 1995) (

Figure 1). It has been postulated that the degeneration of these cholinergic efferents, relative to other structures, is so prominent in AD due to the CBF nuclei

having an inordinately high susceptibility to NFT pathology, possibly due to their position and connectivity rather than any innate property (Mesulam, 2004).

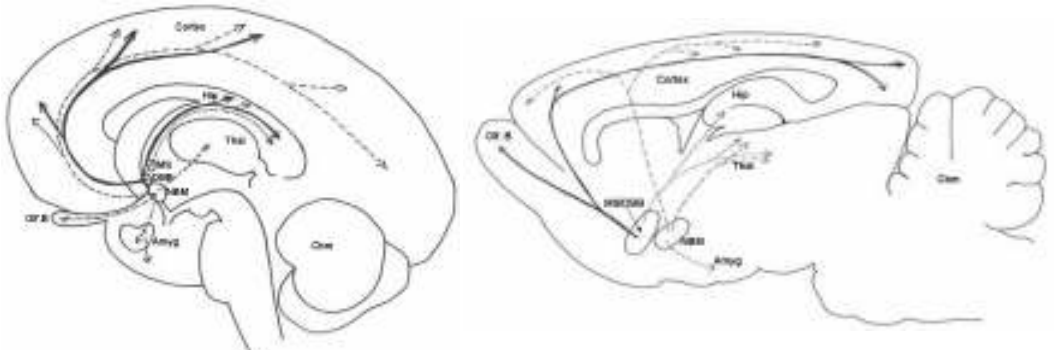

Figure 1: Cholinergic projections from the CBF in human (left) and rat (right). It should be noted that in the rat the CBF system is less differentiated than in higher orders of mammals (Gorry, 1963), thus the attribution of projections is rather less strict than shown here. NBM = Nucleus basalis magnocellularis (Nucleus basalis of Meynert); MS = Medial septum; DBB – Diagonal band of Broca; Thal = Thalamus; Hip = Hippocampus; Cbm = Cerebellum; Olf. B = Olfactory bulbs

Two key features of the CBF are essential to highlight for the purposes of this thesis: i) despite their discrete localisation, the CBF nuclei can be thought of as constituting a “physical continuum" since they share many physical characteristics (eg expression of the $p75^{NGFr}$ [p75 nerve-growth-factor receptor]) (Everitt *et al.*, 1997). However, from a psychological (functional) standpoint it is necessary to consider the nuclei as separate entities which affect cognition differently according to the targets to which they “more or less” discretely project (Everitt *et al.*, 1997); ii) Within each structure, cholinergic cell bodies are heterogeneously distributed, being intermingled with non-cholinergic cell bodies (Everitt *et al.*, 1997).

The degeneration of CBF neurons was first noted thirty years ago (Bowen *et al.*, 1976; Davies *et al.*, 1976) and for a period afterwards this degeneration was hypothesised to contribute to the symptoms of AD. With the magnitude of the deficit being observed to correlate with the degree of cognitive impairment (Francis *et al.*, 1985; Perry *et al.*, 1978; Tiraboschi *et al.*, 2000), the so-called 'cholinergic hypothesis' gained support.

1.2.2 Discovery, cholinergic hypothesis development

Over the previous decade however, the cholinergic hypothesis has been increasingly questioned. The initial investigations which first uncovered the cholinergic deficit were performed in subjects with advanced disease (Bowen *et al.*, 1976; Davies *et al.*, 1976). Subsequent investigations in subjects with only mild AD have shown cholinergic markers to be relatively preserved (Gilmor *et al.*, 1999). For this reason and because there have only been modest results seen following pro-cholinergic therapies, eg (Lopez-Pousa *et al.*, 2005), plus apparently conflicting results of behavioural studies in animal models (see below), the cholinergic hypothesis appeared "headed for oblivion," (Mesulam, 2004). This appears to have been an over-reaction because whilst the symptoms of AD can no longer be thought of purely as manifestations of a cholinergic deficit, it is undoubted that the cholinergic system plays a significant role in aspects of cognitive function which are impaired in AD, albeit within a complex context as described below. Overall, evidence suggests the cholinergic system must still be considered more than a mere

bystander to AD pathogenesis, though the role it does play may no longer be considered as pivotal as it once was (Mesulam, 2004).

The cholinergic system in AD is the foundation of the work performed in this project. A further elucidation of the role its degeneration plays in the morbidity of AD and the evaluation/development of specific tools for its molecular imaging are the two themes of this thesis, introduced in turn below.

1.2.3 Effects on attention and memory (human and animal studies)

The cholinergic system plays a significant role in attentional and mnemonic processes. Dysfunction of these processes are two of the features of AD which contribute greatly to the impairment in day-to-day living and evidence exists for both that they occur relatively early in the disease progression.

Attention, the ability to detect, select and process relevant stimuli (Sarter *et al.*, 2000), is a foundation for many higher cognitive functions and disorders of attention rapidly escalate into major cognitive symptoms (Sarter *et al.*, 2001b). It thus follows that attentional dysfunction is considered to be one of the earliest manifestations of AD (Foldi *et al.*, 2002; Perry *et al.*, 1999). Corticopetal cholinergic afferents from basal forebrain nuclei have in the last decade been shown to be necessary for proper attentional processing (Everitt *et al.*, 1997; Sarter *et al.*, 1997; Sarter *et al.*, 2001b). The mechanisms of this are complex and well reviewed (Sarter *et al.*, 1997; Sarter *et al.*, 2001b). Briefly, efferents from the CBF

project throughout the cortical mantle. Cholinergic afferents to the fronto-parietal cortex, and particulary the pre-frontal cortex, contribute to the activation of knowledge-driven attentional–processes whereby the subject is biased in its attention on the basis of its memory and/or learned strategies. This mechanism complements that driven by the CBF projections to other parts of the cortex which are driven from the perception of the stimulus (Sarter *et al.*, 2001b). Though there are different types of attention classified, they all share this commonality.

In contrast to its role in attentional processes, the interaction between the basal forebrain cholinergic system and memory is nebulous. In the early days of the cholinergic hypothesis the role cholinergic neurons played in memory seemed straightforward. It was well demonstrated that anti-cholinergic interventions had the ability to mimic in young subjects the memory impairments observed in both aging and dementia and these effects could be (partially) reversed by pro-cholinergic therapy (Bartus *et al.*, 1982). The preliminary work examining the effect of pro-cholinergic therapy in dementia produced results which were limited but still promising (Bartus *et al.*, 1982). For example, the acetylcholinesterase (AChE) inhibitor physostigmine was shown to improve memory performance in aged primates and humans, however the optimal dose varied dramatically between individual subjects within these groups (Bartus, 1979; Bartus *et al.*, 1982; Christie *et al.*, 1981; Davis *et al.*, 1979; Drachman *et al.*, 1980; Muramoto *et al.*, 1979; Peters *et al.*, 1979; Smith *et al.*, 1979). In addition, treatment with the muscarinic receptor (mAChR) agonist arecoline enhanced mnemonic performance in AD

patients (Bartus *et al.*, 1982; Christie *et al.*, 1981), the improvement being however only slight in the majority of patients.

These inconsistencies in the cholinergic hypothesis increased. Later work with various animal models, particularly an animal model of specific cholinergic depletion, conclusively demonstrated that the original cholinergic hypothesis was oversimplified and highlighted the complexity of the molecular bases of the cognitive deficits observed in AD. The knowledge of the role of the CBF in cognition and behaviour will be further discussed in the chapters of this text dealing with behavioural studies (Chapters 2 and 3).

Whilst the relationship of the basal forebrain cholinergic system to memory, attention and other cognitive deficits is still in need of further elucidation in AD, it is nonetheless established that this system is perturbed in AD and is thus an important target for molecular imaging.

1.3 Potential role for molecular imaging

1.3.1 General

Molecular imaging is, in its broadest definition, the imaging of dynamic, biochemical processes. These processes can include perfusion (eg cerebral blood-flow); metabolism (eg cellular glucose usage); excretion (eg biliary or renal

excretion); formation (eg the osteoblastic activity of bone remodelling); and specific molecular interactions (eg receptor-ligand binding).

Molecular imaging requires radiopharmaceuticals (RPs). These RPs range from tracers to measure blood flow to specific biochemical and chemical substrates measuring basic physiological function (eg receptor-mediated interactions, energy metabolism, amino acid transport and protein synthesis, DNA synthesis, cell signalling and proliferation and hypoxia). RPs are analogous to regular pharmaceuticals except they are used in much smaller concentrations and they have incorporated into them (for diagnostic purposes) gamma (γ) ray emitting radionuclides (RNs). γ-rays are similar to visible light in as much as they are weightless and without charge; however, they are energetic enough to pass readily through human tissue, making them more closely analogous to x-rays, from which they differ only in terms of a differing origin and energy. RNs which emit γ-rays can be imaged with a γ-camera. Either planar images, (2-dimensional views), dynamic images (a series of planar images) or Single-Photon/Positron Emission (Computed) Tomography (SPECT / PET, a 3-dimensional picture) images can be acquired. As RPs interact in a molecular fashion *in vivo,* imaging which utilises RPs is referred to as 'molecular imaging.'

1.3.2 Molecular imaging in neurology

Molecular imaging of the brain, which is still in a developing stage, has the potential to provide two main benefits to AD sufferers. The first benefit of

improved molecular brain imaging in AD is the provision of early, accurate diagnoses. Confirmation of AD in its early stage will allow the patient and their family to plan for the future implications of the disease. It will also enable current therapeutic interventions to begin sooner, thus improving their efficacy so as to maintain the patient's independence and quality of life as much as possible.

The second benefit to be derived from improved molecular brain imaging in AD will be better treatment of the disease. Apart from studies using current (limited) imaging and electroencephalographic techniques, the bulk of the neurophysiological knowledge of AD has been gained from *post-mortem* studies. Whilst *post-mortem* studies allow good characterisation of more advanced AD, a relative lack of knowledge exists regarding early events in AD and the subsequent pathogenesis. Compared to advanced AD, fewer subjects have been available for investigation of early and even sub-clinical AD *post-mortem*, and obviously *post-mortem* studies cannot be used in a longitudinal (serial) manner. The relative lack of information regarding the aetiology and early pathogenesis of AD, the two areas where intervention would be of most benefit, is a handicap to the design of treatments. Improved molecular imaging will expand knowledge of these areas, thus allowing new directions for treatment to be investigated. For example a better understanding of the mechanisms of cell loss and the natural life of the disease, as well as earlier detection, could enable neuroprotective therapeutic interventions aimed at slowing down or halting the disease process.

1.3.3 Current practice

The current state of molecular imaging of AD is reviewed below. Although molecular imaging of the brain shows much potential it has had relatively limited clinical acceptance. The current clinical criteria for AD support the routine use of structural imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI), but call for further prospective studies of molecular imaging techniques to establish whether they bring a clear advantage over the established investigative tests (Knopman *et al.*, 2001).

A number of molecular imaging techniques are currently available which have the ability to depict molecular systems which are perturbed in neurodegeneration (and AD). RPs have been developed for the dopaminergic system, central and peripheral benzodiazepine receptors and various aspects of the cholinergic system. The most widely evaluated molecular imaging techniques for neurodegeneration are ones which investigate the correlated processes of regional cerebral blood flow (rCBF) and glucose metabolism. It is widely agreed that both of these are reduced in a characteristic fashion in the brain in AD with the most severe reductions being in tempoparietal regions (Asenbaum, 2002; Villemagne *et al.*, 2005). The value that molecular imaging of these processes adds to current clinical diagnosis and monitoring of AD is of primary importance. In subjects clinically diagnosed with "possible" AD, the addition of a SPECT scan to assess rCBF improved the likelihood of a correct diagnosis (as confirmed pathologically); a positive scan allowed a likelihood of 84% compared to 67% for the clinical diagnosis alone (67%

of subjects with a positive clinical diagnosis had AD confirmed at autopsy). A negative SPECT scan reduced this percentage to 52 % (Jagust *et al.*, 2001). Correspondingly, PET studies assessing glucose metabolism have begun to demonstrate such promise that they have been described as yielding the highest prognostic value for providing a diagnosis of presymptomatic AD (Villemagne *et al.*, 2005). This can be two or more years before the full dementia picture is manifested.

Although it appears of be of some benefit, targeting of rCBF and glucose metabolism is still far from ideal for the molecular imaging of AD. Apart from its rather poor negative predictive value (in the study above, 52% of patients clinically diagnosed with "possible" AD with a *negative* SPECT scan went on to have pathologically confirmed AD) (Jagust *et al.*, 2001), it is likely the events targeted are secondary or even tertiary to the primary pathogenic events in AD, however there is debate on this (Cullen *et al.*, 2005). There is also an uncertain connection to AD symptomology, leaving room for complementary molecular imaging techniques which target either primary events in AD pathogenesis and/or entities which can be more closely correlated with the symptoms of AD.

The current state-of-the-art in the case of AD pathology are RPs which target the Aβ plaques characteristic of AD. Based upon molecules originally used for histopathological assessment of Aβ plaques and NFTs, ^{11}C-labelled Pittsburgh Compound-B (PIB) and 2-(1-(6-[(2-[18F]fluoroethyl)(methyl)amino]-2-

naphthyl)ethylidene)malononitrile (^{18}FDDNP) demonstrate properties which make them suitable for molecular imaging of Aβ alone (Klunk *et al.*, 2004) and Aβ and NFTs (Shoghi-Jadid *et al.*, 2002), respectively. PIB has been observed to delineate a much greater deficit than ^{18}F-deoxyglucose (^{18}FDG, an RP for cellular glucose metabolism) in the same subjects clinically diagnosed as having "probable" AD. Klunk and colleagues (Klunk et al., 2004) reported a 40% reduction in glucose metabolism in the parietal cortex of AD subjects compared to controls, whereas the same patient group had on average a 90% greater retention of PIB in the frontal cortex than the same controls. The two subject groups had similar levels of PIB binding in areas of the brain known from post-mortem studies to not possess Aβ plaques. PIB binding has also been shown in a separate study to correlate positively with scores on the mini mental-state exam (MMSE) (Rowe, 2006). Similar results have been reported for ^{18}FDDNP (Shoghi-Jadid *et al.*, 2002). The benefits of molecular imaging regarding the ability to longitudinally examine the disease course and to monitor the effect of treatments were highlighted by these authors (Klunk *et al.*, 2004; Shoghi-Jadid *et al.*, 2002).

In the case of RPs which target entities which can be more closely correlated with the symptoms of AD, current research is focused on the targeting of elements of the cholinergic system, since this degenerates in AD. As reviewed above, the precise effect of the cholinergic system on the areas of cognition perturbed in AD is still in need of elucidation. However this system is the closest we have so far come to a

neurochemical correlate of AD symptomology and is thus currently the molecular imaging target most closely linked to AD morbidity.

1.4 Nicotinic receptor in AD

1.4.1 Review

Consistently observed in AD is a loss of neuronal nicotinic acetylcholine receptors (nAChRs) (Court *et al.*, 2001; Nordberg, 2001). nAChRs are members of the multisubunit neurotransmitter-gated superfamily of ion channels. Thus far, numerous subunits have been identified: α2 to α10 and β2 to β4 which are expressed in the CNS. Different combinations of α and β subunits can form different nAChR sub-types whose activation can elicit a number of functional responses (Paterson *et al.*, 2000; Tribollet *et al.*, 2004). Of the possible sub-types, the $\alpha_4\beta_2$ nAChR sub-type is the most predominant, along with smaller proportions of $\alpha_3\beta_2$ and $\alpha_3\beta_4$, amongst others (Perry *et al.*, 2002). A functionally significant population of the α_7 sub-type is also present (Paterson *et al.*, 2000). nAChRs are known to exhibit a complex distribution. They are distributed heterogeneously in the brain with highest levels found in the thalamus (and, in the case of the α_7 sub-type, the hippocampus), moderate levels in the parietal cortex, striatum and cerebellum and lowest levels in frontal, occipital and temporal cortices and hippocampus (Paterson *et al.*, 2000). nAChRs are to be found mainly pre-synaptically (Bednar *et al.*, 1998; Wonnacott, 1997) but also with significant post-, peri- and extrasynaptic locations (Paterson *et al.*, 2000; Sihver *et al.*, 1999). The

pre-synaptic (and somatodendritic) nAChRs are further known to exist on a number of non-cholinergic cell populations facilitating the release of many neurotransmitters in addition to acetylcholine such as dopamine, 5-hydroxytryptamine, noradrenaline, γ-amino butyric acid (GABA) and glutamate (Paterson *et al.*, 2000; Wonnacott, 1997).

Both the $\alpha_4\beta_2$ and α_7 sub-types are of interest in AD research (Mazurov *et al.*, 2006; Paterson *et al.*, 2000). The α_7 is of interest as it is known to be of importance in cognitive functioning (Mazurov et al., 2006; Wonnacott, 2007) and is known to interact with Aβ (Nagele *et al.*, 2002). It is thus regarded to have much therapeutic potential (Kem, 2000). Its' use in therapy and imaging however is limited currently by a lack of suitable ligands, though the requisite medicinal chemistry is in progress (Mazurov et al., 2006). Due to this lack of ligands for the α_7 and the fact the $\alpha_4\beta_2$ sub-type is considered to be the predominant one lost in AD (Paterson *et al.*, 2000; Warpman *et al.*, 1995), it was decided here to concentrate upon this latter sub-type.

1.4.2 Ligands

Ligands used to investigate the nAChR have included radiolabelled nicotine, cytisine and epibatidine (Paterson *et al.*, 2000). In recent years the 3-pyridyl ether 5-iodo-A-85380 (I-A-85380), when radioiodinated, has been proposed to be a more suitable ligand with greater sub-type selectivity and very high affinity for the $\alpha_4\beta_2$ sub-type (Mukhin *et al.*, 2000). Later studies (Kulak *et al.*, 2002a; Kulak *et al.*, 2002b; Perry *et al.*, 2002) have demonstrated *I-A-85380 (*I = radioiodine) to also

have high affinity for other β_2 sub-unit containing nAChRs, including $\alpha_3\beta_2$ and $\alpha_6\beta_2$ receptors. In spite of this and due to the preponderance of the $\alpha_4\beta_2$ in the brain, *I-A-85380 is still preferred for the investigation of nAChRs and has been used to measure *in vivo* changes in nAChR density in the brain (Kassiou *et al.*, 2001). It has a Kd of around 10pM in the rat which is 15-20 times better than the Kd of ^{3}H-cytisine (Anderson *et al.*, 1994; Gopalakrishnan *et al.*, 1996; Sihver *et al.*, 1998), and though it is comparable to the Kd of ^{3}H-epibatidine (≈8pM) (Gnadisch *et al.*, 1999) it has a much greater sub-type selectivity (Mukhin *et al.*, 2000). Possibly due to this greater selectivity, *I-A-85380 is also significantly less toxic than epibatidine and whilst the amounts of compound used for imaging are well below pharmacological doses, it has been thought prudent to develop less toxic compounds (Vaupel *et al.*, 1998).

1.5 VAChT in AD

1.5.1 Review

Another useful target for functional imaging is the Vesicular Acetylcholine Transporter (VAChT) which is located mainly in the pre-synaptic terminal of cholinergic neurons (Masson *et al.*, 1999). In addition the VAChT has also been demonstrated in the CBF nuclei from which cortical (NBM) (Debeir *et al.*, 1999) and hippocampal (MS) (Gilmor *et al.*, 1998) cholinergic projections originate, probably reflecting a known expression of the VAChT in the soma and dendrites of neurons (Masson *et al.*, 1999). The expression of the VAChT in AD is variable,

dependent upon the severity of disease and the age of the subject at disease-onset. Whilst it has been shown to be relatively preserved in the NBM in mild cases of late-onset AD (Gilmor *et al.*, 1999), more severe and/or early-onset cases showed statistically significant reductions in cortical, particularly temporal, areas (Efange *et al.*, 1997; Kuhl *et al.*, 1996; Sihver *et al.*, 1999).

1.5.2 Ligands

The radioligand used in this study to detect the VAChT *in vivo* was [^{123}I]iodo-benzovesamicol (^{123}IBVM). Previous work with *IBVM has shown it to match the known distribution of cholinergic terminals in the rat brain (Sorger *et al.*, 2000) and in human brain (Kuhl *et al.*, 1996). Blocking studies have also shown *IBVM to have very good selectivity for the VAChT (Sorger *et al.*, 2000). It has therefore been considered to be suitable for detecting changes in cholinergic terminal density *in vivo* (Efange, 2000).

1.6 SAP model

1.6.1 Description

An animal model which displayed a cholinergic deficit similar to AD was essential to the exploration of the dual themes of this project. This cholinergic deficit can be produced by specifically destroying cholinergic neurons in the basal forebrain. A neurotoxin that selectively destroys cholinergic neurons (Messer Jr *et al.*, 2000) by targeting the p75 receptor (Potter *et al.*, 1999), 192 IgG-saporin (SAP), has been

employed by different groups. These "immunotoxic lesions represent a significant advance above and beyond the use of excitotoxins," ((McGaughy *et al.*, 2000), p.256). Compared to excitotoxic lesions, they produce more substantial cholinergic lesions and concurrently more selective attentional deficits (McGaughy *et al.*, 2000).

The basis of the development of SAP was the finding that of the mixed neurons in the basal forebrain, it was only the cholinergic ones which expressed the p75 (low-affinity nerve growth factor) receptor (Book *et al.*, 1992; Hefti *et al.*, 1986). It was subsequently reported that Purkinje cells of the cerebellum also express the p75 receptor, however they are less susceptible to SAP (Waite et al., 1995). 192 IgG having already been characterised as a suitable monoclonal antibody to the p75 receptor (Chandler *et al.*, 1984), it was the group of Wiley who disulphide-coupled it to saporin, a toxin which acts by catalytically inactivating ribosomes and thereby inhibiting protein syntesis (Wiley *et al.*, 1991).

1.6.2 Advantages over previous models

As mentioned earlier, in the CBF cholinergic cell bodies are intermingled with non-cholinergic, particularly GABAergic ones (Everitt *et al.*, 1997). This made specific lesioning of the CBF very difficult and in fact, until 1991 (Wiley *et al.*, 1991), there was no specific chemical neurotoxin for cholinergic neurons (Everitt *et al.*, 1997). Prior to this, lesion studies of the behavioural role of the cholinergic system were performed using animals which had non-cholinergic-specific lesions which led

researchers to misleading conclusions about the nature of the role of the cholinergic system in cognition. The evolution of cholinergic lesioning can be described as follows, taken mainly from the excellent review of Everitt and Robbins (Everitt *et al.*, 1997): electrolytic lesions, which not only destroyed both cholinergic and non-cholinergic cell bodies, but also their axons → excitotoxic lesions with *N*-methyl-*D*-aspartate (NMDA) agonists, mainly ibotenic acid, which preferentially destroyed non-cholinergic neurons but which were probably, but not certainly, cell-body specific (ie axon-sparing) → non-NMDA excitotoxins, the best of which was α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). AMPA is specific for cholinergic cell bodies in the NBM, however in the MS it has been shown to lesion GABAergic neurons as well (McAlonan *et al.*, 1995). Now, the best means of CBF cell lesioning is SAP. SAP is an excellent tool for creating lesions of the CBF as it specifically destroys the cholinergic cells whilst having no known effect on other systems with the exception of Purkinje cells in the cerebellum (discussed below). The other drawback of SAP is that it leaves intact a small population of CBF neurons, those projecting from the NBM to the amygdala (McGaughy *et al.*, 2000). This seems to be of minor concern to studies of AD symptomology however, one of the few paradigms this projection underpins is one of passive avoidance, a test of aversive conditioning (Everitt *et al.*, 1997), whilst the areas of cognition more relevant to AD, ie attention and memory, appear to be subserved by projections other than amygdalar ones (see Chapters 2 and 3). With regards to molecular imaging, it would appear SAP is near ideal; considering that the amygdala is a relatively very small structure and as such would be very difficult to image in the

rat which is itself a relatively small animal (SAP is specific only for the rat), it is therefore of little importance that these projections are not lesioned in the present study. Even the destruction of cerebellar Purkinje cells is of little concern to molecular imaging once one is aware of it. This is because the cerebellum is a relatively large region and rat Purkinje cells are known to express nAChRs (Nakayama *et al.*, 1997). Thus in the molecular imaging evaluation of RPs, the cerebellum actually provides an important region to test the visualisation of some RPs (see Chapter 4).

1.6.3 Work investigating nAChR and VAChT density in SAP model

With regards molecular imaging, the SAP model is a satisfactory tool to evaluate potential RPs targeting aspects of the cholinergic system. This project has focussed on two RPs which, from the literature, it was decided had both the most usefulness and the most potential to become clinical molecular imaging tools. Whilst they have advanced to different extents along the path to clinical acceptance, this project aimed to evaluate them in a systematic manner so as to allow validation of *in vivo* data with *in vitro* measurements. As described below the biochemical changes resulting from SAP lesioning, though very good for evaluating ^{123}IBVM, present a challenge to molecular imaging of the nAChR.

1.6.3.1 nAChR

Previous studies of nAChR density in animals with SAP lesions have demonstrated no abnormalities, either when measured with ^{3}H-cytisine following intracerebroventricular (icv) administration (Rossner *et al.*, 1995a; Rossner *et al.*, 1995b) or with ^{3}H-cytisine and ^{3}H-epibatidine following intranuclear administration of SAP to the NBM (Bednar *et al.*, 1998). The likely reason for this is the great number of nicotinic heteroreceptors, nAChRs which are located on non-cholinergic cells (Paterson *et al.*, 2000; Wonnacott, 1997). Given the specificity of SAP for cholinergic cells, it follows that the nAChR reduction would be relatively small overall. However the RP used in this project has never been reported to have been assessed in an icv SAP animal model before and with its superb affinity and selectivity, it was believed radioiodine-labelled A-85380 would be able to delineate a reduction in nAChR density which until now other RPs could not.

1.6.3.2 VAChT

Following icv administration of SAP an immunocytochemical study of VAChT in the medial septum demonstrated a near complete abolition of this marker (Gilmor *et al.*, 1998). Using an immunoblotting technique, the same group found a reduction of up to 90% in the hippocampus, the target field of the MS (Gilmor *et al.*, 1998). In the same animal model, another group demonstrated using *ex vivo* autoradiography a reduction of ^{125}IBVM binding of 61% in the hippocampus and 51-55% in various cortical regions (Sorger *et al.*, 2000). This smaller reduction of

VAChT binding observed when using *IBVM probably represented a high level of non-specific binding of ^{125}IBVM *in vivo* (Sorger *et al.*, 2000).

1.6.3.3 Behaviour

The SAP cholinergic depletion model is thus the best animal model currently known to investigate the role of the cholinergic system in cognition and by extension, AD. Much work has been performed with this model already, however there remains much to learn about the role of the CBF that this model can be used for. With regards to molecular imaging, it can be seen above that the SAP model is a very good tool for evaluation of RPs which target the VAChT. For the nAChR it presents a greater challenge, however one which an RP should be able to surmount if it is to have potential clinical application.

1.7 Aims of project

1.7.1 To add to the body of knowledge surrounding the role of cholinergic neurons in the symptoms of AD

As stated above, this project focuses upon the cholinergic system in AD: a further elucidation of the role its degeneration plays in the morbidity of AD and the evaluation/development of specific tools for its molecular imaging. The aims of this project are therefore directed towards these ends.

Firstly, given the significant role impairments in attention and memory play in the morbidity of AD, behavioural work in this project has focussed on these two areas. Attention was evaluated in two different varieties of SAP model with a test which has not been used previously with SAP models. Memory was assessed in the same two varieties of SAP model with a paradigm which is relatively unique compared to other memory tests. Though this test has been used in the past to evaluate the performance of SAP-lesioned animals, it has not been tested in the icv SAP model, whilst the previous study to use an intracortical administration lesioned a different part of the cortex.

1.7.2 To validate the model with ChAT immunohistochemistry

The efficacy of the SAP lesioning technique was verified with immunohistochemistry (IHC) for the choline acetyltransferase (ChAT) enzyme. ChAT is an enzyme used in the making of acetylcholine and is thus a specific marker for cholinergic cells. Two different analysis techniques both based on the principle of densitometry were applied to the data.

1.7.3 To evaluate I-A-85380 and IBVM localisation in vitro / ex vivo

In order to evaluate/develop specific tools for molecular imaging, work in this project concentrated on two fields; evaluation of RPs which target aspects of the cholinergic system (*I-A-85380 and *IBVM) and development/testing of hardware for molecular imaging.

Evaluation of the RPs was performed with autoradiography. Autoradiography is a technique with a relatively simple analysis method where slices of tissue are exposed directly to sheets of film, as opposed to molecular imaging where radioactivity detection must be performed *in vivo* via a complex γ-camera. It was aimed to quantify the biochemical loss associated with SAP lesioning identified by each RP with autoradiography, with this data subsequently being able to serve as validation when it is compared to the loss observed by each RP with small-animal molecular imaging.

1.7.4 To develop in vivo imaging capabilities

Quantitative molecular imaging of single-photon emitting radioisotopes is a developing field. The ability to perform this type of imaging in small animals such as rodents would be of great benefit to a great number of areas of medicine, beyond just the neurological applications discussed in this thesis. Small-animal molecular imaging allows a greater number of RPs to be trialled in a greater variety of animal models and its continued development is thus of importance.

In this project a novel small-animal molecular imaging systems, in the prototype stage, was used and its different performance characteristics assessed/tuned. The prototype molecular imaging system, TOHR, is a system based upon a completely novel principle. The work performed with it was thus necessarily of a more preliminary nature. The aim of this project with regards this system was to develop

the TOHR to a stage where it could be used for the molecular imaging of the SAP model with the RPs ^{123}I-A-85380 and ^{123}IBVM.

1.8 Outline of thesis

To address these aims, this thesis is broken into 6 chapters. The next chapter, Chapter 2, addresses the role of the CBF cholinergic system in attention, describing work performed with two varieties of the SAP model. The first was the icv SAP model, where the toxin was administered into the lateral cerebral ventricle and thus destroyed many CBF neurons as well as cerebellar Purkinje cells. The second model was created serendipitously when one group of surgical animals had the toxin injected into the cortex dorsal to the lateral ventricle. Thus only the parts of the CBF nuclei which project to this region were affected by the SAP. Attention was assessed in both these models with a vibrissal-stimulation task which has not been used at all in the SAP animal model before. Chapter 3 addresses the role of the CBF cholinergic system in memory. The same groups of animals were used for these studies as used in the attention studies described above. The test used to assess animals' mnemonic function was an elegant test which, unlike many tests of learning and memory used in the past, didn't rely on any form of motivation, reward or aversive stimulus. It has been used in the past in the SAP model, however not with either the icv model or with the cortical lesion introduced in this project.

Chapter 4 describes work performed using the two RPs in autoradiography. It describes a protocol designed to take advantage of the different physical half-lives of ^{123}I and ^{125}I to perform autoradiography with both RPs in the same animals. In this way the results could be directly compared to each other. These studies were performed in icv SAP lesioned animals with the lesioning technique validated with ChAT IHC. Chapter 5 details work performed in the development of the molecular imaging system used in this project. The prototype system TOHR required much basic testing and the progress of this system is described through sensitivity, linearity, resolution and contrast performance assessments. Chapter 5 describes these studies. Chapter 6 will comprise the general discussion and conclusion for all the work in this thesis.

2 Chapter 2 - Attention

2.1 Introduction

An impairment in attentional function is a significant contributing factor to the morbidity of AD. It has been reported that attention is the first non-memory domain to be impaired in the course of AD (Perry *et al.*, 1999) and that the integrity of attentional processing is an essential determinant of a subject's learning and memory (Sarter *et al.*, 2003). This chapter will address the role of the CBF in attention and describe two studies which were performed to help further elucidate the role of this system in attention.

