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**Attentional effects of modulation  
of cholinergic function**

**A thesis for the degree of PhD**

**Submitted September 2003**

**by**

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# Abstract

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Few would disagree with the claim that the neurotransmitter acetylcholine is involved in the control of attention. The studies reported in this thesis investigate the effects of direct and indirect manipulations of cholinergic function on attention in rats.

Chapters II and III investigate the effects of manipulation of cholinergic function through systemic administration of one of two, non-cholinergic agents, on covert orienting of attention. There is evidence that both the serotonergic (5-HT<sub>6</sub> receptor-selective) antagonist SB-271046 (Chapter II) and prolactin releasing peptide (PrRP) (Chapter III) have cholinergic-modulating properties. Neither SB-271046 nor PrRP administration affect covert orienting in rats, suggesting, and supported by other recent studies, that these administrations do not modulate cholinergic function in brain regions relevant to task improvement.

Chapters IV and V investigate the effects of cholinergic denervation (Chapter IV) and serotonergic denervation (Chapter V) on another type of attention, namely attentional set-shifting. Selective cholinergic lesions of basal forebrain (BF) do not induce performance deficits in attentional set-shifting as are observed after non-selective lesions of regions receiving BF neuronal projections, suggesting that such

deficits are due to non-cholinergic neuronal loss. Selective serotonergic lesions also did not result in improvements in performance in attentional set-shifting as was observed after administration of the 5-HT<sub>6</sub> antagonist, SB-271046, (Hatcher *et al*, 2002), suggesting that serotonergic influence on the task performance is more complex.

These studies demonstrate the complexity of the neuronal mechanisms underlying attention, providing evidence for requirement of future studies investigating attention to consider in their hypotheses that complicated neurotransmitter interactions, as well as several brain regions, may contribute to altered performance in behavioural tasks.

# Introduction

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## 1.1 Varieties of Attention

This thesis is an exploration of the neural mechanisms of attention. The experiments investigate specifically the effects of the neurotransmitter acetylcholine, be they direct effects or via interactions with other neurotransmitter systems.

This thesis will describe attention as a facilitation of information processing of some stimuli at the expense of others. Attention is not a unitary concept or process, and may be categorised along various lines. For example, attention can be conscious and goal-directed (i.e., directed as an executive function to a particular location in space or cognitive attribute of a stimulus) or it can be preconscious and automatic (i.e., elicited, often drawn towards a novel stimulus or location). Alternatively, attention can be categorised on the basis of differing psychological functions, such as selective attention, sustained attention, divided attention, orienting of spatial attention and attentional set. The first part of this introduction will consider the implications of the fact that there are many “varieties of attention” (see Parasuraman, 1984) for explorations of the neural basis of attention. Subsequent sections will consider the evidence for neuroanatomical dissociations.

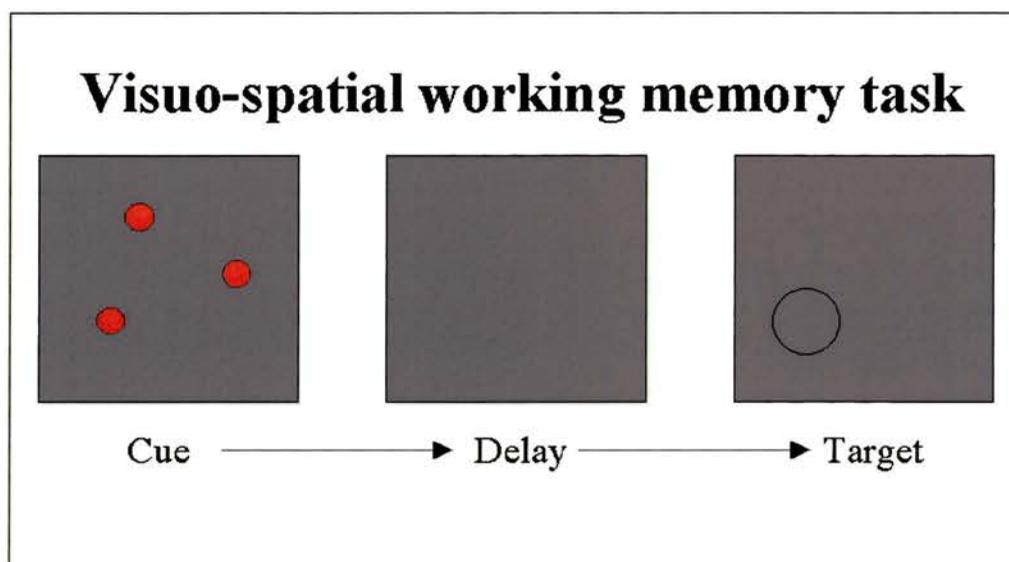
### ***1.1.1 Selective attention***

Selective attention is the process by which attention can be directed to a selected stimulus. It is most commonly investigated in the context of visual selective attention, with the subject required to attend to a feature/spatial location in a visual discrimination task. However, other tasks available for human studies can also involve language, and it is essential to use methods for investigating all forms of sensory input to establish whether attentional processes are common to different sensory modalities.

Selective attention permits the priority processing of relevant information without over-burdening the limited informational processing capacity of the brain. In the absence of such selection, the limited processing capacity of the brain would become overloaded. In goal-oriented behaviour, processing of information from the selected stimulus is enhanced via descending pathways from cortex; specifically, areas that deal with working memory and executive control (Goldman-Rakic, 1987). This form of attentional direction is also known as the “top-down” approach, although this term refers to the concept of the control rather than specifically to descending brain pathways (Sarter *et al.*, 2001). The nature of a stimulus can also affect selective attention and indeed the saliency, as in how much the stimulus stands out (pop-out) with regard to its surroundings, enhances the ability to attend to it. This can be described as a “bottom-up” process, whereby the nature of the stimulus enhances

the ability to detect it by triggering ascending neurons starting in the primary visual cortex. Details of these specific processes and their pathways will be discussed fully later.

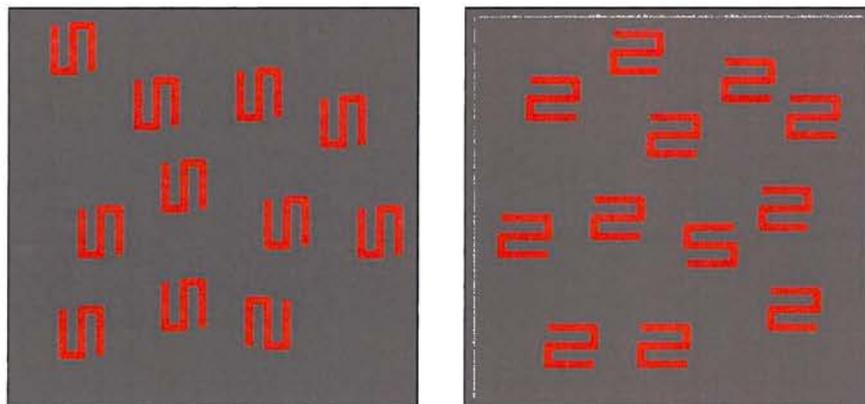
Selective attention is measured using visuo-spatial working memory tasks. Subjects can be instructed, or trained, to respond to a certain stimulus, permitting various manipulations of location of stimulus, combined with addition of distracting elements such as cues. One such example (Jonides *et al.*, 1993; see Figure 1.1) involves requiring the subject to observe the location of three dots on a screen. The dots were placed such that they appeared as if on the circumference of an imaginary circle. The dots would then disappear, and a probe circle would appear on the screen. The subjects were required to identify whether the probe circle had appeared in a location that would permit it to encircle one of the previous three dots. This can be manipulated, making it more difficult by applying a sequence to the task, and requiring the subject to remember several sets of locations before being required to recall one that had occurred previously (Smith *et al.*, 1996). Increasing the number of dots, the length of the sequence, or introducing other factors such as colour to the task increases the difficulty of the task, taxing the mechanisms mediating selective attention.



*Figure 1.1 A schematic of a simple visuo-spatial working memory task. The subject must identify whether the circle on the right screen could surround one of the red dots on the left screen*

Selective attention driven by stimulus salience is measured using tasks that require the subject to look for and identify a stimulus that is present amongst a number of distracting stimuli. Salience of the stimulus can be altered easily in visual tasks by changing the visual properties of the display (e.g., shape or colour). It should be noted that it is hard to separate the concept of stimulus salience driven selective attention from working memory driven selective attention, as subjects involved in tasks where they must identify a target from amongst a group of non-target stimuli must have some knowledge of the nature of the stimuli that they are looking for. Visual search tasks require the subject to identify a stimulus in a group of stimuli that is different from the rest. The salience of the stimuli involved can be manipulated, as in the case in Figure 1.2

## Visual Search Task



*Figure 1.2 A schematic of a visual search task. The subject must identify the “odd one out” on the screen. It is considerably easier to identify the odd stimulus on the right screen than it is on the left. This is because the stimuli on the right screen are recognisable as numbers, and thus the salience of the odd symbol is greater (adapted from Wang et al., 1994)*

where the stimuli on the right screen are difficult to tell apart as they have no significance out with the task. However, turn the stimulus by 90° and they become a stylised “2” or “5”. These stimuli have significance, and thus the salience, or conspicuity of the odd “number” increases, allowing for a faster reaction time to identify the odd stimulus (Wang *et al.*, 1994). It is also noted that, although the stimuli on the left screen may look similar to the capital letter “N”, when the stimuli are replaced by reverse “N”s and one normal “N”, performance is similar to distinguishing a “2” amongst “5”s (and vice versa). This is not the case for the reverse however, with distinguishing a reverse “N” from a group of normal “N”s proving considerably easier. This suggests that, certainly when

recognisable letters are being used, target object salience is enhanced by it being abnormal amongst a group of normal letters.

### ***1.1.2 Sustained attention***

Sustained attention is the ability to maintain attention over a period of time, and is often considered to be vigilance, or a state of alertness. Another term often used in conjunction with sustained attention and vigilance is “arousal”. It is likely that levels of arousal influence the performance of tasks that tax sustained attention, but the two terms are not interchangeable. Sustained attention is mediated primarily by a “top-down” mechanism, in that working memory drives performance through expectation and development of planning strategies (Sarter *et al.*, 2001).

Sustained attention is often tested using time delays in a variety of procedures that tax attentional or other cognitive functions. The most straightforward task involves presentation of a target stimulus a variable length of time after a cue, and subsequent measurement of reaction time: how long does it take from onset of the target stimulus for the subject to respond? Reaction time decreases as the foreperiod, or cue-target delay increases – up to a point. Thereafter, reaction time increases again. This can be taken as a measure of sustained attention, or vigilance; the cue alerts the subject to an impending target, with alertness gradually increasing as time passes. However, this state of alertness is not continuous, and degrades after sufficient time has passed (Posner and

Peterson, 1990). A cue also leads to anticipation of the target, and a subject that is aware of the nature of a task is more inclined to make anticipatory errors as cue-target delay increases. The subject anticipates the target's appearance as more likely the longer he has to wait for it. Capacity to sustain attention over a period of time can be manipulated through varying means. Moving from a simple to go/no-go reaction time task, (in which, after a ready signal, the subject may be presented with either a "signal" target that should be responded to – or a "non-signal" target to which a response must be withheld), increases the difficulty of the task, and taxes sustained attention. Likewise, increasing the rate of stimulus presentation, altering the likelihood of signal to non-signal ratio, promoting uncertainty as to spatial location of target as well as presenting dynamic stimuli all reduce capacity for sustained attention, resulting in reduced detection, or an increase in false detections. Furthermore, requiring the subject to divert his attention to another task that involves working memory, or presenting signals that require further processing also place greater demands on sustained attention (Sarter *et al.*, 2001). Human, monkey and rodent studies of sustained attention tend to focus on the visual or the auditory system. An example of a task that taxes auditory sustained attention would be to identify a target letter from a stream of letters presented over headphones to the subject. If the subject were to be performing a visual search task at the same time, performance in the sustained attention task would be impaired.

Tests of sustained attention do not depend exclusively on the measurement of reaction time to detection of the target. Anticipatory errors as well as errors of omission (the non-detection of a stimulus) are also informative. Indeed, as reaction time increases so detection rate decreases. The faster a subject's responses, the more likely he is to fail to detect a target. Errors of omission also increase over time, outwith factors of reaction time, and this is known as vigilance decrement. The vigilance decrement is often used as evidence that arousal and vigilance/sustained attention are separate psychological processes. Essentially, as a sustained attention task proceeds through time, vigilance decreases and performance lowers. It has been observed through electroencephalograph (EEG) studies that arousal also decreases over the course of a sustained attentional task. However, it has also been observed that arousal decreases over time during tasks where task performance does not (Parasuraman, 1984). Thus although there appears to be some connection between vigilance/sustained attention and arousal, there is good evidence that they are functionally separable.

There is further disagreement as to whether sustained attention and vigilance are usefully regarded as distinct, psychological and/or neural, processes. It has been argued that the decrease in stimulus detection in low frequency presentation tasks can be attributed to vigilance decrement, and is a result of "underload", whereas such decreases in high frequency presentation tasks are "overload" or fatigue based (for review see

Bushnell, 1999). Such low frequency tasks test vigilance, and higher frequency tasks are tests of signal detection. Accordingly, differing results are obtained from the two types of task.

In rats, a discrete trial signal detection task (McGaughy and Sarter, 1995; Bushnell, 1999; McGaughy *et al.*, 1999) involves pressing a lever after an auditory signal to obtain a food reward, or pressing an alternative lever if there is no auditory stimulus, again to obtain a food reward. Auditory signals are 500, 50 or 25ms in length, and signal/no signal trials are pseudo-randomly distributed over 162 trials (81 signal (with each of the three signal lengths presented 27 times pseudo-randomly) and 81 no signal trials). A trial starts with the extension of the two levers 2 seconds after signal or non-signal presentation. Rats had 4 seconds to respond before the levers were retracted. There is an inter-trial interval of  $9 \pm 3$  seconds. Rats' abilities to sustain attention can be challenged by altering rates of stimulus presentation, or by introducing distracting, irrelevant auditory cues. This task can also be used as a test of visual signal detection.

### ***1.1.3 Divided attention***

Divided attention is when attention is directed to more than one task, stimulus or location. For example, a subject might be asked to attend to one area of his visual field and perform a target detection task whilst simultaneously performing a visual discrimination task in another spatial

location (Braun, 1998). However, attention can also be divided between sensory modalities, such as visual and auditory. One common example of this in everyday human life is that of using a telephone whilst driving.