2.1.1 Relationship with AD

The impairment of attention in AD has been known of for some years. An early study measured simple reaction time (RT) (Freed *et al.*, 1989) and showed AD patients to have an impairment in shifting attention. Another study (Parasuraman *et al.*, 1992) examined groups of patients with mild or moderate dementia compared to age-matched controls on a test of reaction time in a cued target detection (CTD) task. Task difficulty was increased where subjects were presented with a letter on a screen which was either to the left or right of centre and which they had to classify as either consonants or vowels. Preceding the letter on the screen was either a valid cue, which directed the subject's attention towards the correct location of the letter to follow, or an invalid cue which directed the subject's attention away from the

location of the subsequent target. AD patients were found to have a performance equivalent to controls with valid cues but to show significant impairments after being presented with invalid cues (Parasuraman *et al.*, 1992), indicating an impairment in the ability to shift attention. Similar findings were reported in a later study (Oken *et al.*, 1994) which also tried to simulate the results seen in AD in normal young subjects by way of an anticholinergic/antihistaminergic intervention used at a dose which provided a sedative effect (but which was thought insufficient to produce an anticholinergic effect). Though the subjects' RTs were diminished, unlike in AD there was no disproportionate impairment in their ability to shift attention, suggesting that the deficit in the AD patients was not related to diminished alertness (Oken *et al.*, 1994).

That the attention deficits described above were (to some degree) due to impairments in the cholinergic system is supported by trials of pro-cholinergic therapies. The acetylcholinesterase inhibitor Tacrine has been shown in randomised, double-blind, placebo-controlled, crossover studies to improve performance on tasks of attention (amongst other tasks) to a mild, yet statistically significant, degree (Eagger *et al.*, 1991; Sahakian *et al.*, 1993). Similarly, the nAChR agonist nicotine has been shown to produce similar improvements (Sahakian *et al.*, 1994). These effects though did not extend to tests of activities-of-daily-living, thus their clinical relevance is uncertain.

When the anti-cholinergic drug scopolamine (an antagonist of the muscarinic acetylcholine receptor - mAChR) was administered to young normal volunteers (mean age 27 years), it was shown to produce attentional impairments (Dunne *et al.*, 1986). In that task, subjects were told the target would appear in one of two quadrants most of the time. Scopolamine decreased target detection (increased RTs) in high-probability areas, indicating an impairment in the ability of the subjects to focus their attention. Scopolamine however *increased* target detection in low-probability areas, probably because the lack of attention focussing allowed for a greater proportion of attention to be available to monitor low-probability areas.

Though invaluable, human studies of attention suffer from some drawbacks. Due to the need for non-invasive techniques, drugs are delivered systemically. This makes it difficult to conclude definitively their location of action. Also, a lack of biochemical specificity means a drug's mechanism of action may also be difficult to elucidate. For example Tacrine, the AChE inhibitor discussed above, is also known to be a monoamine oxidase (MAO) inhibitor (Kaul, 1962) and a potassium channel blocker (Drukarch *et al.*, 1987), both of which may enhance attention indirectly by either increasing the availability of catecholamines (via MAO inhibition) thereby improving alertness, or by increasing the release of a variety of transmitters (via K^+ channel blocking) (Drukarch *et al.*, 1987). As well, it is a ligand for the nAChR and mAChR (Perry *et al.*, 1988) which may simulate the normal function of ACh. Likewise, the nAChR is known to be expressed by many non-cholinergic cells (Paterson *et al.*, 2000; Wonnacott, 1997), meaning the

location of action of nAChR agonists is also difficult to assess. A more detailed elucidation of the role of the CBF in attention therefore requires studies on animal models.

2.1.2 Attention studies in animal models

Similar results have been reported following scopolamine administration to rhesus monkeys as those described above with normal human volunteers (Dunne *et al.*, 1986). In a CTD task, scopolamine was demonstrated to increase all RTs, but to increase those following invalid cues more so than those following valid ones (Davidson *et al.*, 1999). CBF lesions produced with ibotenic acid have also been reported to show this pattern of effects on a CTD task (Voytko *et al.*, 1994). Following ibotenic acid lesions of all CBF nuclei, cynomolgus monkeys were assessed with a range of memory and attention tasks. Whilst no memory effects of these lesions were observed, the above attentional deficit was reported. In addition, lesioned monkeys were also shown to have increased sensitivity to scopolamine, leading this group to conclude the effects produced by ibotenic acid lesioning were cholinergic in origin and that these cholinergic lesions mediate performance in attention but not memory tasks (Voytko et al., 1994, 1996). However, in light of the contrary results described above in human studies following scopolamine administration to normal volunteers (Dunne *et al.*, 1986), the confidence with which a connection has been made between scopolamine and the cholinergic nature of the ibotenic acid lesions is of some concern, since the CBF also contains many non-cholinergic cells which ibotenic acid will also destroy (Voytko *et al.*, 1994).

Data from non-specific lesions will therefore obviously be complemented by studies employing more selective cholinergic immunotoxins.

Though an immunotoxin analogous to SAP does exist for non-human primates and produces the expected behavioural impairments (Fine *et al.*, 1997; Turchi *et al.*, 2005), primate studies are difficult because it is harder to obtain the animals and smaller numbers make statistical analysis more challenging. Studies in rats can overcome these problems. Whilst rodent studies can present their own problems given rodents are further removed biologically from humans than non-human primates, in the analysis of rodent studies the importance of these problems can be assessed according to the specific brain region/system being investigated. Regarding studies of the basal forebrain cholinergic system, it is known that in the higher orders of mammals the CBF nuclei are more discretely located with less intermingling of cholinergic and non-cholinergic cells than in the rat (Gorry, 1963; Wenk, 1997). However, the corticopetal projections from the NBM are known to be similarly topographically organised in both the rat (Wenk, 1997) and humans (and monkeys) (Mesulam *et al.*, 1988) and the use of SAP, a specific neurotoxin, means the poorly differentiated rat CBF can be lesioned as though they were discretely localised. The SAP model thus provides some insight into human cholinergic processes.

Rats with ibotenic acid lesions of the NBM have been reported to have an impairment in divided attention (Olton *et al.*, 1988). The same study reported that

animals with ibotenic acid lesions of the MS had mnemonic impairments. The following year ibotenic acid lesions were reported to impair attentional function in a visual RT task (Robbins *et al.*, 1989a). The latter was a comparative study which found the attentional deficit to persist when the NBM lesion was performed with the more cholinergic specific excitotoxin quisqualic acid. A later study using the same task again demonstrated animals had a persistent deficit following lesion of the NBM with AMPA, which is the most specific of the excitotoxins though it does still cause some collateral damage to non-cholinergic neurons (Muir *et al.*, 1994).

Using the best current anticholinergic neurotoxin, rats with intraventricular lesions of SAP have been shown to be attentionally impaired in a multiple-choice serial reaction time task (MCSRT) (Waite *et al.*, 1999), where an animal receives a reward when it reacts quickly (< 5 secs) and correctly to a light in one of multiple positions ("ports"). Interestingly, icv SAP-lesioned animals performed normally under baseline conditions and only manifested impairments following increases in attentional demands, ie loud static background noise, increased number of ports in use (Waite *et al.*, 1999). This result is not dissimilar to the CTD task described above performed in humans.

The task used in this project to assess animals' attentional function is based on the same rationale (see below). The aim was to investigate whether an attentional deficit would be manifested using a novel test which uses food as a distracter

(rather than a motivator). Two different SAP models were assessed with this task, and it was believed both would manifest some degree of attentional impairment.

2.1.3 Models used in this project

2.1.3.1 ICV

Two methods of SAP administration were used in this project. One was a unilateral icv administration which has been shown to result in significant cell losses in the MS, DBB, and in the NBM (Waite *et al.*, 1994; Wiley *et al.*, 1995). This method was the earliest model of SAP lesioning. In subsequent years however many studies began using interstitial SAP injections in both an effort to create more focused lesions and to avoid damage to the cerebellar Purkinje cells which occurs after icv administration. As this is the first time the simple sensorimotor and disengage tasks (Schallert *et al.*, 1988) have been used to assess the role of the cholinergic system in attention, it was of interest to use a model with a large cholinergic-deficit. Icv administration was also chosen for this project as a comprehensive lesion was desired in order to facilitate radiopharmaceutical studies (Chapter 4 and 5).

2.1.3.2 Intracortical

The second method of SAP administration involved an intracortical injection into the primary motor and primary somatosensory cortex. Intracortical injections have been reported in several studies. The lesions produced are generally not as large as

those following icv lesions or lesions directly into the CBF nuclei. The use of an intracortical lesion in this study was a matter of chance after a group of animals had their stereotaxic surgery mistargeted (see Methods section). Subsequently, data from this serendipitous model was used to address the role of cortical CBF projections in attentional (this Chapter) and mnemonic functions (see Chapter 3). A unilateral lesion in this part of the cortex was expected to impair animals' contralateral performance on the simple component of the sensorimotor task due, not to impaired attentional function, but to impaired sensory and/or motor function. Any additional impairment over and above that caused by the cortical damage, ie an additional impairment on the disengage component of the task, would be an important validation of the sensitivity of the sensorimotor/disengage task as a test of attention.

Further, if the additional impairment was seen bilaterally, having performed the lesion unilaterally, this would help address the question of whether damage to the CBF or to a cortical target modulates attentional performance. Bilateral impairment would suggest that damage to the CBF was the more likely cause. This is possible because it was hypothesised that a known functional link between the NBM and the contralateral cortex (Katsumi *et al.*, 1999; Kiyosawa *et al.*, 1989) would allow bilateral nbm damage to result from a unilateral cortical lesion.

2.1.4 Sensorimotor test

The term 'attention' can be broken into different sub-types. For example, reflexive- or voluntary attention (Beane *et al.*, 2004), the former being a transient, automatic response upon the sensing of a stimulus whilst the latter is a sustained, deliberate process. These attention sub-types can overlap somewhat with sub-types previously described (Perry *et al.*, 1999), where attention was divided into selective-, sustained- and divided- subtypes. Whilst the latter two sub-types require deliberate effort, selective attention is the process of screening out irrelevant stimuli (Perry *et al.*, 1999), a process which may be thought of as being either voluntary or reflexive. Selective attention may be considered reflexive because a closely related process to ignoring irrelevant stimuli is the acceptance of relevant stimuli. That is to say, if one were to take a slightly looser interpretation of selective attention than that previously described (Perry *et al.*, 1999), one would define it as the ability to sort relevant stimuli from irrelevant; a definition which correlates reasonably well to that given for attention as a whole (Beane *et al.*, 2004), ie attention as a system to protect the organism against sensory processing overload.

It is widely accepted that corticopetal projections from the CBF are crucial to attentional function. Along with the studies already described, many other studies have confirmed this deficit and reported pharmacological evidence of the cholinergic basis of it (Jones *et al.*, 1995; Muir *et al.*, 1995; Muir *et al.*, 1992; Sarter *et al.*, 2005). It should be noted that though the cholinergic system is essential to normal attentional processing, it is not the only system involved as, for

example, reflexive attention has been shown with other tasks to be manipulated by not simply the cholinergic system but an interplay between it and the dopaminergic and noradrenergic systems (Beane *et al.*, 2004). In this project a test was used which has not been previously reported in studies investigating the role of the CBF in attention. The sensorimotor task focuses on an animal's ability to shift attention, similar to the CTD task. It is slightly different to many tests of attention with some unique features which may aid the continued understanding of this area of cognition. Whilst many attention tasks reported use a positive motivational stimulus as a reward, for instance the MCSRT (Muir *et al.*, 1994; Waite *et al.*, 1999), the sensorimotor task measures an animals RT to an instinctively salient stimulus without any external motivator. It is feasible that the cognitive basis of paradigms which use a reward as a motivator to complete a task in a certain fashion is different to that of the sensorimotor task. This is because the former rely on the animal learning the rule which will result in the reward, unlike the sensorimotor task which requires no rule-learning/retrieval.

This task has two components. The first component is performed when the animal is in a baseline state (the simple component) and the second when the animal is in a state of attentional loading, that is to say engaged with eating a piece of chocolate (the disengage component). It is the disengage component which is considered to be analogous to invalid cueing in the CTD test described above as it assesses the ability to shift attention (from the chocolate to the vibrissal stimulus rather than from the invalid cue to the target in the CTD). Given the involuntary nature of the

two components of the task and the need to shift attention in the disengage component, it's considered that the sensorimotor task is a test of reflexive selective attention.

2.2 Methods

2.2.1 Surgery

2.2.1.1 Intracortical

Twelve female Sprague-Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 200-300g were housed 3-4 to a cage in a room with a 12h:12h light:dark cycle. Animals had access to standard rat chow and water *ad libitum* both before and after lesioning. All procedures were carried out in accordance with the European Community Council Directive 86/609/EEC for the care of laboratory animals.

Lesioning was performed by stereotactically injecting 4µg of SAP unilaterally into the primary motor and the primary somatosensory cortex. Animals were anaesthetised with inhaled isoflurane (5% induction for ≈5 mins, 2% maintenance thereafter) and positioned in a Stoelting stereotaxic frame. A 1.5cm incision was made in the scalp to reveal the skull surface and a hole was drilled in the bone 0.9mm posteriorally and 1.5mm laterally with reference to Bregma in accordance with a rat-brain atlas (Paxinos *et al.*, 1998). Lesioned animals (n=5) had 4µg of 192IgG-saporin (Advanced Targeting Services, San Diego, USA) injected in a volume of 5µL of 0.9% saline using a Hamilton syringe in a KDS microinfusion

pump at a rate of 1μL/minute and a depth of 3.4mm from skull surface. Sham operated control animals (n=7) were injected with 5μL of normal 0.9% saline. Following the injection the needle was left *in situ* for a further 2 minutes to allow the fluid to disperse. After the needle was withdrawn, the incision was sutured and betadine antiseptic fluid was applied. The animals were then returned to their home cage.

2.2.1.2 ICV

Twenty-six female Sprague-Dawley rats (14 from Janvier, Le Genest-St-Isle, France and 12 from University of Sydney Laboratory Animal Services, Australia) weighing 200-300g were housed 3-4 to a cage in a room with a 12h:12h light:dark cycle. Animals had access to standard rat chow and water *ad libitum* both before and after lesioning. All procedures were carried out in accordance with either the European Community Council Directive 86/609/EEC for the care of laboratory animals or the Australian National Health and Medical Research Council guidelines on the care and use of animals in research and with the approval of the Animal Care and Ethics Committee of the University of Sydney, Australia. Surgery was performed in a manner identical to the above procedure, except with the infusion made at a depth of 3.4 mm from the dural surface, rather than the surface of the skull.

2.2.2 Sensorimotor method

The Simple sensorimotor component was performed according to previously published methods (Henderson *et al.*, 1999; Henderson *et al.*, 2003; Schallert *et al.*, 1988). Briefly, after habituating for 2 mins to the test cage a thin probe was passed through the bars of the lid of the cage. The blunt tip of the probe approached the animal gently from the side and behind in order to minimise auditory and visual cues. The animal's vibrissae were stimulated 3-5 times on each side and the time taken to respond each time (ie turning towards and/or touching the probe) was measured with a stopwatch.

The Disengage component was performed in an identical manner, except vibrissal stimulation was performed whilst the animals were engaged in eating a small (2.5g) piece of chocolate. It was only considered that the animal's attention was engaged when it had the piece of chocolate picked up in its front paws. Both the simple and disengage components of the sensorimotor test were performed at 3- and 6-days post lesion.

In order to account for floor effects, where RTs quicker than the observer's RT become necessarily grouped together, and to reduce variability in the measurements, RTs were scored according to rankings with the scores then used in Kruskal-Wallis non-parametric analysis of variance (ANOVA) statistical measurement with *post-hoc* Mann-Whitney U-tests to evaluate specific group differences. The rankings used to score RTs were as indicated in Table 1.

Table 1: Table of rankings used to transform reaction-times into scores for analysis

RT:	Score:
≤ 0.25	0
0.26-0.30	1
0.31-0.40	2
0.41-0.60	3
0.61-1.00	4
1.01-1.80	5
1.81-3.40	6
3.41-6.60	7
≤ 6.61	8

Analysis with the above rankings provided significant results for both animal models (see Results section below). In the icv animal model however, the simple component of the task yielded an unexpected result (see Discussion section). This data was therefore re-analysed with adjusted rankings (see Table 2) with the aim of clarifying the origin of this result.

Table 2: Alternate table of rankings used to transform reaction-times into scores for analysis

RT:	Score:
≤ 0.40	0
0.41-0.60	1
0.60-1.00	2
1.01-1.80	3
1.81-3.40	4
3.41-6.60	5
≥ 6.61	6

2.2.3 ChAT

ChAT IHC was performed upon all intracortically lesioned animals (n = 12) and 12 of the icv lesioned animals. Animals were sacrificed 7 days post-lesion. The animals were first rendered unconscious by either head trauma or inhaled halothane then transcardially perfused with 0.9% saline followed by 0.4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). The animals were then decapitated and the brains rapidly removed. The brains were kept in 0.4% paraformaldehyde at 4°c overnight followed by a solution of 20% sucrose in PBS until they had sunk (≈ 2 days). Intracortically lesioned brains were removed from the sucrose solution and placed into a -80°c freezer. Once frozen, 50µm thick sections were cut on a cryostat (Leica, Germany), and floated in PBS. Brains from icv lesioned animals were stored in 20% sucrose in PBS with 0.05% thimerasol until cutting. Forty-

eight-micron sections were cut on a microtome (Leica) after brains had been frozen on the apparatus with dry-ice.

For the ChAT immunohistochemistry, the sections were first rinsed in hydrogen peroxide in order to block endogenous peroxidases then incubated overnight in a 1:1,000 dilution of rabbit α-ChAT primary antibody (Chemicon, Temecula, USA). Following incubation in a donkey α-rabbit secondary antibody (Chemicon, Temecula, USA), staining was performed using the Vectastain ABC kit (Vectastain, Burlingame, USA) and the chromagen diaminobenzadine (DAB) (Sigma-Aldrich). Another, parallel, series was treated in the exact same manner except omitting the primary antibody and incubating with vehicle solution only. Once the sections were stained, they were mounted onto gelatine-coated slides, dried and coverslipped for image analysis.

The ChAT staining was analysed with two different methods. The use of the different techniques was a practical decision based upon available facilities. Intracortically lesioned animals' sections were analysed using γ-vision image analysis software (Biospace instruments, Paris). Digital images of the slides were made with a flat-bed scanner. Regions of interest (ROIs) were drawn around the striatum, frontal and parietal cortices, NBM, MS, thalamus, hippocampus and cerebellum. Measures of relative optical density were found in all ROIs with the optical density of the sections incubated without primary antibody taken as a measure of non-specific chromagen binding. This value of non-specific binding

was then subtracted from the values obtained from sections of sham and SAP lesioned animals' brains. All IHC was performed simultaneously with the same solutions, excepting the control solutions which didn't contain the primary antibody. Values were calculated as a ratio to striatal densities, similarly to the studies described in Chapter 4, as striatal cholinergic neurons are unaffected by 192IgG-saporin (Baxter *et al.*, 1995). Ratios from the ipsilateral and contralateral NBM were compared with a Mann-Whitney U-test to evaluate whether the unilateral cortical lesion produced a lateralised lesion in the afferent region of the cortex. Subsequently, SAP- and sham lesioned animal's ratios from each region were compared with Mann-Whitney U-tests to evaluate statistical significance.

In contrast, IHC staining from icv lesioned animals was analysed using a cell-counting technique. Digital micrographs of sections were taken with a Leica DC500 digital camera (Leica, Germany) attached to an Olympus BX51 optical microscope (Olympus, Japan) at a 10x magnification. Using Leica IM1000 image analysis software (Leica, Germany), ROIs were drawn around the NBM and MS and individual cells were counted within them, giving data as cells/mm^2. The mean cell densities in both regions were compared between SAP and sham lesioned animals with Mann-Whitney U-tests. Tests of lateralisation were not performed on these animals. As these animals were icv lesioned, the data for each side was grouped as cerebrospinal fluid would disperse the toxin throughout both hemispheres of the brain.

2.3 Results

2.3.1 Sensorimotor

To assess whether there was any difference in RT between the side ipsilateral and the side contralateral to the lesion, the side-to-side data from intracortically SAP-lesioned animals was compared on both the simple and disengage components of the task with Mann-Whitney U-tests (Figure 2). Neither on the simple nor the disengage component of the task were the lateralisation comparisons statistically significant ($p = 0.83$ and 0.45 for simple and disengage comparisons, respectively).

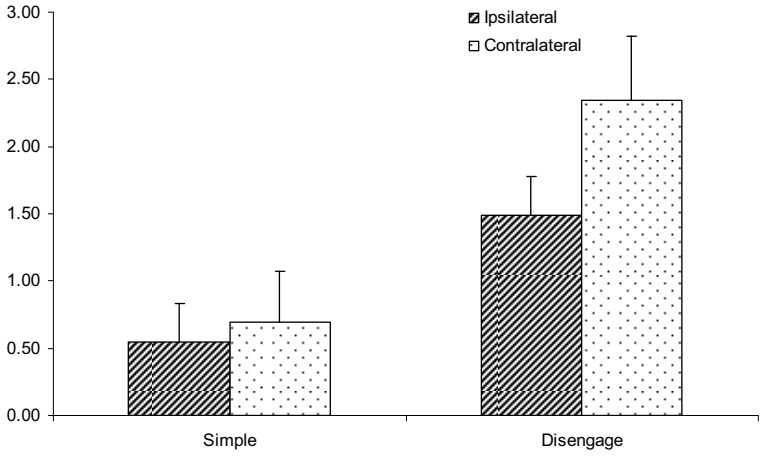

Figure 2: Difference between ipsilateral and contralateral sensorimotor response rankings for intracortically SAP lesioned animals

As no significant difference was observed between ipsilateral and contralateral reaction times (Figure 2) data were grouped. Large, significant differences were found between intracortically SAP- and sham-lesioned animals (Kruskal-Wallis ANOVA $H = 30.03$; $(df = 3)$; $p < 0.0001$). SAP lesioned animals were found to be

impaired on the disengage component of the test relative to the sham-lesioned animals (Mann-Whitney $p < 0.0001$, Figure 3). There was also a significantly longer delay in response in the disengage task in lesioned animals, relative to when they performed the simple component of the test (Mann-Whitney $p = 0.0002$; Figure 3). Also observed in these animals was a smaller, but significant difference between the performances of the two groups of animals on the simple component of the task test (Mann-Whitney $p = 0.015$; Figure 3).

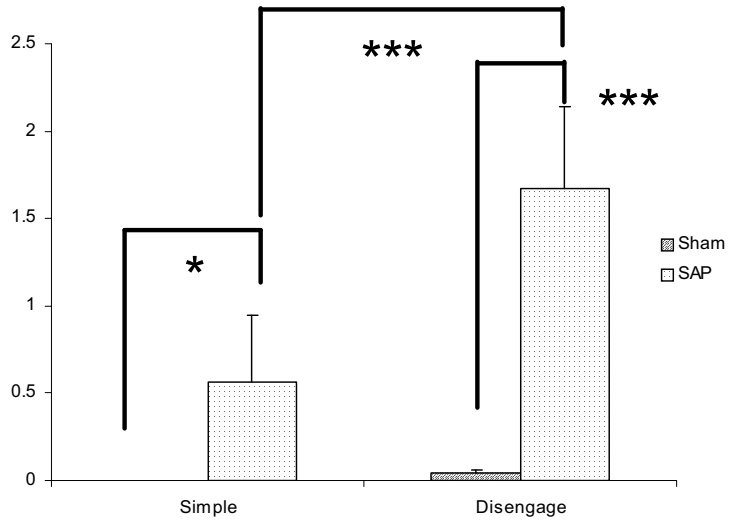

Figure 3: Results of sensorimotor testing on intracortically-lesioned animals using rankings derived from Table 1. * $p < 0.05$, *** $p < 0.001$. Sham n = 7, SAP n = 5.

Icv SAP lesioned animals showed a similar pattern to those lesioned intracortically. SAP lesioned animals appeared impaired on the disengage component of the test relative to the sham-lesioned animals, though this difference was not statistically significant (Mann-Whitney $p = 0.09$, Figure 5). There was a statistically significant longer delay in response in the disengage task in lesioned animals, relative to when

they performed the simple component of the test (Mann-Whitney $p < 0.001$, Figure 5). Again, as no significant difference was observed between ipsilateral and contralateral reaction times with Mann-Whitney p-values for the two components of the study both > 0.99 (Figure 4) data were grouped.

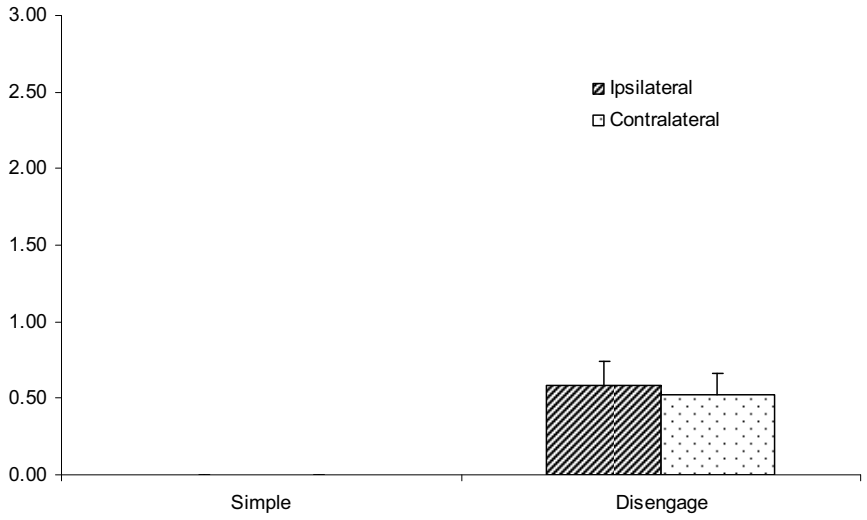

Figure 4: Difference between ipsilateral and contralateral sensorimotor response rankings for icv SAP lesioned animals

Similarly to intracortically lesioned animals, icv lesioned animals displayed differing performances on the simple component of the task. Interestingly, the deficit was due to sham operated controls displaying a deficit (Mann-Whitney $p < 0.05$). Sham animals performance in the simple component was not significantly different to the corresponding performance in the disengage component of the study. An examination of the RTs for icv sham lesioned animals revealed many scores which bordered on the 0 ranking. As it was these data which prevented the relative performances on the disengage component from reaching statistical significance

and as the result on the simple component were unusual, it was decided to re-analyse these data with a higher threshold.

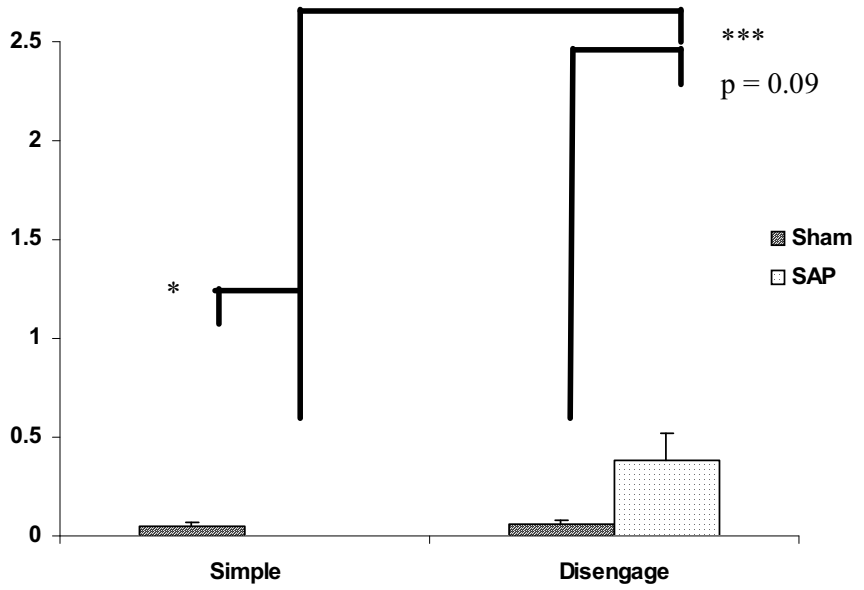

Figure 5: Results of sensorimotor testing of icv-lesioned animals using scores derived from Table 1. * $p < 0.05$, *** $p < 0.001$. Sham n = 12, SAP n = 12

When sensorimotor data from icv lesioned animals was re-analysed according to the scores given in Table 2, the only non-zero result was for SAP lesioned animals performing the disengage test (Figure 6). Between the simple and the disengage components of the task, these data displayed a similar pattern to both intracortically lesioned animals (Figure 3) and icv lesioned animals analysed with the shorter ranking threshold (Figure 5), with SAP animals showing a significant decrease in performance with increasing attentional load (Mann-Whitney $p < 0.01$) and sham-operated control animals performances not differing across the two components. Due to the reduced mean RT rankings for the control animals, the difference

between SAP-lesioned and control animals' performance on the disengage component of the task was also significant (Mann-Whitney $p < 0.01$) whilst the two groups displayed the same performance on the simple component.

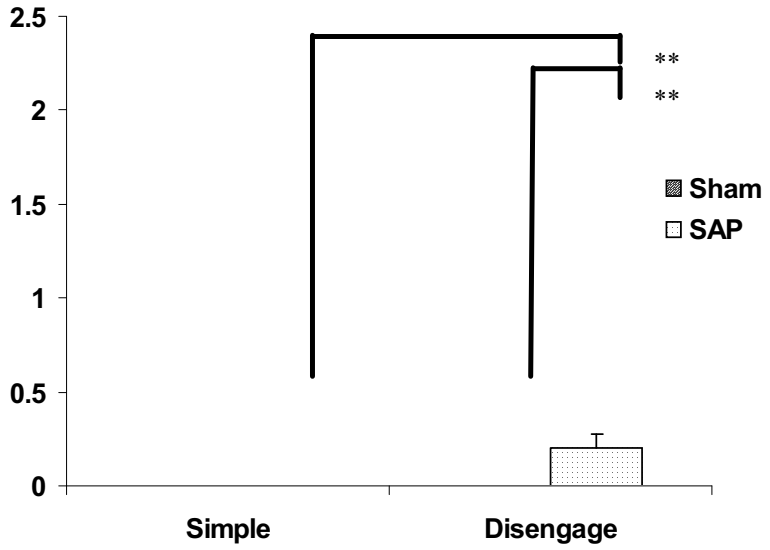

Figure 6: Results of sensorimotor testing of icv-lesioned animals using scores derived from Table 2. * $p < 0.05$, *** $p < 0.001$. Sham n = 12, SAP n = 12

2.3.2 IHC

2.3.2.1 Intracortical

IHC for ChAT confirmed the efficacy of the lesions. Evaluation of the possible lateralisation of the intracortical lesion showed no significant difference between the ipsilateral and contralateral NBM (Mann-Whitney $p = 0.53$). Subsequently, ipsilateral and contralateral data for each group in each region were pooled. Figure 7 shows the relative optical densities (ROD) of DAB chromagen staining in various regions compared to the ROD of chromagen staining in the striatum of

intracortically lesioned animals. The only regions to demonstrate statistically significant differences in the density of ChAT-antibody localisation were the CBF nuclei. The cortex and hippocampus of SAP lesioned animals demonstrated non-significant reductions in ChAT-antibody localisation (p = 0.34 and 0.07, respectively).