Of course, tests of divided attention presumably will also tax selective and sustained attention. It is often the case in tasks that test divided attention that at least one aspect of the task will require both selective and sustained attention. Thus, it may not be possible (or desirable) to make a distinction between divided and selective attention. For example, when distracters are applied to sustained attention tasks, divided attention is also involved. A subject is asked to perform a task that involves sustained attention, with the description of the task bringing in selective attention; the subject becomes aware of what is required of him during the task, and so begins to attend to that aspect of the task. If the subject is informed, and receives a description of, another task to perform during the sustained attention task, then it will divide his attention between the two. Thus the “top-down” processes that mediate selective attention are required, both to maintain attention and to search for the distracter task. “Bottom-up” processes may also be involved depending on the nature of the task (whether discrimination or simply detection is required) and the salience of the stimuli to be attended to.

In testing divided attention across the visual field, it is necessary to ensure that the process of divided attention is involved. It is possible

that attention could be shifted from one task to the other on certain trials (mixed strategy) or that it could be partially devoted to each task on all trials (pure strategy). It has also been argued that attention may be switched rapidly between tasks on each individual trial (see Braun, 1998 for review).

Rodent studies of divided attention have used a simultaneous temporal processing task, requiring the subjects to learn to lever press a fixed time after presentation of a stimulus. Trials can be either simple, with presentation of either a light or a tone as stimulus, or complex with both presented simultaneously. Presentation of the stimuli occurs randomly throughout the task. Activity (from Positron Emission Tomography) in the lateral agranular frontal cortex during this task suggests a role in cognition, supported by observations that lesions of this area result in impairment in task performance (Pang *et al.*, 2001).

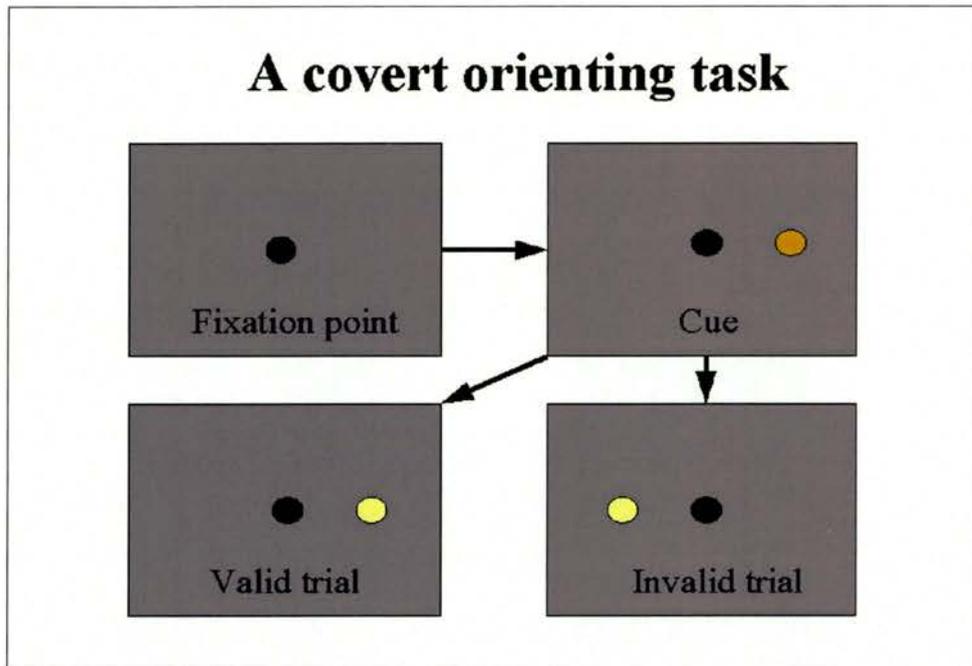
Similarly, Turchi and Sarter (1997) condition rats to respond to both tone and light, then present the subjects with three batches of trials; the first presents 20 trials of one modality (modality certainty or unimodal), the second presents 20 trials of the other modality, and the third presents 60 trials of both modalities (modality uncertainty or bimodal). Subjects are slower to respond to trials during the bimodal batch.

#### ***1.1.4 Orienting of attention***

Orienting of attention is the process where a subject ceases to attend to a currently attended stimulus, moves attention to another stimulus and engages there. As such, orienting of attention can be further split into overt orienting and covert orienting. Overt orienting of attention involves physical movement to bring senses to bear on the stimulus. This can involve actions as simple as moving the eyes to bring the fovea (the densest area of receptors on the retina; to foveate) onto a visual stimulus or to move the head for the same function. Humans and primates also move their heads to orient towards auditory stimuli, although other animals are capable of orienting their ears for this function. Animals (including rodents) which lack a discrete fovea move their heads rather than their eyes during overt visual orienting. Rodents also orient their heads to bring their papillae (whiskers) to bear on touch modality stimuli.

Covert orienting is the process wherein attention is oriented without any associated overt movement. The fovea represents the densest area of visual receptors on the retina and is the location at which most information about a visual stimulus is gathered, thus there does not seem to be a great advantage to 'covertly orient' for animals with a fovea. Nevertheless, Posner (1980) used cued simple reaction time tasks to investigate both overt and covert orienting of attention in human subjects. He established that depending on the nature of the cue and the target stimulus, orienting of attention to a stimulus would be either overt or

covert. Human subjects orient covertly to stimuli that are based on luminance. Thus subjects requested to foveate upon a central visual stimulus direct their attention to target stimuli with luminescence as their most salient feature using covert orienting. Subjects were presented with various forms of visual stimuli, and either cued to the location the target would appear in (valid cue) or away from the location (invalid cue) (see Figure 1.3). It was observed that subjects cued to (or away from) a location with a dim light preceding a brighter target light oriented covertly even when requested to orient overtly. Stimuli based on salient features other than luminescence induce overt orienting, probably due to processing requirements that the fovea best suits. Furthermore, reaction times to validly cued targets are faster than those to invalidly cued targets, creating the “validity effect”, a term coined by Posner to describe the processing cost of an invalid cue on attentional orienting.



*Figure 1.3 A schematic of a covert orienting task. The subject must foveate on the fixation point and await the dimly lit cue light. A variable time after the onset of the cue (foreperiod or cue-target delay) a bright target light will appear either in the location of the cue light (valid trial) or away from it (invalid trial)*

Tests of rodent attentional orienting commonly use overt orienting in the form of the five choice serial reaction task (5CSRT). This task presents the subject with five holes in an operant box, which the subject must nose-poke when lit. There are many elements of the task that can be manipulated, including duration of stimulus presentation and rate of stimulus presentation (Robbins *et al.*, 1989; McGaughy *et al.*, 2002). More recently a rodent version of Posner's covert orienting task has been developed (Ward and Brown, 1996). Rats are trained to maintain a nose-poke before being presented with a dimly lit cue to one of either side. A variable time after cue onset a target light appears on either of the two

sides and the rat must then withdraw and nose-poke the lit hole to obtain a food reward.

Covert orienting can be argued to be a better measure of attentional orienting in rodent studies that rely on light as stimuli as the task requires a nose poke to initiate each trial. Data from the 5CSRT, and other orienting tasks that do not account for the rat's position at trial initiation, may be affected by inattention when the trial begins.

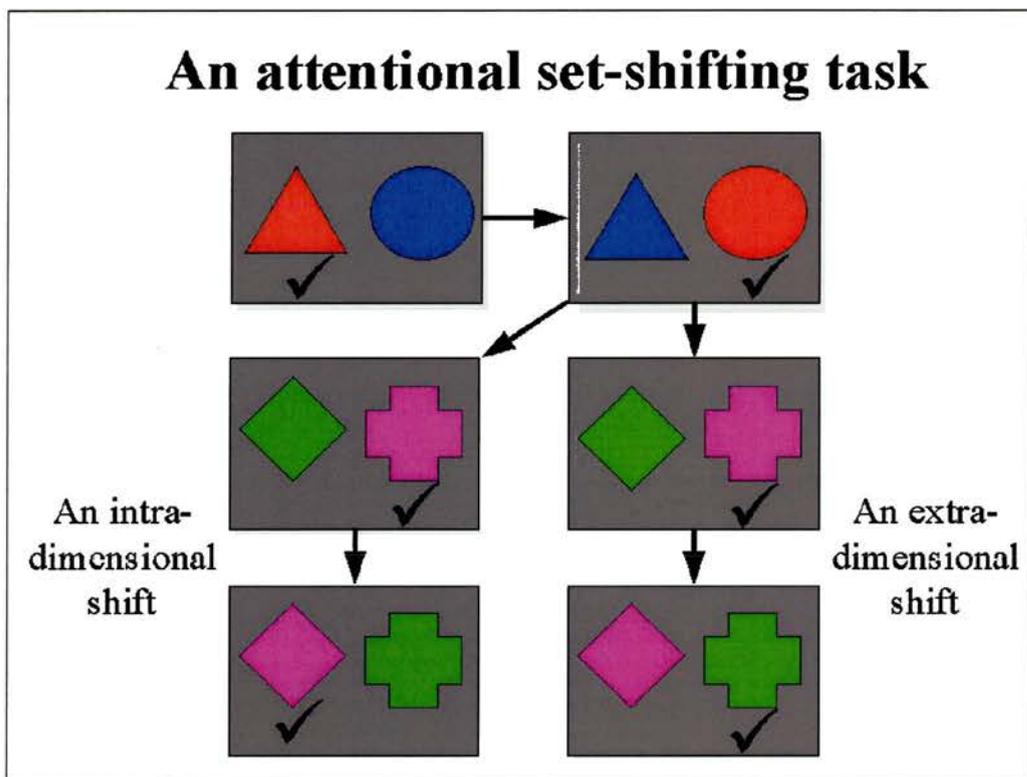
#### ***1.1.5 Attentional set***

Attentional set is a predisposition to attend to a stimulus or stimulus feature. Complex stimuli are more rapidly discriminated if attention is directed to a particular, relevant aspect of them. Thus in a visual discrimination task in which a subject must detect and respond to a particular colour, when presented with a series of complex stimuli varying in, for example, both shape and colour, responses are more rapid if the subject attends selectively to the colour and ignores the shape of the stimuli. In everyday life, it is easier to scan a crowd for someone wearing a red hat than to consider all the features of the searched for individual. This in itself is not attentional set, but simply an example of selective attention, involving both working-memory (selective attention driven by an executive decision) and salience of the stimulus (selective attention driven by the conspicuity of the stimulus). Attentional set arises when a category is attributed to the sensory feature and attended to (Roberts *et*

*al.*, 1988). Thus in the above task with red as the attended to sensory feature, the attentional set would be for colour. It is possible to demonstrate the formation of an attentional set by testing a subject's ability to shift attention within and between stimulus dimensions. In the above example, a subject is rewarded every time they select red (correct) in the series of presented stimuli. They are reinforced to ignore blue and, for example, square and triangle when presented with a series of coloured shapes. If the two colours and two shapes were changed, and the subject asked to indicate which is correct, he would most likely select one of the two new colours. If the subject's initial choice of colour was incorrect, then he would select the other colour as correct. This would be an intra-dimensional (ID) shift of attentional set: the new rewarded feature is in the same sensory dimension as the previously rewarded one. A subject performing a series of such discriminations is thrown, if, during a shift, one of the shapes becomes the rewarded feature: this is an extra-dimensional (ED) shift (see Figure 1.4). This reduced performance after an ED shift demonstrates the existence of a category of sensory feature attended to, or, in other words, attentional set (Roberts *et al.*, 1988).

As formation, maintenance and shifting of set require selective attention, they are also likely influenced by “top-down” pathways in the brain that govern directed selective attention. Studies have demonstrated the involvement of the prefrontal cortex in marmosets (see Chapter IV for review; Dias *et al.*, 1996) and in rats (Birrell and Brown, 2000). The

marmoset version of the task uses a monitor to display complex visual stimuli that can be discriminated by two features: shapes and lines. Marmosets are reinforced with food reward to touch the screen to indicate the correct stimulus. The rat version of the task presents the rat with complex stimuli that can be discriminated by two factors: scent and digging medium. Rats are reinforced to dig in the correct bowl by the presence of a food reward at the bottom of the correct bowl.



*Figure 1.4 A schematic of an attentional set-shifting task or ID/ED task. The subject must identify which of the stimuli is correct based on previous knowledge. In this example, initially red is correct, making the attended to dimension colour. In the new discrimination, the subject must again decide which is correct. If he follows the original rule, he will select either green or pink. The intra-dimensional shift on the left shows an example where the original rule stays the same and pink is correct. However, the example on the right is of an extra-dimensional shift, and the subject would get the second trial wrong if he followed the original rule, and continued to respond to colour (the “✓”s are only present in the schematic to show which is correct in this example).*

A commonly used task in humans that utilises the concept of attentional set is the Wisconsin Card Sort Test (WCST; Milner, 1964). The WCST presents the subject with a pile of cards bearing a number of coloured shaped stimuli. The cards can be sorted according to three rules: number of stimuli, colour of the stimuli or shape of the stimuli. During the card sorting process the rule can change and the subject must adapt his strategy accordingly. The WCST and ID/ED tasks have been regularly used as a measure of executive function and working memory in patients with neurodegenerative disorders (Paolo *et al.*, 1995; Gauntlett-Gilbert *et al.*, 1999), frontal cortex damage (Barceló and Knight, 2002), obsessive compulsive disorder, schizophrenia, unipolar depression (Moritz *et al.*, 2002) and epilepsy (Igarashi *et al.*, 2002).

## **1.2 Neural Systems and Pharmacology of Attention**

The concepts of “top-down” and “bottom-up” have already been mentioned in the descriptions of attention above, but these terms only barely cover the complexity of the systems involved, and are used to define forms of attention as controlled by executive function (in the case of “top-down”) or by autonomic responses to perceived stimuli (in the case of “bottom-up”).

The neural basis of attention is attributed to the cholinergic system of the basal forebrain (BF) and the terminal regions to which these

neurons project. However, it is clear that other regions of the brain are involved, with the most obvious being those areas that project to BF, as well as those that influence BF neuron terminal regions. In order to understand what is currently known about the pharmacology of attention, it is necessary to look not only at the anatomy of the brain regions involved in attention, but also at the mechanisms of control that those regions exert over attentional function.

### *1.2.1 Anatomy of acetylcholine systems*

The cholinergic neurons of the basal forebrain (BF) project to the cortex, the thalamus, the thalamic reticular nucleus (Rt), the amygdala and the hippocampus. However, BF itself is composed of several nuclei, all with different afferents and efferents. The nuclei that make up BF are defined here as those being predominantly cholinergic. The cortex is also divided into multiple areas, each again with their own role, afferents and efferents, as well as containing differing layers of cells within these areas. The thalamus likewise contains several nuclei, and Rt projects topographically to them, as well as receiving input from cortex.

It is worth noting that other areas of the brain also contain cholinergic neurons: the caudate putamen, nucleus accumbens and the olfactory tubercle are areas rostral and lateral to BF. Rather than consist of projection neurons, these cholinergic neurons are interneurons (Woolf, 1991), receiving glutamatergic projections from the neocortex, and

dopaminergic projections from the substantia nigra, and only participating directly in local circuits. Other cholinergic projection neurons do exist, arising in the laterodorsal and pedunculo-pontine tegmental nuclei. The projections of these neurons will be discussed in relation to BF cholinergic neurons and their projections.

Within BF can be identified the medial septum (MS), and horizontal and vertical limb of the diagonal band of Broca (HDB and VDB) which collectively are referred to as the septal complex. The magnocellular BF consists of the nucleus basalis of Meynert (Magnocellularis in rats; nbM), the substantia innominata (SI) and the magnocellular preoptic nucleus (MCPO) (Semba, 2000). The ventral pallidum is also considered part of BF due to the cholinergic nature of its neurons.

The MS receives afferents from the medial prefrontal cortex (mPFC) (Gaykema *et al.*, 1991) with reciprocal cholinergic projections to mPFC (Mesulam *et al.*, 1983). Afferents originating in the medial hypothalamus also synapse with cholinergic cells in MS (Cullinan and Zaborszky, 1991) as do those from the posterior hypothalamic nucleus (Vertes *et al.*, 1995) and median raphe nucleus (Azmitia and Segal, 1978; Hjorth and Sharp, 1991). The MS also receives afferents from locus coeruleus, currently considered to be noradrenergic (Berridge and Foote, 1996), although previously thought to be glutamatergic and/or

aspartatergic (Carnes *et al.*, 1990). Other than mPFC, the MS also has cholinergic projections to hippocampus (Ferencz *et al.*, 2000), visual cortex (Calarco and Robertson, 1995) and to posterior lateral hypothalamus, although the majority of MS projections to lateral hypothalamus are GABAergic (Gritti *et al.*, 1994).

The VDB also receives projections from the mPFC and, as with MS, has reciprocal cholinergic projections back to mPFC (Mesulam *et al.*, 1983; Gaykema *et al.*, 1991) The medial portion of the HDB also receives projections from mPFC, with the majority of HDB projections from PFC arising in orbital prefrontal cortex (Gaykema *et al.*, 1991). In addition, both VDB and medial HDB receive projections from medial hypothalamic cells, with HDB and MCPO also receiving afferents from medial lateral hypothalamus (Cullinan and Zaborszky, 1991), and both VDB and HDB receive projections from posterior hypothalamus (Vertes *et al.*, 1995) and the medial ventral tegmental area (Gaykema and Zaborszky, 1996). There are moderately dense, presumably serotonergic, projections from the dorsal raphe nucleus to HDB, VDB and MCPO (Vertes, 1991; Vertes *et al.*, 1999) and noradrenergic projections from locus coeruleus (Berridge and Foote, 1996), caudal nucleus tractus solitarius and caudal ventrolateral medulla (Senatorov and Renault, 1999).

Other than its cholinergic projection to hippocampus (Ferencz *et al.*, 2000), VDB has similar, mostly cholinergic, efferent connections to HDB. Both project to visual cortex (Dinopoulos *et al.*, 1989), the thalamic nuclei, including the Rt, and the zona incerta (ZI) (Kolmac and Mitrofanis, 1999). As with the MS and MCPO, however, there are projections, mostly GABAergic with some cholinergic, to posterior lateral hypothalamus (Gritti *et al.*, 1994). The HDB-MCPO also projects to the piriform cortex (Rosin *et al.*, 1999) and sends GABAergic projections to the olfactory bulb (Paolini *et al.*, 1996).

The predominantly cholinergic nbM (see Tables 1.1 and 1.2 and Figure 1.5) receives projections from laterodorsal tegmental nuclei and pedunculopontine tegmental nuclei (Semba and Fibiger, 1992; Rasmusson *et al.*, 1994)), orbital, medial prefrontal, and agranular insular cortex (Gaykema *et al.*, 1991; Carnes *et al.*, 1990), dorsal raphe nucleus (Gasbarri *et al.*, 1999), hippocampus, locus coeruleus, and lateral septum (Carnes *et al.*, 1990).

In turn cholinergic efferents originating in nbM project to, as with V/HDB, the various thalamic nuclei, including Rt (Kolmac and Mitrofanis, 1999), various subdivisions of the primary and secondary somatosensory and primary motor cortices (parietal cortex) (Mesulam *et al.*, 1983; Baskerville *et al.*, 1993), the visual cortex (Dinopoulos *et al.*,

1989) and the amygdala (Bergersweeney et al., 1994; Heckers and Mesulam, 1994; Ferencz et al., 2000).

*Table 1.1 Projections to the various basal forebrain nuclei with corresponding neurotransmitters known or suspected to be involved.*

<b>Nucleus</b>	<b>Origin of BF afferents</b>	<b>Afferent cell type</b>
<b>Medial septum</b>	Medial prefrontal cortex (Cingulate areas 1 and 2 and prelimbic cortex)	Glutamatergic
	Medial hypothalamus	
	Posterior hypothalamus	Serotonergic
	Locus coeruleus	Noradrenergic
	Median raphe nucleus	Serotonergic
<b>Vertical limb of the diagonal band</b>	Medial prefrontal cortex	Glutamatergic
	Medial hypothalamus	
	Posterior hypothalamus	
	Locus coeruleus	Noradrenergic
	Dorsal raphe nucleus	Serotonergic
	Ventral tegmental area	Dopaminergic
<b>Horizontal limb of the diagonal band</b>	Medial prefrontal cortex (medial HDB only)	Glutamatergic
	Medial hypothalamus (medial HDB only)	
	Posterior hypothalamus	
	Orbital prefrontal cortex	Glutamatergic
	Medial lateral hypothalamus	Neurotensinergic
	Dorsal raphe nucleus	Serotonergic
	Ventral tegmental area	Dopaminergic

<b>Nucleus basalis of Meynert (Magnocellularis)</b>	Laterodorsal tegmental nuclei	Cholinergic
	Pedunculopontine tegmental nuclei	Cholinergic and Glutamatergic
	Hippocampus	
	Amygdala	
	Locus coeruleus	Noradrenergic
	Orbital, medial prefrontal, and agranular insular cortex	Glutamatergic and/or aspartatergic
	Lateral septum	
	Dorsal raphe nucleus	Serotonergic
Ventral pallidum		
<b>Ventral pallidum</b>	Nucleus accumbens	GABAergic
	Substantia nigra pars compacta	Dopaminergic
<b>Magnocellular preoptic nucleus</b>	Medial lateral hypothalamus	Neurotensinergic
	Dorsal raphe nucleus	Serotonergic
	Ventral pallidum	
<b>Substantia innominata</b>	Medial lateral hypothalamus	Neurotensinergic
	Far lateral hypothalamus	Neurotensinergic
	Dorsal raphe nucleus	Serotonergic
	Lateral ventral tegmental area	Dopaminergic
	Substantia nigra pars compacta	Dopaminergic
	Medial and ventral prefrontal, insular and piriform cortices	Glutamatergic
	Ventral pallidum	

*Table 1.2 Projections from the various basal forebrain nuclei with corresponding neurotransmitters known or suspected to be involved.*

<b>Nucleus</b>	<b>Terminal region of BF efferents</b>	<b>Efferent cell type</b>
Non-specific basal forebrain	Medial prefrontal cortex (Cingulate areas 1-3)	Cholinergic, GABAergic and non-cholinergic/GABAergic
Medial septum	Medial prefrontal cortex Visual cortex Posterior lateral hypothalamus  Hippocampus	Cholinergic Cholinergic GABAergic (with some Cholinergic)  Cholinergic
Vertical limb of the diagonal band	Medial prefrontal cortex Visual cortex Posterior lateral hypothalamus  Thalamic reticular nucleus Intralaminar, midline and mediodorsal nuclei of dorsal thalamus Zona incerta Hippocampus	Cholinergic Cholinergic GABAergic (with some Cholinergic)  Cholinergic Cholinergic Cholinergic Cholinergic
Horizontal limb of the diagonal band	Posterior lateral hypothalamus  Thalamic reticular	GABAergic (with some Cholinergic)  Cholinergic

	nucleus Intralaminar, midline and mediodorsal nuclei of dorsal thalamus Zona incerta Medial prefrontal cortex Piriform cortex Visual cortex	Cholinergic  Cholinergic Cholinergic Cholinergic
Nucleus basalis of Meynert (Magnocellularis)	Parietal Cortex (Primary and secondary somatosensory and primary motor cortices) Prefrontal Cortex (Agranular insular cortex) Visual cortex Amygdala Thalamic reticular nucleus Intralaminar, midline and mediodorsal nuclei of dorsal thalamus Zona incerta	Cholinergic   Cholinergic  Cholinergic Cholinergic Cholinergic  Cholinergic Cholinergic
<b>Ventral pallidum</b>	Magnocellular basal forebrain Ventral striatum	

<b>Magnocellular</b>	Piriform cortex	Cholinergic
<b>preoptic nucleus</b>	Posterior lateral hypothalamus	GABAergic
	Olfactory bulb	GABAergic
<b>Substantia innominata</b>	Thalamic reticular nucleus	Cholinergic
	Intralaminar, midline and mediodorsal nuclei of dorsal thalamus	Cholinergic
	Zona incerta	Cholinergic

The SI, in similar fashion to the HDB receives afferent input from the medial lateral hypothalamus, as well as from the far lateral hypothalamus (Cullinan and Zaborszky, 1991; Morin and Beaudet, 1998), dorsal raphe nucleus (Vertes, 1991), the lateral VTA and the substantia nigra pars reticular (Gaykema and Zaborszky, 1996) (although some of the projections from VTA and substantia nigra terminate on parvalbumin-immunoreactive neurons (Gaykema and Zaborszky, 1997) and are possibly GABAergic). The SI also receives limited afferent input from the medial and ventral prefrontal, piriform and insular cortices (Zaborszky *et al.*, 1997)

As with much of the BF, the SI projects cholinergic efferents to thalamic nuclei, again including the Rt and the ZI (Kolmac and Mitrofanis, 1999). It is also worth noting that the SI and the nbM have in the past been considered a single nucleus, and that projections to and from

what is now identified as the nbM may have synapses with, or be projections from SI neurons.

The ventral pallidum receives projections from the nucleus accumbens (Zaborszky and Cullinan, 1992) and substantia nigra pars compacta (Gaykema and Zaborszky, 1996) and is described as projecting to the magnocellular BF (Carnes *et al.*, 1990), although the type of neuron involved is not known, only that they are neither glutamatergic nor aspartergic. Ventral pallidum also sends non-cholinergic projections to ventral striatum (Kuo and Chang, 1992)

It is likely that there are non-cholinergic/non-GABAergic projections from the BF to the various terminal regions already mentioned. Both medial prefrontal cortex and parietal cortex receive afferents from BF, some of which are cholinergic, some of which are GABAergic and some are neither. Other than tracing studies, in which neuronal labelling is observed after injection of retrograde tracer into terminal regions, the majority of investigations of the BF's projections come from lesions with an immunotoxin selective for a receptor mainly found on the BF's cholinergic neurons.

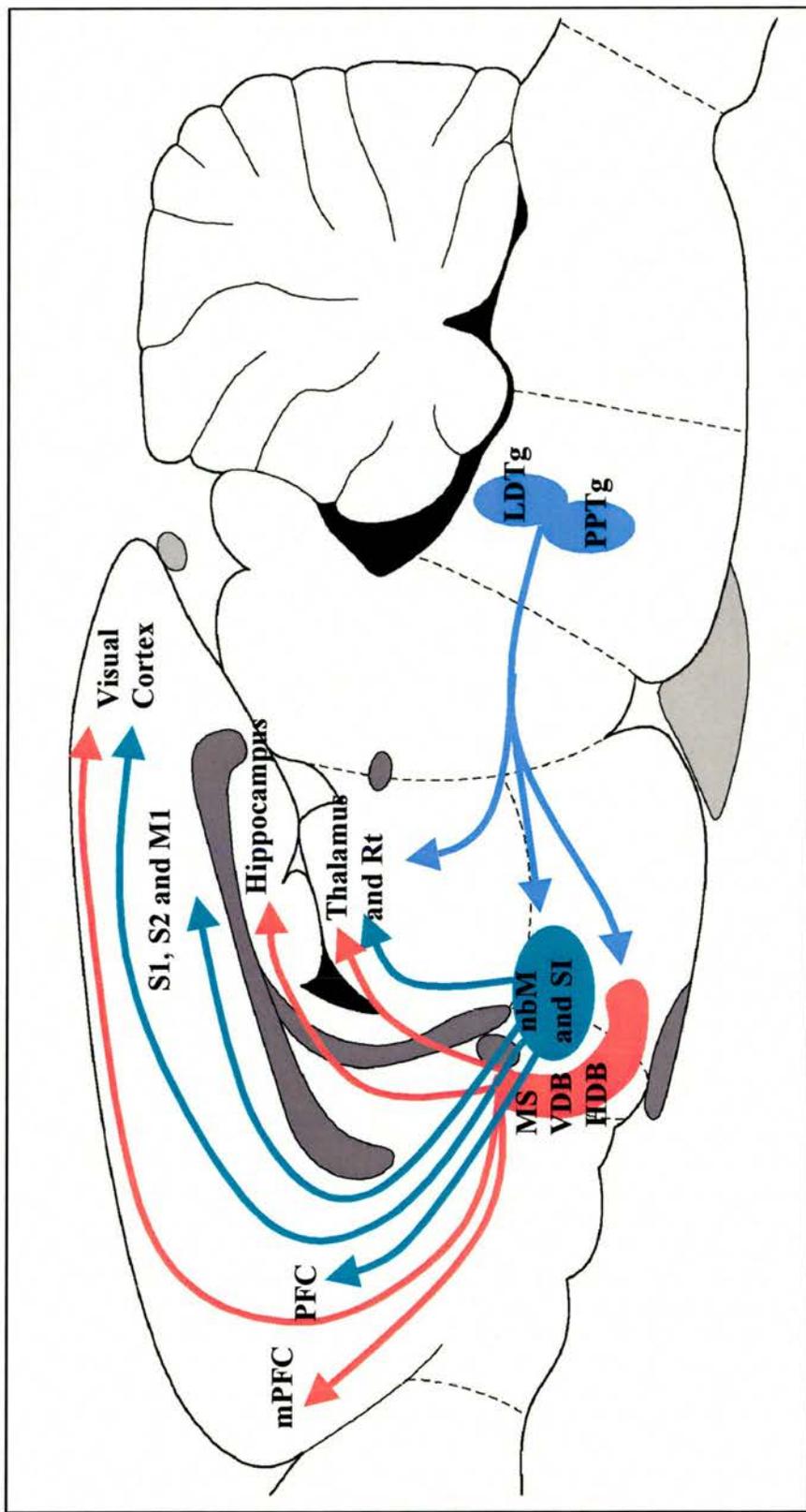


Figure 1.5 Sagittal schematic of the rat brain showing cholinergic projection pathways important in attentional function. The basal forebrain (BF) consists of the nucleus basalis of Meynert (Magnocellularis) (nbM), the substantia innominata (SI) and the septal complex: medial septum (MS), vertical and horizontal limb of the diagonal band of Broca (V/HDB). The BF projects to prefrontal cortex (PFC, including mPFC), primary and secondary somatosensory and primary motor cortex (S1, S2 and M1; also known as parietal cortex), thalamus and thalamic reticular nucleus (Rt) and visual cortex. The laterodorsal and pedunculopontine tegmental nuclei (LDTg and PPTg) also send cholinergic projections to BF (adapted from Paxinos and Watson, 1998 and Woolf, 1991).