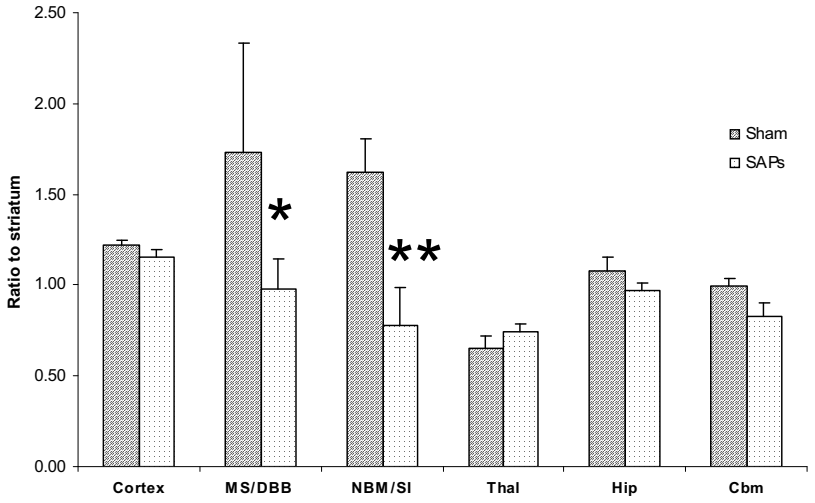

Figure 7: Ratio of ChAT expression in various regions to ChAT expression in the striatum as assessed by immunohistochemistry in intracortically lesioned animals. MS/DBB = Medial Septum / Diagonal Band of Broca; NBM/SI = Nucleus Basalis Magnocellularis / Substantia Inominata; Thal = Thalamus; Hip = Hippocampus; Cbm = Cerebellum. * $p < 0.05$, ** $p < 0.01$. Sham n = 7, SAP n = 5.

2.3.2.2 ICV

The results of ChAT IHC are presented in Figure 8 and Figure 9. Following icv SAP lesioning, there were significant reductions in ChAT immunopositive cell densities in both CBF nuclei. The reduction in the MS/DBB was 74% whilst the reduction in the NBNM/SI was 63%. As the lesion was performed icv, no test of

lateralisation was performed on these animals. Only ChAT staining in the CBF was examined as no readily discernible specific chromagen localisation was seen in any of the terminal fields, in accord with previous reports (Geula *et al.*, 1996).

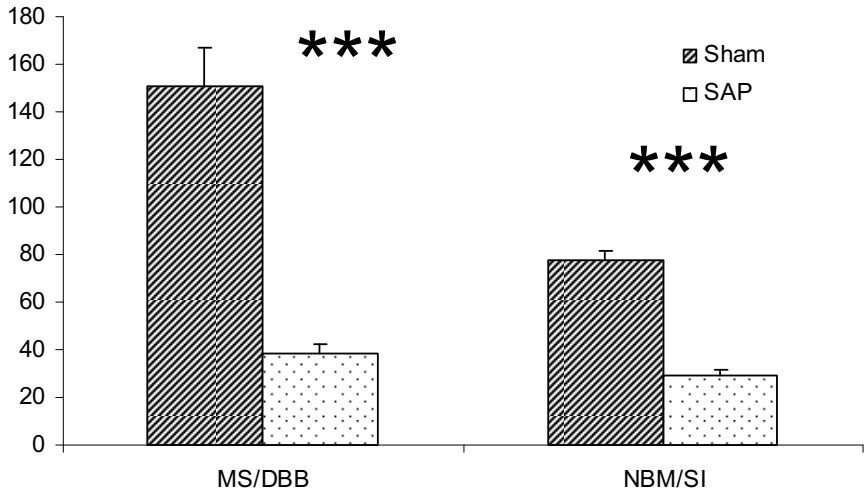

Figure 8: Immunohistochemistry for ChAT in ICV lesioned animals. Data are give as the mean number of cells per mm^2

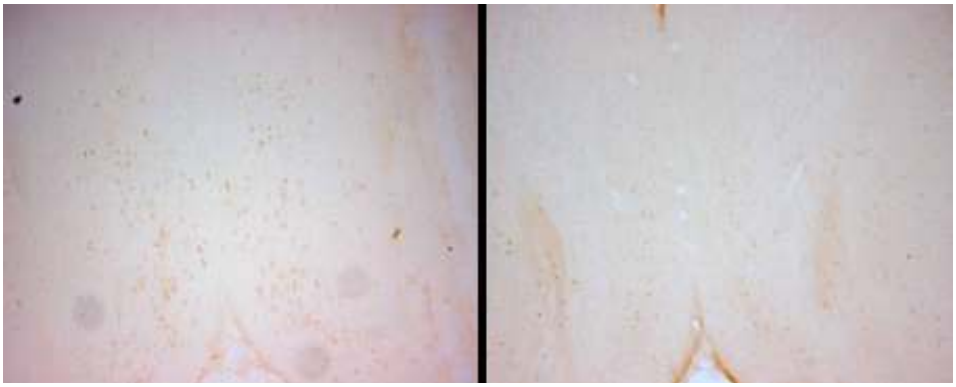

Figure 9: Immunohistochemistry for ChAT. The image on the left is a 50µm section at the level of the MS/DBB from a sham-lesioned animal. The image on the right is an equivalent section from an animal lesioned icv with 4µg of SAP.

2.4 Discussion

As stated, one of the aims of this project was to contribute to knowledge of the role that degeneration of the CBF plays in the morbidity of AD. The evidence of the general role of the CBF, and thus of the impact of its degeneration, in attention is great. To add precision to this knowledge there are a large number of studies focussing on various specific aspects of attentional function and their relation to the CBF projection system. The present study examined the effect of two SAP lesions on the sensorimotor task, using its simple and disengage components to assess reflexive selective attention. It was found that animals which were lesioned with SAP intracortically displayed a poorer performance on the simple component than controls. This poor performance became significantly worse when the attentional demands on the animals were increased in the disengage component. Control animals performance on the other hand was the same across both components of the test. Icv lesioned animals also displayed this pattern moving between the simple and the disengage components, though icv SAP lesioned animals were not impaired on the simple component. However when RT rankings were used with a 250ms threshold, the data for control animals was unexpectedly high. The results were therefore re-analysed with a 400ms threshold. These data showed icv SAP lesioned animals to be significantly impaired on the disengage component whilst all other measures showed no impairment.

2.4.1 Sensorimotor task

In the context of this project, the term 'sensorimotor' test is slightly misleading. Of the original reports of this test, one of the most striking features was the disparate performances of lesioned animals on the simple and disengage components, a disparity not observed in control animals. Following unilateral 6-hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle (MFB) in rats, used as an animal model of Parkinson's disease, there was an increase in contralateral response latency to simple vibrissal stimulation, with a complete abolition of contralateral responses in the disengage component of the task, whilst ipsilateral RTs were normal in both tasks (Schallert *et al.*, 1988). From 28 days post lesion animals began developing adaptive movements which allowed them to orient to the probe such that at 226 days post lesion contralateral RTs on the simple component of the task were back to baseline level, whereas contralateral RTs in the disengage component remained extinguished (Schallert *et al.*, 1988). The great disparity between animals' abilities to perform the simple and disengage components of the task, in addition to the recovery of a fast contralateral orienting ability, is indicative of an impairment beyond that of sensorimotor function.

These observations led the authors to postulate the disengage deficit they saw was a manifestation of damage to the basal ganglia, leading to an impaired ability to disengage attention from one activity to respond to a stimulus (Schallert *et al.*, 1988). In light of results described above with CTD tasks (Davidson *et al.*, 1999; Freed *et al.*, 1989; Oken *et al.*, 1994; Parasuraman *et al.*, 1992) it was logical to

investigate the relationship of lesions of the cholinergic system to the impairment in the ability to disengage attention via the combined simple and disengage sensorimotor tasks.

2.4.2 Scoring

The use of the scoring tables (Table 1 and Table 2) was necessitated by the nature of the data. All 'normal' RTs would fall into a narrow range of values close to zero, and thus would become grouped together and show little variability. Such a 'floor' effect artificially reduces these data's variability. 'Abnormal' RTs on the other hand could fall into an infinite number of values. These values, though they all represent RT deficits, would therefore have a large variability, likely rendering any statistical computation null. The scoring system, by pooling 'abnormal' RTs (and 'normal' RTs) into discrete groups, artificially reduces these data's variability, allowing for more meaningful comparisons.

The basis of the scoring system is the hypothesis that there must be some maximum time it would take for a normally functioning animal to react, whilst all RTs longer than this represent some type of deficit. On an analogue scale it would be possible to have a sample of RT values such that the hypothesis they came from the same population of values as 'normal' RTs couldn't be rejected. Using the above hypothesis, two possible populations of values are created, with one group representing a population of 'normal' values and 5-7 groups representing an 'abnormal' population of values.

2.4.3 Lesion

Animals lesioned intracortically with 4µg of SAP in 5µL in this study demonstrated reductions of ChAT density of 44% in the MS/DBB and 52% in the NBM/SI. This loss is only slightly larger than that reported previously for other intracortical lesions made with much smaller amounts of SAP and measured at various later timepoints (10 days to several weeks) (Bucci *et al.*, 1998; Fadel *et al.*, 1996; Gill *et al.*, 2000; Holley *et al.*, 1994; McGaughy *et al.*, 1998; Winters *et al.*, 2005). The non-linearity in dose-effect has been evidenced previously amongst these groups. Whilst other groups used amounts of SAP of generally < 0.05µg and found cholinergic marker reductions of 26-75%, one group (Bucci *et al.*, 1998) used 10 times the concentration (0.56µg) and reported a 32% reduction of AChE staining in the posterior parietal cortex. Therefore, though the intracortical lesion used in this project was created by chance, it is believed to be a valid model, consistent with previously reported ones.

One serious consequence of the chance nature of the intracortical lesion used in this project is that the infusions (SAP or saline) were of a large volume (5µL). Though SAP has an almost perfect specificity for cholinergic neurons the possibility exists that this volume, which was intended for the ventricle, could have caused non-cholinergic damage via mechanical forces. This scenario highlights the importance of the sham-lesioned control animals. These animals had an equal volume of vehicle alone injected into the same part of the cortex as other animals but

exhibited neither sensorimotor deficits on the simple component of the task nor an exacerbated response latency on the disengage component. That these were observed in the SAP lesioned animals is indicative of the cholinergic nature of these animals' impairments on this task.

It would appear sham lesioning either didn't produce significant cellular damage or, if it did, the damage it produced wasn't relevant to performance of the sensorimotor task. Some evidence for this latter hypothesis was recently reported (Bailey *et al.*, 2004) where excitotoxic lesioning (with NMDA) of various regions of the frontal cortex was performed in order to differentiate the cognitive functions that they are implicated in. Attentional impairments were found following lesions of the dorsal pre-frontal cortex, a lesion which impinged upon the secondary motor cortex. However a more caudal lesion which affected both the primary and secondary motor cortex produced different results. This motor cortical lesioning produced an impairment on the task which was independent of the attentional load applied (Bailey *et al.*, 2004) which led the authors to conclude that the motor cortex plays a role in the generation and control of 'intentional actions guided by external stimuli' and that lesions of this area reduce performance on the task without 'producing signs of attentional or sensory impairment'. The lesion produced in our study was also placed in the primary motor cortex (albeit in a more posterior portion than that produced in the report above (Bailey *et al.*, 2004)) and likely also produced damage in the secondary motor (and primary somatosensory cortex). Any non-cholinergic damage produced by our lesion would likely be analogous to the above lesion and

thus be unlikely to produce impairment on the sensorimotor test of reflexive attention.

The icv SAP model is more closely analogous to others reported in the literature. This model has been shown previously to reduce ChAT activities in the CBF nuclei by 80-90% at 7 days (Waite *et al.*, 1994). In this study slightly smaller reductions were observed, with a reduction of 74% in the MS and 63% in the NBM (Figure 8). The difference is probably due to the difference between measuring ChAT-positive neuron density as opposed to ChAT activity. The method used in this project may be more accurate than the test of ChAT activity as it has been shown that it is possible for ChAT activity to be reduced without concurrent loss of neurons, as represented by VAChT density (Kuhl *et al.*, 1996).

2.4.4 Task performance

For intracortically lesioned animals, a small, significant difference in RT was seen during the simple component of this test between SAP-lesioned animals and controls (Figure 3). In contrast a marked, statistically significant difference was seen between the two groups during the disengage task, when the animal was engaged with eating a piece of chocolate and had to 'disengage' it's attention to orient to the probe. The difference between SAP animals' performances on the simple and disengage parts of the test were also highly significant, whereas the difference between control animals' performances on the two components of the task was negligible. These results are interpreted to indicate that intracortical SAP

lesioned animals had two deficits impairing their performance on the disengage component of the test: a small sensorimotor deficit as evinced in the simple component and a relatively large attentional-deficit as demonstrated by the large statistically significant difference between SAP lesioned animals performance when comparing performance on the two different tests.

ICV SAP lesioned animals displayed a similar pattern of impairment across the two components of the task as intracortically lesioned ones (Figure 5). Their response latency measured in the disengage component was significantly higher than that measured in the simple component. SAP animals RTs in the disengage component also tended to be greater than those of sham-operated control animals in the same component. Unlike intracortically lesioned animals, icv SAP lesioned animals did not display an impairment on the simple component of the task. In addition, despite both the intracortically lesioned animals (Figure 3) and the first analysis of the icv lesioned animals (Figure 5) using the scores listed in Table 1, with a lower threshold of 250 msec, it could be seen the icv SAP lesioned animals had much quicker average RTs than intracortically SAP lesioned animals on both components of the task. This supports the hypothesis that intracortically lesioned animals had a sensorimotor deficit in addition to an attention deficit, whilst the icv lesioned animals displayed only an attentional deficit.

That the attention deficit in intracortically SAP lesioned animals was bilateral is evidence it was damage to the CBF that caused the impairment. This is based on

the hypothesis that a unilateral cortical deficit, especially one of such a relatively large magnitude, would cause bilateral CBF deficits. One early report of a unilateral interstitial CBF lesion with ibotenic acid found that only the ipsilateral cortex was subsequently affected (London *et al.*, 1984). This would seem to oppose the above hypothesis. However, a later study which also performed lesions with ibotenic acid in the CBF found a unilateral lesion could reduce cortical metabolic rate bilaterally in animals which had been previously lesioned (4 weeks earlier) in the contralateral CBF and allowed to recover function (Katsumi *et al.*, 1999). Additionally, in the earlier study, although all animals were found to have statistically significantly lower metabolic rates in the hemisphere ipsilateral to the lesion compared to the contralteral hemisphere, the lesioned animals did however all demonstrated a lower metabolic rate in the contralteral hemisphere when compared to the hemisphere of control animals contralateral to their sham lesions. These studies are however not totally comparable to the intracortical SAP model used in the present study as ibotenic acid is known to cause damage to both cholinergic and non-cholinergic cells and has been shown to cause much less damage to cholinergic cells than SAP as assessed by ChAT densitometry (Everitt *et al.*, 1997). 3-Bromopyruvate is a compound which has been shown following administration to the CBF to reduce tissue AChE content by up to 90% for up to 18 days, but to only partially and transiently reduce ChAT activity (Arendt *et al.*, 1990). When administered unilaterally, it has been shown to reduce metabolic rate in the contralateral frontal cortex compared to sham operated controls, however the reduction was not as great as that reported in the ipsilateral cortex (Ouchi *et al.*,

1996). The result of ChAT IHC in the NBM in the present study, where no significant difference was found between the nucleus ipsilateral and contralateral to the cortical lesion, supports the hypothesis that a unilateral cortical administration may cause bilateral CBF lesions.

In the analysis presented in Figure 5, sham-operated icv control animals displayed non-zero mean scores in both components of the task. An examination of the raw data revealed these results to stem from a relatively few individual measurements with RTs between 0.26 and 0.29 seconds. As it was considered that these results were operator-induced errors, ie the threshold for a zero score was possibly below the reaction ability of the experimenter, it was decided to analyse them with a higher threshold to see if the significant findings demonstrated in Figure 5 were still evident. When the RT scores from icv-lesioned animals were re-analysed with a zero score threshold of 400 msec (Table 2), the pattern of performance across the groups and the components of the tasks remained the same as for the lower threshold (Figure 6). Though the mean score for SAP lesioned animals on the disengage task was lower on this analysis, the same comparisons on this analysis as on Figure 5 were statistically significant. These results therefore support and extend upon the basic observations made from icv lesioned animals analysed with a 250 msec threshold.

2.4.5 Motivation

It is proposed in this project that the Sensorimotor task is a test of attention, however alternative explanations for the performances observed must be addressed, though the evidence discounting them is great. For example, a possible alternative explanation is that lesioned animals had a greater preference for chocolate than control animals and it has in fact been shown in a microdialysis study that frontal cortical ACh is increased in animals anticipating a chocolate reward (Inglis *et al.*, 1994). This effect remained significant into and throughout the consumption of the reward (Inglis *et al.*, 1994). The increase was linked to a role of the NBM cortical projection in the recognition of cues which signal a reward. As it relates to the sensorimotor task, it would appear that SAP lesioned animals would have had no augmentation of ACh concentration during the disengage component of the task, whereas control animals may have. Any increased attentional capacity the control animals may have had as a result of an increased ACh concentration in the cortex in the disengage component over the simple component of the task would not have been able to be detected due to the floor effect of such fast RTs. A lack of ability to augment ACh concentration in the frontal cortex in the anticipation and consumption of chocolate would not however be able to explain the differing performances of SAP lesioned animals in the simple and disengage components of the sensorimotor task.

Also seen in the present study was a diminished density of ChAT staining in the MS/DBB, nuclei which project to the hippocampus. Dysfunction of the

hippocampus is not postulated to impact on the disengage component of the sensorimotor task. This is because whilst the hippocampus has been implicated in the acquisition of rule-based behaviour, it has been reported to not be involved in positive reward association (Ridley *et al.*, 1989). The authors of the latter study believe the stimulus-reward association has the amygdala as a critical component. No specific damage to the amygdala could have been done in the present study however as the Ch4 neurons from the NBM to the amygdala are not lesioned by SAP (McGaughy *et al.*, 2000). Although it is possible the amygdala could have been damaged non-specifically following mechanical damage to the cortical target, the closest that amygdala projections come to the site of lesions in the present study are projections from the medial frontal cortex to the central amygdaloid nucleus which originate rostrally to the injection site used in the present study (Ottersen, 1982).

It is necessary to point out that the processes above implicating the cholinergic system in motivation demonstrate ACh augmenting the desirability of the reward. In order to have an alternate explanation for the discrepant performances of SAP animals on the simple and disengage components of the sensorimotor task, it is necessary to find a rationale for how an *impairment* in the cholinergic system would increase the saliency of the chocolate used in the disengage task. Each of the above processes above indicates an opposite effect. Thus, the simplest explanation with strong theoretical support by which cholinergic destruction can

produce the results observed in the sensorimotor task is an attentional dysfunction caused by a lesioned corticopetal projection system.

As stated earlier, previous studies to use the disengage test have demonstrated a deficit on the disengage task and a divergence of performance between the simple and disengage components in rat models of PD (Henderson *et al.*, 1999; Henderson *et al.*, 2003; Schallert *et al.*, 1988). One of the models studied used an infusion of 6-OHDA into the (MFB) which was subsequently shown histologically to produce a mean 71% dopaminergic cell loss on the lesioned side, as assessed by the marker tyrosine hydroxylase, in the substantia nigra pars compacta. A similar magnitude diminution of dopamine was found in the striatum of these animals (Henderson *et al.*, 2003). Whereas these studies attribute the disengage deficits seen to damage to the basal ganglia, the impairments on the sensorimotor task seen following SAP lesioning in this project are attributed to cholinergic dysfunction in the basal forebrain. Though these mechanisms may be independent, it is possible that they are subtly interconnected.

Dopaminergic dysfunction is considered the major neurochemical dysfunction underpinning the attention abnormalities in attention-deficit hyperactivity disorder (ADHD) and schizophrenia. In ADHD, the efficacy of dopaminergic stimulating drugs like methyphenidate and D-amphetamine in ameliorating symptoms strongly suggest a dopaminergic basis for this disorder. Lateralization of ^{18}F-fluorodopa uptake in ADHD patients is also consistent with this conclusion (Nieoullon, 2002).

In the other direction, increases in striatal dopaminergic transmission have been found consistently in schizophrenia patients. This hyperactivity has been particularly noted in the nucleus accumbens (NAC) (Sarter *et al.*, 2001a). This increase in NAC dopaminergic activity is thought to be the reason for hyper-attention, one of the core symptoms in schizophrenic patients. It has been proposed (Sarter *et al.*, 2001a) that the mechanism for this is that increases in NAC dopamine activity, when the NAC is in certain states of excitation, increase the activity of the CBF via a decrease in inhibitory GABAergic projection activity to the CBF. In light of this hypothesis, it is possible that the disengage deficit found previously (Henderson *et al.*, 1999; Henderson *et al.*, 2003; Schallert *et al.*, 1988) in the dopamine depleted rat is a result of decreased CBF corticopetal projection activity, resulting from dopamine depletions in the NAC.

These results provide evidence that cholinergic corticopetal projections from the basal forebrain are essential for normal performance (of the disengage component) of the sensorimotor task. Specifically, this work supports the proposal that this population of neurons is recruited during periods of higher attentional demand on a test of reflexive selective attention. That the task relied upon instinctive responses rather than rule → reward learning demonstrates with greater precision the attentional, more so than a motivational, role of this population of neurons.

The other cognitive decline contributing to the morbidity of AD which is addressed in this project is memory. The role of the CBF projection system in mnemonic

processes was one of the earliest examined, and subsequently it was one of the first shadows of doubt to fall on the cholinergic hypothesis. However some of these early, more general, results are being re-examined recently with behavioural paradigms designed to focus more upon more specific aspects of mnemonic function. Chapter 3 will describe one such study.

3 Chapter 3 - Memory

3.1 Introduction

Impairments in mnemonic function are a significant contributory factor to the morbidity of AD and are the symptom most widely associated with the disease. Though a widespread role for CBF projections in memory is no longer generally accepted, specific cholinergic lesions have still been shown to cause impairments in particular mnemonic tasks of relevance (Winters *et al.*, 2005). The studies described in this chapter were performed as part of a more focussed examination of the role of the CBF in memory, using lesion/model combinations which have not been previously reported.

3.1.1 Cholinergic system in AD

As previously discussed, in the first several years after its inception the cholinergic hypothesis considered the mnemonic dysfunction characteristic of AD to be straightforward, symptomatic of the loss of this neurotransmitter; analogous to the motor deficits following dopaminergic dysfunction in Parkinson's disease (Mesulam, 2004). The evidence supporting the role of the cholinergic system in memory up to that time was reviewed then by Bartus *et al.*, (1982).

That learning and memory were cognitive domains subserved by the cholinergic system had been postulated even before a cholinergic deficit was reported in AD

(Deutsch, 1971). In the subsequent decade, as the cholinergic hypothesis developed, many studies were performed investigating the effects of muscarinic receptor antagonists and agonists and AChE inhibitors in young and aged humans, young and old rats and non-human primates, along with studies of AD patients relative to age-matched controls (Bartus, 1978; Bartus, 1979; Bartus *et al.*, 1980; Bartus *et al.*, 1976; Christie *et al.*, 1981; Davis *et al.*, 1979; Davis *et al.*, 1978; Drachman, 1977; Mewaldt *et al.*, 1979; Risch *et al.*, 1981; Sitaram *et al.*, 1978). The results of all these pharmacological studies strongly indicated a specific role for the cholinergic system in mnemonic processes (Bartus *et al.*, 1982). As mentioned in the previous chapter, studies employing systemic administration of cholinergic modulating drugs cannot define the location and/or mechanism by which the drugs exert an effect (Everitt *et al.*, 1997). In addition, the known neurodegeneration and cell-loss in various brain regions crucial for memory (ie entorhinal region, amygdala, hippocampus) in AD renders the elucidation of the specific role of the CBF projection system in memory very difficult (Mesulam, 2004). As for attention, more precise knowledge of the role of the CBF in mnemonic processes requires the use of animal lesioning techniques. It is these more precise studies which have uncovered the complexity of the link between diminished CBF neuron function and memory.

3.1.2 Previous work in animal models of AD

In contrast to the results observed in rats (see below), non-human primates with non-cholinergic-specific lesions have shown relatively little impairment on tests of

mnemonic function. Following ibotenic acid lesions of CBF nuclei in the marmoset and squirrel monkeys, some studies have reported either no impairments in memory (Voytko *et al.*, 1994) or, when effects were observed, they were transient (Ridley *et al.*, 1985; Ridley *et al.*, 1986; Roberts *et al.*, 1990; Voytko *et al.*, 1996). It has also been reported that lesioned animals did show an apparent increase in sensitivity to scopolamine in some studies (Ridley *et al.*, 1985; Ridley *et al.*, 1986; Voytko *et al.*, 1994) but not others (Roberts *et al.*, 1990). The sensitivity of lesioned animals to scopolamine is however not necessarily indicative of the involvement of the CBF because, as stated above, the effects of systemically administered drugs are widespread. Therefore differential deficits between lesioned and control animals could be due to an interaction of non-specific CBF damage and non-CBF cholinergic suppression (Voytko *et al.*, 1996).

Differential effects of lesions of the different CBF nuclei in the non-human primate have been reported. On tests where non-cholinergic-specific NBM lesions do not disrupt mnemonic performance, similar lesions of the DBB have been demonstrated to prevent the learning of a new visuospatial task, albeit without impairing the performance of the same task where the learning of it had occurred prior to lesioning (Ridley *et al.*, 1988; Voytko *et al.*, 1996). These differences are likely due to an interaction of the lesion and the specific test used. It was hypothesised by Ridley and co-workers (Ridley *et al.*, 1989; Ridley *et al.*, 1988) that the hippocampus, the region to which efferent projections from the MS/DBB project, is involved in tasks based upon rule-based behaviour rather than positive reward

associations. Thus, whereas previously the cholinergic hypothesis had postulated a general role of the CBF in learning and memory, its actual role began to be seen as being more complex.

CBF lesions in the rat made with ibotenic acid, contrary to those described above in non-human primates, have been shown on a range of mnemonic tasks investigating many facets of memory to produce profound impairments (Dunnett *et al.*, 1991; Everitt *et al.*, 1997). Following NBM lesions with the more cholinergically-selective neurotoxin AMPA, impairments in most of these tasks were no longer observed (Dunnett *et al.*, 1991; Everitt *et al.*, 1997; Muir *et al.*, 1993; Page *et al.*, 1991; Robbins *et al.*, 1989b; Wenk *et al.*, 1992). A deficit which persisted was that in a passive-avoidance task, thought to be due to damage to projections from the NBM to the amygdala, the amygdala being a region known to modulate aversive conditioning (Everitt *et al.*, 1997).

Despite the AMPA studies seemingly supporting that the results reported following ibotenic acid lesions were not cholinergically based, it has been shown that deficits produced by this non-cholinergic-specific lesion can be reversed by pro-cholinergic therapy. For example, performance deficits in the Morris water-maze following ibotenic acid lesions could be reversed by AChE inhibition (Ohara *et al.*, 1997). Like the scopolamine-sensitivity discussed above, the amelioration of task performance of lesioned animals by pro-cholinergic therapy however need not be

considered evidence of a role for cholinergic neurons of the lesioned region in the task.

Similarly to non-human primate studies, differential effects of lesions of particular CBF nuclei have been reported in the rat as well. Like in the NBM, AMPA lesions of the MS didn't produce impairments on many tasks. These lesions did however produce deficits on a visual discrimination task and a delayed non-matching to position task (Johnson *et al.*, 2002), though this latter deficit could be due to attentional impairments (Everitt *et al.*, 1997). Interestingly, the AMPA lesion of the MS has been reported to enhance animals' performance in a test requiring learning about an aversive stimulus (McAlonan *et al.*, 1995).

With the exception of spatial deficits following icv administrations of SAP which are generally agreed to be caused by damage to cerebellar Purkinje cells (cerebellar damage has been shown to impair maze learning independently (Lalonde *et al.*, 2003; McGaughy *et al.*, 2002)), very few studies have reported memory deficits following SAP lesions of the CBF (Everitt *et al.*, 1997).

Whilst it cannot be said the CBF projection system is generally critical for mnemonic performance, it has been shown to play an important role in some mnemonic studies. For example, on an object recognition task using rhesus monkeys, the cholinergic-specific immunotoxin ME20.4-saporin, a primate analogue of SAP, has been also shown to cause impairments following direct

injection to the rhinal cortex (Turchi *et al.*, 2005). In the novel object recognition task (NORT), a version of the object-recognition task which doesn't involve any kind of motivation/reward component, SAP lesion of the perirhinal cortex (PRh) has also been shown to cause a mnemonic impairment (Winters *et al.*, 2005). These studies demonstrate certain aspects of the CBF projection system can impact mnemonic function. The studies presented in this chapter aim to aid the clarification of the role of the CBF in memory, using the NORT which has been shown to be able to be modulated by the cholinergic system (Puma *et al.*, 1999; Winters *et al.*, 2005) and lesions which have not been assessed with this task.

3.1.3 NORT

The NORT is unique amongst the memory investigation paradigms in that it places no motivational requirements on the animal, requires no rule-learning and unlike maze tests it is non-spatial (de Lima *et al.*, 2005; Morley *et al.*, 2001) and therefore less likely to be affected by cerebellar Purkinje cell damage. It is thus a more "pure" test of memory as it contains fewer confounding variables. This also makes it a more reproducible test (Ennaceur *et al.*, 1988). The memory assessed in the NORT is object-recognition memory, which shares the characteristics of and is thus thought to be analogous to primate episodic memory (Bartolini *et al.*, 1996).

3.1.4 Previous work with the cholinergic system in the NORT

The effect of modulations of the cholinergic system on performance of the NORT has mainly shown task performance to vary directly with cholinergic system function. For example the muscarinic receptor antagonist scopolamine has been shown to consistently impair animals performance in this task in both rats (Bartolini *et al.*, 1996; Rispoli *et al.*, 2004) and mice (Dodart *et al.*, 2000). Unlike in many of the memory tests described above, non-cholinergic-specific lesioning studies on rats using quisqualic acid (Bartolini *et al.*, 1996) or AMPA (Rispoli *et al.*, 2004) to lesion the NBM also demonstrated impaired performance on the NORT. Acute administration of nicotine to normal animals on the other hand has been shown to improve performance on the NORT (Puma *et al.*, 1999). Given a 24 hour interval between T1 and T2 (the inter-trial interval, ITI), control animals displayed no discrimination between the novel and familiar objects. A separate group of animals administered with nicotine before or after T1 or before T2 showed significantly better performance in this task (Puma *et al.*, 1999).

One group who lesioned CBF nuclei with SAP reported lesioned animals' performance on the NORT to be essentially the same as control animals' (Paban *et al.*, 2005). The significance of this report is difficult to assess though as the study used ITIs of 1 min, 1 day and 1 week which makes it incomparable to studies, such as are cited above, which generally use ITIs of 1-3 hours. Another group, introduced above, who assessed what effect SAP lesions would have on the NORT (Winters *et al.*, 2005) aimed to discern what role the perirhinal cortex plays in

object recognition memory. To this end, they administered SAP intracortically to the PRh and observed its effect on object-recognition and spatial-working memory. They found that performance on the NORT was impaired whilst spatial-working memory was normal (Winters *et al.*, 2005). The authors give strong evidence that the most reasonable explanation for the impaired performance in the NORT displayed by these animals is that cholinergic PRh circuitry is essential to object-recognition memory and that SAP lesions to the PRh lesioned this circuitry leading to the impairment (Winters *et al.*, 2005), an explanation which also is supported by the results of another study (Abe *et al.*, 2004) which found NORT impairments following scopolamine infusions into the PRh. An alternative hypothesis that was proposed by Winters *et al.*, (2005) but which could not be completely dismissed was that SAP damage to the CBF was responsible for the NORT deficits as the PRh SAP lesion was found to produce a small (23%) but significant reduction in ChAT immunoreactive (IR) cells in the CBF (Winters *et al.*, 2005).