In considering the anatomy of BF, it is necessary to isolate those nuclei with predominantly cholinergic projections to the areas of cortex and thalamus that are reported as involved in attentional function. Neither MCPO nor ventral pallidum have cholinergic projections to either cortex or thalamus, so their contribution to cholinergic modulation of attentional function, if any, is likely to be indirect.

The mPFC receives considerable innervation from BF, as well as sending glutamatergic projections back to BF as has already been described. The PFC is defined as areas of the cortex with reciprocal projections to the medial dorsal nucleus (MD) of the thalamus, and comprises of the mPFC (prelimbic area, cingulate areas 1-2, the agranular insular cortex) and the ventral, dorsal, medial and dorso-lateral orbital cortex (Zilles and Wree, 1995), although these areas of cortex also project to other thalamic nuclei. Projections from thalamus to PFC are glutamatergic or aspartatergic in origin (Pirot *et al.*, 1994), as are those from PFC to thalamus. Further, projections from thalamus to PFC are topographically organised, so that those from central and medial areas of MD project to lateral orbital cortex and agranular insular cortex, whilst the more medial MD projections terminate in caudal areas of PFC (Ray and Price, 1992; Ray *et al.*, 1992). Likewise, reciprocal projections from PFC to thalamus are topographically organised, with anterior cingulate and prelimbic cortices projecting to medial thalamic nuclei, and agranular

insular cortex projecting to rostral interlaminar nuclei, ventromedial and ventrolateral nuclei (Kuroda and Price, 1991; Vertes, 2002). It is also worth noting that the hippocampus (CA1 field), which receives cholinergic projections from MS and VDB, sends projections to the prelimbic and medial orbital areas of the PFC (Jay and Witter, 1991). These projections are distributed across the layers of the cortex, and so likely modulate cholinergic input to PFC from BF.

Other than the cholinergic projections from BF, cortex receives negligible cholinergic input from pedunculopontine tegmental nuclei and laterodorsal tegmental nuclei (Mesulam *et al.*, 1983).

The thalamus receives cholinergic projections from BF as well as from the hindbrain (Hallanger *et al.*, 1987). Thalamus also receives glutamatergic projections from PFC and parietal cortex (Wise and Jones, 1977), as well as GABAergic projections from Rt (Jones, 1975). The thalamus projects to PFC and parietal cortex with glutamatergic projections (Jones, 1985). From an anatomical point of view there are ascending projections from BF to cortex and thalamus, as well as descending projections from cortex to BF and thalamus. The Rt wraps around the thalamus receiving axon collaterals from both thalamo-cortical projections and cortico-thalamic projections (Jones, 1975).

There are, however, varying forms of cholinergic receptor subtype involved in these projections. Differing forms of both nicotinic (nAChR; at which nicotine is an agonist) and muscarinic (mAChR; at which muscarine is an agonist) receptors types have been identified and their distribution in the brain established. Central nAChRs are pentameric, consisting of five subunits. The subunits are designated either  $\alpha$  or  $\beta$ , with seven  $\alpha$  subunit types ( $\alpha 2-8$ ) and three  $\beta$  subunit types ( $\beta 2-4$ ) (Sargent, 1993). Distribution studies show that subunit types are expressed in specific areas of the brain. The  $\alpha 4\beta 2$  subunit combination is implicated in learning and memory, and is distributed both pre-synaptically and post-synaptically in the cortex, where nAChRs bind nicotine with high affinity (initial classification of nAChRs depended on affinity to ( $^3\text{H}$ )(-)-nicotine or ( $^{125}\text{I}$ ) $\alpha$ -bungarotoxin). Those containing the  $\alpha 7$  subunit have higher binding affinity to  $\alpha$ -bungarotoxin (for review see Levin and Simon, 1998; Court *et al.*, 2000). ( $^{125}\text{I}$ ) $\alpha$ -bungarotoxin binding is high in hippocampus, but also present in the cortex. The  $\alpha 7$  subunit is expressed post-synaptically in the Rt, where the  $\alpha 4\beta 2$  subunit is less heavily distributed. Despite this, the thalamus shows high density ( $^3\text{H}$ )(-)-nicotine binding, which is attributed to the presence of the  $\alpha 3$  subunit.

There are five classified mAChR subtypes (M1-5) in the rat, and five in the human (HM1-5). It is possible that there are further, yet to be differentiated mAChRs, as genes have been uncovered that code for up to nine mAChR subtypes (for review see Van der Zee and Luiten, 1999).

Localisation studies have demonstrated mAChRs expressed post-synaptically on both pyramidal cells and GABAergic interneurons in cortex and hippocampus, as well as pre-synaptically on MS and V/HDB cholinergic projection neuron terminals. Immunostaining studies suggest there are few mAChRs on nbM cholinergic neurons, although they are present on terminals from PPTg and LDTg. These data correlate with the idea that the majority of connections from PPTg and LDTg to nbM are glutamatergic in origin (Rasmusson *et al.*, 1994). This also suggests a low degree of autoregulation by nbM cholinergic projection neurons via mAChRs.

When considering the cholinergic system, it is important to remember that acetylcholine binds to all forms of these receptors, and that only during targeted manipulations of the cholinergic system (including smoking), or neurodegenerative disorders with specific patterns of degeneration (such as AD) do such delineations of receptor subtype become relevant.

### ***1.2.2 “Top-down” versus “bottom-up”***

Beyond the purely anatomical description of the basal forebrain (BF) cholinergic system, this section considers the means by which the cholinergic BF influences attentional function. The terms “top-down” and “bottom-up” have already been used to describe the psychological mechanisms of attentional control. The question that now must be

answered is how the described anatomy relates to the effects observed. The best means to do this is through analysis of central manipulations that modify attentional function.

The “top-down” form of attentional control relates to executive function and planning. It is involved in selective attention, permitting attention to be directed to relevant stimuli/tasks to which a subject may direct attention. “Top-down” refers directly to the modulation of central neural function through medial prefrontal cortex (mPFC) efferents to thalamus and BF and those areas’ subsequent modulations of each other and cortex (Sarter *et al.*, 2001). mPFC is considered the cortical region responsible for working memory and executive function. Specific deficits resulting from mPFC lesions will be discussed later, but to summarise at this stage, mPFC lesions impair performance in tasks such as attentional set-shifting that tax working memory in humans, monkeys and rats (Barceló and Knight, 2002; Dias *et al.*, 1996; Birrell and Brown, 2000), Data from electrophysiological (Levy and Goldman-Rakic, 2000) and microdialysis (Izaki *et al.*, 1998) studies support the involvement of mPFC in attentional function, demonstrating neuronal activation or increased neurotransmitter efflux when working memory is involved in a task. Thus means of examining and manipulating mPFC efferents to thalamus and BF, as well as the reciprocal efferents to mPFC provide valuable insight into attentional function and working memory.

The “bottom-up” form of attentional control refers to an autonomic control of attention, regulated by sensory cortical projections to thalamus. Rather than prior knowledge controlling attention, overriding stimuli direct attention. As mentioned previously, the salience, or conspicuity of a stimulus dictates the level of regulation of attention by this route.

In both cases that involve thalamus, the thalamic reticular nucleus (Rt) is also involved, receiving axon collaterals from both thalamo-cortical and cortico-thalamic pathways. As Rt projects only to thalamus, it is considered that its role is that of an attentional filter (Crick, 1984), in that it modulates thalamic function through both cortical influence and thalamic influence.

### ***1.2.3 Studying pharmacology***

Studies of how acetylcholine (ACh) modulates attentional function vary according to subject. There are methods that are available to study animal modulation of attention that are not available in studies of human attention. A general *in vivo* method that can be applied to all subjects is administration of selective agonists and antagonists of ACh central receptors. Behavioural tasks already described can then be used to observe induced impairments or improvements in attentional function. There are also *in vivo* visualisation techniques available, such as positron emission tomography (PET), magnetic resonance imaging (MRI) and

functional magnetic resonance imaging (fMRI). The complexity and expense of these methods lends them more to human and non-human primate work; there are other methods that can be used in rodents, although these are more invasive. However, as these techniques on rodents demonstrate functional homology/analogy between the primate brain and the rodent brain, MRI, fMRI and PET are increasingly used to study rodents. *In vivo* microdialysis combined with high pressure liquid chromatography (HPLC) permits delivery and measurement of minute amounts of extra-cellular chemicals in brain tissue using semi-permeable membranes and a constantly renewing concentration gradient. The study of electrophysiology is also available to study non-human pharmacology. This can be *in vitro* slice work, where-in electrodes are used to record synaptic potentials in slices of brain tissue after stimulation elsewhere in the slice. This permits a better understanding of excitatory and inhibitory effects mediated by projection neurons and interneurons. It is also possible to perform *in vivo* implantation of electrodes, again permitting recordings of synaptic potentials, but in a whole animal. This has obvious advantages over *in vitro* slice preparations in that data obtained from an intact animal is likely to be more informative than from a slice preparation. The converse of this is that *in vitro* slice preparations are easier to set-up and operate with.

There are several identified agonists and antagonists to the two main forms of cholinergic receptor. As mentioned previously, nicotine

non-selectively stimulates nAChRs, binding to the receptors and having the same effect as ACh. Nicotine is heavily studied in human subjects due to its presence in tobacco, and to nAChR expression alteration in disease such as Alzheimer's disease, Parkinson's disease and schizophrenia (Court *et al.*, 2000). Mecamylamine (Zevin *et al.*, 2000) and hexamethonium (Malin *et al.*, 1997) are both antagonists at nAChRs, in that they bind to the receptor and block it from either ACh or nicotinic agonists.

Muscarine non-selectively stimulates mAChRs in the same fashion as nicotine does to nAChRs. Atropine and scopolamine are antagonists at mAChRs, blocking them so that neither ACh, nor any muscarinic agonist can bind to the receptors. Unlike nAChRs, expression of cortical mAChRs is not as reduced in neurodegenerative disorders such as Alzheimer's disease, and natural aging. Nor do lesions of nbM in rats produce reductions in cortical mAChRs as are seen in nAChRs (Van der Zee *et al.*, 1999)

#### ***1.2.4 Human***

A vast wealth of information on human pharmacology of attention comes from the study of neuropathology. The neurodegenerative disease, Alzheimer's disease (AD), causes a reduction in basal forebrain (BF) neurons, which in turn impairs attentional function, as well as memory, in patients with the disease. The neuropathology of AD will be discussed in

a later section, although it should be considered that considerable study of the involvement of acetylcholine in attention stems from study of this disease and knowledge of the affected systems after autopsies of deceased subjects' brains.

Another important motivation behind and source of study of acetylcholine (ACh) pharmacology arises in smokers. It has already been mentioned that nicotine is present in tobacco, and so a variety of studies use chronic smokers as source material for analysis of ACh pharmacology. Mecamylamine has been studied for its efficacy in assisting in aiding stopping smoking. Transdermal administered mecamylamine significantly reduces cardioacceleration and adrenaline release induced by intravenously administered nicotine (Zevin *et al.*, 2000). Smokers and non-smokers have been used to explore the attentional and memory benefiting effects of nicotine. During an immediate and delayed verbal free recall study in which subjects smoked a nicotine containing cigarette, inhaling once between each of eight blocks of four presented words, nicotine enhanced performance in the immediate recall of the first block of four words and in the delayed recall of latter blocks. This demonstrates both an attentional facilitation as well as a post-learning facilitation of memory (in that subjects first self-administration of nicotine occurred after exposure to the first block of four words) (Warburton *et al.*, 1992). Further exploration of post-learning memory facilitation shows that it is attenuated when a subject is required

to perform a second, post-trial, attentional task prior to recall (Rusted and Warburton, 1992), suggesting that nicotine's effect in this task is to increase attentional processing capability. Further attentional tasks, in the same fashion as divided attention, reduce resources available for the primary memory task.

Chronic smokers participating in a covert orienting task after nicotine inhalation through a cigarette are able to orient their attention more quickly. Furthermore, reaction times to report targets after invalid cues are also decreased significantly as compared to those to valid cues (Witte *et al.*, 1997).

As to how this can be tied to BF pathways to cortex and thalamus, positron emission tomography (PET) observations of regional cerebral blood flow after intravenous nicotine administration show a decrease in the anterior cingulate cortex and an increase in the occipital cortex during a maze-solving task in both non-smokers and smokers (Ghatan *et al.*, 1998). This demonstrates the involvement of nicotine, and thus acetylcholine in these areas, and points towards a possible effect of nicotine in facilitating a change of strategy away from working memory based selective attention, and more towards saliency dependent, "bottom-up" driven attention.

Further evidence to suggest that nicotine improves saliency driven attention over selective, working-memory driven attention comes from administration of nicotine transdermally, via a patch, in the Stroop test, and an attentional flexibility task. The Stroop test requires the subject to identify a colour from two sets of displays: one bearing colour patches (control), the other bearing letters of one colour that spell out a different colour (interference). The flexibility of attention test requires subjects to fixate on a central target, either side of which will randomly appear a letter and a number. The subject must indicate with a response key whether the letter is on the right or the left. During the task, the subject will have to alter between identifying the side of the letter and that of the number. Nicotine is not observed to affect attentional switching in the flexibility task, although it is observed to decrease time taken to identify colours in both control and interference displays in the Stroop test. Thus nicotine is not observed to enhance the ability to switch between processing of one task to another, but is observed to potentiate processing of colour determination, even with interference from distracting letters (Mancuso *et al.*, 1999).

Further study of transdermal nicotine administration in non-smokers shows improvement of sustained attention in a Conner's computerised continuous performance test (subjects must depress a key as a response to the presentation of a target visual stimulus, but not rarer, non-target visual stimulus). In this task, vigilance decrement is attenuated,

as subjects after nicotine administration show decreased errors of omission (Levin *et al.*, 1998), with no corresponding increase in errors of false target identification, nor increase in reaction time to respond.