3.1.5 Aim of this study

This study assessed the impact of intracortical and icv SAP lesioning on object-recognition memory, using the same animals as the studies described in Chapter 2. Due to the rather unique nature of the NORT, which similarly to the sensorimotor task has no reward component and relies on the animal's instinctual behaviour, and the specificity of the SAP lesion, the contribution of the CBF to mnemonic processes could be more precisely defined. Previous work in this field has provided interesting hypotheses as to the role of the CBF in memory, hypotheses

which require additional data to confirm. Specifically, this project investigated the hypothesis stated above from Winters *et al.*, (2005) of a role for PRh cholinergic afferents in object-recognition memory by addressing an alternative explanation for the reported results. Additionally, as an icv SAP model's performance in the NORT has not previously been reported, this study reports novel results of the largest, most generalised cholinergic-deficit model known.

3.2 Methods

3.2.1 Surgery

Animals used in these studies were the same as those used for the studies reported in Chapter 2.

3.2.2 NORT method

The NORT was performed at 3 and 6 days post-lesion and was modified from the test developed by Ennaceur *et al.*, (1988). It consisted of two trials, separated by an interval, the ITI. In the first trial (T1) following a 2min habituation to the empty field, animals were put into an apparatus with two identical objects, either dice or marbles, affixed to the floor with blu-tac (an adhesive gum to which they had never had any motivational attraction). T1 lasted for 15 minutes to ensure the animals developed a thorough familiarity with the objects. The time the animals spent exploring each object during the first 5 mins was recorded with a video camera. After a 1 hour ITI, the second trial (T2) commenced. Once more, the animals were

placed in the apparatus with two objects. In T2 however, only one of the objects was unfamiliar to the animal (N), the other being the same as in T1 (F). The time animals spent exploring each object in T2 was also recorded for 5 mins. It has been demonstrated that if animals' memories were normal, they will remember F and spend more time exploring N, at ITIs of up to 6 hours (Dodart *et al.*, 1997). The ITI used in this project was 1 hour, at which it was expected that normal animals would retain memory for F (recognition for N).

The apparatus used consisted of an open field, ~2,800 cm^2 in area, surrounded by a wall 40cm high. The floor of the field had lines drawn on it dividing it into 6 segments. After every trial, except the habituation phase, the apparatus was washed with 100% ethanol and allowed to dry to prevent olfactory cues interfering with the animals' natural exploration. The positions of the objects in the field and the roles the objects play (novel or familiar), were counter-balanced in order to control for animals having a preference for a particular object or a particular part of the field.

The dependent variable in this test is the T2 discrimination index (d2). The time the animal spent exploring each object (defined as having its nose < 2.5cm from and directed towards the object) was measured minute-by-minute over the 5 minutes filmed in T2. d2 was calculated as the amount of time the animal explored N minus the amount of time the animal explored F divided by the total T2 exploration time {(tN-tF)/(tN+tF)}. The differences between sham- and SAP-lesioned animals' d2 values were evaluated with the non-parametric Mann-Whitney

U-test for a T2 of both 1 minute and 5 minutes duration at both 3 and 6 days post-lesion.

As a complement to the discrimination data, total exploration time in T2 was also analysed. The non-parametric Kruskal-Wallis test was used initially to analyse separately T2 data over 1 min and 5 mins, with lesion type (SAP or sham), lesion location (icv or intracortical) and time post-lesion as independent variables. As there is no systematic non-parametric *post-hoc* test, ANOVA and Fisher's PLSD (protected least squares difference) were used to describe individual group differences. Meaningful comparisons which tended towards statistical significance on these tests (ie those comparisons which had a p-value < 0.30 on the PLSD) were then assessed with individual Mann-Whitney U-tests to see if they achieved statistical significance ($p < 0.05$) on the non-parametric test.

3.2.3 ChAT IHC

Histological data are the same as reported in Chapter 2.

3.3 Results

3.3.1 Intracortical

Measures of d2 at 3 and 6 days post lesion, with exploration times measured over the first one minute and five minutes, for intracortically sham and SAP lesioned animals are given in Figure 10. At 3 days post-lesion, SAP animals' average d2

was almost zero (0.03 and -0.01) for each of the T2 durations measured; a d2 of zero indicating no difference in the amount of time spent exploring the novel and familiar objects. Corresponding d2 values for sham-lesioned animals were higher on average (0.15 and 0.10). Both groups displayed such large standard errors however that the difference in their means was not significant. At 6 days post-lesion, SAP animals' mean d2 values were similar to those at 3 days post-lesion (0.04 and 0.00) and the values again were associated with very large standard errors. Of the two sham values (T2 = 1 min or 5 mins), the value for a 5 min T2 duration was similar to the means observed at 3 days post-lesion (0.19). Differences in d2 between sham and SAP lesioned animals were assessed with Mann-Whitney U-tests. Only when exploration time was measured over the first minute of T2 at 6 days post lesion was a reasonable difference discerned between the SAP and sham lesioned animals. Whilst under all conditions sham-operated animals spent a greater average time exploring the novel object in T2 than the familiar one relative to SAP lesioned animals, this was the only condition under which these differences neared statistical significance.

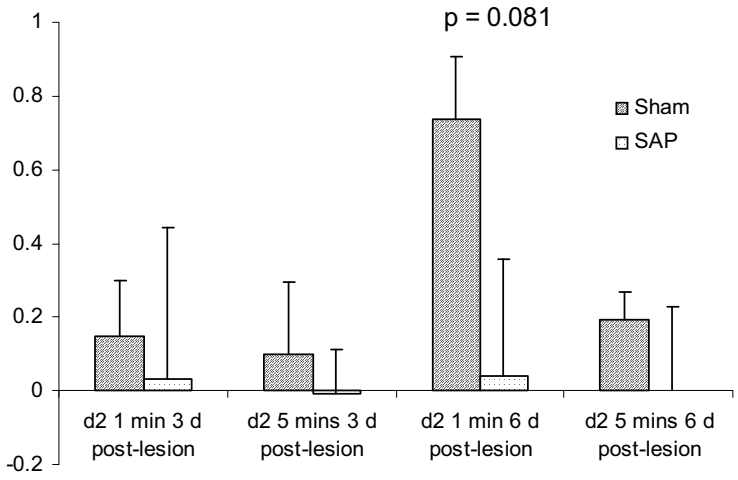

Figure 10: d2 NORT values for intracortically-lesioned animals

Total exploration times in T2 were very small. For instance, in control animals over a 1 min T2, mean total exploration time was 4.86±0.77 secs at 3 days post-surgery and 3.57 ± 1.78 at 6 days post surgery, whereas Winters *et al.*, (2005) found a total exploration time of 11.01±1.45 secs for control animals at approximately 14 days post-surgery. In control animals over a 5 min T2, mean total exploration time was 16.71±2.77 at 3 days post-surgery and 14.29 ± 3.58 at 6 days post-surgery, whereas Bartolini et al., (1996) found total exploration times ranging between 17 and 24 secs for the various studies they employed.

No significant difference was found for either SAP- or sham-lesioned groups' performances between 3- and 6-days post-lesion over either T2 duration (Table 3). At both T2 durations 3 days post-lesion however, it was found lesioned animals

spent significantly less time exploring the objects than control animals did. These differences were not present at 6 days post-lesion (Table 4).

Table 3: Comparison of exploration times for intracortically-lesioned groups between 3 days and 6 days post-lesion. P-values (Mann-Whitney U-test) are shown if a comparison is significant ($p < 0.05$ = *).

	Exploration, 1 min T2 duration: secs ± S.E (p-value)		Exploration, 5 min T2 duration: secs ± S.E (p-value)	
SAP	3 days	2.00 ± 0.63	3 days	7.00 ± 1.67
	6 days	1.80 ± 0.97	6 days	15.20 ± 4.35
	Difference	0.20 ± 1.16	Difference	8.20 ± 4.67
Sham	3 days	4.86 ± 0.77	3 days	16.71 ± 2.77
	6 days	3.57 ± 1.78	6 days	14.29 ± 3.58
	Difference	1.29 ± 1.94	Difference	2.43 ± 4.52

Table 4: Comparison of exploration times for each timepoint post-lesion between intracortically lesioned and control animals. P-values (Mann-Whitney U-test) are shown if a comparison is significant ($p < 0.05$ = *).

	Exploration, 1 min T2 duration: secs ± S.E (p-value)		Exploration, 5 min T2 duration: secs ± S.E (p-value)	
3d post-lesion	SAP	2.00 ± 0.63	SAP	7.00 ± 1.67
	Sham	4.86 ± 0.77	Sham	16.71 ± 2.77
	Difference	2.86 ± 0.99*	Difference	9.71 ± 3.23*
6d post-lesion	SAP	1.80 ± 0.97	SAP	15.20 ± 4.35
	Sham	3.57 ± 1.78	Sham	14.29 ± 3.58
	Difference	1.77 ± 2.03	Difference	0.91 ± 5.64

3.3.2 ICV

Measures of d2 at 3 and 6 days post lesion, with exploration times measured over the first one minute and five minutes, for icv sham and SAP treated animals are given in Figure 11. All differences in d2 between sham and SAP lesioned animals were assessed with Mann-Whitney U-tests. At 3 days post-lesion, SAP animals' average d2's indicated a very good memory for the novel object (d2 = 0.45 → N:F = 2.6:1) over a 1 min T2 and a milder memory effect when measured over a 5 min T2. Corresponding d2 values for sham-lesioned animals were not significantly different. At 6 days post-lesion, SAP animals' mean d2 values showed a similar pattern as those observed at 3 days post-lesion, with the d2 values again not differing significantly from control values. Results of sham lesioned animals at 6

days, and SAP lesioned animals at both 3 and 6 days post lesion, show a trend of higher d2 values over the 1 min T2 than the 5 min T2.

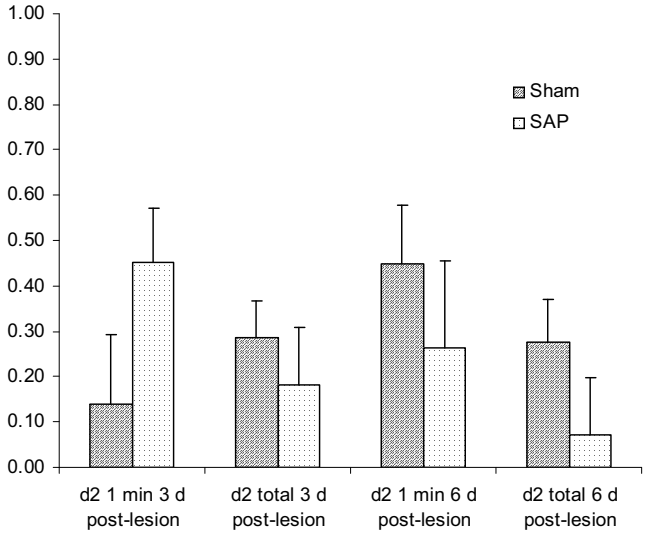

Figure 11: d2 NORT values for icv-lesioned animals

Similarly to intracortically lesioned animals, icv lesioned animals' exploration times in T2 were very small. For instance, in control animals over a 1 min T2, total exploration time was 6.00±0.75 secs at 3 days post-surgery and 6.83±1.54 at 6 days post surgery. In control animals over a 5 min T2, total exploration time was 14.33±1.11 at 3 days post-surgery and 15.75±2.56 at 6 days post-surgery.

Sham icv-lesioned animals didn't show any significant difference in total T2 exploration time between 3- and 6-days post-lesion over either T2 duration, whilst over the 1 min T2 duration neither did icv-lesioned SAP animals. Over a 5 min T2 duration, icv SAP lesioned animals spent significantly longer exploring objects at 3

days post-lesion than at 6-days post-lesion (Table 5). Over the 1 min T2 duration, icv SAP lesioned animals spent significantly less time exploring the objects than control lesioned animals did at both times post-lesion. At 3 days post-lesion, the difference between SAP- and sham lesioned animals' exploration times normalised when the T2 analysis was extended to 5 mins, whereas at 6 days post-lesion the difference remained highly significant (Table 6).

Table 5: Comparison of exploration times for icv-lesioned groups between 3 days and 6 days post-lesion. P-values (Mann-Whitney U-test) are shown if a comparison is significant ($p < 0.05$ = *).

	Exploration, 1 min T2 duration: secs ± S.E (p-value)		Exploration, 5 min T2 duration: secs ± S.E (p-value)	
SAP	3 days	3.67 ± 0.81	3 days	12.67 ± 1.90
	6 days	1.83 ± 0.37	6 days	6.00 ± 1.20
	Difference	1.83 ± 0.89	Difference	6.67 ± 2.25*
Sham	3 days	6.00 ± 0.75	3 days	14.33 ± 1.11
	6 days	6.83 ± 1.54	6 days	15.75 ± 2.56
	Difference	0.83 ± 1.71	Difference	1.42 ± 2.79

Table 6: Comparison of exploration times for each timepoint post-lesion between icv lesioned and control animals. P-values (Mann-Whitney U-test) are shown if a comparison is significant ($p < 0.05$ = *; $p < 0.001$ = ***).

	Exploration, 1 min T2 duration: secs ± S.E (p-value)		Exploration, 5 min T2 duration: secs ± S.E (p-value)	
3d post-lesion	SAP	3.67 ± 0.81	SAP	12.67 ± 1.90
	Sham	6.00 ± 0.75	Sham	14.33 ± 1.11
	Difference	2.33 ± 1.10*	Difference	1.67 ± 2.20
6d post-lesion	SAP	1.83 ± 0.37	SAP	6.00 ± 1.20
	Sham	6.83 ± 1.54	Sham	15.75 ± 2.56
	Difference	5.00 ± 1.58***	Difference	9.75 ± 2.82***

3.3.3 Both models

Overall, very little difference in total T2 exploration time was observed between intracortically and icv lesioned animals. Sham, icv lesioned animals at 6 days post-lesion spent more time exploring the objects in the first minute of T2 than intracortically lesioned animals did, an effect which disappeared over the longer duration. Conversely intracortically SAP lesioned animals spent longer exploring the objects over the 5 min T2 duration, an effect which wasn't present during the first minute (Table 7).

Table 7: Comparison of exploration times between intracortically- and icv-lesioned animals for each timepoint post-lesion and each lesion type. P-values (Mann-Whitney U-test) are shown if a comparison is significant ($p < 0.05$ = *).

	Exploration, 1 min T2 duration: secs ± S.E (p-value)		Exploration, 5 min T2 duration: secs ± S.E (p-value)	
3d SAP	Intracortical	2.00 ± 0.63	Intracortical	7.00 ± 1.67
	ICV	3.67 ± 0.81	ICV	12.67 ± 1.90
	Difference	1.67 ± 1.03	Difference	5.67 ± 2.53
6d SAP	Intracortical	1.80 ± 0.97	Intracortical	15.20 ± 4.35
	ICV	1.83 ± 0.37	ICV	6.00 ± 1.20
	Difference	0.03 ± 1.04	Difference	9.20 ± 4.51*
3d Sham	Intracortical	4.86 ± 0.77	Intracortical	16.71 ± 2.77
	ICV	6.00 ± 0.75	ICV	14.33 ± 1.11
	Difference	1.14 ± 1.07	Difference	2.38 ± 2.98
6d Sham	Intracortical	3.57 ± 1.78	Intracortical	14.29 ± 3.58
	ICV	6.83 ± 1.54	ICV	15.75 ± 2.56
	Difference	3.26 ± 2.36*	Difference	1.46 ± 4.40

3.4 Discussion

3.4.1 NORT

As described in section 3.1.3, the NORT is unique amongst the memory investigation paradigms in that is both a more "pure" test of memory, containing

fewer confounding variables, and a more reproducible test (Ennaceur *et al.*, 1988). These properties of the NORT contrast with various other memory paradigms.

For example, learning impairments in non-human primates have been measured with a visual object discrimination task (Ridley *et al.*, 1985; Ridley *et al.*, 1986). This task, like the NORT, relies upon the ability of the animal to recognise and discriminate objects. However, unlike the NORT, the ability to discriminate the objects is re-enforced with a food reward. As discussed in Chapter 2, Ridley *et al.* provide evidence that such a stimulus-reward association has the amygdala as a critical component (Ridley *et al.*, 1989). The delayed-matching-to-position memory task similarly requires the animal to respond to the motivation of a food reward (Steckler *et al.*, 1995). The NORT was preferred for the studies presented in this chapter both because it was desired to elucidate the role of the cholinergic system in purely mnemonic processes and because, due to the SAP model being chosen as the best possible one to assess the radiopharmaceuticals discussed in Chapter 4, a test which had the amygdala as the critical substrate would have been unlikely to yield valuable results given the amygdala is unaffected by the SAP immunotoxin (McGaughy *et al.*, 2000).

Pertinent to this study of the cholinergic system, it has been suggested that the NORT could be particularly sensitive to an alteration of attentional processes (Dodart *et al.*, 1997). This opinion however is in conflict with those groups who espouse the NORT as a paradigm purely for working memory (Abe *et al.*, 2004;

Ennaceur *et al.*, 1988) as it doesn't require the learning of any rule. As described in Chapter 2, attention is critically dependent on the CBF projection system, therefore it is very important to address this issue. Sarter *et al.*, (2001b) describe different aspects of sustained attention as being either a knowledge-driven process where the subject responds according to previously learnt rules or a process dependent on the sensory saliency of the target. It can be confidently assumed that neither of these processes is the subject of the NORT paradigm as i) with no reward (or punishment) there is no opportunity for the animal to learn a rule and ; ii) the objects were chosen so as they could not be displaced, gnawed or played with, removing from them as much sensory saliency as possible.

3.4.2 Intracortical results

For both intracortically- and icv-lesioned animals, differential exploration times in T2 were measured separately over both the first minute and the first 5 mins. The two analysis times were needed because though data has shown that discrimination performance is typically maximal in the first minute of T2 (Dix *et al.*, 1999; Winters *et al.*, 2005), the low levels of exploration times in these animals meant that a longer sampling period was also desired.

In intracortically lesioned animals, the mean sham value for the 1 min T2 duration at 6 days post-lesion was very different to all other values. This made it sufficiently different to the SAP value for 1 min d2 at 6 days post-lesion that despite large standard errors, the difference between the SAP- and sham-lesioned

animals bordered on statistical significance. In the context of the large standard errors, other NORT results in this study and other groups' results assessing the mnemonic effects of SAP lesions using both the NORT (Paban *et al.*, 2005) and other memory tests (Baxter *et al.*, 1995; Dornan *et al.*, 1996; Vnek *et al.*, 1996), this difference must be interpreted cautiously. These data therefore do not necessarily indicate an impairment in object-recognition memory in these animals.

The trend towards statistical significance is nevertheless worthy of comment. The d2 of the control group at 6 days post-lesion was much greater when measured over 1 min in T2 than over 5 mins in T2, though the data at 3 days post-lesion neither support nor contradict this. This accords with data which has shown that discrimination performance is typically maximal in the first minute of T2 (Dix *et al.*, 1999; Winters *et al.*, 2005).

3.4.3 ICV results

No significant impairment in object discrimination was exhibited by icv SAP lesioned animals. The closest a comparison came to rejecting the null hypothesis was in fact the first one, at 3 days post-lesion with a 1 min T2 duration, where it was the SAP lesioned animals, rather than controls, which appeared to display a greater interest in the novel object than the familiar one (Figure 11). This apparent difference was reversed when the T2 data was analysed over 5 mins. At 7 days post-lesion and both T2 durations, SAP lesioned animals tended to display less mean object-discrimination than control animals.

Like intracortically lesioned animals, the d2 of the control group at 6 days post-lesion was much greater when measured over 1 min in T2 than over 5 mins in T2. Unlike the other group, the data from 3 days post-lesion don't support this observation. As well, the object discrimination of SAP lesioned animals at 6 days post-lesion tended to be lower than that observed at 3 days post-lesion for both T2 durations. This may be related to timecourse data (Waite *et al.*, 1994) which shows that ChAT immunoreactivity in the CBF is diminished to ≈ 50% of control levels at 3 days post-lesion before reaching a maximal diminution of ≈ 90% at 7 days post-lesion with no further change for up to 28 days. However, as neither the reduction in SAP animals' d2 between 3 and 6 days post-lesion nor the absolute difference in d2 between SAP-lesioned and control animals' d2 were statistically significant, it is likely this apparent relationship (between SAP animals' performance and published SAP-lesion timecourse data (Waite *et al.*, 1994)) is mere co-incidence.

3.4.4 Both models

The study by Winters *et al.*, (2005) assessing what effect SAP lesions would have on the NORT aimed to discern what would be the mnemonic effect of a lesion of the PRh. They found that performance on the NORT was impaired whilst spatial-working memory was normal (Winters *et al.*, 2005). The authors give strong evidence that the most reasonable explanation for the impaired performance in the NORT displayed by these animals is that cholinergic PRh circuitry is essential to object-recognition memory (Winters *et al.*, 2005), an explanation which also is

supported by the results of another study (Abe *et al.*, 2004). An alternative hypothesis that was proposed Winters *et al.*, (2005) was that it was generalised damage to the CBF which was responsible for the NORT deficits. The results presented here support the former hypothesis. Rats in the first study described in this chapter had intracortical injections of SAP to the primary motor- and primary somatosensory-cortices of a much larger magnitude than rats in the study of Winters *et al.*, (2005) (4µg in the present study v. 0.024µg in total [for 6 injections]), which had the effect of producing a ChAT-IR deficit in the CBF twice the size of the previous study. That a significant deficit in object-recognition memory was not seen despite this CBF damage is evidence to support the conclusion of Winters *et al.*, (2005) that cholinergic PRh circuitry is critical to object-recognition memory. In the icv study described in this chapter, where ChAT density was reduced by up to 75%, there was still no mnemonic impairment observed on the NORT, further strengthening the support for the PRh hypothesis for object-recognition memory.

3.4.5 Exploration

3.4.5.1 Intracortical

The conclusions which can be drawn from the NORT results must be analysed in light of the relatively low levels of absolute exploration time being analysed. For example, compared to previous studies (Bartolini *et al.*, 1996; McGregor *et al.*, 2003; Winters *et al.*, 2005), control intracortically lesioned animals' exploration

times in this study were approximately one-quarter to one-half of these other results. Smaller differences are seen when data are compared to other work (Ennaceur *et al.*, 1988). Possible differences in methodology which contributed to these differences are discussed on p. 94. In the study of Ennaceur *et al.*, (1988), normal animals' total exploration times in T2 were 15.0 secs and 11.7 secs over 3 minutes in T2 in studies 48 hours apart, which are 20-50% greater than exploration times over 3 mins in T2 in this study (data not shown). The effect of this diminished exploration time is to increase the floor effect, limiting the sensitivity of the study to detect discrimination between objects because the *difference* in exploration times must always be less than *total* exploration time. A similar effect can be considered to apply to icv lesioned animals as there was generally no difference between the total T2 exploration times for control intracortical and sham lesioned animals (Table 7).

As seen in Table 3, neither group of intracortical animals displayed significantly different exploration times between studies at 3- and 6-days post-lesion. Additionally, Figure 10 shows positive d2 values for sham animals at 6 days post-lesion. These are evidence that a 3 day interval between studies is appropriate. As the same objects were used at both timepoints, though their roles were reversed, the possibility existed that the animals would remember both objects (and the task itself), thus neither object would be novel, therefore there would be neither differential exploration nor as great a degree of total exploration. The results in Table 3 indicate that between the two studies the task regained its novelty. This

conclusion is supported by previous work which has shown normal animals to be unable to discriminate objects in T2 following a 24 hr ITI (Puma *et al.*, 1999).

The previous observations based upon control animals' total T2 exploration times remain equally valid when considering lesioned animals' results from 6 days post-lesion. As shown in Table 4, at 6-days post-lesion the difference between lesioned and control animals' total T2 exploration times were not statistically significant. That there was a significant difference observed in the exploration data for intracortically SAP- and sham-lesioned animals at 3 days post-lesion may indicate some transient effect of the SAP lesion in these animals. The effect could have been a specific deficit in locomotor activity caused by the immunotoxin, though given the SAP lesion has been shown to remain stable (ie not reverse) for up to 30 days (Waite *et al.*, 1994) it is unlikely such an effect would have been transient. Alternatively, it may be that the intracortical SAP-lesioned animals took longer to recover generally from the surgery and at the 3 day timepoint the apparently reduced exploratory activity was due to the after-effects of the surgery. However, given the data shown inTable 3, the 3 day results in Table 4 are considered to be of only mild interest.

3.4.5.2 ICV

Similarly to intracortically lesioned animals, each group of icv lesioned animals generally did not display different levels of exploration in T2 between 3 and 6 days post-lesion. One comparison which did attain statistical significance was for icv

SAP lesioned animals over the 5 min T2 duration (Table 5). In this comparison, SAP lesioned animals had half the amount of total exploration at 6 days post-lesion than as at 3. This difference may be due to the progressive nature of the lesion for the first week post-lesion (Waite *et al.*, 1994), where the CBF lesion impairs the animal's exploratory activity. That icv control animals and intracortically lesioned animals (Table 3) did not display the same pattern may be explained by the smaller CBF lesion produced following intracortical lesions (see above).

The results presented in Table 6 are the inverse of those in Table 4 where SAP lesioned animals displayed significantly less exploration relative to controls at 6 days post-lesion than at 3. Given the large differences and the high significance of the comparisons, it appears that the icv SAP lesion progressively impaired animals' exploratory activity. In light of these animals' normal performances on the simple-sensorimotor task presented in Chapter 2, the apparent decrease in exploratory activity observed in the NORT must not necessarily be attributed to a diminished ability to explore. Rather, this could be a result of diminished activity, whereby a lesioned animal will spend a relatively large proportion of the time not moving.

3.4.5.3 Both models

There was no overall pattern of significant differences when comparing the exploration of intracortically- and icv lesioned animals (Table 7). The two comparisons which did achieve significance had the differences in different directions and were not supported by other observations. The significant difference

found for control animals at 6 days post-lesion over a 1 min T2 duration was not present over the 5 min T2 duration. This indicates that both icv and intracortically lesioned animals had the ability to explore to a similar level, however their time course of exploration was slightly different.

The difference found for SAP lesioned animals at 6 days post-lesion over a 5 min T2 was statistically significant, whereas neither that found over a 1 min T2 at 6 days post-lesion nor a 5 min T2 at 3 days post-lesion were (Table 7). In comparison to the former measure, both intracortically and icv lesioned animals demonstrated greater exploration times over a 5 min T2 than a 1 min T2, however the increase in exploration was more marked for intracortically lesioned animals. In comparison to the latter measure, between 3 and 6 days post-lesion the exploration time of intracortically lesioned animals doubled whereas that for icv lesioned animals halved, allowing for the large difference between these measures at 6 days post-lesion. Whereas this pattern of exploration level change mirrors that observed over a 1 min T2 for icv lesioned animals, it doesn't for intracortically lesioned animals. Given the lack of time-course effect on the data for the 1 min T2, and the opposite effect observed for icv lesioned animals over the 5 min T2, it is difficult to draw a conclusion from this observation.

There are a couple possible explanations for the overall diminished exploratory activity in all these animals relative to those in other studies. For intracortically lesioned animals it could be due to damage to the primary motor cortex. Though

the exploratory data discussed here relate equally to sham-operated animals, the mechanical damage caused to this part of the cortex would be the most likely cause of diminished exploratory activity. However, the results of the sensorimotor testing (Chapter 2) and a published study (Himmelhaber *et al.*, 2000), where microdialysis probes were placed into the same cortical area without mechanical-damage-induced impairment, do not support this explanation.

For both models, the decrease in exploration may be due to strain/sex differences, this study using female SD rats whilst the others used either male Wistar (Bartolini *et al.*, 1996; Ennaceur *et al.*, 1988) or Lister Hooded rats (Winters *et al.*, 2005). Alternatively, the decrease in exploratory activity could be related to the objects used in the task. Similarly to prior studies (Bartolini *et al.*, 1996; Ennaceur *et al.*, 1988; Winters *et al.*, 2005), the objects used were made of materials the animals could not chew, were affixed to the floor so they couldn't be moved and had no motivational significance for the animals. However the objects (glass marbles and plastic dice) were much smaller than those used previously (Bartolini *et al.*, 1996; Winters *et al.*, 2005). It seems either that smaller objects are not as 'interesting' to rats and thus receive only perfunctory examination, or that rather than being more 'interesting,' big objects receive more attention simply because they occupy a relatively larger proportion of the space in the testing field. In either case, it appears larger objects, ≈ the size of the rat's body, may provide more robust object-recognition data.

Additional factors which may have resulted in animals in this study displaying less exploratory activity than that reported in other studies are the size of the apparatus and the time testing was conducted. Animals in this study spent around half as much time exploring the objects in the first minute of T2 as the animals reported by Winters *et al.*, (2005). Whereas all testing in this study was conducted during the animals' light phase, the published study performed testing during the animals' dark phase. As rats are known to be more active during the dark phase of the diurnal cycle (Altman, 1962; Wallace *et al.*, 2001), this seems like a viable explanation. However, animals reported in another published study (Bartolini *et al.*, 1996) also displayed twice the exploratory activity of animals in the present study despite being tested during the light phase of the cycle.

A similar pattern is seen when considering whether the size of the apparatus used in the present study is responsible for the decreased exploratory activity observed in T2. Specifically, it is possible that the height of the walls of the apparatus may affect the amount of exploratory behaviour measured. High walls isolate the animal from the outside environment, having the effect of focussing the animal's attention into the test area and therefore maximising the amount of time spent exploring the objects. Lower walls on the other hand allow the animal to perceive a wider environment, diluting its exploration into a wider area. However, whereas animals in both the studies mentioned above (Bartolini *et al.*, 1996; Winters *et al.*, 2005) displayed twice the exploratory behaviour of animals in the present study, the former reported use of an apparatus with higher walls than used in this study,

whereas the latter used lower walls than in this study. The effect of low exploratory activity on the d2 calculation and analysis of the NORT however is not considered to be significant. Whereas animals in the Winters study (Winters *et al.*, 2005) had exploration times of ~11 seconds over the first minute of T2, animals in the present study (both SAP and sham lesioned) had comparable exploration times (7.0 – 16.7 secs) in the 5 minute T2. Though it is usually preferred to use data from a 1 min T2 as it can be felt that after this the novel object loses its novelty, given neither object was explored to a great extent it is felt the d2 values used in this study are valid.

3.4.6 The cholinergic system in memory

The results from intracortically lesioned animals presented here support the study of Winters *et al.*, (2005). Rats in our study had intracortical injections of SAP to the primary motor- and primary somatosensory-cortices of a much larger magnitude than rats in the prior study (Winters *et al.*, 2005) (4µg in the present study v. 0.024µg in total [for 6 injections]), which had the effect of producing a ChAT-IR deficit in the CBF twice the size of the previous study. That a significant deficit in object-recognition memory was not seen despite this CBF damage is evidence to support the conclusion of Winters *et al.*, (2005) that cholinergic PRh circuitry is critical to object-recognition memory.

Further support for a role of the PRh in object-recognition memory is provided by the results from icv lesioned animals. These animals had ChAT deficits of a larger

magnitude than the intracortically lesioned animals (74% vs 44% for the MS/DBB, 63% vs 52 % fir the NBM/SI), yet tended to show better object-discrimination performance than intracortically lesioned animals.

Aside from the study of Winters *et al.*, (2005), only one other group has published work investigating the effect of SAP lesioning in the NORT (Paban *et al.*, 2005). That group performed an interstitial SAP lesion of the NBM and reported no subsequent mnemonic dysfunction. The only time robust deficits became apparent in that study was at 17 months post-lesion, a time when the authors concede it is likely the impairments were not cholinergic in origin (Paban *et al.*, 2005). The significance of that study is difficult to assess however because of two methodologies they use which make their study very different to both this study and that of Winters *et al.*, (2005). Firstly, they used ITIs of 1 day (and 1 week), at which normal animals have been shown to be unable to perform the task (Bartolini *et al.*, 1996; Puma *et al.*, 1999), and an ITI of 1 minute which may not challenge animals' memories enough to demonstrate a deficit. Secondly, they reported NORT data as difference in exploration times (tN - tF) rather than as a ratio ([tN – tF] / [tN + tF]). Giving data as an absolute value rather than as a proportion of total exploration time makes it impossible to assess the possible confounding effects of differing/limited total exploration times.

As stated earlier, pharmacological manipulations of the cholinergic system have previously demonstrated effects on the NORT (Bartolini *et al.*, 1996; Dodart *et al.*,

1997; Puma *et al.*, 1999; Rispoli *et al.*, 2004). Whilst these generalised, systemic manipulations have been shown to have effect, it is generally accepted that SAP lesions of CBF corticopetal projections rarely cause memory deficits and in the instances where they do the deficits can be ascribed to either cerebellar damage (Lalonde *et al.*, 2003) or to attentional dysfunction (Galani *et al.*, 2002; Mesulam, 2004). That neither group of animals in this study displayed a significant deficit in the NORT is congruent with these data, as are the studies of Winters *et al.*, (2005) and Paban *et al.*, (2005). As discussed previously, the difference between the pharmacological manipulations and the CBF lesion studies is likely due to either non-specific effects of the drugs, acting at non-cholinergic sites (eg nicotinic heteroreceptors (Wonnacott, 1997)), or to specific effects of drugs at sites outside the CBF projection system (eg the striatum or brainstem).

As stated above, it has been reported that attentional impairments can contribute to apparent memory dysfunction (Galani *et al.*, 2002; Mesulam, 2004; Sarter *et al.*, 2003). Though such impairments have been observed in these animals, as described in Chapter 2, it is unsurprising no memory dysfunction was observed in these studies. One of the reasons for choosing the NORT as a test of mnemonic function was its seeming ability to demonstrate mnemonic function with few confounding variables meaning it would have a very low attentional component. When considered in light of the results of the sensorimotor testing described in the previous chapter where the animals only displayed attentional deficits during the

test with higher attentional demands, it can be seen as unlikely these animals' attentional deficits would impact on their performance of the NORT.

It is also possible the CBF projection system contributes to memory, albeit only in combination with other systems. For example, the GABAergic system also plays a role in learning and memory which may either totally or partially depend on CBF activation of the neocortex. As discussed in Chapter 2, it has been shown that GABAergic projections from the nucleus accumbens have an inhibitory effect on CBF activity (Sarter *et al.*, 2001a). This is a possible mechanism to explain the results of Pitsikas *et al.*, (2003b) who investigated the effects of a $GABA_B$ receptor agonist (Baclofen) and antagonist (CGP 35348) on rats' performance in the NORT. The group found the agonist impaired performance in a dose-dependent manner, an effect which was countered by the antagonist. The same antagonist has been used in a passive-avoidance task (Bianchi *et al.*, 1993). This group found in mice that following intra-peritoneal administration the antagonist reversed the deficits produced in this task by scopolamine, supporting an interaction between the GABAergic and cholinergic systems and memory. Thus far, the role of the GABAergic system in learning and memory is far from being conclusively elucidated however.

Additionally, it is clear that besides the cholinergic system other neurotransmitter systems also have roles to play in learning and memory. It was found by Pitsikas *et al.*, (2003a) that the 5-HT_{1A} antagonist WAY 100635 both ameliorated the memory

impairment in rats induced by scopolamine and, when given to normal animals, allowed for object discrimination at a 24 hour ITI. These results are in contrast with the study of Dringenberg *et al.*, (1999) who did not find any effect with WAY 100635, though the highest dose they used was half the effective dose found by Pitsikas et al., (2003a). Deficits of serotonergic and dopaminergic neurons induced by a neurotoxic methamphetamine regimen have been found to significantly impair performance of the NORT at a 90 min ITI (Schroder *et al.*, 2003). As nAChRs are known to exist has heteroreceptors (Wonnacott, 1997), an action of nicotine on these non-cholinergic transmitter systems is a likely mechanism to explain the results of Puma et al., (1999) who observed nicotine to improve the performeance of rats in the NORT. It can thus be seen that though the results of work with scopolamine and nicotine suggest some important role for the cholinergic system in memory, it is far from alone in its interaction with mnemonic processes.

3.4.7 Conclusion

The results of studies on both intracortically and icv lesioned animals add further evidence to the hypothesis of the critical role of the PRh in object-recognition memory. That selective and complete destruction of the CBF via icv lesioning didn't produce an impairment in the NORT supports the work of Paban et al., (2005) who concluded that damage to the NBM doesn't impair performance of the NORT. By using a 1 hour ITI and by concurrently lesioning the MS/DBB, nuclei which project to the hippocampus, it has extended upon that previous study to indicate

that neither do CBF projections to the hippocampus appear to be critical for object-recognition memory.

These studies using the NORT, a test with unique properties amongst memory tests, add substance to the current status of the cholinergic hypothesis of memory loss in AD. To ameliorate the mnemonic component of the morbidity of AD other transmitter systems, particularly the serotoninergic, dopaminergic and GABAergic systems, will need to be modulated, perhaps in combination with cholinergic modulations given the possibility of transmitter system interactions in the control of memory. In contrast, the results from Chapter 2 support the current paradigm that the CBF performs an integral, unique and direct role in attention, indicating that to ameliorate the attentional component of the morbidity of AD it is modulation of the basal forebrain cholinergic system which should be concentrated upon.

The studies in this chapter investigated the NORT with 2 animal models which had not before been assessed in this paradigm whilst the studies in Chapter 2 investigated these animal models with a paradigm which has never been used in cholinergically-modified animal models before. Given the small group numbers obtained and the limited number of tasks able to be used, these results must necessarily be considered as preliminary, however within this limiting context, they may nonetheless be considered to have contributed to the further understanding of the role of the CBF projection system in the morbidity of AD in a novel way. Whilst further studies using larger groups and/or more sophisticated behavioural

tests are awaited, (eg utilising electronic timing for reflexive attention or by improving the exploratory motivation and/or object saliency in the NORT), such a further understanding as a basis for the diagnosis and treatment of AD is the foundation of the rationale for targeting the cholinergic system in efforts to develop tools for the molecular imaging of AD.

4 Chapter 4 – Cholinergic Radioligand Evaluation

4.1 Introduction

4.1.1 Potential role for molecular imaging in AD

As described in Chapter 1, molecular imaging of the brain, which is still in a developing stage, has the potential to provide two main benefits to AD sufferers. Firstly, the provision of early, accurate diagnoses and secondly, improved molecular brain imaging in AD may lead to better treatment of the disease through better understanding of disease processes. As one of the hallmark biochemical abnormalities found in AD is cholinergic in origin, novel RPs which can be used for the molecular imaging of the cholinergic system are sought. Before an RP can be used in molecular imaging, its suitability must be rigorously validated. The procedure for this includes assessment of its *in vitro* properties and a comparison of these with an independent measure, eg histology, with further validation requiring assessment of its *in vivo* characteristics, (it should be noted that these validations are in addition to studies of an RP's safety etc which would only occur after the potential of an RP has been established). The work described in this chapter was performed to assess some *in vitro* and *in vivo* characteristics of two RPs which, by targeting different cholinergic entities which are abnormally expressed in AD, show promise to become RPs for the molecular imaging of AD.

4.1.2 VAChT and nAChRs in AD

One useful target for the molecular imaging of AD is the Vesicular Acetylcholine Transporter (VAChT). The VAChT is a transmembrane protein which pumps ACh into secretory vesicles in the synaptic terminal, from where the ACh can thence be discharged by exocytosis (Eiden *et al.*, 2004). This localisation makes the VAChT a marker unique for cholinergic neurons. Located mainly in the pre-synaptic terminal of cholinergic neurons, the VAChT has also been demonstrated in the CBF nuclei (Debeir *et al.*, 1999; Gilmor *et al.*, 1998). As described in Chapter 1, whilst the VAChT has been shown to be relatively preserved in the NBM in mild / late-onset AD (Gilmor *et al.*, 1999), more severe and/or early-onset cases showed statistically significant reductions in cortical, particularly temporal, areas (Efange *et al.*, 1997; Kuhl *et al.*, 1996; Sihver *et al.*, 1999). It has also been hypothesised that the VAChT is a measure of actual neuronal number, rather than neuronal function. This would make the VAChT a very interesting target for molecular imaging as it means neurodegeneration would be able to be assessed directly, rather than inferred from other measures.

The prototypical ligand for investigating the VAChT is vesamicol, which has been reported to bind to the VAChT with a Kd of ~5nM (Parsons, 2000). Vesamicol however has relatively poor selectivity for the VAChT as it has also been shown to bind to a nonvesicular protein called the vesamicol-binding protein (VBP) (Hicks *et al.*, 1991). Derivatives of vesamicol have been made which not only show little affinity for the VBP, but which also have greater affinity for the VAChT than

vesamicol itself (Rogers *et al.*, 1993). Amongst these derivatives is 5-substituted benzovesamicol, which has been reported to be the optimal positional isomer of iodinated benzovesamicol (Jung *et al.*, 1996). It was this radioligand (^{123}IBVM) which was used in this project to detect the VAChT *in vivo* (Figure 12).

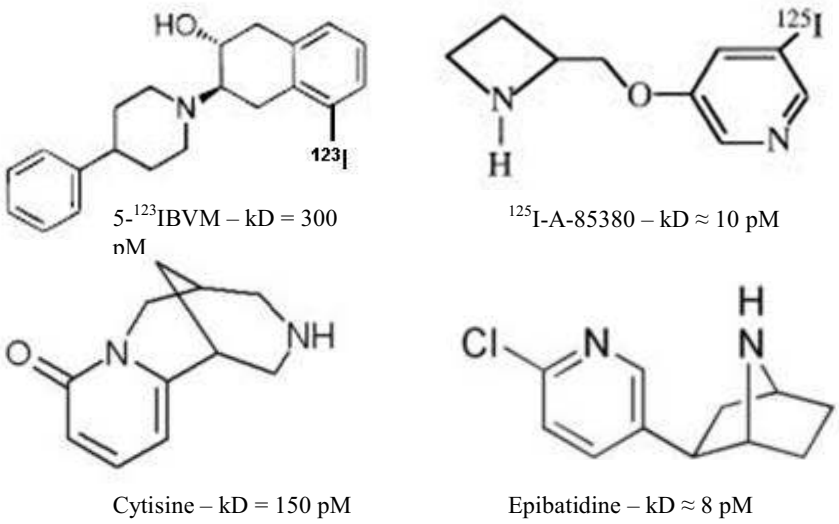

Figure 12: Chemical structures and binding affinities of radioligands for the cholinergic system.

Previous work with *IBVM has shown it to match the known distribution of cholinergic terminals in the mouse and rat brain *ex vivo* (Jung *et al.*, 1990; Sorger *et al.*, 2000), and human brain *in vivo* (Kuhl *et al.*, 1996). In the mouse brain, *IBVM has been reported to have 5 times better extraction into the brain than tritiated vesamicol and 2.6 and 6.8 times better selectivity in the cortex and striatum, respectively (region : cerebellum) with the cerebellum taken as a measure of non-specific uptake (Jung *et al.*, 1990). In the rat brain, it has been reported that *IBVM

localisation is significantly diminished in the cortex and hippocampus following SAP lesion (see below) (Sorger *et al.*, 2000).

That group also found, with blocking studies, *IBVM to have very good selectivity for the VAChT over sigma-receptors (Sorger *et al.*, 2000). When animals were pre-treated with either non-radioactive vesamicol or non-radioactive IBVM, uptake of radioactive *IBVM was decreased by 55-70% in cholinergically rich brain regions, whereas it remained unchanged in the cerebellum. Following pre-treatment with various dopamine and sigma drugs, the uptake of *IBVM remained unchanged (Sorger *et al.*, 2000). These results are important as they provide evidence that *IBVM is a purely cholinergic RP, rather one with mixed affinity for cholinergic and non-cholinergic systems.

In a human SPECT study, the cortical binding of *IBVM was found to be inversely correlated to the degree of AD-related dementia (ie more demented patients had lower VAChT densities). This relationship though was also co-dependent upon the age of disease onset; amongst subjects with a lesser degree of dementia those with early-onset disease had less *IBVM binding than those subjects with late-onset disease; amongst subjects with a severe degree of dementia, those with both early- and late-onset disease displayed a similar reduction of binding (Kuhl *et al.*, 1996). The reductions were however not as great as the known reductions of ChAT activity in AD would suggest, eg (Geula *et al.*, 1996), implying impaired cholinergic cell function occurs prior to actual neuronal loss. Studies such as these

have therefore led to *IBVM being considered suitable for detecting changes in cholinergic terminal density *in vivo* (Efange, 2000). Its' use in the autoradiographic study described in this chapter serves at once as a validation for both the subsequent studies with ^{125}I-A-85380 and any subsequent ^{123}IBVM *in vivo* molecular imaging studies performed in this same animal model, as well as allowing comparison to ChAT IHC.

Another biochemical abnormality which is consistently observed in AD is a loss of neuronal nicotinic acetylcholine receptors (nAChRs) (Court *et al.*, 2001; Nordberg, 2001). nAChRs are members of the multisubunit neurotransmitter-gated superfamily of ion channels. Thus far, numerous subunits have been identified: $\alpha 2$ to $\alpha 10$ and $\beta 2$ to $\beta 4$ which are expressed in the CNS. Different combinations of α and β subunits can form different nAChR sub-types whose activation can elicit a number of functional responses (Paterson *et al.*, 2000; Tribollet *et al.*, 2004). Of the possible sub-types, the $\alpha_4\beta_2$ nAChR sub-type is the predominant, along with smaller proportions of $\alpha_3\beta_2$ and $\alpha_3\beta_4$, amongst others (Perry *et al.*, 2002). These nAChR sub-types are located both pre- and post-synaptically (Paterson *et al.*, 2000; Sihver *et al.*, 1999) and are distributed heterogeneously in the brain with highest levels found in the thalamus, moderate levels in the parietal cortex, striatum and cerebellum and lowest levels in frontal, occipital and temporal cortices and hippocampus (Paterson *et al.*, 2000). Unlike the VAChT, nAChRs are also know to exist on a number of non-cholinergic cell populations (Paterson *et al.*, 2000; Wonnacott, 1997). *In vivo* and *post-mortem* studies have demonstrated marked

reductions in cortical nAChR density in AD, relative to age-matched controls, and the level of nAChR density measured *in vivo* by PET has been shown to correlate significantly with cognition (Nordberg *et al.*, 1995; Paterson *et al.*, 2000; Whitehouse *et al.*, 1986). Strong evidence suggests that the $\alpha_4\beta_2$ nAChR is the predominant sub-type lost in AD (Sihver *et al.*, 1999) and that its loss may be an early phenomena in the course of the disease (Paterson *et al.*, 2000). Additionally, whereas in control subjects nAChR binding exhibited a laminar pattern with maximal binding in layers I, III and V, in AD this pattern was not observed (Sihver *et al.*, 1999). However no direct correlation has been observed between NFT or Aβ pathology and nAChR losses in the cortex and other correlational studies have given rise to the idea that most of the nAChRs lost in AD are pre-synaptic and those lost are not exclusively located on cholinergic neurons (see below) (Bednar *et al.*, 1998; Marutle *et al.*, 1999; Wonnacott, 1997). Notwithstanding its varied and heterogeneous distribution and manner of loss in AD, the fact that nAChR impairment occurs early in the course of AD and is likely to be intrinsically involved in the morbidity of AD makes the nAChR a compelling target for molecular imaging.

Ligands used to investigate nAChR sub-types include radiolabelled nicotine, cytisine and epibatidine (Paterson *et al.*, 2000) (see Figure 12). All these RPs have demonstrated similar relative impairments in nAChR density in AD compared to age-matched controls (Sihver *et al.*, 1999). Nicotine was the first ligand for the nAChRs and it is this compound for which they are named. Radioactive carbon-11

labelled nicotine was the first RP used to investigate the nAChR *in vivo* and it demonstrated with PET reduced accumulation in frontal and temporal cortices of AD patients (Nordberg *et al.*, 1995; Paterson *et al.*, 2000), areas where much of the cortical degeneration of this disease is known to occur (Geula *et al.*, 1996). It has however been shown *in vivo* to be readily metabolised and to have much non-specific binding (Grunwald *et al.*, 1996) and, not surprisingly, it shows poor subtype selectivity. Thus, other nAChR RPs were developed. An RP which has often been used in *in vitro* studies of the nAChR is radiolabelled cytisine. When it was used *in vivo* however it was found to have poor blood-brain barrier penetrability (Paterson *et al.*, 2000). Moreover, its Kd in the rat is 15-20 times worse than the Kd of the following two ligands (Anderson *et al.*, 1994; Gopalakrishnan *et al.*, 1996; Sihver *et al.*, 1998).

Epibatidine is a compound originally isolated from the poisonous tree frog *Epipedobates tricolor* (Fisher *et al.*, 1994). Epibatidine has a very high affinity for the nAChR ($\approx$8pM (Gnadisch *et al.*, 1999)) though it has poor sub-type selectivity (Mukhin *et al.*, 2000). This may perhaps not be considered a serious problem given the overall preponderance of the $\alpha_4\beta_2$ subtype in the brain (Perry *et al.*, 2002), however a more selective ligand would still be preferred to a less selective one as in selected regions, particularly the midbrain, spinal cord and some thalamic nuclei, it is not the $\alpha_4\beta_2$ sub-type which predominates (Perry *et al.*, 2002). In recent years the 3-pyridyl ether I-A-85380, when radioiodinated, has been proposed to be a more suitable ligand than epibatidine with similarly high affinity and greater selectivity

for the $\alpha_4\beta_2$ sub-type (Mukhin *et al.*, 2000). Later studies have demonstrated *I-A-85380 to also have high affinity for other β_2 sub-unit containing nAChRs, including $\alpha_3\beta_2$ and $\alpha_6\beta_2$ receptors (Kulak *et al.*, 2002a; Kulak *et al.*, 2002b; Perry *et al.*, 2002). Like epibatidine, this may lead to uptake in some areas where it is not the $\alpha_4\beta_2$ which predominates, however the number of regions where this occurs would be smaller than for epibatidine (Perry *et al.*, 2002). For this reason and due to the preponderance of the $\alpha_4\beta_2$ in other brain regions, *I-A-85380 is preferred for the investigation of nAChRs and has been used to measure *in vivo* changes in nAChR density in the brain (Kassiou *et al.*, 2001).

4.1.3 nAChR and VAChT in SAP models

Studies investigating VAChT densities following SAP lesion have demonstrated significant abnormalities in the expression of this cholinergic marker. Following icv administration of SAP, an IHC study of VAChT in the MS demonstrated a near complete abolition of this marker (Gilmor *et al.*, 1998). Using an immunoblotting technique, the same group found a reduction of up to 90% in the hippocampus, the target field of the MS (Gilmor *et al.*, 1998). In the same animal model, another group demonstrated using *ex vivo* autoradiography a reduction of ^{125}IBVM binding of 61% in the hippocampus and 51-55% in various cortical regions (Sorger *et al.*, 2000). This smaller reduction of VAChT binding observed when using *IBVM was thought to represent a high level of non-specific uptake of ^{125}IBVM *in vivo* (Sorger *et al.*, 2000).

Unlike studies of VAChT density, studies of nAChR density in animals with SAP lesions have reported no reductions in this marker. For example following icv administration of SAP, whilst levels of ChAT, sodium-dependent high-affinity choline uptake and ACh release were all reduced, no change in nAChR density was observed with ^{3}H-cytisine autoradiography (Rossner *et al.*, 1995a; Rossner *et al.*, 1995b). Given the relatively poor affinity of ^{3}H-cytisine for the nAChR however, it may be that nAChR density was reduced in this model but the RP wasn't able to delineate it.

Another group has looked at nAChR density following SAP lesion with ^{3}H-epibatidine (and ^{3}H-cytisine) following administration of SAP to the NBM (Bednar *et al.*, 1998). That group also did not detect any significant reduction in nAChR density. The implication of this is limited however as the animal model had only a restricted lesion and the reductions in ChAT it produced were only moderate. Additionally, the authors found only 60-67% of ^{3}H-epibatidine binding could be blocked by a saturable concentration of non-radioactive cytisine (Bednar *et al.*, 1998), which is relatively selective for the $\alpha_4\beta_2$ nAChR (Perry *et al.*, 2002). The authors suggested this was evidence of a large degree of binding of ^{3}H-epibatidine to non-$\alpha_4\beta_2$ nAChRs (Bednar *et al.*, 1998), though this conclusion is not supported by a later study which showed > 98% of ^{125}I-ebibatidine binding sites in the bulk of the cortex to be at $\alpha_4\beta_2$ nAChRs (Perry *et al.*, 2002). This contradiction is likely because an insufficient concentration of cytisine was used as a blocking compound. Whereas Bednar et al., (1998) used 4nM of cytisine to block 0.1nM of ^{3}H-

epibatidine, Perry *et al.*, (2002) calculated 200nM cytisine would be needed to block 99% of $\alpha_4\beta_2$ nAChRs following 0.08nM ^{125}I-epibatidine and demonstrated $\approx$ 60nM cytisine is needed to block ^{125}I-epibatidine used in concentrations of 0.10-0.47nM.

It is not surprising reductions in nAChR density have been difficult to delineate because, as discussed above, a large proportion of nAChRs are thought to exist on non-cholinergic cells (Marutle *et al.*, 1999; Wonnacott, 1997). Due to the fact a high affinity RP has not been evaluated in the icv animal model, one of the aims of this project was to investigate whether ^{125}I-A-85380 could demonstrate a loss amongst the relatively small proportion of nAChRs which are located on cholinergic cells. To my knowledge *I-A-85380 has not been used previously to assess nAChR expression in such a model.

Evaluation of the RPs was performed with autoradiography, a technique which is used to investigate and measure the localisation of an RP in a tissue section with great sensitivity and resolution. Two autoradiographic techniques were used in this study. In *ex vivo* autoradiography, an animal is injected intravenously (iv) with an RP. Following a suitable uptake time, the animal is sacrificed and the organ-of-interest removed, cut into thin sections, mounted onto slides and apposed to photographic film which detects the emitted radiation. In *in vitro* autoradiography, the RP isn't administered until after the organ has been sliced and slide-mounted. After a suitable incubation time the slides are rinsed, dried and apposed to

photographic film. The *ex vivo* method assesses the *in vivo* characteristics of the RP, demonstrating it's biodistribution in the presence of factors such as metabolism, excretion, perfusion, extraction, plasma protein binding etc, thus it is more analogous to the role an RP is designed play in molecular imaging. The *in vitro* method assesses in contrast the physical interaction of the RP and its binding sites, as well as the distribution of those binding sites in the tissue studied. This method can be used to assess the specificity and affinity of the RP's localisation, as well as accurately portraying the density of binding sites within the tissue section.

4.1.4 Aim of this study

The aim of the work presented in this chapter was to quantify with autoradiography the biochemical loss associated with SAP lesioning for each RP. Integral to the design of this work was the utilisation of the different half-lives of ^{123}I and ^{125}I (13 hours and 60 days, respectively). By labelling IBVM with ^{123}I and I-A-85380 with ^{125}I, both radioligands could be assessed in the same animals, enabling data on each to be readily comparable. These data would serve as an indication of the potential utility of the RPs for molecular imaging and could subsequently serve as validation when compared to the loss observed with each RP with small-animal molecular imaging.

4.2 Methods

4.2.1 Animals

The animals used for autoradiography in this chapter are the icv-lesioned animals used in Chapters 2 and 3. Additionally, there are 5 animals (3 SAP and 2 sham) included which were experimented upon as part of a pilot study. These animals were operated upon in an identical manner to the other animals. For the ChAT IHC, a parallel group of 12 animals was used to test the lesion's efficacy.

4.2.2 Radiolabelling

Chloramine T was purchased from Sigma-Aldrich (France). The 5-tributylstannyl-3-[1-t-butoxycarbonyl-2(S)azetidinyl methoxy]pyridine and (-)-5-(tri-n-butyl tin)benzovesamicol were purchased from ABX (Germany). For radioiodination [^{123}I]NaI from Schering (Cis Bio International) and [^{125}I]NaI from GE Healthcare Bio-sciences (Europe) was used.

4.2.3 IBVM ex vivo

[^{123}I]Iodobenzovesamicol (^{123}I-IBVM) was prepared according to a published procedure (Sorger *et al.*, 2000). The radiochemical purity was evaluated by HPLC and was determined to be greater than 98%.

Ex vivo ^{123}IBVM autoradiography was performed similarly to a previous study (Sorger *et al.*, 2000). Briefly, seven days after the stereotaxic surgery animals were

injected with 22.2MBq (600μCi) of ^{123}IBVM via the tail vein. The radioligand was injected in 600μL of 50:50 ethanol:0.9% saline. Two hours post-injection, animals were sacrificed by decapitation after cranial trauma. The brains were rapidly removed and frozen in isopentane chilled with dry-ice to -35°C. Six series of 20μm thick sections were cut on a cryostat and mounted on gelatine coated slides. One of the series was exposed to Kodak β-max film for 3 days whilst the others were stored at -80°C for > 7 days to allow for the decay of ^{123}I in preparation for ^{125}I-A-85380 *in vitro* autoradiography.

^{123}IBVM *ex vivo* autoradiography images were digitised and analysed with either Biocom (France) or γ-Vision (Biospace, Paris) image analysis software. ROIs were drawn in the following regions: striatum (caudate putamen; CPu), grouped frontal/parietal cortex (Ctx), MS/DBB, NBM / substantia inominata (NBM/SI), thalamus (Thl), hippocampus (Hip) and cerebellum (Cbm). An analysis method similar to that reported previously (Sorger *et al.*, 2000) was used. Essentially, the ROD of each region was divided (after subtraction of non-specific binding) by the ROD of the CPu so that results are expressed as a ratio to striatum. Activity in the cerebellum was taken to be a measure of non-specific binding. The use of the CPu as an internal control is based on the fact that though the striatum is cholinergically rich, it is unaffected by the SAP lesion (Waite *et al.*, 1994). Data in each region were compared between sham- and SAP-treated animals using Mann-Whitney U-tests, with p-values < 0.05 considered to be significant.

4.2.4 I-A-85380 in vitro

5-[^{125}I]iodo-3-(2-azetidinylmethoxy)pyridine (^{125}I-A85380) was prepared according to published procedures (Chefer *et al.*, 1998; Horti *et al.*, 1999; Kassiou *et al.*, 2001). The radiochemical purity was evaluated by HPLC and was > 98%.

The procedure used for ^{125}I-A-85380 *in vitro* autoradiography was similar to one previously described (Kulak *et al.*, 2002a). Briefly, brain sections were thawed and dried at room temperature then incubated for 60 mins in buffer (50mM Tris, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.0 mMMgCl2) containing one of 5 different concentrations of ^{125}I-A-85380 (30, 50, 100, 210 500pM). Non-specific binding was performed with the same solutions but also containing 10μM nicotine. After the incubation, slides were rinsed twice for 5 mins in ice-cold buffer and 1 min in ice-cold deionised H_2O then dried at room temperature overnight. When dry, slides were exposed for 2-3 days to Kodak β-max film alongside ^{125}I standards (Amersham).

4.2.5 ^{125}I-A-85380 data Analysis

^{125}I-A-85380 images were analysed with specialised image analysis systems (Biocom (Biospace, France)/MCID (InterFocus, UK)). Briefly, ROIs were drawn on the ^{125}I standard and a calibration curve derived. ROIs from regions in each image were compared to the calibration curve and values calculated in fmol/mg. Parallel slides incubated with radioligand and 10 μM nicotine were used as a measure of non-specific activity. Mean binding in each region for each

concentration, corrected for non-specific binding, were analysed with Prism software (GraphPad Software Inc., USA) and B_{max} values were derived using non-linear regression (one-site model; $y = B_{max} . x / (Kd + x)$). B_{max} values for each region were compared between sham- and SAP-treated animals using Mann-Whitney U-tests, with p-values < 0.05 considered to be significant.

Subsequently, the binding values (fmol/mg) for only the 210pM concentration were compared to values from the CPu to derive ratios to striatum. This concentration of RP was chosen as it should theoretically saturate 95% of binding sites (Mukhin *et al.*, 2000). The analysis method here is similar to the analysis of the *ex vivo* ^{123}IBVM autoradiography, except slides incubated in the presence of 10µM nicotine were used to assess non-specific activity, rather than the use of a reference region. Data in each region were compared between sham- and SAP-treated animals using Mann-Whitney U-tests, with p-values < 0.05 considered to be significant.

4.2.6 ChAT immunohistochemistry

The efficacy of the lesion technique was assessed in a parallel group of 12 animals with ChAT IHC. The method and results are described in Chapter 2 (Figure 8).

4.3 Results

4.3.1 ^{123}IBVM ex vivo autoradiography

The results of ^{123}IBVM *ex vivo* autoradiography are presented in Figure 13. In sham-lesioned animals, ^{123}IBVM uptake displayed a pattern similar to that previously demonstrated (Sorger *et al.*, 2000). The highest specific uptake was in the CPu (striatum), with the next most intense specific uptake in the CBF nuclei which displayed 30-40% of the striatal uptake. Uptake was then seen in the cortex > hippocampus > thalamus (Figure 13). Uptake in the cerebellum was by far the lowest with uptake averaging half that observed in the thalamus (data not shown).

In SAP lesioned animals, the cortex, MS/DBB and NBM/SI along with the hippocampus each displayed a significantly reduced uptake of ^{123}IBVM. The cortex displayed a 30% reduction in relative uptake in SAP animals compared to sham-treated animals, MS/DBB a 41% reduction, NBM/SI a 35% reduction and hippocampus a 60% reduction. The amount of non-specific uptake, as defined by uptake in the cerebellum (see discussion), was not significantly different between the groups.

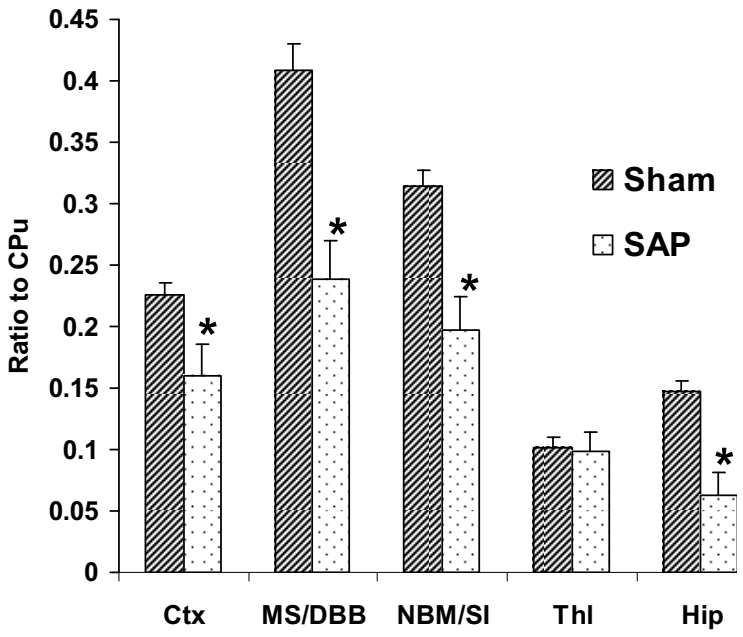

Figure 13: Uptake of ^{123}IBVM as measured by *ex vivo* autoradiography, ratio of ^{123}IBVM uptake in various regions. * $p \leq 0.05$

4.3.2 ^{125}I-A-85380 in vitro autoradiography

The preliminary results of ^{125}I-A-85380 *in vitro* autoradiography are presented in Figure 14. Figure 15A and B show saturation binding curves for sham lesioned animals. Each curve represents an autoradiography study performed with a different batch of ^{125}I-A-85380. It can be seen that the saturation curves vary markedly between the groups, making meaningful comparisons impossible. This effect can be seen in Figure 14 where sham lesioned animals, all from the same surgical group, experimented upon with different batches of ^{125}I-A-85380, displayed very large differences in derived B_{max} values. Despite the variability which exists between animals evaluated with different batches of ^{125}I-A-85380, the

pattern of binding in each instance is similar to the published distribution of the ^{125}I-A-85380 distribution (Saji *et al.*, 2002), with highest levels being found in the thalamus, moderate cortex and striatum and milder levels in the CBF, cerebellum and hippocampus (Figure 14). Due to the difficulty of making comparisons with this data, a subsequent analysis was performed using only the data from the 210pM concentration of RP.