As nicotine is demonstrated to improve attentional function, so abstinence of nicotine in chronic smokers is observed to decrease attentional function. Electroencephalographs (EEG) permit the study of brain activation through recording electrical currents in the surface of the brain. Event-related potentials (ERPs) are time-differentiated voltage changes on the scalp that correlate with information processing. Non-smokers, smokers and 12 hour deprived smokers were tested on a serial-probe recognition task (subjects are presented with a series of sets of five five-letter words presented individually every 1800ms for about 300ms each; between each set a probe word is introduced and the subjects must identify with a key press whether it is from the last observed set (in-set) or not (out-of-set)). Both smokers and deprived smokers were observed to have a faster reaction time to the probe words than were non-smokers. Both smokers and deprived smokers were observed to be even faster if an in-set probe word matched the first or the last word of the set. From the EEG data, recorded P300s (a positive-polarity potential occurring between 250-750ms following onset of a meaningful event, assumed to measure mnemonic, attentional and decision making activity) were larger for in-set probes than out-of set probes in smokers, although this difference was less in the deprived smokers. Furthermore, latencies to in-

set probe P300s in smokers were shorter than those of non-smokers, and longest in deprived smokers (Pineda *et al.*, 1998).

Not all data observed from nicotine studies are consistent however, as some report that nicotine enhances working memory (Pineda *et al.*, 1998) whereas others report that nicotine facilitates saliency driven attention at the expense of working memory (Levin *et al.*, 1998; Mancuso *et al.*, 1999). It is clear from these observations that the effects of nicotine on attentional function are far from defined, and also that some tasks employed in the analysis of attentional function may utilise diverse attentional strategies that further complicate the understanding of nicotine's role in them.

However, as nicotine is observed to potentiate attentional function, so cholinergic antagonists are observed to attenuate attentional function. Scopolamine, the muscarinic antagonist, is observed to increase decision making in a visual search matching to sample task, as well as decreasing accuracy performance in a delayed matching to sample test of visual recognition memory (Robbins *et al.*, 1997). Scopolamine is also observed to induce deficits in a verbal free recall task (30 word lists and 10 word lists) that are not reversed by nicotine (Rusted and Eatonwilliams, 1991).

Systemic stimulation of cholinergic function through use of physostigmine, an anticholinesterase drug (inhibits acetylcholinesterase,

the enzyme that breaks down acetylcholine) has been used, in conjunction with scopolamine to visualise in a PET study (measuring cerebral blood flow with  $H_2^{15}O$ ) the effects of stimulation of nAChRs and mAChRs on brain activity in human patients exposed to flashed visual pattern stimuli. On its own, physostigmine stimulates cholinergic function in the brain, whilst in conjunction with scopolamine, only those processes mediated by nAChRs are potentiated. Therefore, regions with altered cerebral blood flow after administration of physostigmine can be attributed a role in cholinergic function. Combination of physostigmine and scopolamine allows dissociation between regions under the influence of nAChR activation and those under mAChR activation influence. mAChRs mediate synaptic activity in striate cortex and lateral visual association areas. nAChRs mediate synaptic activity in thalamic and inferior parietal lobules (Mentis *et al.*, 2001).

It is clear then from studies of human ACh pharmacology that attention and memory are mediated in some fashion by cholinergic function. As already stated, there is considerable further evidence for these observations in the study of the neurodegenerative disorder, Alzheimer's disease. There is also, however, considerable evidence amongst non-human animals that ACh mediates attentional function – both in primates and rodents.

### ***1.2.5 Animal research***

As mentioned, there are various ways in which the effects of acetylcholine (ACh) and agonists/antagonists to the cholinergic receptors can be measured in animals. Studies involving monkeys are the closest analogy to human studies, as basal forebrain (BF) structure and projections are very similar, with most brain regions receiving BF innervation sharing functional homology (Mesulam *et al.*, 1983).

As in human studies, nicotine administered to monkeys induces a reduction in reaction times in the covert orienting task to both valid and invalidly cued targets, with a reduction in validity effect caused by a larger reduction in reaction times to invalidly cued targets (Witte *et al.*, 1997). In the same study, atropine, the muscarinic antagonist, also reduced reaction times, but had no effect on the validity effect, an effect also reported in a human study where scopolamine was administered. In double-cued trials where no attentional orienting was required, atropine slowed reaction times. The authors suggest that these data support previously observed suggestions that nAChRs play a role in saliency driven attention, and that mAChRs play a role in alerting. Scopolamine administered to monkeys in this task slows reaction times to both valid and invalidly cued targets, with a disproportionate slowing of reaction time to validly cued targets. Scopolamine has no effect on reaction times to targets with no cued spatial information (Davidson *et al.*, 1999).

The selective agonist for nAChRs (human  $\beta 4$  subtype), SIB 1553A, improves accuracy in a delayed matching to sample task with a distracter in monkeys in the short delay interval, indicating a reduction in the effect of the distracter (Terry *et al.*, 2002) and providing further evidence to support the role of acetylcholine in attention and the similarities between central control of human and monkey attentional function.

The effects of scopolamine on a task can also be reversed by administration of the agonist, milameline (which has equal affinity to all five human mAChRs), to mAChRs. Milameline, administered to monkeys in combination with the acetylcholinesterase inhibitor, tacrine, reverses scopolamine induced deficits in a continuous performance task (Callahan, 1999).

*In vivo* microdialysis in rats has shown that muscarinic antagonists, such as atropine, induce a long term increase in cortical ACh (Quirion *et al.*, 1994). Antagonists (AF-DX 116, AF-DX 384 and AQ-RA 741) selective for the mAChR subtype, M2, potently stimulate cortical ACh release, and to a greater extent than antagonists selective for other mAChR subtypes. It can therefore be argued, combined with data from receptor distribution studies (Vilaró *et al.*, 1992), that a number of muscarinic M2 receptors are self-regulatory autoreceptors located pre-synaptically on BF ACh neurons. ACh binding to these receptors would

subsequently reduce ACh release, but with the muscarinic antagonists blocking the receptor, ACh continues to be released. Nicotinic agonists (nicotine and RJR-2403 (selective for the  $\alpha 4\beta 2$  subunit) also induce cortical ACh release, although to a lesser degree and for a shorter duration than the muscarinic antagonists (Summers *et al.*, 1996). This suggests a role for pre-synaptic nAChRs acting as autoreceptors, but potentiating ACh release rather than attenuating it.

From electrophysiological studies further data can be added to characterise the role of ACh in modulation of cortical function. In *in vitro* slices of the rat prelimbic area of mPFC, nicotine was observed to have no effect on resting membrane potential of layer II and layer III pyramidal cells. However, during stimulation of superficial layers, nicotine was observed to induce (an effect blocked by nicotinic antagonists) an increase in amplitude of monosynaptic excitatory post-synaptic potentials mediated by non-N-methyl-aspartate glutamate receptors in 14% of tested cells. In contrast, muscarine reduced potentials in 100% of cells tested (Vidal and Changeux, 1993). It is concluded that pre-synaptic cholinergic neurons are mediating the effects observed. These conclusions are supported by observations from an *in vivo* microdialysis study where nicotine induces an increase in extracellular prelimbic prefrontal cortex (PFC) glutamate. Furthermore, the same study utilised *in vivo* electrophysiological stimulation of medial-dorsal nucleus (MD) to show that nicotine potentiates short-latency responses, mediated by the MD-

Prelimbic pathway, and long-latency responses mediated by cortical pyramidal neuron activation (Gioanni *et al.*, 1999).

There have been numerous behavioural studies that use microdialysis to monitor ACh release during attentional tasks. Most have observed rats performing in a task that requires sustained, or divided attention. The five choice serial reaction time task (5CSRT) has been discussed already, and is employed to measure both divided and sustained attention. ACh release is increased in mPFC during performance of the task (Passetti *et al.*, 2000), although there is no observed correlation between ACh release and performance in the task. Likewise, in a sustained attention task during which subjects must lever press left or right according to the presence of a cue or not, there is an overall increase in ACh release in frontoparietal cortex. Furthermore, during a manipulation of the task resulting in an increase in attentional load, there was observed an initial reduction in ACh release, concurrent with reduced attentional effort as the subjects developed a response bias. During continued exposure to this increased demand on attentional function, the response bias attenuated, and ACh release increased again (Himmelheber *et al.*, 2000). Complicating matters, rats performing a low demand version of this task that does not tax sustained attention are also observed to have increased frontoparietal cortex ACh release (Himmelheber *et al.*, 2001), and ACh levels during shifts between these two tasks are not significantly altered beyond the initial increase.

Thus, from the evidence to date, there is an indisputable role for ACh in attentional function. This thesis will explore this role further, in particular to identify neural mechanisms mediating the role of ACh in attention through ligands with ACh modulating properties, or probable ACh modulating properties, and through lesion studies that reduce ACh innervation of brain regions previously noted as being involved in attention.

### **1.3 Lesions**

Apart from the pharmacological study of acetylcholine (ACh) receptor function, there are other ways to explore the relationship between ACh and attention. It has already been commented upon that the neurodegenerative disorder, Alzheimer's disease (AD) is both a means and a reason to study the BF cholinergic system in human subjects. There are also various ways to simulate AD in animal models through use of selective lesions. Areas of the brain that are involved in attention can be selectively damaged using neurotoxins, permitting observation of behaviour resulting from such damage. These methods prove invaluable in the study of both neurodegenerative disorders in general, and also central neural function.

Studies of human lesions are based on extant damage to central neural pathways, either caused by accidental injury, such as closed head

injury, or as a result of a neurodegenerative disorder. These cannot be manipulated in the same fashion as can animal subjects for ethical reasons. Thus, any data arising from studies of human subjects is dependent on a variable outwith the control of the observer. Studies involving animals can be more tightly controlled, as specific manipulations are possible, permitting a better understanding of target neural pathways.

### ***1.3.1 Alzheimer's Disease (AD)***

The neurodegenerative disorder most commonly associated with the BF cholinergic system is AD. AD has various causes, in that it can be inherited, referred to as familial AD (wherein several identified genetic mutations leads to onset of AD), or it can be sporadic (non-familial), wherein genetic variation accounts for increased susceptibility to AD. Familial AD tends to have an earlier onset than sporadic AD. The effects are, however, the same, from the point of view of neural degeneration and subsequent symptomology.

Degeneration occurs in the basal forebrain (BF) cholinergic system in the presence of abnormal structures called senile plaques and neurofibrillary tangles. The nbM is most significantly affected, and so cholinergic projections to cortex, thalamus and amygdala are reduced. The septal complex, including medial septum (MS), vertical and horizontal limbs of the diagonal band of Broca (V/HDB) are also affected,

so cholinergic innervation of hippocampus is also reduced. Plaques and tangles are not confined to BF however, nor to areas to which BF projects. Dorsal raphe nucleus (DRN), locus coeruleus and some hypothalamic nuclei are affected as well as BF and its projection terminals. Interestingly, not all areas of the cortex are equally affected, and indeed the cingulate and visual cortices are among the least affected areas (Feldman *et al.*, 1997). This would suggest that processes governed by these regions would be least affected by the disease, assuming that it is cortical damage, and not BF degeneration that results in cognitive impairment.

The effects of AD on cognition are quite marked, and become more severe as the disorder progresses. Memory is one of the most significant and immediately noticeable deficits that a patient suffers. Loss of memory is also associable with age related or senile dementia, and does not necessarily indicate AD in a patient however. Loss of semantic memory in patients is observed in a reduced ability to word-find: patients have difficulty in either retrieving words from their semantic memory, or suffer a loss of knowledge such that the word is not there to retrieve. The specific mechanism behind reduced word-finding ability in AD patients is unclear. There are observations to support both theories. Subjects with AD required to provide a word in a category-fluency task (e.g. name animals) are worse than if they must provide a word in a letter-fluency task (e.g. words beginning with the letter "A"). This is not the case for

dementia brought on by age or deficits observed in other neurodegenerative disorders such as Huntington's disease patients. If this were a case of an impairment in retrieval ability, then it has been argued that cueing should help. Patients with AD receive no benefit from cues, unlike patients with either Huntington's or Parkinson's disease. These data suggest that loss of knowledge is responsible for this word-finding impairment.

The main argument in favour of retrieval impairment lies in on-line semantic priming. AD patients presented with semantically related words are faster to provide a word than those presented with a non-related word (for review see Parasuraman and Martin, 1994). It is possible that on-line semantic priming is aided by working memory function however. With minimal damage to cingulate areas of prefrontal cortex, the cueing effect of on-line semantic priming may benefit more than the cueing effect of category priming.

Visual attention is also impaired in AD patients. In a test of covert orienting of attention similar to that described by Posner (1980), AD patients were observed to fit into three subgroups of deficit: one group was impaired in responding to all targets presented to the left visual field; one subgroup was impaired in responding to all targets presented to the right visual field; one subgroup was impaired in responding to all targets irrespective side of presentation. These data show a progression of the

symptomology of AD, with asymmetric impairment likely as a result of a differential in hemispheric degeneration.

Further evidence exists to suggest that AD patients are impaired in shifting attention from one spatial location to another. An overt attentional orienting task requiring subjects to shift attention from one spatial location to another, based on letter discrimination. Subjects with AD are slower to respond to a target when cued to an incorrect spatial location than age matched controls. Also, AD patients cannot shift attention between identifying stimuli in the Navon task (subjects are presented with a large letter or number constructed from smaller letters or numbers and must identify either the large stimulus or the smaller ones). Evidence suggests that AD patients are impaired in shifting their attentional focus between small and large regions (for review see Parasuraman and Martin, 1994).

#### ***1.3.1.1 Understanding neuropathology in Alzheimer's Disease***

AD-induced degeneration of basal forebrain (BF) neurons causes a reduction in cholinergic receptors. As cholinergic receptors are located both pre-synaptically and post-synaptically, there is observed a more significant loss of nicotinic and M2 mAChRs than M1 mAChRs (which are located post-synaptically). Despite this, there is evidence to suggest abnormality in the function of the intact post-synaptic M1 receptors, resulting in all cholinergic function being impaired (Feldman *et al.*,

1997). More recent observations in the rat (Bednar *et al*, 1998) indicate that there are marked differences in cortical nAChR survival after BF lesion depending on toxin used. Tritiated ligands with differing selectivity for nAChR subtypes show no reduction in binding in hippocampus, frontal and parietal cortex after 192-IgG-saporin lesions of BF, compared to up to 40% loss after ibotenic acid BF lesions. This difference in vulnerability suggests that a significant number of nAChRs are located post-synaptically to cholinergic projection neurons in cortex and hippocampus. The fact that AD patients show such loss of nAChRs may be explained by the fact that regions with high densities of post-synaptic nAChRs are less affected by the degeneration than are those with pre-synaptic receptors.