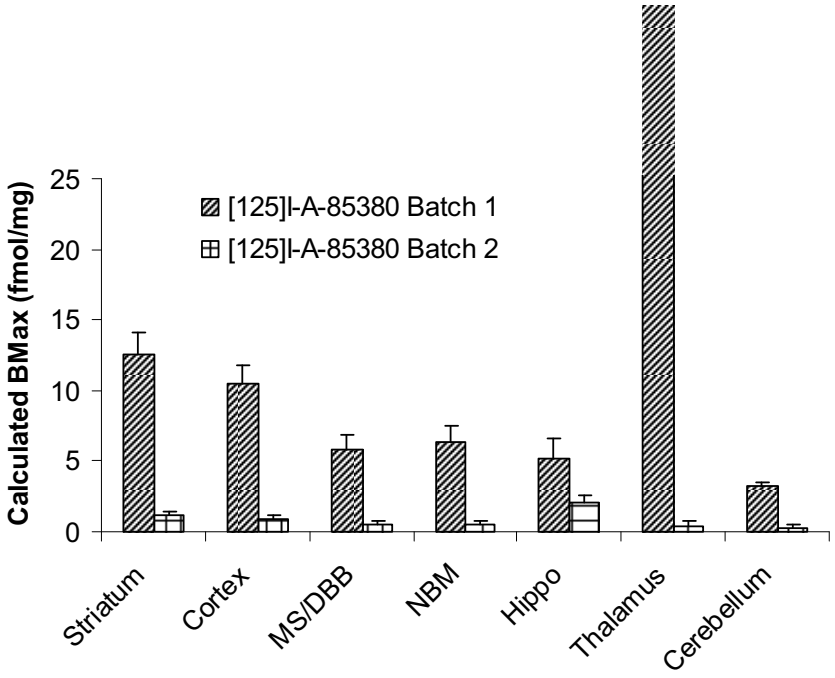

Figure 14: B_{max} data from the same groups represented in Figure 15. 'Batch 1' is data from Figure 15A and 'Batch 2' is from Figure 15B.

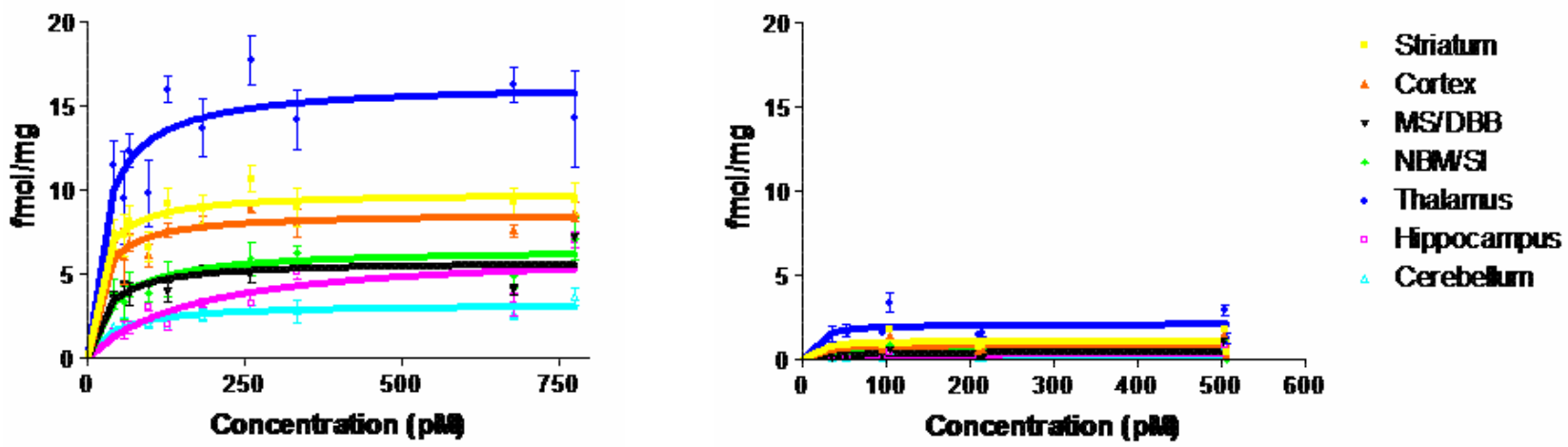

Figure 15: A) Sham animals studied with Batch 1 of 125I-A-85380; B) Sham animals studied with Batch 2 of 125I-A-85380

The results of 210pM ^{125}I-A-85380 *in vitro* autoradiography when analysed as a ratio to the striatum are presented in Figure 16. They are presented in a similar fashion to the results of the ^{123}IBVM *ex vivo* autoradiography. Sham-operated animals displayed an uptake which is consistent with the known distribution of $\alpha_2\beta_4$ nAChRs in the brain. Highest uptake was observed in the thalamus, followed by the CPu and cortex > CBF nuclei > hippocampus > cerebellum. This is similar to results reported previously (Mukhin *et al.*, 2000) in normal male Fischer-344 and Sprague-Dawley rats studied with ^{125}I-A-85380 *in vitro* autoradiography, however with slightly lower binding in the hippocampus and cerebellum which may be due to gender differences.

Whilst all regions studied displayed reduced binding of ^{125}I-A-85380 following SAP-lesioning, only in the hippocampus (49%) and cerebellum (28%) were these reductions statistically significant. Non-specific uptake, as measured by co-incubation of sections with 10μM nicotine, was very low in both groups and was unable to be visualised with image analysis software.

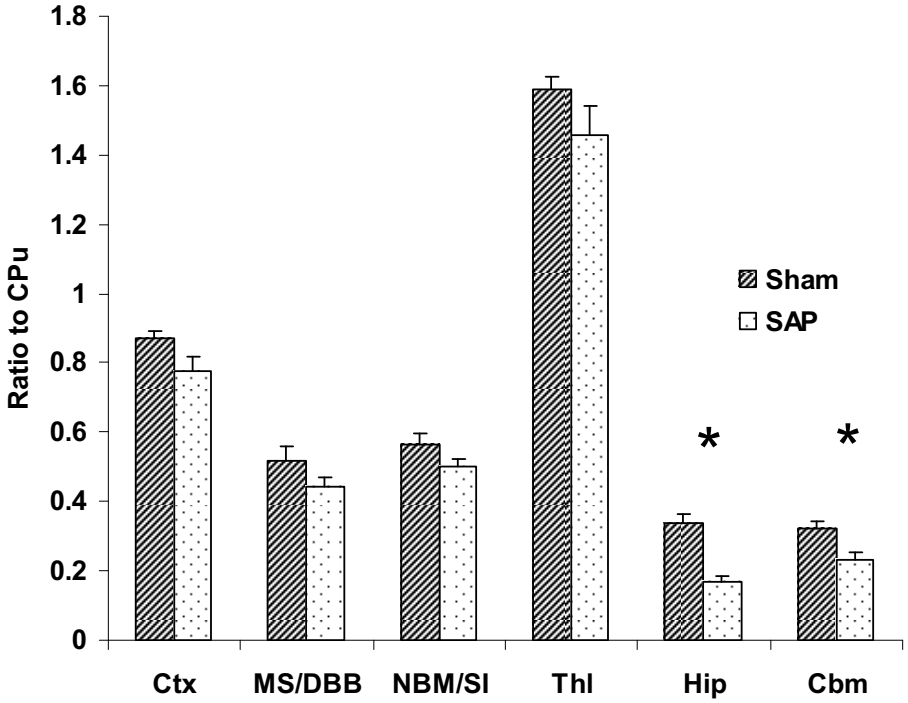

Figure 16: Binding of ^{125}I-A-85380 as measured by *in vitro* autoradiography. Ratio of ^{125}I-A-85380 localisation in various regions : uptake in CPu . * $p \leq 0.05$

4.4 Discussion

4.4.1 IBVM

^{123}IBVM *ex vivo* autoradiography results followed a similar general pattern to that previously reported by Sorger et al., (2000), however with some differences. Whilst in this project there was observed a significant 60% *reduction* in relative binding in the hippocampus in SAP-treated animals compared with control animals, compared to the previously published study of Sorger et al., (2000) only half the

total amount of relative binding in both groups of animals was observed. This 60% reduction is significantly less than the reduction observed in the hippocampus of the same animal model when VAChT density was measured immunohistochemically (Gilmor *et al.*, 1998). This finding could be considered to indicate a weakness of the radioligand *IBVM however it more likely simply highlights the difficulties involved with targeting biochemical markers *in vivo*. Unlike the results observed in the hippocampus, control animal data in this study found a similar level of relative binding of ^{123}IBVM in the cortex as the report of Sorger et al., (2000), though the % reduction observed in SAP animals in this study was 30%, as opposed to 50% previously reported.

Notwithstanding these variations between various observations of VAChT density in the SAP animal model, the results of ^{123}IBVM *ex vivo* autoradiography presented here are consistent with what was expected following SAP immunotoxin. The CBF nuclei NBM/SI and MS/DBB project both to the cortex and the hippocampus (Everitt *et al.*, 1997) and the presence of VAChT has been detected in both CBF nuclei (Gilmor *et al.*, 1998; Gilmor *et al.*, 1999) as well as in the terminal fields in the hippocampus (Gilmor *et al.*, 1999) and the cortex (Efange *et al.*, 1997; Wong *et al.*, 1999). VAChT density in these four regions was therefore expected to be strongly affected by SAP and that a significant reduction in ^{123}IBVM was observed in these four regions confirms the efficacy of the SAP lesion in these animals. These observations in the NBM/SI and MS/DBB are an extension upon previous work (Sorger *et al.*, 2000). The use of the cerebellum as a marker of non-specific

binding was justified by the findings that ^{123}IBVM uptake in the cerebellum was extremely low and not significantly different between the groups (data not shown) and also by the previous report that ^{125}IBVM uptake in the cerebellum cannot be blocked with either non-radioactive IBVM or vesamicol following icv SAP lesioning (Sorger *et al.*, 2000).

4.4.2 I-A-85380

4.4.2.1 Binding

The likely explanation for why the *in vitro* autoradiography binding study didn't provide meaningful data is probably related to the RP. That such widely varied results could have been observed in sham-lesioned animals from the same surgical group (Figure 14 and Figure 15) is indicative the problem encountered was not related to the animal model. Given that the pattern of RP distribution (Figure 14 and Figure 15) matches the known distribution of ^{125}I-A-85380 binding (Paterson *et al.*, 2000; Saji *et al.*, 2002), and that RP binding was completely blocked in the presence of 10μM nicotine, it can be confidently asserted that each study, using the various batches of RP, all contained only the complete ^{125}I-A-85380 RP (ie no fragments of the original RP). Thus, the most likely explanation for the variability between different batches of RP is that each had different specific activities.

The specific activity of a radioactive compound is its activity per amount of compound (Ci/mmol). The facilities used for the production of the batches of ^{125}I-

A-85380 used in *in vitro* autoradiography were not equipped for measurement of specific activity. However, because the [125I]NaI purchased from GE Healthcare Bio-sciences (Amersham Biosciences Europe, France) was carrier free, ie contained no isotopes of iodine other than ^{125}I, the specific activity of the ^{125}I-A-85380 was taken to be that of the carrier free [125I]NaI, 2,200 Ci/mmol. This value for specific activity could overestimate the actual value however. For example, if the high-performance liquid chromatography column used in the preparation of the RP had non-radioactive I-A-85380 in it, this could have mixed in with the radioactive ^{125}I-A-85380, reducing its effective specific activity. Alternatively, if non-radioactive iodine was introduced into the reaction mixture in some way, it would have been introduced onto the molecule in place of the radioiodine, having the same effect on the specific activity. The effect of this would be the radioactive ^{125}I-A-85380 having its binding sites competed for by non-radioactive I-A-85380, reducing its receptor-binding. Different batches of RP having different specific activities would therefore display variable levels of binding depending upon the proportion of competing ligand mixed in with the RP. It is not known for certain the reason behind this problem, however the impact of it upon the study was able to be minimised by the implementation of an alternative analysis method.

4.4.2.2 Ratio

The rationale for the alternative analysis method is the same as that for the ^{123}IBVM *ex vivo* autoradiography. That is to say that as the CPu both strongly

expresses the nAChR and is also unaffected by the SAP lesion, it can act as a very good reference region. Sections incubated with 210pM ^{125}I-A-85380 were chosen as this is consistent with that reported in previous studies (Kulak *et al.*, 2002a; Kulak *et al.*, 2002b; Mukhin *et al.*, 2000; Perry *et al.*, 2002) and because 210pM should theoretically saturate 95% of binding sites (Mukhin *et al.*, 2000). Though the data were not fit for the quantitative analysis above, the fact that the pattern of uptake was consistent with the known distribution of nAChRs in the brain (Figure 14) and that the binding could be completely eliminated in the presence of 10μM nicotine support the use of the data in an analysis which is independent of specific activity variability. Because both the target region and the CPu would experience a similar level of interference by any non-radioactive I-A-85380, the ratio between them would not be perturbed. For example, if competing ligand displaced half the uptake in both regions, it would halve both the numerator and the denominator in the ratio calculation, leaving the result unchanged.

In vitro autoradiography with ^{125}I-A-85380 displayed mild to moderate reductions of ligand binding in all regions investigated, however only in the hippocampus and cerebellum did the reductions achieve statistical significance. These results contrast to previous studies using other nAChR ligands which have not found an effect of SAP on nAChR density (Bednar *et al.*, 1998; Rossner *et al.*, 1995a; Rossner *et al.*, 1995b). The likely reason for this discrepancy between studies is related i) to the distribution of nAChRs in the brain; ii) to the properties of *I-A-

85380 as opposed to other nAChR radioligands; and iii) to differences between the animal models used.

Neuronal nAChRs are known to exhibit a complex distribution. They are to be found mainly pre-synaptically (Bednar *et al.*, 1998; Wonnacott, 1997) but also with significant post-, peri- and extrasynaptic locations (Paterson *et al.*, 2000). The pre-synaptic (and somatodendritic) nAChRs are further known to exist on a number of non-cholinergic cell populations facilitating the release of many neurotransmitters in addition to acetylcholine such as dopamine, 5-hydroxytryptamine, noradrenaline, γ-amino butyric acid and glutamate (Paterson *et al.*, 2000; Wonnacott, 1997). Following lesioning therefore, any nAChR not located on a cholinergic neuron affected by SAP would be spared. However, as some nAChRs do exist on neurons affected by SAP, it stands to reason that there should be a reduction in nAChR density in the SAP model, albeit a very small one in relation to the entire nAChR population. Given this, it is not surprising that a reduction in nAChR density arising from a specific cholinergic neurotoxin would be difficult to observe.

As mentioned above, *I-A-85380 is one of the best radioligands currently available for investigation of the nAChR. It has a Kd of around 10pM in the rat which is 15-20 times better than the Kd of ^{3}H-cytisine (Anderson *et al.*, 1994; Gopalakrishnan *et al.*, 1996; Sihver *et al.*, 1998), and though it is comparable to the Kd of ^{3}H-epibatidine (≈8pM) (Gnadisch *et al.*, 1999) it has a much greater sub-type selectivity (Mukhin *et al.*, 2000). However given that the $\alpha_4\beta_2$ nAChR sub-type

makes up the vast majority of nAChRs in the forebrain (> 90% in most forebrain structures) (Perry *et al.*, 2002), it would be logical to think ^{3}H-epibatidine would also be capable of detecting small nAChR changes.

Only one group has published a study using ^{3}H-epibatidine to measure nAChR changes in the rat following SAP lesioning and they however discovered no difference in nAChR density in either the cortex or hippocampus of lesioned animals compared to controls following membrane homogenate binding studies with ^{3}H-epibatidine and ^{3}H-cytisine (Bednar *et al.*, 1998). Similarly, nAChR density in the cortex was discovered in the present study using ^{125}I-A-85380 to be reduced by only a small, statistically insignificant, magnitude (11%). The largest reduction in nAChR density observed in this study was in the hippocampus where a statistically significant reduction of 50% was demonstrated. Again the previous study (Bednar *et al.*, 1998) didn't observe a difference in nAChR density in this region. The likely reason for this is not so much related to the radioligand or the model, but to the fact in the ^{3}H-epibatidine study, the lesion was made discretely into the NBM. It is well established Ch4 neurons arising in the NBM project to the neocortex, with essentially no projection to the hippocampus which receives its innervation primarily from the MS/DBB (Rye *et al.*, 1984; Wenk, 1997; Wenk *et al.*, 1980). Though the previous study did produce some collateral damage to the hippocampus (Bednar *et al.*, 1998), probably by diffusion of SAP from the NBM to the MS, the subsequent reduction of hippocampal ChAT activity was small (21%) and therefore not equivalent to the damage observed in this study subsequent to

SAP lesioning which produced a reduction of ChAT immunoreactive cells in the MS/DBB of 74% (Figure 8). Had the group of Bednar et al., (1998) used ^{3}H-epibatidine with an icv SAP model instead of an interstitial one, it is possible they also would have demonstrated a reduction in hippocampal nAChR density as well.

It is unlikely that the differences between the above results (using male SD rats) (Bednar *et al.*, 1998) and those presented in this report (using female SD rats) is due to gender. Previously, MS/DBB ChAT immunoreactive neuron density and hippocampal ChAT activity following SAP lesioning have been reported to be not-significantly different between male and female Long-Evans rats (Jonasson *et al.*, 2004), suggesting a similar susceptibility to the SAP immunotoxin between the sexes. Whilst previous studies have reported some divergent effects of SAP lesions (behavioural effects) or nAChR agonism (behavioural and biochemical effects) between the sexes (Damaj, 2001; Jonasson *et al.*, 2004; Koylu *et al.*, 1997; Pogun *et al.*, 2000), studies of nAChR densities in control animals have demonstrated no sex differences in ^{3}H-epibatidine binding (Donny *et al.*, 2000; Rada *et al.*, 2003). Finally, the effect of oestrus cycle stage is not considered significant in this study. As group-housed SD rats are not considered to synchronise their oestrus cycles (Schank, 2001), the control and lesioned groups are considered to have had a similar proportion of animals at various stages randomly distributed within them. For comparison with previous work examining *IBVM binding in the SAP model, both this study and the previous one (Sorger *et al.*, 2000) used female rats of unknown oestrus status.

4.4.3 Comparison

Comparison of the binding intensity of ^{123}IBVM and ^{125}I-A-85380 yields some observations. In the terminal projection fields of the CBF nuclei, ie the cortex and hippocampus, a similar pattern of radioligand distribution was observed with SAP lesions producing mild reductions of uptake in the cortex (30% for ^{123}IBVM and 11% for ^{125}I-A-85380 [this reduction was non-significant, p=0.13]) and moderate reductions of uptake in the hippocampus (60% and 50%). The differences in the magnitude of the reductions observed following SAP lesion probably reflect the fact that VAChT is a purely pre-synaptic cholinergic marker whilst, as discussed above, the nAChR is a pre- and post-synaptic marker with mixed cholinergic and non-cholinergic distributions.

That specific uptake of ^{125}I-A-85380, but not ^{123}IBVM, was observed in the cerebellum is in accord with current knowledge. It is known that *IBVM uptake in the cerebellum cannot be blocked (Sorger *et al.*, 2000) and thus no specific uptake would be expected (see above). It is known as well that the α_4 nAChR sub-unit is expressed in Purkinje cells of the cerebellum of rats (Nakayama *et al.*, 1997), that there is specific binding of ^{3}H-epibatidine in human Purkinje cells (Lee *et al.*, 2002) and that there is specific ^{3}H-epibatidine and ^{125}I-A-85380 uptake in both rat and human cerebellum homogenates (Turner *et al.*, 2005). In light of the fact that icv SAP has been shown consistently to damage Purkinje cells (Heckers *et al.*, 1994;

Waite *et al.*, 1995; Waite *et al.*, 1999), it is not surprising a reduction of ^{125}I-A-85380 binding was observed in the cerebellum. The other discrepancy between the binding of ^{123}IBVM and ^{125}I-A-85380 is in the MS/DBB and NBM/SI nuclei. Whilst ^{123}IBVM uptake was significantly reduced in these two nuclei following SAP lesion (by 41% and 35% respectively), ^{125}I-A-85380 binding in these nuclei only showed non-significant, mild reductions (15% and 11% in these regions; p=0.14 and 0.08 respectively). Given the known somatodendritic localisation of some nAChRs (Clarke, 1993; Wonnacott, 1997), it was expected a statistically significant reduction would be seen in these regions. Similar to binding in the cortex and hippocampus, it would appear that the discrepancy in reductions revealed by ^{123}IBVM and ^{125}I-A-85380 in the CBF nuclei is due to there being a greater proportion of all VAChT located in the soma and dendrites of cholinergic neurons than the proportion of all β_2 containing nAChRs similarly located.

The results presented here confirm and extend previous work investigating *IBVM uptake in the SAP-lesioned rat. They are also indicative of the excellent biochemical properties of *I-A-85380 and are the first evidence that administration of the immunotoxin SAP can result in a demonstrable loss of nAChRs in the rat. It is therefore considered that the two RPs investigated in this chapter show satisfactory properties such as to be appropriate to be evaluated as tools for molecular imaging in the future.

The work described in Chapter 5 was performed to enable this evaluation. Though molecular imaging is a well-established imaging modality, until recently there was no capacity to perform it in small animals. As much of the technology was developed for or adapted from clinical systems, molecular imaging in animals was restricted to only large animals, rodents were much too small to be adequately visualised. The advent of small-animal molecular imaging systems has enabled work to be performed on animal models which until recently were unable to be imaged in such a fashion. Chapter 5 describes work performed for the development of a novel molecular imaging system. The final aim of the study was to develop this system to a stage where it could be used for the evaluation of the RPs used in the studies described in this chapter for molecular imaging.

5 Chapter 5 – Development of *in vivo* imaging

5.1 Introduction

This chapter describes ameliorative work which was performed on a small-animal molecular imaging system such as to enable it to be used to image SAP-lesioned and sham-operated control rats with the cholinergic RPs *IBVM and *I-A-85380. As shown in Chapters 2 and 3, the cholinergic lesion produced by SAP can mimic in rats some of the important symptoms of AD and the studies described in Chapter 4 demonstrated that the chosen cholinergic RPs were able to detect post-mortem the biochemical abnormalities caused by this SAP lesion. Therefore it was reasonable to assess whether these RPs were able to detect *in vivo*, with small-animal molecular imaging techniques, the biochemical abnormalities caused by SAP with a view to their possible use in the investigation of AD. However, as circumstances eventually precluded these *in vivo* studies, the final aim of the work described in this chapter was thus to develop the molecular imaging system so as these latter studies may be done in the future.

The ability of molecular imaging to investigate the integrity of the cholinergic system *in vivo* in AD would be of great clinical benefit. These benefits would derive from both the improved diagnostic ability such imaging would provide, as well as the advances in the understanding of the underlying disease processes which could, in turn, facilitate ongoing research into therapeutic products to

ameliorate the symptoms of AD. As discussed in Chapter 1, although molecular imaging of the brain shows much potential utility, to date it has had relatively limited clinical acceptance. One reason for this is that the processes targeted by current RPs are one or two levels removed from those which are directly involved in the morbidity of AD. For example, the PET RP ^{18}FDG allows the *in vivo* measurement of glucose metabolism in cells. Though it has been described as an RP with very good diagnostic value (Villemagne *et al.*, 2005), regional reductions of cerebral glucose metabolism are an event downstream of the processes which directly modulate the symptoms of AD.

Regional cortical atrophy, demonstrable with structural imaging techniques such as CT and MRI, can be considered in a similar manner, though it is these techniques which are part of the current criteria for AD diagnosis (Knopman *et al.*, 2001). Additionally, though reductions of glucose metabolism and cortical atrophy both provide some degree of diagnostic utility, investigation of neither would advance in any meaningful way the understanding of the underlying disease processes of AD.

The recent development of RPs which enable the investigation of the Aβ protein and NFTs (Klunk *et al.*, 2004; Shoghi-Jadid *et al.*, 2002) represent an advance on the investigation of the two processes above. As accumulation of the Aβ protein and NFTs are biochemical hallmarks of AD, their investigation with molecular imaging would allow a closer examination of the processes underlying AD, as well

as providing a definitive diagnostic tool. It would therefore be reasonable to expect this imaging to become a part of AD clinical criteria.

Though Aβ plaques and NFTs are characteristic pathologies in AD, they are processes which are upstream of the biological events directly responsible for the symptomology of AD (Bennett *et al.*, 2006), and their direct involvement with these responsible events is in fact debated (Cullen *et al.*, 2005). That is not to say they are irrelevant to the pathogenesis of AD, far from it, however it is possible to focus more specifically upon events leading to cognitive decline. The events which are directly responsible for the cognitive declines associated with AD are impairments of neuron function and/or cell death. As discussed throughout this report, amongst the cell groups whose perturbations are directly responsible for AD associated cognitive decline, impairment/loss of cholinergic neurons is a longstanding characteristic. RPs which target the cholinergic system are thus desired.

The cholinergic RPs *IBVM and *I-A-85380, as characterised both in this project and in many previous studies (eg (Chefer *et al.*, 1998; Kassiou *et al.*, 2001; Kuhl *et al.*, 1996; Saji *et al.*, 2002; Sorger *et al.*, 2000), show good potential to become tools for molecular imaging. Prior to their evaluation for this role however, much work had to be performed on refining the small-animal molecular imaging systems to enable this evaluation. This chapter describes the studies performed for the

refinement of these systems. It was initially aimed to include evaluations of the RPs in the SAP model in this chapter, however this has not been possible.

5.1.1 Imaging

Molecular imaging sets challenges beyond that of *in vitro* and *ex vivo* studies such as were described in Chapter 4. *In vitro* studies, whilst allowing the investigation of some important pharmacological RP characteristics such as the binding affinity and selectivity, do so in the absence of the many confounding factors present in biological systems. *Ex vivo* studies, such as the autoradiography study with ^{123}IBVM described in Chapter 4, impose greater challenges. For example, whereas in *in vitro* studies the capacity exists to completely assess non-specific binding (by blocking specific binding, as for example was done with 10μM nicotine in the *in vitro* ^{125}I-A-85380 autoradiography described in Chapter 4), if it is desired to measure the amount of non-specific binding in an *ex vivo* study, the concentration of the blocking compound administered must be a compromise to ensure it causes no adverse pharmacological effect on the animal. Because of this, specific binding may not be able to be completely blocked. Other additional challenges of *ex vivo* rather than *in vitro* studies are the ability of the RP to access its binding site and the effect of an animal's metabolism. For neurology RPs, this means firstly that it is essential they be able to cross the blood-brain barrier. In addition, sufficient quantities of the RP must reach the binding site intact to provide an image, and of the parts of the RP which are metabolised the radioactive parts must be excluded

from the region of the binding sites, ie (for neurology) they should be unable to cross the blood-brain barrier.

Once satisfactory *ex vivo* properties have been met, an RP needs to meet few further criteria to be suitable for molecular imaging. However, molecular imaging still imposes great challenges beyond those of *in vitro* and *ex vivo* studies. Specifically, the great challenge of molecular imaging, besides RP development, is related to optimisation of the instrumentation. It is to this end that the studies described in this chapter were performed.

5.1.2 Overview of γ-camera principle

The external detection and localisation of the *in vivo* RP in these studies was achieved with γ-cameras. To detect the photon emitted by an RP, the γ-camera has a crystal which scintillates when a photon emitted by the RP interacts with it. The light subsequently strikes photo-multiplier tubes (PMTs) where it interacts in a photoelectric manner to release electrons. A voltage is then applied to the PMTs which accelerates the electrons past various dynodes within the PMT, causing more ionising events, which creates an amplified electric signal which can eventually be detected (Eberl *et al.*, 1994). The mechanism to localise the photon depends on the molecular imaging system used.

5.1.3 Overview of TOHR principle

The studies described in this chapter, with a view to performing evaluations of the RPs *IBVM and *I-A-85380, were all performed with a small-animal molecular imaging system named the High-Resolution Tomograph (TOHR) (Pinot *et al.*, 2003; Ploux *et al.*, 1997). In the TOHR, the tomographic (3-D) image is acquired by a ring of 10 scintillation crystals coupled to 20 PMTs. Before reaching the crystal, photons emitted by the RP must first pass through a collimator, which is a layer of photon-shielding material with defined pathways set into it. These pathways ensure only certain photons reach the detector. The 10 collimators (one for each crystal) used with the TOHR were converging (focussing) collimators. This means that the pathways along one of which a photon must travel in order to pass through the collimator and reach the detector all converge on a single point, the focal point. This is illustrated in Figure 17, where visible light shone through the back of a focussing collimator takes on a cone shape. In the TOHR, all 10 collimators are focussed upon the same point.

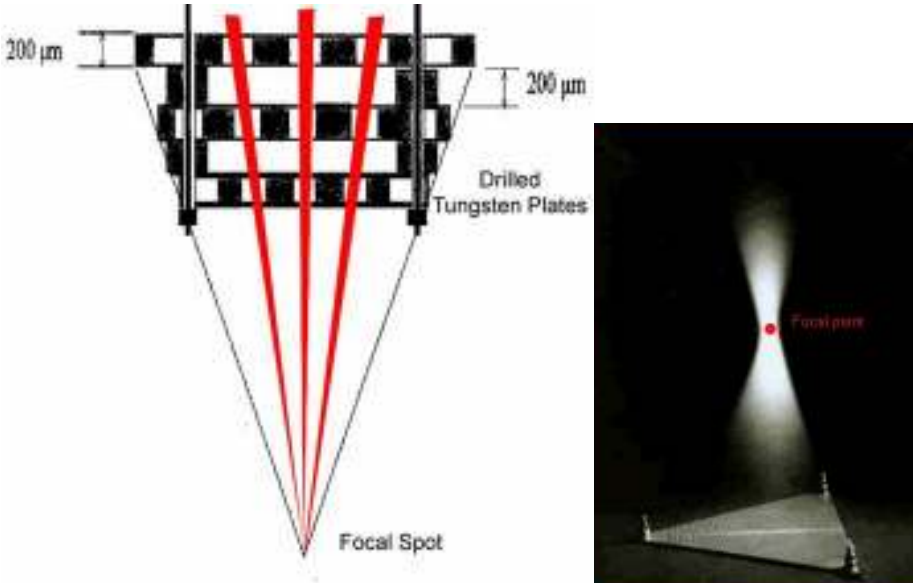

Figure 17: Illustration of principle of the focusing collimator used in the TOHR

To acquire a spatial volume-representation image, the object in which the RP is distributed (either an anaesthetised animal model or an *in vitro* physical 'phantom'), is placed on a motorised bed in the centre of the ring of detectors. The bed is then moved in a systematic fashion such that the entire area of interest within the object passes through the focal point of the 10 collimators. The detectors behind the collimators measure the amount of radioactivity coming from the focal point at each discrete stage of the object's passage through it and use this data to build an image (Ploux *et al.*, 1997; Valda Ochoa *et al.*, 1997). The physical size of the discrete data points the TOHR measures can be either 200µm, 500µm or 1mm, with these becoming the pixel size in the final image. Smaller pixel sizes give better images (higher resolution) however they permit the collection of fewer counts (lower sensitivity). To maximise the number of photons collected for the final

image the systematic pattern the object moves in to build the image can be repeated any number of times (each repetition called a passage). This can effectively increase the sensitivity of the system without concurrently decreasing the resolution, however it extends the time needed to acquire the image, which in turn can compromise the temporal information able to be gained regarding the biokinetics of the RP within the subject.

The TOHR has two acquisition modes, simple and co-incidence. In simple mode, all photons from the RP which enter a detector are counted in the measurement of that point's radioactivity. For radioisotopes which emit photons of more than one energy, co-incidence mode can be used. Of the two radioisotopes used in these *in vitro* molecular imaging studies, ^{99m}Tc and ^{123}I, only ^{123}I was suitable for both simple and co-incidence detection. When ^{123}I decays it emits multiple photons which have, on average, energies of either 28 keV (kilo-electron-volts) or 159 keV (McLean *et al.*, 1994). The amount of light the crystal in the detector releases is proportional to the incoming photon's energy, thus the TOHR can discriminate between these two photon energies. When the TOHR is set to co-incidence mode, it only counts activity in a point when it receives simultaneously the different energy photons (Valda Ochoa *et al.*, 1997). Imaging in co-incidence mode results in a higher quality image (higher resolution), however one which takes much longer to acquire (lower detection efficiency).

The TOHR imaging principle is unique in the field and allowed earlier prototypes of the TOHR to achieve a spatial resolution of 1.4mm (ie the system can differentiate between two discrete points which are 1.4 millimetre apart). This is comparable with some commercially available small-animal molecular imaging systems (Larobina *et al.*, 2006).

A more advanced prototype of the TOHR, which was thought (similar to a mouse-only system (Beekman *et al.*, 2005)) to have sub-millimetre resolution was acquired for RP evaluation studies in this project. However, initial *in vivo* studies were inconsistent with expectations of this system's performance and subsequently a large number of individual experiments, described in this chapter, were performed in order to optimise its performance parameters. These studies included *in vivo* studies examining the ability of the system to depict the thyroid, striatum, and cerebral blood flow in the rat and *in vitro* studies examining the system's detection geometry, sensitivity and resolution. These studies enabled the system to eventually attain its expected performance characteristics. However soon after this a significant software failure occurred with the system and the decision was made in the laboratory to discontinue work with the TOHR. The studies which were performed however support the conclusion that, in principle, the TOHR is suitable to perform a variety of targeted neurological studies in rats.

5.2 Methods

5.2.1 Rat thyroid studies

The initial study performed upon acquisition of the TOHR was an *in vivo* study using the RP $^{99m}TcO_4^-$ in the rat. $^{99m}TcO_4^-$ (and $^*I$) are RPs known to accumulate avidly in the thyroid gland and are widely used in clinical practice for investigations of this gland (McLean *et al.*, 1994). The thyroid is one of the simplest organs to visualise with molecular imaging as its avid accumulation of these RPs gives it excellent contrast to background. In addition, the small size of the thyroid in the rat (~3mm) makes this organ ideal to test the resolution of an imaging system.

All procedures were carried out in accordance with the European Community Council Directive 86/609/EEC for the care of laboratory animals. Either male Wistar (~400g) or Sprague-Dawley (250-300g) rats were used in the *in vivo* portions of this study. In the first study, an animal was injected with 3.5mCi $^{99m}TcO_4^-$ in the penile vein then immediately anaesthetised with an i.p. injection of pentobarbital (60mg/kg). Once the animal was sufficiently anaesthetised, as assessed by tail- and pedal-pinch reflexes, it was positioned supine on the motorised bed of the TOHR. Imaging was commenced 25 minutes after the injection with an area 10mm x 10mm square in a coronal plane and 5mm thick along the dorsal-ventral (DV) axis. The focal point of the TOHR was in the centre

of this area, over the neck of the animal and ~10mm from the ventral surface of the neck. Four, 6 min acquisitions were performed, with a 1mm pixel size.

In the second study, the same protocol as above was used except the animal was injected with 1.1mCi $^{99m}TcO_4^-$ and the area imaged was 8mm x 5mm x 8mm (ie 8mm square in the sagittal plane). One, 96 min acquisition was performed, with a 200µm pixel size. The third study was performed in the same fashion as these previous animals, except 575µCi of [^{123}I]NaI was used as the RP and the area imaged was 15mm x 7mm x 15mm (ie 15mm square in the sagittal plane) with a 500µm pixel size. These parameters made for an 81min acquisition time. This ^{123}I study was performed in co-incidence acquisition mode.

The final thyroid study was performed on a male Sprague-Dawley rat. This animal was injected with 4.1mCi of $^{99m}TcO_4^-$ and sacrificed 30 mins later with an overdose of pentobarbital. Five acquisitions were subsequently performed with this animal. The first acquisition was used to precisely localise the thyroid and consisted of an area of 20mm x 10mm x 10mm (ie an area 10mm square in the transaxial plane and 20mm long in the rostral-caudal direction). This acquisition used a 1mm pixel size and was two passages. The second and third acquisitions used a 7mm cubic area, a 500µm pixel size and 4 passages, whilst the fourth and fifth acquisitions used a 2-D 8mm x 7mm transiaxial area and a 200µm pixel size.

5.2.2 Rat striatum ^{123}I-PE2I study

[*I]PE2I is an RP analogue of cocaine which has been shown to have good affinity and specificity for the dopamine transporter (Guilloteau *et al.*, 1998). Importantly here, the same study reported [*I]PE2I to accumulate avidly in the striatum with very low fixation in the surrounding cortex (Guilloteau *et al.*, 1998). In comparison to thyroid imaging, the target-to-background contrast is not as great, however the striatum is also much bigger than the thyroid and it was therefore believed the images should be of a similar quality.

[^{123}I]PE2I imaging was performed in two male Wistar rats. The first rat was administered with 630 μCi [^{123}I]PE2I via the penile vein then immediately anaesthetised with 60mg/kg pentobarbital. A 1 x 15 x 15 mm co-incidence image was acquired in the transaxial plane, 9.5mm rostral to the inter-aural line (in accordance with a rat-brain atlas, (Paxinos *et al.*, 1998)), with a 500 μm pixel size and 18 passages. Imaging commenced 30 mins after RP injection and continued for 56 mins. The second rat was injected with 2,530 μCi [^{123}I]PE2I and imaging was performed in an identical manner except 20 passages were acquired.

5.2.3 DatSCAN study

Like [*I]PE2I, another radioiodinated cocaine analogue RP with affinity for the dopamine transporter is [^{123}I]DaTSCAN (GE Healthcare, UK) which, unlike [*I]PE2I is commercially available. [^{123}I]DaTSCAN has been shown in rats to bind

specifically to dopamine transporters and to demonstrate very good target-to-background ratios (Booij *et al.*, 1997; Lavalaye *et al.*, 2000) and clinically it has been shown to be able to differentiate Lewy-Body dementia from AD (Costa *et al.*, 2003).

[^{123}I]DaTSCAN imaging was performed in a single male Wistar rat. The animal was injected with 2.9 mCi of the RP via the penile vein and was then sacrificed with an overdose of pentobarbital following a 2 hour RP distribution time. A 4 x 15 x 15 mm transaxial image was acquired 9.5mm rostral to the inter-aural line (Paxinos *et al.*, 1998) using a 500 μm pixel size, co-incidence detection and 10 passages. When the image was first started the computer crashed and was not able to be used again for 24 hours (during which time the carcass was refrigerated). Thus, the image finally acquired contained almost ¼ the activity originally intended to be used.

5.2.4 Rat cerebral blood flow ECD study

[^{99m}Tc]-Bicisate (^{99m}Tc-ECD) (Bristol-Myers Squibb, USA) is an RP used for the molecular imaging of cerebral blood flow, (Encinas *et al.*, 2003; Siitonen *et al.*, 2003). It is a lipophilic compound which readily diffuses across the blood-brain barrier and cell membranes and which, once inside the cells, is metabolised into polar compounds which do not readily diffuse out (Bristol-Myers Squibb Medical Imaging, 2003). The localisation of ^{99m}Tc-ECD is thus a function of perfusion and

cell function. In this study, as normal animals are used cell function was assumed to be also normal and thus RP localisation taken to be a measure of perfusion.

A male SD rat was injected with 4.2 mCi ^{99m}Tc-ECD via the penile vein then sacrificed 30 mins later with an overdose of pentobarbital. A 20 x 20 mm 2-D transaxial image with a 200 μm pixel size was acquired 10.0 mm rostral to the interaural line (Paxinos *et al.*, 1998) with 20 passages for a total acquisition time of 273 mins.

5.2.5 Sensitivity / linearity studies

The efficiency and geometry of the detectors' measurement of radioactivity was assessed with a point source. The point source used was a small drop of $^{99m}TcO_4^-$, with an activity of ~1mCi, sealed in a narrow plastic cylinder. The small size of this RP source allowed investigation of the ability of the detectors to measure activity at the focal point only, without extraneous activity entering the detector. The absolute number of photon events detected by the system as a proportion of the activity of the source is the measure of the detector's efficiency. By displacing the point-source along the axis of the bed, the geometry of the system could also be assessed. Thirty, 1 min acquisitions were performed, each at 1 cm intervals from the focal point.

5.2.6 Phantom studies

To investigate the qualitative resolution of the TOHR system, a series of acquisitions was performed with a Micro Deluxe phantom (Data Spectrum Corporation, USA). The Micro Deluxe phantom consists of a solid Perspex cylinder with 6 groups of holes of varying sizes bored through it which contain the RP solution (2-5mCi in 15mL) (Figure 18). 2-D (ie 1 pixel thick) transaxial images were acquired with either a 500μm or 200μm pixel size and 1-5 passages.

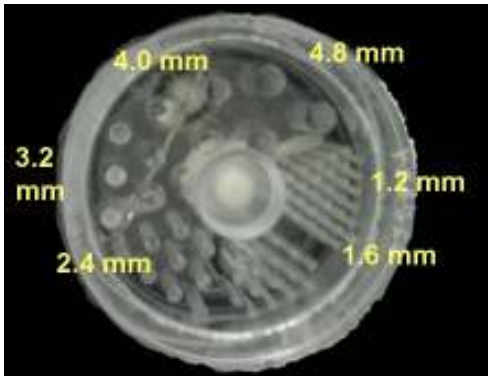

Figure 18: The Micro Deluxe Phantom (Data Spectrum Corporation, USA). Phantom external diameter = 50 mm.

5.2.7 Contrast phantom studies

To assess the ability of the system to differentiate between regions with different activities, a contrast phantom was used. The study used a 10 μL point source with 20 μCi of [^{123}I]NaI (2 mCi/mL). This point source was inside a glass bulb which was immersed in 35 mL of H_2O in a cylindrical vessel (15 x 50 mm [d x l]). In the first contrast phantom study, the H_2O in the vessel contained 700 μCi ^{123}I (0.02

mCi/mL), giving a contrast of 100:1. For the second study the vessel was drained and rinsed and refilled with H_2O and no activity, giving a contrast of 1:0. These acquisitions were performed in both simple and co-incidence mode with a 1mm pixel size and an imaging area of 10 x 20 x 20 mm.

5.3 Results

5.3.1 Rat thyroid studies

In the first *in vivo* thyroid study, from neither the animal injected with the low (1.1 mCi) nor the high (3.5 mCi) activity of $^{99m}TcO_4^-$ were images acquired which allowed visualisation of the thyroid. The images produced had counts homogeneously distributed throughout their entire fields (data not shown). Given it was thought unlikely that either a mistargeting of the area to be imaged or an inadequate dose of RP were the reason these images failed (see Discussion), studies were performed to measure the sensitivity and geometry of the detection system.

5.3.2 Sensitivity / linearity studies

When a ^{99m}Tc point image was acquired at the focal point, the efficiency of the detection system was measured to be 767 cps/MBq (counts-per-second/megabecquerel (1 MBq = 10^6 cps)), or 0.08%, comparable to some commercially available systems (Beekman *et al.*, 2005; Meikle *et al.*, 2005). The geometry of the detector system is shown in Figure 19. It can be seen that an

enormous number of photons, (603% of that detected at the system's focal point) were able to be detected at 7cm from the focal point. As the TOHR builds the image, plotting a map of the spatial radioactivity distribution, on the basis of counts from the focal point, such an excess of counts at a position removed from the focal point would greatly degrade the image. Thus, in light of this experiment, ameliorative work had to be performed on the system.

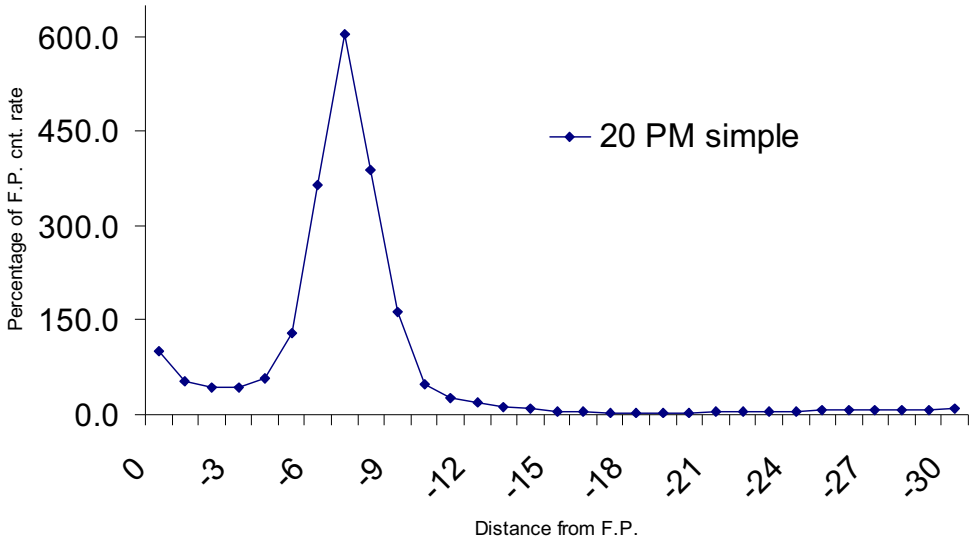

Figure 19: Geometry of detector efficiency for a ^{99m}Tc point source

Following the addition of extra shielding along the borders of each collimator, the detector geometry study was repeated (Figure 20). It could be seen now that photon detection was optimal at the focal point. However, a not-insignificant fraction (20-35%) of the activity could still be observed from 1-7 cm from the focal point.

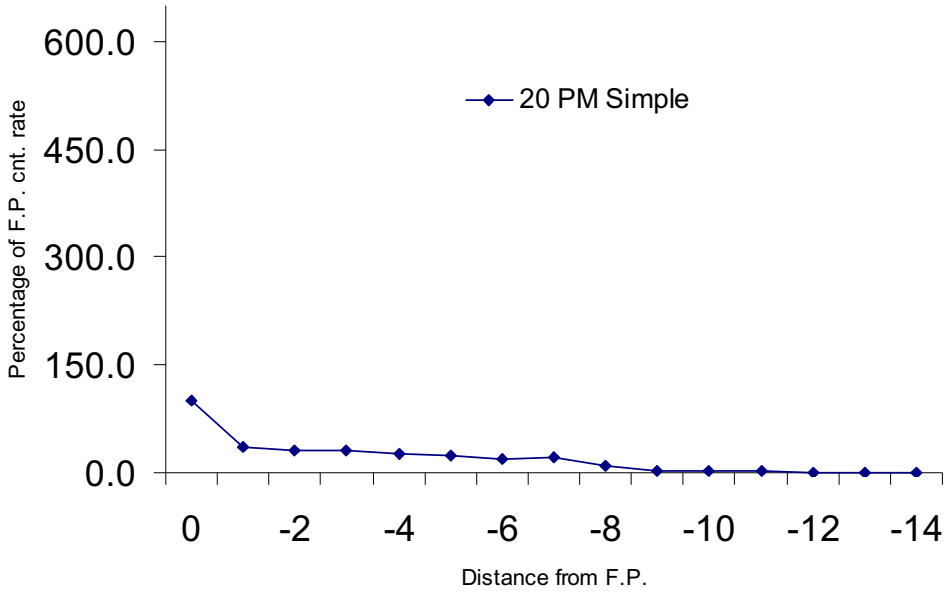

Figure 20: Geometry of detector efficiency for a ^{99m}Tc point source following inter-collimator shielding repairs

5.3.3 Phantom studies

An image of the mid-sized bars of the Micro-Deluxe phantom was performed with 2 mCi ^{99m}Tc in the phantom (Figure 21). The image acquired had a 500 µm pixel size and was acquired over 135 mins. The image shows that the TOHR system could clearly delineate bars of 2.4 mm diameter but not of 1.6 mm.

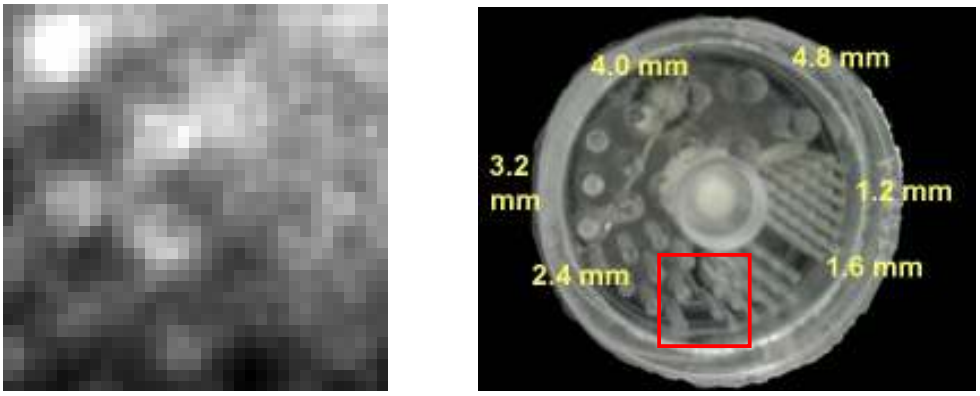

Figure 21: Image of Micro-Deluxe phantom, of an area indicated by the red box on the right

Later studies, following the addition of further shielding and a re-alignment of the collimators, produced images such that not only could the 1.6 mm bars be discerned but the 1.2 mm ones as well (Figure 22).

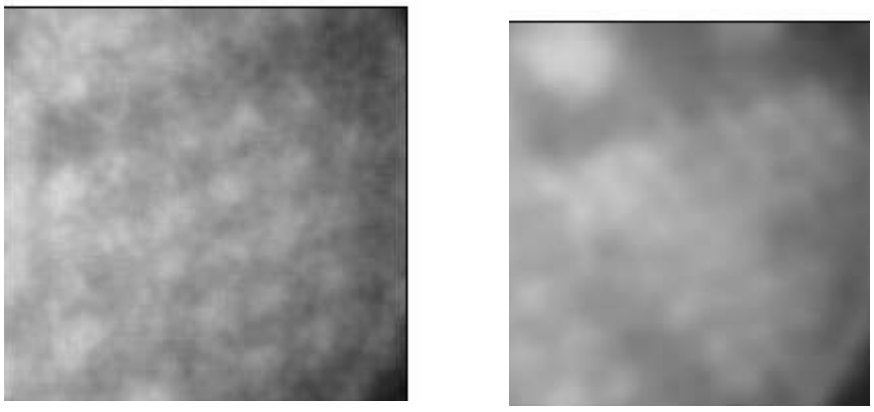

Figure 22: Images of Micro-Deluxe phantom: 2-D, 20 x 20 mm, 200 µm pixel size, 10 passages, 3.5 mCi $^{99m}TcO_4^-$ in phantom, 150 min acquisition time. The image on the left is of the group bars of 1.6 mm diameter whilst the image on the right is of the group of bars of 1.2 mm diameter.

5.3.4 Contrast phantom studies

The images in Figure 23 were performed with a contrast phantom. In the image on the left, though it was slightly mistargeted, the point source can be seen clearly against the background. In the image on the right, where activity had been added in the background, the point source can still be seen, however the contrast is subjectively poorer than the image on the right.

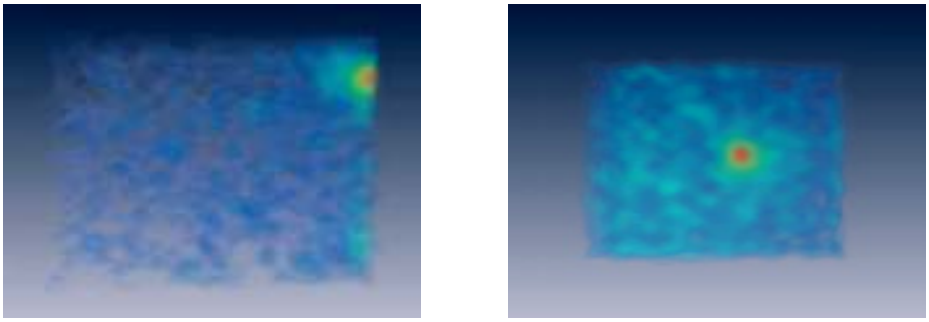

Figure 23: Images of a contrast phantom. The image on the left is of a point source of 20 μCi ^{123}I in plain water whilst the image on the right is the same point source in water with 1% the activity concentration of ^{123}I.

5.3.5 ^{123}I-PE2I study

Similarly to the thyroid imaging described above, [^{123}I]PE2I imaging of the striatum resulted in a negative image (Figure 24). The image is homogeneous and no features can be discerned.

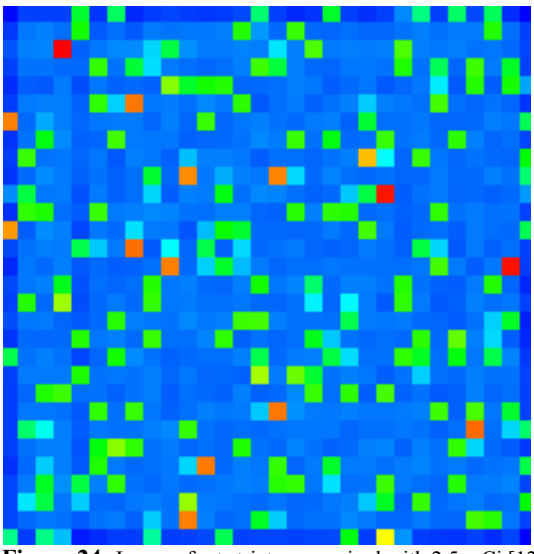

Figure 24: Image of rat striatum acquired with 2.5 mCi [123I]PE2I

5.3.6 ^{123}I thyroid study

Shown in Figure 25 is a series coronal views taken of a rat injected with 575 µCi of the thyroid-seeking RP [^{123}I]NaI. As in the previous thyroid acquisitions performed with the RP $^{99m}TcO_4^-$, the thyroid is unable to be visualised.

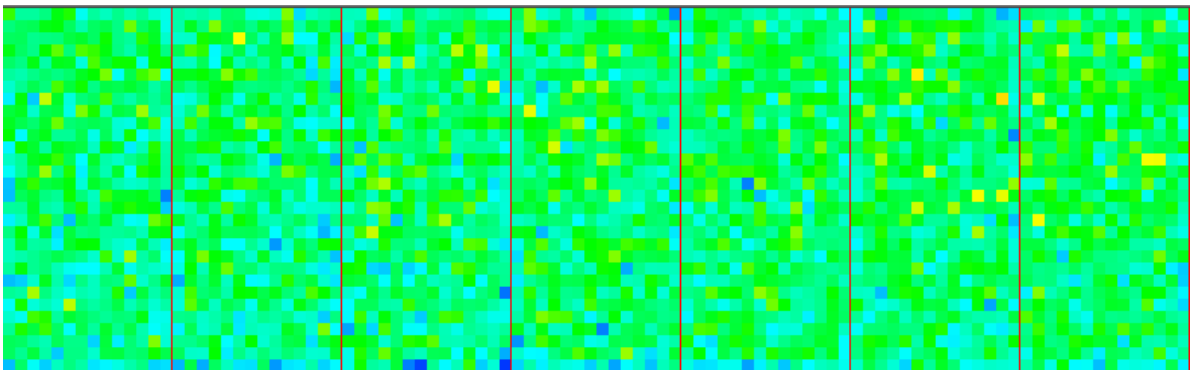

Figure 25: [^{123}I]NaI coronal images in the regions of a rat thyroid.

5.3.7 Thyroid images

Molecular imaging performed on the carcass of a rat which had been sacrificed 30 mins following the injection of 4.1 mCi $^{99m}TcO_4^-$ was able to demonstrate clearly the thyroid in situ (Figure 26).

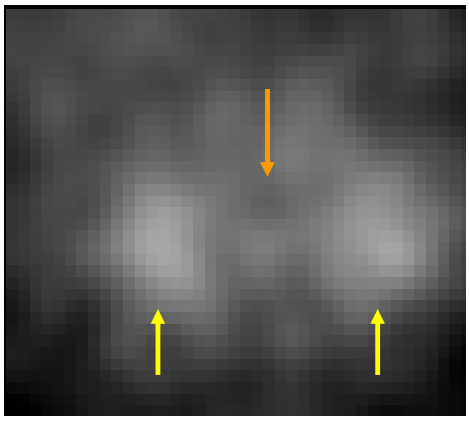

Figure 26: Post-mortem image of a rat thyroid. The animal was injected ante-mortem with 4.1 mCi $^{99m}TcO_4^-$ and sacrificed 30 mins later. Image is taken from the carcass 5 hours post-mortem: 2-D, 8 x 7 mm, 200 µm pixel size, 1 passages, 66 min acquisition time. Yellow arrows indicate the lobes of the thyroid, the orange arrow indicates the isthmus.

5.3.8 ECD study

The result of post-mortem imaging of ^{99m}Tc-ECD distribution is shown below (Figure 27). Whilst the outline of the brain can be clearly seen, the resolution of the system was insufficient to outline any of the underlying architecture. A published ^{18}FDG study from a group in the United States (see caption) is also

shown in Figure 27 for comparative purposes. This image from a PET system shows some heterogeneity of uptake within the organ.

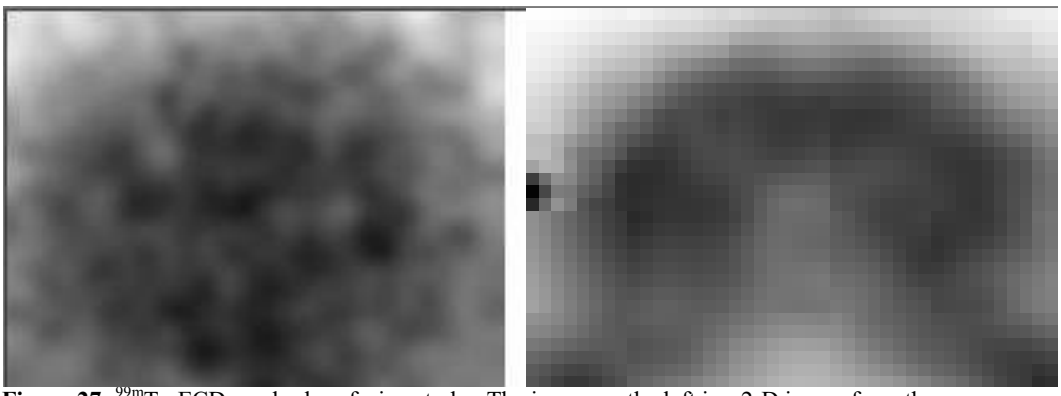

Figure 27: ^{99m}Tc-ECD cerebral perfusion study. The image on the left is a 2-D image from the TOHR at ~10.0 mm rostral to the interaural line. The image on the right is, by way of comparison, an ^{18}FDG PET image of cerebral metabolic function in a rat in the same region (image taken from the following website: http://www.crump.ucla.edu/user-files/resprojects/microPET/images_toc.html)

5.3.9 DaTSCAN study

The result of imaging with the dopamine reuptake transporter RP [^{123}I]-DaTSCAN (GE Healthcare, UK) is shown in Figure 28. In spite of the acquisition being compromised by instrumentation failures, an heterogeneity of RP distribution and an apparent appearance of two symmetrical regions of uptake (at the bottom of the image) are demonstrated in the image.

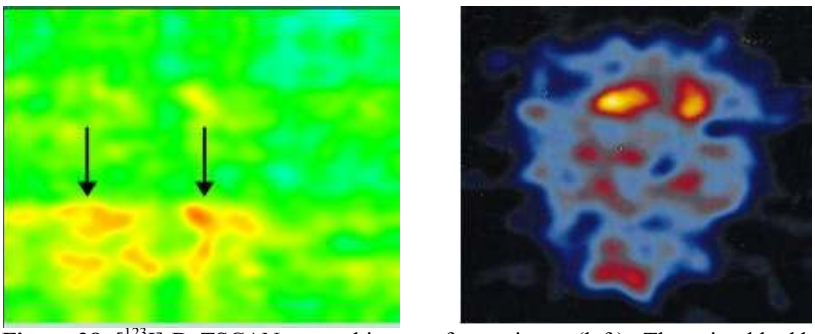

Figure 28: [^{123}I]-DaTSCAN coronal image of rat striatum (left). The animal had been injected with 2.9 mCi of the RP, however due to instrumentation problems, imaging was not able to be commenced until 24 hours later. By this time the absolute amount of activity in the rat would have been ~800 μCi. The image on the right is a transaxial [^{123}I]PE2I image in a cynomolgus monkey, taken from Guilloteau et al., (1998), for the purposes of comparison. In the image on the right, the two bright spots near the top of the image represent RP binding in the striatum. These are equivalent to the spots in the left image indicated by the arrows, (imaged in the coronal ([^{123}I]-DaTSCAN) rather than the transaxial ([^{123}I]PE2I) plane).

5.4 Discussion

Similarly to Chapter 4, the work described in this chapter was designed to evaluate / develop specific tools required for the molecular imaging of the cholinergic system, with a view to potential utility in clinical AD. The original aim of this study was to assess whether the TOHR system was capable of demonstrating *in vivo* the reductions in *IBVM and *I-A-85380 revealed by the autoradiographic studies described in Chapter 4. Following the initial studies with this system, it was realised much work would need to be directed towards the development the imaging system before it would be capable of this assessment. Multiple *in vivo* and *in vitro* studies, elaborated below, were performed to this end. Unfortunately, at the point where the performance characteristics of the system appeared to be developed

to such a point where pilot molecular imaging studies in the SAP model with the cholinergic RPs would have been possible, a significant failure of the system's control software occurred and it was decided by the laboratory in which I was working to discontinue further studies with the system. Discussed below is evidence that the novel principle of the TOHR would be capable of a variety molecular imaging studies, particularly with regard to studies of CBF integrity, analogous to the autoradiography studies described in Chapter 4.

5.4.1 Thyroid studies

The initial studies performed with the TOHR were thyroid studies in the rat. These studies were chosen as results reported for simulations and the prototype system had indicated the TOHR would have satisfactory detection efficiency and resolution for this structure and it was desired to validate this (Ploux *et al.*, 1997; Valda Ochoa *et al.*, 1997). The failure of the TOHR system to visualise the thyroid could have been due to a number of causes.

For instance, it was thought possible that the thyroid may not have been visualised in the image from the first animal because it was a coronal image and thus the thyroid may not have been accurately targeted by the area imaged. The second animal was thus imaged in the sagittal plane. As the thyroid can be confidently localised in the lateral direction, a sagittal image maximised the area viewed in the other two directions. In this image too however the thyroid was not visualised. It

should be noted that though the sagittal image had one-third the activity of the coronal image, it is not thought likely to be a reason the thyroid wasn't visualised in the sagittal image. Clinical $^{99m}TcO_4^-$ thyroid imaging uses a dose of ~5 mCi, eg (Ozdogan *et al.*, 2006). This value makes the doses used in these animals, even the lower dose (1.1 mCi), proportionally very large.

It was thus thought the likely reason for the observed homogeneity of count distribution in the studies would be the detection of photons originating from outside the focal point of the system, ie from the body of the animal. Such counts would be detected in a consistent manner throughout the image, thus would greatly diminish the contrast between the thyroid and the background. To assess the degree of this phenomenon, the detection efficiency and geometry of a point source of activity was investigated.