In order to further study AD, it is useful to create a neurotransmitter-based model, so that manipulations using selective agonists and antagonists can isolate cognitive impairments and establish probable causes. AD consistently involves degeneration of the ACh system, and so one model of AD uses the muscarinic antagonist, scopolamine to study deficits. Some effects of scopolamine have already been discussed, although not in light of AD deficits. Scopolamine is observed to have no effect on cued responses in a simple reaction time task (subject is required to respond to a target with a key press: either with a pre-target cue (a tone with variable cue-target delay) or with no cue) indicating no effect on alerting. Scopolamine causes an increase in

reaction time in a simple/choice reaction time task (subject is required to indicate with a key press which of four boxes on a monitor lights red: subject is aware of which box will light in the simple condition; there are no cues) irrespective of whether the trial was simple or choice. Scopolamine has no effect on a selective visual attention task (subjects are required to verbally identify an orange letter from a green and orange letter superimposed on each other under three levels of difficulty), but does affect Posner's covert orienting task: reaction times to both validly and invalidly cued targets are slowed. Scopolamine affects vigilance in a sustained attention task (subjects must listen to a series of blocks of beeps and report the number of beeps in the set previous to that just heard: frequency ranges from 5-14 beeps per set), impairing subjects at low frequency but not at high frequency (Broks *et al.*, 1988). Certainly the covert orienting observations match those observed in AD patients. There is conflicting evidence to suggest that sustained attention is impaired in mild cases of AD. Rizzo *et al* (2000) report deficits in a visual sustained attention task in patients with mild AD, and similar has been observed in auditory alertness (Brazzelli *et al.*, 1994), although other evidence suggests sustained attention is left relatively intact in mild cases of AD (Perry and Hodges, 1999).

It is likely that the scopolamine model of AD models some of the deficits observed in AD, but does not cover all the impairments that are

observed during progress of the disorder, specifically in light of the differential losses in AD of nAChRs and mAChRs.

### ***1.3.2 Head injury***

It is also possible for human subjects to suffer lesions after head injury, and it is often the case that attentional disorders arise after such. Subjects with damage to the posterior parietal lobe show a reduced ability to shift attention (Petersen *et al.*, 1989; Posner and Petersen, 1990), and it is likely that similar neurodegeneration in Alzheimer's patients is responsible for attentional shifting impairments. However, rather than show an increase in reaction times to both validly and invalidly cued targets, patients with closed head injury show only an increase in reaction time to validly cued targets (compared to control performance, and also in relation to neutral cues (alerting the subject, but with no spatial information). The cost of the invalid cue is not affected, but the benefit of the valid cue is reduced (Cremona-Meteyard *et al.*, 1992).

### ***1.3.3 Other neurodegenerative disorders***

It is not however the case that Alzheimer's disease is the only neurodegenerative disorder to result in impaired attentional function. Parkinson's disease (PD) has also been observed to lead to impaired attentional function. PD involves degeneration of dopaminergic neurons, and it is likely that loss of dopaminergic innervation to cortical regions is

responsible for observations of increased difficulty in maintaining attention in Posner's covert orienting task (Wright *et al.*, 1990). Interactions between dopamine and acetylcholine, as well as dopaminergic modulation of attentional function will be discussed later.

Likewise, Huntington's disease (HD), which involves degeneration of neurons in the striatum, subsequent dopamine depletion to subthalamic nucleus and ventral anterior nucleus and further degeneration of cortical regions receiving innervation from these basal ganglia nuclei (Feldman *et al.*, 1997) also leads to considerable cognitive impairment, including attentional, in patients with HD (Lawrence *et al.*, 2000).

#### ***1.3.4 Basal forebrain lesions in rats***

Damage resulting from neurodegenerative disorders or injuries that lead to lesions are imprecise, and although valuable for study of cognition in humans, it is difficult to specify which brain regions are involved in which function when several regions are affected. Lesion studies in animal subjects provide a means by which an observer can manipulate specific regions of a subject's central nervous system and observe the effects on both neurochemistry and behaviour/cognition.

In studying cholinergic modulation of attention, the area of most interest is basal forebrain (BF). Thus means by which targeted areas of

BF can be lesioned are important to develop for use in such studies. Behavioural/cognitive effects of BF damage in animal subjects provides observers with a means to draw parallels between animal and human modulation of attention, as well as creating models for AD and uncovering precise neural control mechanisms that study of human subjects alone cannot achieve.

To this end, there have been many studies aimed at lesioning the various nuclei of BF, as well as targeting those areas that BF projects to in an effort to uncover the specifics of BF neural control of attention. The rat is one of the most widely used subjects in these studies and can be trained to perform a variety of behavioural tasks designed to explore attentional function. Availability of comparison with other studies, combined with optimised laboratory methodologies means that this is likely to continue to be the case.

Initial studies of BF lesions in rats relied on non-selective amino acid neurotoxins, also known as excitotoxins. Without the availability of a selective neurotoxin, these studies require precise intraparenchymal infusion of the excitotoxin into the targeted brain region. Excitotoxins work by causing prolonged neuronal depolarisation, resulting in excitation of the neuron and an influx of water via osmosis caused by ion flux during prolonged activation. Eventually the neuron swells to the point of lysis, or bursting, removing that neuron and its ability to

influence neurons in the terminal fields of its projections. Although neurodegenerative disorders result in neuronal loss through different mechanisms, the ability to induce neuronal loss with excitotoxins is a valuable tool in exploring models of neurodegeneration.

Lesions of nucleus basalis magnocellularis (nbM) with the excitotoxin quisqualic acid (primarily an agonist at the AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) glutamatergic receptor subtype) were observed to result in a reduction in choline acetyltransferase (ChAT: the enzyme that catalyses synthesis of acetylcholine (ACh)) immunoreactivity in cortex, as well as a reduction in glutamatergic activity in both cortex and hippocampus (with no corresponding reduction in ChAT), demonstrating BF modulation of cortical glutamatergic neurons (Reine *et al.*, 1992). Quisqualic acid induced lesions in BF also result in impaired visual attention, with lesioned rats showing reduced choice accuracy and increased response latency in the five choice serial reaction time task (5CSRT) (Muir *et al.*, 1992).

The efficacy of a selection of excitotoxins on parvocellular neurons (small peptidergic neurons) in BF has been tested (Winn *et al.*, 1991). Kainic acid is observed to be the most potent excitotoxin, followed by ibotenic acid, then quinolinic acid and N-methyl-D-aspartate. Quisqualic acid was not observed to lesion parvocellular neurons within

BF, suggesting a degree of selectivity of quisqualic acid for cholinergic neurons in BF (Dunnett *et al.*, 1987).

There are means other than excitotoxins that have been used to create lesions of BF though. A conjugate of rat nerve growth factor (rNGF) and diphtheria toxin has been used to target the cholinergic neurons of BF which bear the rNGF receptor. As this is a selective neurotoxin it was possible to infuse it into the terminal regions of the BF projections (cortex) without fear of significant damage to the glutamatergic neurons present. Imaizumi *et al* (1991) observed a retention deficit in a passive avoidance test in mice, as well as a memory deficit in mice lesioned after acquisition of a passive avoidance response.

However, a significant breakthrough in targeting cholinergic BF neurons came with the development of 192-IgG-saporin. The immunotoxin saporin is conjugated to the monoclonal antibody 192-IgG which is selective for the rNGF receptor (p75 neurotrophin receptor) found almost exclusively on the cholinergic neurons in BF of the rat (Korsching *et al.*, 1985; Wiley *et al.*, 1991). The immunotoxin, once in the neuron, prevents the transcription of mRNA, ultimately leading to cell death. There is considerable evidence for the selectivity of 192-IgG-saporin, as well as for the effects of lesions of 192-IgG-saporin in rats, making it a very useful tool in the analysis of BF function and anatomy (for review see Chapter IV).

### ***1.3.5 Basal forebrain lesions in primates***

There is also evidence of the BF's involvement in attentional function in primates, although manipulations of primate BF cholinergic neurons have not been as selective as has been reported in rats. The 192-IgG monoclonal antibody does not bind to the p75 neurotrophin receptor in primates, and only limited experimental data is available for administration of the conjugate ME20.4 IgG-saporin (selective for primate p75 receptor). It has been reported in marmosets that the p75 neurotrophin receptor is located on most BF cholinergic projection neurons in medial septum (MS), horizontal and vertical limb of the diagonal band of Broca (H/VDB) and nucleus basalis of Meynert (magnocellularis in rats; nbM). p75 neurotrophin receptor immunoreactivity is observed in those regions, with immunoreactivity in cortex, hippocampus and amygdala, corresponding with the cholinergic projections from BF to those terminal regions (MacLean *et al.*, 1997) It is worth noting that unlike in rats, cholinergic projections to amygdala from BF do bear the p75 receptor, and would therefore be damaged after administration of ME20.4 IgG-saporin. The amygdala's role in emotion/anxiety/stress could thus become a factor in any manipulation and behavioural task involving ME20.4 IgG-saporin.

Marmosets administered ME20.4 IgG-saporin into BF have been observed impaired in the acquisition stage of a visual attentional task.

That it was also noted that density of immunostaining for ME20.4 in nbM corresponded to the density of acetylcholinesterase (AChE) staining in frontal and temporal cortex, and that there is an inverse correlation between levels of staining and performance in task acquisition (Fine *et al.*, 1997) provides further support for the role of the nbM in attentional function.

Further evidence supports a role for the BF in attentional function in primates after excitotoxic (ibotenic acid) lesions of BF neurons in cynomolgus monkeys. Although not selective for cholinergic neurons, it is possible to some degree to dissociate observed attentional deficits mediated by loss of cholinergic projections from the BF from those induced by loss of GABAergic neurons also present in the BF. Certainly in rats, comparisons of attentional impairment after lesions with ibotenic acid or 192-IgG-saporin have shown that attentional impairments mediated by GABAergic neurons tend to indicate an executive role for GABA in attentional modulation. Ibotenic acid lesions of BF have a far larger effect on cortical GABAergic projections (from reductions in parvalbumin-positive cortical immunoreactivity) than they do on cortical acetylcholine activity (from AChE-positive cortical immunoreactivity). Conclusions drawn from these data are forcibly vague as ibotenic acid is not selective for GABAergic neurons, indicating that deficits may result from non-cholinergic/non-GABAergic neuronal degeneration in the BF (Burk and Sarter, 2001). Likewise assuming that non-cholinergic

projections from rat BF to cortex are similar to those in primates will also lead ultimately to speculative error. However, it is reasonable to consider deficits in primate attentional function that appear executive in origin may stem from loss of GABAergic innervation to cortex rather than cholinergic. Indeed, monkeys with ibotenic acid lesioned BF are impaired in attentional focusing, but not in accuracy to a variety of visual attentional tasks (Voytko *et al.*, 1994). That the BF cholinergic system was impaired was confirmed after lesioned monkeys were seen to be more sensitive to administration of the cholinergic antagonist scopolamine (a muscarinic-receptor specific antagonist) in a delayed non-matching-to-sample task.

Marmosets with BF lesions after administration of N-methyl-D-aspartate (NMDA) were impaired in an attentional set-shifting task. This task requires visual discrimination of a stimulus as well as the ability to attend to varying dimensional properties of the stimulus, shift attention from one stimulus to another based on the same dimensional properties, and from one stimulus to another based on differing dimensional properties previously ignored. Furthermore, the task also tests the marmoset's ability to learn to ignore a previously rewarded stimulus in favour of a previously ignored stimulus (reversal). Marmosets with BF lesions were impaired in their ability to learn the reinforcement value of new stimuli and also in their ability to ignore previously reinforced stimuli. That they were impaired in the reversal suggests that they were

either perseverating on the previously rewarded stimulus or were unable to learn the strategy of reversal. It is hypothesised that a certain behavioural rigidity could account for the inability to learn the reversal, as performance in a well-learned discrimination of marmosets with BF lesions was unaffected by the introduction of novel stimuli of the irrelevant dimension (probe test) (Roberts *et al.*, 1992).

These data provide strong evidence for the involvement of BF in attentional function, as well as for the importance of ACh in modulating attention. They suggest that BF serves a similar function in humans, non-human primates and rodents.

#### **1.4 Other acetylcholine modulating factors**

Although acetylcholine (ACh) is clearly very important for mediating attentional function through basal forebrain (BF), it is also the case that other neurotransmitters are involved in attention. As some ACh receptors are located post-synaptically as heteroceptors for other neurotransmitters, so other neurotransmitters can modulate ACh function through non-cholinergic receptors on cholinergic neurons.

In considering the manipulation of ACh function through either drugs that directly affect ACh or those that affect neurotransmitter systems with ACh modulating effects, the mechanism by which ACh

modulates attention must be considered. Antagonists of ACh receptors can result in attentional impairment, but cholinergic agonists do not reliably enhance cognitive function (Sarter *et al.*, 1996). It is considered that the phasic activity (rhythmic bursting rather than continuous firing maintaining a tone; see Détári *et al.*, 1999 for review) of cholinergic neurons cannot be replaced or maintained by either cholinergic agonists or acetylcholinesterase inhibitors. Attempts to modulate attentional function through manipulations of the cholinergic system must address the mechanisms by which the phasic activity of these cholinergic neurons arises and is maintained (Sarter *et al.*, 1996).

#### ***1.4.1 GABA ( $\gamma$ -aminobutyric acid)***

The neurotransmitter GABA is an amino acid, and, as previously mentioned, GABAergic neurons are present in basal forebrain (BF), sending projections to lateral posterior hypothalamus (Gritti *et al.*, 1994), and to other regions that receive cholinergic innervation from BF, such as cortex (Freund and Gulyas, 1991), with a subpopulation of interneurons synapsing with cholinergic projection neurons in BF (Gritti *et al.*, 1993; Gritti *et al.*, 1997). GABAergic influence is normally inhibitory, although Alreja and Liu (1996) report evidence to suggest that GABAergic interneurons in BF have excitatory synapses with the cholinergic projection neurons

Contrary to this is a report that stimulating the GABA<sub>A</sub> or the GABA<sub>B</sub> receptor subtype in nucleus basalis magnocellularis (nbM) through intraparenchymal administration of either muscimol (GABA<sub>A</sub> receptor subtype-specific agonist) or baclofen (GABA<sub>B</sub> receptor subtype-specific agonist) impairs working memory but not reference memory in a double Y-maze task. This effect was not seen when the GABA<sub>B</sub> receptor subtype-specific antagonist is co-administered with baclofen (Desouza *et al.*, 1994). This suggests that GABA-activation in nbM impairs attentional function, and that BF cholinergic projection neurons to cortex are inhibited, not excited. Supporting these data, studies of effects of benzodiazepine receptor (BZR) inverse-agonists (ligands that produce the opposite effect to an agonist) on cholinergic neurotransmission reported in Sarter *et al.* (1996) demonstrate that, rather than blocking GABAergic neurotransmission, BZR inverse-agonists modulate the extent of GABAergic neurotransmission, and thus GABAergic inhibition of cholinergic neurons, depending on the activity of the GABAergic neurons. Thus, where GABAergic agonist and antagonist administration would amount to “noise”-producing neuronal signals to a phasic bursting pattern, BZR inverse-agonists can manipulate cholinergic neurotransmission whilst maintaining cholinergic phasic activity through this allosteric modulation of GABAergic function. Likewise, where administration of cholinergic agonists might be unlikely to alleviate attentional deficits induced after BF lesion, administration of BZR

inverse-agonists could attenuate deficits by potentiating activity in still functional cholinergic projections.