5.4.2 Sensitivity / linearity

The study of detector efficiency found the sensitivity of the TOHR to be comparable to that claimed for some other small-animal molecular imaging systems. Its geometry however, as illustrated in Figure 19, confirmed the hypothesis that extraneous counts were entering the detector. The most likely reason for this was that there was a gap between the focussing collimators allowing extraneous photons to enter the detectors. Hence, extra shielding was added along the borders of each collimator. Following the addition of this shielding, the geometry study was again

performed (Figure 20). After the addition of this shielding, a study of the geometry of the system demonstrated the large fault initially observed 7 cm from the focal-point had been repaired, though some residual non-focal-point activity was still being detected. An *in vitro* phantom experiment to test the resolution of the system was therefore performed.

5.4.3 Phantom studies

Imaging of the mid-sized bars of the Micro-Deluxe phantom produced a satisfactory image of the 2.4 mm diameter bars but a poorly resolved image of the 1.6 mm ones (Figure 21). This result was a lot better than that derived from the initial thyroid studies, however it was still not as good as had been originally indicated for the system (Ploux *et al.*, 1997; Valda Ochoa *et al.*, 1997). That the resolution of the 1.6 mm bars was poor was thought to be due, in addition to contrast being compromised by the much-reduced but still unacceptably high activity being detected from outside the focal point, to a (slight) misalignment of the collimators. The effect of this, each collimator being focussed on a (slightly) different point, would have been to reduce contrast in the image as some detectors would be measuring low-activity areas whilst other detectors were measuring high-activity areas, averaging the detected photons across the image.

The collimators were thus re-aligned and yet more shielding was added between them and then images of the Micro-Deluxe phantom were once more acquired.

These images (Figure 22) not only showed the 1.6 mm bars clearly, but also 1.2 mm diameter bars, the smallest of the phantom. Though a pleasing result, indicative of the very good resolution of the TOHR, the 2-D images in Figure 22 took 2 ½ hours to perform. In the context of small-animal molecular imaging, where larger volumes are often imaged and information regarding dynamic processes is often sought, the time needed to obtain high-quality images such as these would limit the types of studies able to be performed with the system.

5.4.4 Contrast phantom studies, PE2I and I-123-thyroid images

The phantom and the point-source studies described above shared a common feature in that the contrast in activity between the target and the background was 1:0. *In vivo* this contrast would almost never exist as areas outside that of interest to a study would be expected to contain some blood-borne RP, contributing activity to the final image. To model non-perfect activity contrasts, a contrast phantom was used.

One image was acquired with perfect contrast, whilst another had a target : background contrast of 100:1 (Figure 23). In the image with perfect contrast the point source is clearly visible whereas, as would be expected, the addition of a small amount of activity to the background made the delineation of the point source in the second image subjectively more difficult. However, an inspection of this latter image shows the background activity to be relatively homogeneously

distributed and the point-source to be still discretely resolved. These images of the contrast phantom are evidence that the collimators were accurately aligned and that there was adequate inter-collimator shielding. Thus further biological studies were performed.

Neither the [^{123}I]PE2I dopamine transporter study nor the [^{123}I]NaI thyroid study yielded desirable images (Figure 24 and Figure 25). Given that studies of the phantoms had resulted in good images, the poor *in vivo* images could not have resulted from contrast reductions due to collimator misalignment or the detection of non-focal-point activity between the collimators. Rather, due to the animals being much larger than the phantoms, the most likely explanation for the lack of contrast in the *in vivo* images was non-focal-point activity reaching the detectors not between the collimators but around them, from parts of the animal's body more distant than assumed following the original thyroid study. Extra shielding was thus added outside the ring of detectors.

When subsequent *in vivo* images were acquired the results were significantly better than previously. $^{99m}TcO_4^-$ thyroid imaging produced a clear image of this organ, allowing both lobes and the isthmus to be distinctly observed (Figure 26). With a 2-D image acquisition time was 66 mins, indicating the TOHR can perform high-resolution imaging of small volumes in a satisfactory time-frame. This contrasts with Figure 22 where a larger image was acquired, necessitating an overly long acquisition time.

Further evidence that the TOHR, given sufficient acquisition time, is capable of imaging RP distribution in biological models comes from the results of the ^{99m}Tc-ECD and the [^{123}I]-DaTSCAN studies (Figure 27 and Figure 28). The ^{18}FDG image on the right in Figure 27, taken from a published source for the purposes of comparison, is indicative of the homogeneity expected during imaging of a perfusion (and cellular function) RP. The ^{99m}Tc-ECD image on the left in Figure 27, whilst not delineating as clearly the different cerebral regions as the ^{18}FDG image, clearly shows the outline of the brain. Additionally, this image also shows some heterogeneity of activity within the brain, indicating the potential of the TOHR to resolve to a small degree the variable distribution of a perfusion RP in the brain.

The image acquired with the RP [^{123}I]-DaTSCAN equally demonstrates the potential of the TOHR system. This image was acquired 24 hours after the injection of the RP and the sacrifice of the animal, due to the computer controlling the system crashing, resulting in a very small equivalent dose of activity being imaged (~800 μCi). In spite of this, the image was able to delineate the striatum against the background. This image was of a larger volume than the *in vivo*/*post-mortem* studies above (4 x 15 x 15 mm) with an acquisition time of 200 mins. Had the image been able to be acquired 24 hours earlier, with the full dose of RP, this acquisition time would have been significantly shorter (< 1 hr). To enable this reasonably short acquisition time, in comparison to the ^{99m}Tc-ECD study for

example, a larger pixel size was used (500 μm), effectively trading resolution for detector efficiency.

5.4.5 Imaging performance of the TOHR

The detection parameters required of a system to perform a small-animal molecular imaging study depend upon the size of the regions studied and the pharmacology / pharmacodynamics of the RP used. Generally though; a resolution of 1-2 mm may be considered satisfactory, with < 1 mm resolution considered excellent; a detection efficiency such that imaging may be performed in ≤ 1 hr is desirable; additionally, a field-of-view (FOV) large enough to image all regions of interest, including target region, control region (if any) and any region required for image analysis - for neurology imaging in rats a transaxial FOV of ~3-4 cm is required. Resolution and detector efficiency must generally each be a compromise for the other (ie increased detector efficiency results in decreased resolution, and vice versa). Both these parameters are further modulated by the FOV used.

The studies described in this chapter, in conjunction with the criteria described above, support that the TOHR, using a novel detection principle, has the capability to perform a variety of neurological imaging studies in rats. Imaging of the Micro-Deluxe phantom, after adjustments of the collimators and inter-collimator shielding, demonstrated very good resolution. The smallest bars on the phantom, with the bars 1 diameter apart (2x diameter centre-to-centre) were distinctly resolved, giving

a resolution for the TOHR of < 1.2 mm. This very good resolution of the TOHR was further demonstrated in the final thyroid study where the individual lobes of the thyroid (each ~1 mm in size) were clearly visualised. The resolution of the various brain regions in the ^{99m}Tc-ECD study was diminished by the relatively low contrast provided by the cerebral perfusion RP, whereas the higher regional contrast afforded by the dopamine-transporter ligand [^{123}I]DaTSCAN allowed a distinct image to be acquired despite the technical problems experienced.

The images with the highest resolution however each required a very long acquisition time. Though the detection efficiency of the TOHR (in simple acquisition mode) is in the mid-range of molecular imaging system efficiencies (see p. 149), this efficiency is only in the very small region of the focal point, rather than over the entire FOV of the system. This means that overall the TOHR has a much lower detection efficiency than systems based upon other imaging principles. This low detection efficiency is highlighted by long acquisition times. The images of the Micro-Deluxe phantom which showed very good resolution took 150 mins to acquire. Similarly the ^{99m}Tc-ECD image took 273 mins and the [^{123}I]-DaTSCAN image took 200 mins whilst the smaller image of the rat thyroid took a reasonable 66 mins. These acquisition times are inconsistent with the desirable criteria described above.

However the TOHR system may still be considered suitable to perform a variety neurological imaging studies in rats with the following considerations; i) because of

technical problems the [^{123}I]-DaTSCAN study was delayed 24 hours, meaning the final image was acquired with ~¼ the intended activity - without the delay the image would have been able to be acquired in ~¼ of the time, ie 50 mins; ii) this study also provided an image with good resolution using a pixel size of 500 µm, whereas the phantom, thyroid and ^{99m}Tc-ECD studies used a 200 µm pixel size; iii) the [^{123}I]-DaTSCAN study imaged a 3-D volume of 4 x 15 x 15 mm, much larger than the 2-D volumes imaged in the above studies. In sum, these considerations indicate that the 500 µm pixel size provides sufficient detection efficiency to image a 3-D volume, including the entire lateral and dorsal / ventral extremities of the brain, within a suitable timeframe (≤ 1 hr).

With regards cholinergic RP evaluation in the SAP model, imaging to assess the relative densities of RP in the cortex, thalamus, hippocampus, cerebellum and CBF to that in the striatum (analogous to the analysis of the autoradiography studies described in Chapter 4) may, according to the results discussed above, be able to be performed with the TOHR. The autoradiography studies described in Chapter 4 used coronal brain sections from 4 different levels (~ Bregma +0.5, -1.3, -4.0 and -12.0 mm AP). Assuming an activity of ^{123}IBVM or ^{123}I-A-85380 was administered to the rat such that the amount extracted into the brain was the same as that in the [^{123}I]-DaTSCAN study, the same acquisition parameters as this latter study would enable 4 virtual coronal 'sections' to be imaged (each 1 x 15 x 15 mm), with images of reasonable quality able to be obtained in ~1 hr. Thus, the density of

^{123}IBVM and ^{123}I-A-85380 uptake in various cerebral regions of the SAP model would be able to be explored *in vivo*.

Further, as the SAP lesion has been shown to be stable from 7 days post lesion (Waite *et al.*, 1994), a number of other RPs (eg RPs for the mAChR or AChE etc) would also be able to be investigated in the same animals. Molecular imaging studies of other RPs, in different animal models, may also be suitably performed with the TOHR, if the regions pertinent to the goals of the study could be targeted within the relatively limited FOV of this system. If studies were designed which only required much smaller FOV's than discussed here then higher resolution images may be achieved, using techniques which demonstrate much lower detection efficiency such as a 200 μm pixel size or co-incidence acquisition mode.

Overall, the studies described in this chapter support the capability of the novel molecular imaging principle of the TOHR to perform a variety of targeted neurological studies in rats. This capability may ultimately be invaluable to the development of RPs for the investigation of the biochemical profile of diseases such as AD.

6 Chapter 6 – Discussion / Conclusion

6.1 Overview

This project focussed upon the cholinergic system in AD. Specifically, the work performed in this project aimed to extend the elucidation of the role of this system in the debilitating morbidity associated with AD and to contribute to the evaluation and development of specific tools for the molecular imaging of this disease.

Chapters 2 and 3 describe studies performed investigating the effect of highly specific lesions of the cholinergic system on attention and memory, two areas of cognition which are profoundly diminished in AD. These chapters investigating behavioural abnormalities following cholinergic lesioning used two different SAP models. One of the models was created when every animal in a surgical group had their lesions, which had been intended for the ventricle (icv), mistakenly placed into a region at the border of the primary motor and primary somatosensory cortices. The other model used was the successfully created icv model.

In Chapter 2, an important role for the CBF projection system in attention was demonstrated in the performance of a task which had not before been used to investigate cholinergic dysfunction. This finding is in accord with the current consensus that the CBF projection system plays a critical role in normal attentional function, eg (Sarter *et al.*, 2003; Sarter *et al.*, 2001b). The studies described in

Chapter 3 provide further evidence that certain aspects of normal memory function, assessed here with the specific mnemonic paradigm of the NORT for the first time in the icv model, can be sustained in spite of very large impairments in this system. This also is not inconsistent with current knowledge, eg (Mesulam, 2004).

The degree of cholinergic impairment associated with the behavioural observations, in the icv SAP model, was demonstrated in Chapter 4 to be able to be discovered using RPs which target 2 different aspects of the cholinergic neuron *in vitro* or *ex vivo*. Whereas Chapter 5 had initially aimed to subsequently investigate the ability of these RPs to delineate the same impairment *in vivo*, prevailing circumstances precluded this. Ultimately, rather than the *in vivo* studies originally desired, the associated development work performed with the unique small-animal molecular imaging system is described. This original work in sum strongly indicates that the TOHR would have the capability to perform the *in vivo* imaging described above, such that in future studies *in vivo* measures of cholinergic system integrity would be able to be performed concurrently with behavioural studies investigating different aspects of the functional role of this system, such as those studies described in Chapters 2 and 3.

The RPs chosen to investigate the degree of cholinergic impairment associated with the behavioural observations were ones which target the VAChT and nAChRs, ^{123}IBVM and ^{125}I-A-85380 respectively. The VAChT is a purely cholinergic marker and its relationship to the SAP model, which causes cholinergic-specific

neuronal degeneration is straightforward. nAChRs in contrast exist on cholinergic neurons in only relatively small proportions (Wonnacott, 1997). They thus provide a challenge for detecting the cholinergic degeneration in the SAP model. Additionally, the relationship between nAChR loss and behavioural dysfunction, inferred in AD from investigations with the SAP model, is more difficult to postulate. As described in Chapters 2 and 3 other transmitter systems, which are known to express nAChRs, can impact on attention and memory. It is in this context that the report of the effect of the nAChR agonist ABT-418 is important (McGaughy *et al.*, 1999). In that study it was found, unexpectedly to the authors, that nAChR agonism improved the performance of control animals on an attention task but not the performance of SAP lesioned animals. That report indicates that, with regards to attention, nAChRs expressed on cholinergic neurons rather than nAChR heteroreceptors are critical to normal function.

6.2 Implications

The implications of the work described in these chapters are multiple. That SAP lesioning caused impairments on the sensorimotor task is an extension upon previous work in that it provides evidence that the CBF system is implicated in a more refined test of attention. This is because it is not thought to involve any reward-stimulus association which may be a confounding variable in other tests of attention. When viewed in context of the large body of work implicating the CBF system in all facets of attention, it is entirely consistent with the hypothesis that the

cholinergic dysfunction evident in AD underpins the attention dysfunction evident in this disease.

6.2.1 Role of attention in mnemonic processes

Though superficially it may seem that an attentional dysfunction would not be a very debilitating symptom, this dysfunction may subserve many other symptoms. Whilst for the purposes of the studies in this project attention and memory were investigated as separate entities, in a broader context they should be viewed in a more connected manner. In a recent review, Sarter et al., (2003) have argued that "a fundamental dissociation between attention and learning and memory appears unlikely," (p247). They present evidence that for every step of the (learning and) memory process attentional resources are required and predict that persistent attentional dysfunction in a subject would produce escalating impairments in learning and memory.

From this perspective it would appear that cholinergic-lesion animal models which produce attentional deficits, including the SAP models, would be a more analogous model of AD than (negative) studies of memory alone in these models would suggest (Dunnett *et al.*, 1991; Muir *et al.*, 1993; Robbins *et al.*, 1989b; Voytko *et al.*, 1994). Similarly to the NORT used in this project, the reason attentional impairments in the animal models used in these cited studies did not manifest impairments on mnemonic tasks is most likely due to the fact that the mnemonic

studies did not sufficiently tax attentional resources (Sarter *et al.*, 2003). A similar phenomenon was observed in the CTD (Freed *et al.*, 1989; Parasuraman *et al.*, 1992) and sensorimotor attention studies described in Chapter 2. In these attention studies it was only when attentional capacities were challenged, eg by the introduction of a distracter, that attentional impairments were observed. Thus, whilst a large body of evidence supports the theory that the CBF projection system is not involved in mnemonic processes *per se*, that this system is critical for normal attentional processes and that attentional processes have the ability to modulate aspects of learning and memory processes (the latter symptoms being ones most widely associated with AD morbidity) provides a foundation for investigations of the cholinergic system in AD. As described in the Introduction (Chapter 1), the degeneration of the CBF projection system is one of the most significant changes observed in AD (Bartus *et al.*, 1982; Bowen *et al.*, 1976; Davies *et al.*, 1976). The degree to which this degeneration can be attributed in the early stages of AD to the disease rather than to age-related changes is difficult to elucidate (Mesulam, 2004), however it is difficult to dispute that this system plays an integral, though complex, role in the morbidity of AD.

6.2.2 Molecular imaging

For this reason, the development of tools critical for the molecular imaging of various aspects of the cholinergic system is of great potential benefit. The envisaged benefit is twofold. From a research standpoint, advances in the

molecular imaging of the cholinergic system would provide a number of tools for probing, non-invasively, the integrity of this system in various stages of AD in humans and the effect of different challenges to this system in animal models. Such imaging would also be useful in the assessment of novel therapeutic strategies. From a clinical standpoint, novel molecular imaging strategies will aid in the diagnosis of AD, bringing to the patient the attendant benefits of an earlier, more accurate diagnosis (and treatment). In this setting too, molecular imaging has the potential to evaluate the response to therapy.

6.2.3 Molecular imaging in current practice

The current focus of molecular imaging in AD is a refinement of studies investigating cerebral perfusion/activity with ^{18}FDG and ^{99m}Tc-HMPAO and the development of imaging for the Aβ pathology (Klunk *et al.*, 2004; Shoghi-Jadid *et al.*, 2002; Villemagne *et al.*, 2005). Both of these techniques, in different ways, contribute to similar goals as those for the molecular imaging of the cholinergic system outlined above. When considered in conjunction with assessments of cognitive function, molecular imaging with ^{99m}Tc-HMPAO has been shown to improve the accuracy of AD diagnoses (Jagust *et al.*, 2001). In a clinical setting this is of great benefit to patients. Similarly, ^{18}FDG has been described as an RP with very good diagnostic value (Villemagne *et al.*, 2005).

With regards to benefits in a research setting however, the cerebral perfusion / activity RPs appear to have less potential for expanding the body-of-knowledge surrounding AD than those RPs which target Aβ or aspects of the cholinergic system. This is because the processes they target are neither widely believed to be a root cause of AD morbidity nor objects of investigation for novel therapeutic strategies such as immunisation against Aβ or the use of drugs to modify either the production of Aβ or to modulate various neurotransmitter systems, eg cholinergic, GABAergic, serotoninergic systems (Jacobsen *et al.*, 2005; Vasilevko *et al.*, 2006).

Molecular imaging of cerebral Aβ and NFT densities on the contrary has the potential to make a great impact in AD research. Numerous therapeutic strategies are currently in the process of development which target aspects of fibrillar pathology; including compounds which inhibit the enzymes which convert APP into Aβ (Jacobsen *et al.*, 2005), metal chelators which inhibit the binding of zinc and copper to Aβ and hence destabilise it (Ritchie *et al.*, 2003), and Aβ immunisation (Vasilevko *et al.*, 2006). Concurrent with measures of cognitive function the ideal method to assess the efficacy of any of these therapies, clinically and serially in animal models, is molecular imaging. Additionally, such imaging will aid knowledge of the timecourse of Aβ and NFT pathogenesis. Particularly, knowledge needs to be expanded as to the correlation between these pathologies and dementia symptoms. The presence of gross cortical plaque formation in non-demented subjects has been documented as early as 1968 (Tomlinson *et al.*, 1968). More recent reports have confirmed the presence of widespread neuritic as well as

diffuse Aβ plaques and NFT in both cortical and limbic structures in non-demented subjects (Bennett *et al.*, 2006; Hulette *et al.*, 1998; Price *et al.*, 1999). These findings are sometimes interpreted as representing a preclinical stage of AD, known as mild cognitive impairment (MCI), particularly as they can be observed concurrently with mild, non-dementing, cognitive impairments. On a different tack, these and similar observations have been hypothesised to represent solely secondary considerations of the pathology of AD, with an emphasis placed upon the selective neuronal vulnerability apparent in AD (Schmitt, 2005).

Though the benefits from such research aided by Aβ molecular imaging may undoubtedly flow to the clinical arena, molecular imaging in clinical practice of Aβ and NFT pathology is unlikely to provide the same potential benefits as those described above for imaging of the cholinergic system. The rationale for this assertion is that a molecular imaging study positive for Aβ pathology is not necessarily an early and/or accurate predictor of future AD occurrence. Whether the presence of Aβ/NFT pathology is always an indicator of pre-clinical AD, even in the absence of any cognitive impairment, is a matter for debate. Evidence shows that large numbers of people can exhibit significant AD neuropathology without manifesting AD symptoms (Bennett *et al.*, 2006). However one can only speculate as to whether any, or all, of these subjects would have progressed to severe AD had they not died. In a clinical setting, until such research has been performed so as to be able to identify which non-demented subjects with AD neuropathology will

progress to AD within a relevant time-span and who will not, molecular imaging of Aβ will be of relatively minor interest to clinicians.

Furthermore, whereas the presence of Aβ and NFT pathology is not, as seen above, a direct influence on the cognitive declines associated with AD, as can be seen by the conclusions of Chapters 2 and 3 the presence of deficits in the CBF projection system can be considered of primary importance to AD symptoms. Thus, as described earlier, molecular imaging which targets aspects of the cholinergic system has much potential to aid research into AD and to ameliorate clinical management of this disease.

6.2.4 Radiopharmaceutical evaluation

RPs are essential tools for molecular imaging and studies contributing to the evaluation and development of two RPs which target aspects of the cholinergic system are described in Chapter 4. The emerging field of small-animal molecular imaging provides tools which are to be indispensable for the continued evaluation and development of these and other RPs. The studies described in Chapter 5 describe a number of aspects of a novel small-animal molecular imaging system which were assessed so as to enable it to assist in the continued evaluation of the two cholinergic RPs discussed in this project.

The process of RP evaluation and development is a long and convoluted one. Following the need to assess an RPs strict *in vitro* and *in vivo* properties, such as receptor binding affinity, lipophilicity, biological clearance rate etc, further studies need to be performed to investigate whether these properties extend into allowing useful information to be derived about various disease states. Without this additional data, the advancement of an RP to widespread use would have little justification.

In this project, the two RPs chosen have been used previously under a variety of conditions. For example, as described in Chapter 4, the VAChT RP *IBVM has been used in human subjects to investigate the degree of VAChT loss in AD and Parkinson's disease, finding various patterns of uptake reduction (Kuhl *et al.*, 1996). This RP has also been used to look at VAChT density in the rat following SAP lesion (Sorger *et al.*, 2000). Similarly, the nAChR RP *I-A-85380 has been used as a SPECT agent in a number of studies of non-human primates, evaluating its distribution in normal animals and assessing the effect of acute administration of non-radioactive cytisine or chronic administration of nicotine (Chefer *et al.*, 1998; Kassiou *et al.*, 2001; Saji *et al.*, 2002). It has also been shown in a normal human subject to accumulate in a manner consistent with the known distribution of $\alpha_4\beta_2$ nAChRs (Ueda *et al.*, 2004). An analogue of *I-A-85380 labelled with radioactive fluorine has been shown in monkeys to demonstrate useful properties for molecular imaging and in *post-mortem* human studies to demonstrate reduced uptake in AD (Schmaljohann *et al.*, 2004; Valette *et al.*, 2005). In humans it has been used both

in normal volunteers and to demonstrate nAChR occupancy in smokers (Brody *et al.*, 2006; Mitkovski *et al.*, 2005).

The studies performed in the present project were thus aimed not at being basic biological evaluations, but rather as targeted investigations of the RPs' potential utility for studying a specific aspect of a particular disease state, namely the cholinergic deficit associated with AD. The central goal of this project was to investigate the potential of the RPs for molecular imaging of AD in a well validated and systematic manner. To this end, the use of the same animal model, and with some studies the same animals, in several studies allowed for some conclusions to be drawn regarding the validity of each study based upon the results of its preceding study.

Specifically, the creation of the cholinergic deficit was confirmed with IHC for the specific cholinergic cell marker ChAT. The IHC studies (icv and intracortical model) were performed on groups of animals parallel to the animals used for autoradiography. Ideally for the purposes of validation the autoradiography studies and the IHC would have been performed in the same animals. This was initially planned, however a successful protocol was not able to be developed in a suitable time-frame to allow this. Thus, rather than being a direct validation of the lesion created in the animals used for autoradiography, it provided a validation of the technique used in this project to create the animal model used for both IHC and autoradiography studies. Thus the utility of the lesion in the animals for the

autoradiography study is inferred from validity of the lesion technique. It is not thought the conclusions drawn in this project are compromised by this distinction, but it must be kept in mind.

The behavioural studies described in Chapters 2 and 3 were performed with animals from both the IHC and the autoradiography studies. It is thus posited that these studies are well validated and that the findings from them can be thought of as due to the SAP cholinergic deficit. That the findings from both the attention and memory studies are so readily consistent with previously reported behavioural studies following modulations of the cholinergic system in turn provides additional validation for the autoradiography studies.

For an evaluation of an RPs potential for molecular imaging, ideally small-animal molecular imaging with an appropriate animal model would be performed. Following autoradiographic validation and with *post-mortem* IHC such studies would have provided much interesting data. It is unfortunate for this project such imaging eventually proved unfeasible. The studies which were necessary in preparation for such imaging however advance knowledge in this field and it is hoped in the future that, building on data such as that described in this project, this imaging will become feasible.

The findings of the studies described in Chapter 4 support further evaluation of both the RPs studied with a view to their use in the molecular imaging of AD. The

excellent properties of the specific VAChT RP ^{123}IBVM were demonstrated by its reduced binding in the cortex, CBF and hippocampus of SAP lesioned animals, a pattern of reduction consistent with that reported previously (Sorger *et al.*, 2000). The excellent properties of the specific $\alpha_4\beta_2$ nAChR RP ^{125}I-A-85380 were displayed as its uptake was consistent with the known nAChR distribution in control animals and, additionally, that in SAP animals this RP, unlike previously used nAChR RPs, identified a statistically significant reduction of binding in the hippocampus and cerebellum and a pattern of reduced binding in the cortex and CBF, consistent with the known (and inferred) toxicity of SAP (Heckers *et al.*, 1994; Lee *et al.*, 2002; Waite *et al.*, 1994). This latter finding is of particular interest as it provides evidence that ^{125}I-A-85380 has excellent properties for potential molecular imaging, given its ability to delineate a very small deficit.

6.2.5 Future studies for RP evaluation

To aid the continued, well validated, evaluation of these RPs in the future, the studies described in Chapter 5 were performed to develop a novel small-animal molecular imaging capability. The system upon which these studies were performed, the TOHR, is not the only small-animal molecular imaging system available. However it's unique detection system, which theoretically would enable sub-millimetre resolution to be achieved, made it a promising system for studies in this project where, for comparison with the autoradiography studies described in Chapter 4, visualisation of the relatively small structures of the CBF was desired.

Whereas the initial studies with the TOHR revealed a number of challenges would have to be overcome before it would be suitable for such imaging, the final studies performed showed the TOHR to eventually be suitable for this imaging. Extrapolating from the results of the ^{123}I-DaTSCAN study (Figure 28) and the other *in vivo* images, the TOHR reached a point, immediately prior to its crash and the laboratory's subsequent decision to suspend studies with the TOHR, where it showed the capability to perform the studies envisaged for the continued biological evaluation of the two RPs used in this project. If such imaging were to be performed and it was found one, or both, of the RPs to be able to delineate the cholinergic denervation caused by the SAP lesion; and the correlation of the magnitude of this deficit measured *in vivo* with the magnitude of the deficit measured with an *in vitro* technique, such as described in Chapter 4, was high (ie the *in vivo* data was validated by the *in vitro* data); then it would provide a strong rationale for the use of that (or those) RP(s) in human studies.

As discussed earlier (p. 177), both of the RPs (or analogues thereof) used in this project have been used previously in *in vivo* research studies in humans. The results of these studies have generally shown them able to demonstrate the expected distribution, and the expected deficit, following molecular imaging. The implications of the studies described in this project therefore go, as they must, further than simply providing justification for the use of the two RPs described for molecular imaging. If small-animal molecular imaging studies are performed, in

continuation of the work described in Chapters 2 through 4 and founded upon the work described in Chapter 5, then the correlation of data derived from *in vivo* studies with the gold-standard of *in vitro* derived biochemical data, in addition to behavioural data, may be systematically validated. Thus conclusions regarding the actual biochemical environment within a subject, inferred from data provided by *in vivo* molecular imaging such as discussed above, may be drawn more confidently.

As described earlier, molecular imaging of the cholinergic system in AD has the potential to advance both research into this disease as well as the clinical management of patients. Specific potential benefits of molecular imaging with the RPs *I-A-85380 and *IBVM in AD have been highlighted in a few published studies. For example, in a study of *post-mortem* brain tissue from patients with AD, Parkinson's disease, dementia with Lewy bodies and vascular dementia, ^{125}I-A-85380 binding was found to be able to discriminate between the different dementias (Pimlott *et al.*, 2004). If this RP can provide a similar differentiation in a clinical situation it would obviously impact greatly on patient management. Whether the results of that study can be translated to molecular imaging is difficult to predict though as the reported technique would require the imaging system to be able to delineate very small regions, ie to separate the caudate and the putamen, the external and internal globus pallidus and various thalamic nuclei. The general potential utility for imaging of the nAChR in AD has also been well reviewed elsewhere (Court *et al.*, 2001; Nordberg, 2001; Paterson *et al.*, 2000; Pimlott *et al.*, 2004; Warpman *et al.*, 1995; Whitehouse *et al.*, 1986).

In a research setting, molecular imaging with ^{123}IBVM has been used, as described earlier, to image differences in its binding in subjects with AD, Parkinson's disease and normal aging (Kuhl *et al.*, 1996). Additionally, it has been used to investigate whether there is a cholinergic dysfunction associated with the morbidities in multiple-system atrophy (Gilman *et al.*, 2003a; Gilman *et al.*, 2003b) and the effect on the cholinergic system of gonadal hormone therapy (Smith *et al.*, 2001). Though ^{123}IBVM has provided positive results in all these studies, a search of the literature suggests it is relatively under-utilised in the research setting. Given the many disease states in which the cholinergic system has been implicated, and given the apparently very good ability of *IBVM to target this system, it is hoped that the validations offered by the studies performed and proposed in this project may encourage further work with this RP.

Besides for RPs targeting the cholinergic system, the techniques used in this project (senorimotor test, NORT, *ex vivo* and *in vitro* autoradiography, molecular imaging) would be useful for evaluating and developing RPs which target a number of other systems as potential molecular imaging agents for AD. As well as the cholinergic system, it is known that the serotonergic, noradrenergic and histaminergic systems are also severely impaired early in the course of AD (Bondareff *et al.*, 1987; Fernandez-Novoa *et al.*, 2001; Higuchi *et al.*, 2000; Schmitt, 2005; Yang *et al.*, 1999). The development of well-validated molecular imaging techniques for

aspects of these other systems would serve, alongside techniques for imaging the cholinergic system, to build a more complete biochemical picture of AD.

6.3 Conclusion

In conclusion, the studies performed in this project and described here have:

- added support to current evidence that the cholinergic system is critical to the maintenance of normal attentional function, using a technique designed to reduce extraneous variables stemming from rule-learning or reward-based behaviour;

- added to the evidence that the cholinergic system, rather than being directly responsible for the mnemonic deficit characteristic of AD, may modulate it indirectly and in collaboration with other transmitter systems;

- demonstrated the excellent properties of two cholinergic RPs with potential utility in the molecular imaging of AD, with reference to the behavioural deficits manifested in the same animal models and;

- have demonstrated the potential of a novel small-animal molecular imaging system, founded upon a unique principle, to achieve the necessary characteristics to continue the evaluation of such RPs as described in this

project, in a manner where the results of the imaging may be correlated with and validated by the studies preceding it such as those described in this project.

Overall, it has been found that the SAP model demonstrates behavioural abnormalities as a result of lesions of the cholinergic system, the biochemical abnormalities within these animals can be detected and measured with the RPs ^{123}IBVM and ^{125}I-A-85380, and that the small-animal molecular imaging system TOHR has the performance characteristics essential to the *in vivo* detection of these RPs.

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