In a previously described sustained attention task (see Introduction: Sustained Attention), Sarter and Bruno (1997) discuss how 192-IgG-saporin BF lesion-induced impairments in rats' performance is alleviated by administration of BZR inverse-agonists. This effect is demonstrated in subjects with 50-70% BF neuronal loss, but not in subjects with greater than 90% cell loss. The influence of GABAergic neurons on the remaining BF cholinergic neurons is able to compensate for the loss only up to a point. In the cases where performance impairments were alleviated, activated cortical ACh efflux was also increased.

In a study of cortical and hippocampal GABA/ACh release in rats exposed to a novel environment, it was observed that cortical and hippocampal ACh is increased on initial exposure to the novel environment. The increase in ACh levels is reduced during a second exposure, although remains higher than baseline levels, with GABA levels increased above baseline levels. During the second exposure, ACh cortical and hippocampal increases are correlated to motor activity, as are hippocampal GABA levels, with cortical GABA levels inversely correlated to motor activity. This suggests that ACh is involved in both an initial increase in attentional requirements during exploration, as well as

in subsequent motor activity. GABA release may be related to a habituation process (Giovannini *et al.*, 2001).

#### ***1.4.2 Serotonin (5-hydroxytryptamine; 5-HT)***

Serotonin's role in the central nervous system involves mediating satiety, aggression and anxiety. Numerous serotonergic nuclei in the brainstem (including dorsal and median raphé nuclei) send ascending projections to the forebrain (Feldman *et al.*, 1997). 5-HT receptors are located both pre- and post-synaptically, and there are currently fourteen identified subtypes (5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-ht<sub>1E</sub> and 5-ht<sub>1F</sub>; 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub>; 5-HT<sub>3</sub>; 5-HT<sub>4</sub>; 5-ht<sub>5A</sub> and 5-ht<sub>5B</sub>; 5-HT<sub>6</sub>; 5-HT<sub>7</sub>). Nomenclature is decided by the serotonin subcommittee of the International Union of Pharmacology (IUPHAR). Receptor subtypes are designated with lower case letters until they are sufficiently studied for some functionality to be understood (Hoyer *et al.*, 1994; Hoyer *et al.*, 2002). Of the 5-HT receptor subtypes, six of them (namely, 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub>) are reported to influence cholinergic function (for review see Lucki, 1992; Boess and Martin, 1994; Barnes and Sharp, 1999; Meneses and Terrón, 2001; Hoyer *et al.*, 2002).

Microdialysis studies have shown that agonists of the 5-HT<sub>1A</sub> receptor induce a reduction of rat forebrain serotonin levels – an effect that is negated by 5-HT<sub>1A</sub> receptor antagonists such as WAY-100635 (Fletcher *et al.*, 1993, 1995; Dourish, 1995). Administration of 5-HT<sub>1A</sub>

receptor antagonists alone does not increase serotonin levels however (Sharp *et al.*, 1996). The 5-HT<sub>1A</sub> agonist 8-OH-DPAT induces increased acetylcholine (ACh) release in the cortex and hippocampus of rats, although the cortical increase appears to be mediated by the 5-HT<sub>1B</sub> receptor. 8-OH-DPAT has a mild affinity for the 5-HT<sub>1B</sub> and 5-HT<sub>7</sub> receptors (Middlemiss and Fozard, 1983; Nakai *et al.*, 1998; Meneses and Terrón, 2001), and the increased cortical ACh is antagonised by joint 5-HT<sub>1A/1B</sub> antagonists, but not by WAY-100635 (Izumi *et al.*, 1994; Consolo *et al.*, 1996). ACh increases appear to be mediated by post-synaptic 5-HT<sub>1A</sub> receptors, but the specific location of these is unknown (Consolo *et al.*, 1996). The septal 5-HT<sub>1A</sub> receptor possessing cholinergic neurons are likely to project to the hippocampus and the cortex, but these are known to be inhibitory (Van Den Hooff and Galvan, 1992).

There is some evidence that 5-HT<sub>1A</sub> antagonists have cognition enhancing effects. Spatial learning impairments induced by cholinergic antagonists (Carli *et al.*, 1997) and visual attention impairments induced by lesions (Balducci *et al.*, 2003) have been reversed and attenuated, respectively, by WAY-100635. It is thought that 5-HT<sub>1A</sub> inhibitory input to hippocampal/cortical pyramidal neurons is reduced by 5-HT<sub>1A</sub> antagonists, although there is a lack of confirmatory evidence for this mechanism (Dijk *et al.*, 1995; Hajós *et al.*, 1998), compensating for the loss of excitatory cholinergic input to these neurons.

5-HT<sub>1B</sub> receptors are located pre-synaptically on 5-HT neurons and they function as autoreceptors (Engel *et al.*, 1986). 5-HT<sub>1B</sub> receptors found pre-synaptically on non-5-HT neurons are thought to act as heteroreceptors. Cassel *et al.* (1995) performed in vitro studies on rat hippocampal tissue, observing that CP 93129, a 5-HT<sub>1B</sub> receptor agonist, inhibited ACh release. Usually seen as inhibitory, it is likely that any facilitatory effect by 5-HT<sub>1B</sub> receptor agonists stems from an indirect route. Microdialysis studies have shown that CP 93129 administered locally induces increased ACh levels in the rat frontal cortex (Consolo *et al.*, 1996).

There are many 5-HT<sub>3</sub> (Maricq *et al.*, 1991) receptor-selective ligands that can be used to observe receptor pharmacology, although it is worth noting that several of these show species-specific variations in affinity. Ondansetron is a potent 5-HT<sub>3</sub> receptor antagonist in both humans and rats and phenylbiguanide, PBG, is a potent 5-HT<sub>3</sub> receptor agonist in both humans and rats.

Ondansetron has also been used to demonstrate 5-HT<sub>3</sub> involvement in cognition. Following systemic administration of the cholinergic antagonist scopolamine, marmosets were impaired in task acquisition in the Wisconsin Card Sort Test. Systemic administration of ondansetron negated the scopolamine induced deficit (Carey *et al.*, 1992). Since ACh is long acknowledged as important in learning and memory,

and as Ondansetron reverses a specifically cholinergic deficit, understanding the interaction of 5-HT<sub>3</sub> receptors and ACh release becomes of fundamental importance. Stimulating the 5-HT<sub>3</sub> receptor has been shown to inhibit cortical ACh release (Barnes *et al.*, 1989), although there is some indication that ACh release mediated by 5-HT<sub>3</sub> receptor is not direct, but also involves GABA. For example, in vitro microdialysis studies on rat entorhinal cortex have shown that 2-methyl-5-HT blocks ondansetron induced ACh release, and that the GABA<sub>A</sub> antagonist bicuculline further potentiates ACh release (Ramirez *et al.*, 1996). Ramirez *et al.* hypothesise that 5-HT activates 5-HT<sub>3</sub> receptors on GABAergic neurons in the cortex (see also Morales *et al.*, 1996; Morales and Bloom, 1997), inhibiting ACh release. Consolo *et al.* (1994b) reported that stimulating the 5-HT<sub>3</sub> receptor in rat hippocampus induces ACh release, although the indirect nature of the cortical interactions, as well as rapid desensitisation, could explain this, and other, apparently contradictory functional evidence.

It is evident from several studies that the 5-HT<sub>4</sub> receptor has a modulatory role on the release of ACh. Consolo *et al.* (1994a) administered a 5-HT<sub>4</sub> receptor agonist intracerebroventricularly in the rat, inducing potentiation of ACh release, which was reduced by concurrent administration of a 5-HT<sub>4</sub> receptor antagonist. Antagonists on their own did not affect ACh levels in the frontal cortex of the rat, indicating that ACh modulation by 5-HT<sub>4</sub> is under phasic, rather than tonic, control. It

has been observed in post-mortem studies of Alzheimer's patients that hippocampal 5-HT<sub>4</sub> receptor density is reduced (Reynolds *et al.*, 1995). As 5-HT<sub>4</sub> receptors are found post-synaptically (Waeber *et al.*, 1994), on hippocampal neurons, this is likely due to hippocampal neuronal loss rather than cholinergic BF neuron degeneration, as it is not just cholinergic neurons that are damaged in AD (Reinikainen *et al.*, 1988).

There is considerable evidence favouring a role of the 5-HT<sub>4</sub> receptor in mediating cognitive function in rats. Stimulating the 5-HT<sub>4</sub> receptor results in reversal of atropine-induced impairments in water maze performance. Pre-treatment with a selective 5-HT<sub>4</sub> antagonist prevents this agonist-induced reversal (Fontana *et al.*, 1997). It has already been pointed out that 5-HT<sub>4</sub> receptor stimulation potentiates cholinergic release, and this is the method by which cognitive enhancement mediated by 5-HT<sub>4</sub> is believed to operate. It is likely that this is an indirect interaction, as there is evidence that it is hippocampal pyramidal neurons that express the 5-HT<sub>4</sub> receptor (Vilaró *et al.*, 1996). These neurons are involved in long term potentiation (LTP), leading to theories that the 5-HT<sub>4</sub> receptor also mediates this.

There is some evidence that the 5-HT<sub>6</sub> receptor also modulates central ACh (for review see Chapter II and Chapter V). Past studies have shown that behavioural observations (stretching and yawning) induced by antisense oligonucleotides (which reduce expression of the receptor)

(Sleight *et al.*, 1996) and the antagonist Ro 04-6790 (Sleight *et al.*, 1998) are attenuated by administration of the muscarinic antagonists atropine and scopolamine (Bourson *et al.*, 1995; Bentley *et al.*, 1999). Furthermore, administration of Ro 04-6790 is reported as inducing a non-significant 50% increase in hippocampal ACh levels (Shirazi-Southall *et al.*, 2002). The 5-HT<sub>6</sub> receptor subtype-specific antagonist, SB-271046, is recently reported as significantly increasing ACh levels in medial prefrontal cortex (mPFC) (Jones, 2002, in communications), and new selective 5-HT<sub>6</sub> receptor subtype-specific compounds (e.g. the antagonists SB-258585 (Hirst *et al.*, 2002)) are being researched for similar effects.

There is also evidence that the 5-HT<sub>7</sub> receptor has acetylcholine modulating properties. The 5-HT<sub>1A/1B</sub> agonist 8-OH-DPAT has mild affinity for 5-HT<sub>7</sub> receptors, as do the 5-HT<sub>2</sub> receptor antagonists LY215840 and ritanserin. 8-OH-DPAT administration facilitates learning consolidation in an autoshaping task (Meneses and Terrón, 2001). This effect is abolished by both LY215840 and ritanserin, but not by selective 5-HT<sub>2A</sub> antagonist MDL100907 or 5-HT<sub>2B/2C</sub> antagonist SB200646. This suggests a role for the 5-HT<sub>7</sub> receptor in learning consolidation. A learning deficit induced by scopolamine was also reversed by both ritanserin and LY215840 at the same dose that abolished the 8-OH-DPAT-induced facilitation. This supports a role for 5-HT<sub>7</sub> in the modulation of acetylcholine. These data add support to the hypothesis that

5-HT<sub>7</sub> receptors are involved in 8-OH-DPAT administration-induced increase in hippocampal ACh (Nakai *et al.*, 1998).

#### ***1.4.3 Dopamine (3,4-Dihydroxyphenylethylamine)***

Dopamine is one of the catecholamine neurotransmitters (the others being noradrenaline (norepinephrine) and adrenaline (epinephrine), so called because of their structure (a catechol nucleus consisting of a benzene ring with two hydroxy groups and a side chain which is a derivative of ethylamine). Dopamine is involved in several areas of neural control, including motor control and motivation. Dopaminergic neural processes are damaged during the progress of the neurodegenerative disorder, Parkinson's disease. Dopamine antagonists induce catalepsy, where-in a subject exhibits difficulty initiating voluntary motor activity; Parkinson's patients suffer a variety of motor difficulties, including tremor, muscular rigidity, akathisia (involuntary movement), bradykinesia (slowing of movement) and postural disturbance (Feldman *et a.*, 1997).

Parkinson's patients are also observed to suffer cognitive deficits, and there is considerable evidence showing how dopamine modulates cholinergic function in areas of the brain receiving cholinergic input from basal forebrain (BF). As has already been reported, vertical and horizontal limb of the diagonal band of Broca (V/HDB) receive dopaminergic input from ventral tegmental area (Gaykema and Zaborszky, 1996). However

VTA also sends dopaminergic projections to hippocampus and medial prefrontal cortex (mPFC) (Thierry *et al.*, 2000), allowing for dopaminergic modulation of acetylcholine (ACh) in these areas.

There is evidence from both autoradiography and microdialysis that dopamine receptors serve as heteroreceptors on cholinergic nerve terminals in hippocampus. After fimbriaectomy, in which 50% of choline acetyltransferase (ChAT) activity was lost, a significant reduction in binding of [ $H^3$ ]-SCH23390 (a D1 receptor subtype antagonist) was observed, with no effect on [ $H^3$ ]-raclopride (a D2/D3 receptor subtype antagonist) binding. Furthermore, administration of the D1 agonist, SKF 38393, induced a dose dependent increase in hippocampal ACh levels in unlesioned freely moving rats. This effect was attenuated by administration of SCH23390. The D2/D3 agonist, quinpirole, did not affect ACh levels, nor did the D2/D3 antagonists sulpiride. This suggests that D1 receptors are located on cholinergic neurons in the hippocampus, and that hippocampal dopamine modulates hippocampal ACh release (Hersi *et al.*, 1995).

Similarly, cholinergic projections to frontal cortex are mediated by dopaminergic input. ACh levels in frontal cortex are increased during tactile stimulation; an effect which is attenuated by simultaneous antagonism of both D1 and D2/D3 receptor subtypes by SCH23390 and Raclopride (although neither one alone has this effect) (Acquas *et al.*,

1998). Atypical antipsychotics with 5-HT<sub>2A</sub> and D2 receptor antagonising properties also increase both DA and ACh in mPFC, although the mechanism by which ACh release is potentiated is still unknown (Li *et al.*, 2003).

There is also evidence to show dopaminergic modulation of ACh in behavioural studies. Microdialysis during acquisition of an operant lever pressing task shows increase in both mPFC ACh and dopamine (Izaki *et al.*, 1998), with only mPFC ACh remaining increased during study of task retention.

Other than at terminals of BF cholinergic neurons in cortex and hippocampus, dopamine also mediates ACh levels in the striatum through inhibitory synapses with cholinergic interneurons present in the striatum (Feldman *et al.*, 1997). Various slice studies of striatum have demonstrated the modulation of ACh release by dopaminergic ligands. D1 receptor antagonists do not attenuate electrically induced striatal ACh release (Tedford *et al.*, 1992) in slice preparations, although D1 agonists do induce striatal ACh release (Consolo *et al.*, 1996). As mentioned previously, there are striatal projections to ventral pallidum, which in turn projects to other BF nuclei. Stimulation of dopamine release in nucleus accumbens (ventral striatum) does not, however, affect sustained attention performance in rats, although antagonising dopaminergic neurotransmission with the non-selective dopaminergic antagonist cis

flupenthixol impairs both detection of signals and non-signals in the task (Himmelheber *et al.*, 2000). Interestingly, systemic amphetamine administration increases cortical ACh levels, although this does not appear to be mediated via BF projection neurons, as antagonising dopaminergic neurotransmission in BF with both D1 and D2/D3 antagonists does not reduce observed amphetamine induced ACh increases. Instead, cortical ACh increases are attenuated by kynureate, a glutamatergic antagonist, administered into BF (Arnold *et al.*, 2001). This complicates theories of dopamine modulated striatal mediation of attention through projections to BF; amphetamine administered systemically induces increased cortical ACh, but when administered into nucleus accumbens does not modulate sustained attention performance, despite previous evidence that sustained attentional tasks involve increases in cortical ACh.

Dopaminergic pathways and prefrontal cortex are also involved in other cognitive functions, including attentional set-shifting. Indeed, attentional set-shifting is used to assess cognitive dysfunction in patients with Parkinson's disease (Antal *et al.*, 1998) and Schizophrenia (Moritz *et al.*, 2002), both of which involve dopaminergic dysfunction (Kaasinen and Rinne, 2002; Goldman-Rakic, 1999). Any interactions between dopaminergic and cholinergic pathways in attentional set-shifting remains to be established however.

#### ***1.4.4 Noradrenaline (norepinephrine; NE) and adrenaline (epinephrine; EPI)***

A catecholamine like dopamine, noradrenergic pathways modulate vigilance and satiety (Feldman *et al.*, 1997). The locus coeruleus noradrenergic neurons project to basal forebrain (BF), in particular the septal complex and nucleus basalis magnocellularis (nbM) (Milner *et al.*, 1995; Berridge and Foote, 1996), as well as a variety of other central brain regions. Electrophysiological study of NE administered into septal complex slice preparation shows activation of GABAergic neurons and cholinergic neurons activated by the GABAergic neurons. The  $\alpha_1$  (NE receptor subtype) agonist, phenylephrine induced similar effects, and these were blocked by  $\alpha_2$  antagonists (Alreja and Liu, 1996). Furthermore, NE administered into septal complex also results in a reduction in activity in the hypothalamic supraoptic nucleus (Cunningham *et al.*, 1993), which receives projections from septal complex, although these are more likely to be GABAergic than cholinergic (Gritti *et al.*, 1994). Microdialysis data show that the  $\alpha_2$  agonist clonidine and the  $\alpha_1$  antagonist prazosin have no effect on baseline forebrain acetylcholine (ACh) levels, although they do attenuate tactile stimulation-induced ACh increases. The  $\alpha_2$  antagonist yohimbine increases baseline forebrain ACh levels (Acquas *et al.*, 1998).

Evidence for behavioural effects of direct modulation of ACh by NE is limited. Monkeys with 6-hydroxydopamine lesions of medial

prefrontal cortex (mPFC) show impairment in attentional set-shifting, as well as a reduction in cortical dopamine and NE, although there is no direct evidence of interaction between cholinergic and noradrenergic pathways (Roberts *et al.*, 1994). Similarly, microdialysis observations in rats undergoing the five choice serial reaction time task (5CSRT) task show increased cortical ACh and NE levels, but no direct interaction between the two systems. Although lesions of locus coeruleus are observed to impair performance in 5CSRT task, this only occurs when unpredicted distracters are present (Dalley *et al.*, 2001).

Central adrenaline (or epinephrine (EPI)) is not observed to mediate either cholinergic function or attentional function. Instead, peripheral EPI is observed to have cardiorespiratory modulating properties, and although it does not cross the blood-brain barrier, there are secondary effects on the CNS, including anxiety and headache (Feldman *et al.*, 1997).

#### ***1.4.5 Glutamate and Aspartate***

Glutamate and aspartate are, like GABA, amino acid neurotransmitters. Glutamate is very important in several descending cortical pathways, including those to the various cholinergic nuclei of basal forebrain (BF) from medial prefrontal cortex (mPFC) (Gaykema *et al.*, 1991). Descending cortical pathways to thalamus that send axon collaterals to thalamic reticular nucleus (Rt) are also glutamatergic, as are

ascending thalamic pathways to cortex (Steriade, 1995). It is clear then that glutamate has a very important role in modulating both attentional function and acetylcholine (ACh).

Observations from microdialysis show that mPFC ACh levels increase after intraparenchymal administration of the AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate (two of three ionotropic receptor subtypes; the other being N-methyl-D-aspartic acid (NMDA)) glutamate receptor subtype agonist, kainate, into BF, and that these effects are attenuated by the antagonist DNQX. Increased mPFC ACh levels induced by external stimulus (exposure to darkness reinforced with food reward) is attenuated by administration of the ionotropic glutamate antagonist, kynurenate. Stimulation of NMDA receptors with NMDA induces increases in ACh levels after external stimulation which are larger and last longer than kainate induced increases. Furthermore, a high dose of NMDA induces a large increase in mPFC ACh release prior to external stimulation, although a smaller dose does not (Fadel *et al.*, 2001). The NMDA receptor antagonist, phencyclidine (PCP), also induces an increase in cortical ACh release (Kim *et al.*, 1999), and induces increased mPFC neuronal firing in freely moving rats (Jodo *et al.*, 2003). These data suggest that differing glutamatergic receptors modulate different cognitive functions mediated by BF cholinergic neurons and mPFC ACh.

It is possible to induce ACh release in cortex by electrically stimulating neurons in the pedunculopontine tegmental nucleus which sends both cholinergic and glutamatergic projections to nbM. Glutamate administered into cortex reduces this electrically evoked ACh release, although it has no effect on spontaneous ACh release. The agonists AMPA and NMDA mimic the effect of glutamate, and the evoked ACh increase is attenuated by glutamate antagonists. The effect was also attenuated by both GABA<sub>A</sub> and GABA<sub>B</sub> antagonists, suggesting that glutamate modulation of cortical cholinergic function is indirect, involving GABAergic interneurons (Materi and Semba, 2001).

If the glutamate antagonist, kynurenate, is administered into BF itself, electrically evoked cortical ACh levels are reduced in a similar fashion to that after non-specific neuronal blocker administration. Neither cholinergic nicotinic nor muscarinic antagonists produce this effect, suggesting that input to BF from pedunculopontine tegmental nucleus is mostly glutamatergic rather than cholinergic (Rasmusson *et al.*, 1994).

There are also cholinergic/glutamatergic interactions in Rt, which receives input from cortex and thalamus as well as BF. However, there is more information on cholinergic modulation of glutamatergic function than vice versa. mAChRs on BF projection neurons to Rt modulate glutamatergic function, antagonising (via scopolamine) facilitatory effects on Rt neurons induced by glutamate in extracellular recordings (Marks

and Roffwarg, 1991). Further evidence in support of this comes after muscarinic antagonists injected into nbM attenuated inhibition of tonic firing of Rt neurons in anaesthetised rats after intrabasis administration of glutamate (Pinault and Deschênes, 1992).

The glutamatergic system is implicated in Schizophrenia (Haroutunian *et al.*, 2003), and it has already been noted that the WCST is used to measure cognitive function in patients with Schizophrenia. The involvement of nAChRs in Schizophrenia is well documented (Levin and Simon, 1998), and nicotine has been shown to induce an increase in prefrontal cortex glutamate release in rat *in vitro* slices as long as thalamocortical terminals are intact (Lambe *et al.*, 2003).

#### ***1.4.6 Neuropeptides***

Neuropeptides are chains of amino acids rather than simple compounds like other neurotransmitters. There are several of them that interact with cholinergic function, although the most significant of these is galanin. Galanin receptors are found on the cholinergic neurons of the medial septum (MS), vertical and horizontal limbs of the diagonal band of Broca (V/HDB) and nucleus basalis magnocellularis (nbM) in basal forebrain (BF) (Pasqualotto and Vincent, 1991; Kitchener and Diamond, 1993) as well as on terminals of unknown origin that synapse with BF cholinergic neurons (Henderson and Morris, 1997).

Microdialysis study of galanin injected into the septal complex shows a reduction of scopolamine-induced acetylcholine (ACh) release in hippocampus. It is considered that galanin terminals synapsing with cholinergic neurons in BF inhibit ACh release in BF neuron terminal regions (Robinson *et al.*, 1996). Galanin administration is also observed to induce deficits in several tasks of learning and memory in rats. Impairments in an operant spatial delayed non-matching to sample task induced by intra-peritoneal administration of scopolamine are potentiated by galanin (Robinson and Crawley, 1993). Impairments are also observed in both acquisition and retention of the Morris water maze task and T-maze delayed alternation (McDonald *et al.*, 1998).

Expression of galanin is also observed to be upregulated in patients with AD, leading to the search for selective galanin antagonists as a potential treatment for cognitive deficits observed in AD (Crawley, 1993). The antagonist M40 has been observed to reduce the effects of galanin on spatial tasks in rats, and in combination with the muscarinic M1 antagonist, TZTP, and 192-IgG-saporin lesions of BF, reduce impairments in delayed non-match to position tasks in rats (McDonald *et al.*, 1998).

Two other neuropeptides, vasopressin and oxytocin, also have receptors distributed through BF. Oxytocin receptors are found in nbM

and V/HDB in an autoradiographic study of the human brain (Loup *et al.*, 1991). Vasopressin receptors found on HDB neurons in rats mediate both increase and decrease in voltage-activated currents in two populations. The decrease is attenuated by a vasopressin receptor subtype, V1 antagonist, and the increase is attenuated by a V2 antagonist, suggesting that these two receptor subtypes have differing roles in modulation of HDB neurons (Easaw *et al.*, 1997).

Although there is little behavioural evidence so far suggesting oxytocin's involvement in attentional function, there are data available supporting a role for vasopressin in modulation of cholinergic function. Vasopressin mediates an increase in ACh release in rat hippocampal slices; an effect attenuated by V1 antagonists but not V2 antagonists, and is also reported as facilitating memory (Tanabe *et al.*, 1999). It is possible, however, given the presence of oxytocin receptors on BF neurons that oxytocin is involved in modulation of cholinergic BF neurons and hence attentional function.

Distribution studies for the mRNA of the GPR10 receptor for the neuropeptide, prolactin releasing peptide (PrRP) show relative high density of distribution in the Rt (Roland *et al.*, 1999). Chapter III discusses this in more detail.

#### ***1.4.7 Summary***

The involvement of ACh in attention is well documented. What remains to be discovered is the exact nature of the role of ACh in attention, and how ACh interacts with other neurotransmitters to govern the neurological processes that underly attentional function. Thus although refinement of procedures to enable more selective targeting of brain regions/neurotransmitter systems will provide greater insight into the mechanisms of attention, it is necessary to consider that observed behavioural effects may arise from indirect as well as direct effects of manipulations.

Central ACh interacts with 5-HT, glutamate, dopamine, GABA and peptide neurotransmitters – both modulating the function of, and in turn being modulated by these systems to varying levels and effects dependent on brain region. In considering the role of ACh in this thesis, it is necessary to be aware that observed effects may be attributable to either the direct effect of ACh, or an indirect effect – or indeed the direct effect of serotonergic manipulations, or an indirect effect through modulation of ACh.

#### **1.5 Experimental questions and thesis outline**

During investigations of the involvement of acetylcholine (ACh) in attention, initial focus is on systemic manipulation of ACh using either a serotonin (5-HT) antagonist or a peptide, both hypothesised to modulate

cholinergic function. Progression of studies lead to targeted lesions of cholinergic systems (basal forebrain (BF)) and regions of the brain receiving cholinergic input (thalamic reticular nucleus (Rt)).

Chapter II looks at the effects of systemic administration of the 5-HT<sub>6</sub> receptor subtype-selective antagonist, SB-271046, on covert attentional orienting. Evidence is presented which suggests that 5-HT<sub>6</sub> antagonists modulate (stimulate) central cholinergic function, and that systemic administration of the nAChR agonist, nicotine, mediates covert attentional orienting.

**Hypothesis:** it would therefore be hypothesised that a similar effect on covert attentional orienting would be observed after systemic administration of SB-271046.

**Result:** there is no observed effect of SB-271046 on covert orienting of attention, most likely due to absence 5-HT<sub>6</sub> receptors in brain regions relevant to nicotine-mediated performance in the task.

Chapter III looks at the effects of intracerebroventricular (i.c.v.) administration of the recently discovered prolactin releasing peptide (PrRP), endogenous ligand to the GPR10 receptor, on covert attentional orienting. Rt involvement in covert orienting has also been observed, and available data for the distribution of GPR10 suggested a high density in Rt.

**Hypothesis:** it was hypothesised that i.c.v. PrRP administration would mediate covert attentional orienting.

**Result:** PrRP administration had no effect on covert orienting, and recent GPR10 receptor distribution studies show lower distribution within Rt than mRNA distribution would have predicted.

Chapter IV looks at the effects of cholinergic deafferentation of the cortex and Rt after administration of 192-IgG-saporin (an immunotoxin selective for neurons bearing the p75 rat nerve growth factor receptor) into BF or Rt on attentional set-shifting. Previous data have shown that excitotoxic lesions of the prelimbic area of the medial prefrontal cortex and orbito-frontal cortex induce deficits in performance in the rat attentional set-shifting task.

**Hypothesis:** investigating attentional set-shifting performance in rats after cholinergic deafferentation of these areas by injection 192-IgG-saporin into BF would establish whether performance deficits recorded after excitotoxic lesions are mediated by cholinergic function.

**Result:** 192-IgG-saporin lesions of nbM did induce a performance deficit in attentional-set-shifting

Chapter V looks at attentional set-shifting after lesions of serotonergic innervation to forebrain induced after i.c.v. administration of 5,7-dihydroxytryptamine (5,7-DHT). The 5-HT<sub>6</sub> antagonist, SB-271046

has been observed to induce an increase in mPFC ACh levels, and also to improve performance in the rat attentional set-shifting task. Furthermore, there is considerable evidence that manipulation of forebrain serotonin modulates forebrain ACh levels.

**Hypothesis:** it is hypothesised that depletion of forebrain serotonin would affect forebrain ACh levels, inducing an effect on the attentional-set-shifting task.

**Result:** no effect of 5,7-DHT lesion was observed on performance in the attentional set-shifting task.

Chapter VI, the discussion, collates the results obtained from the studies described in this thesis, drawing conclusion and presenting suggestions for further investigations in related fields.

Experimental procedures described within this thesis complied with all national (Animal [scientific procedures] act, 1986) and international (European communities council directive of 24<sup>th</sup> November 1986 [86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific experiments.

## Chapter II

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### **2.1 Part A: an examination of 5-HT (5-hydroxytryptamine) mediation of cholinergic activity using 5-HT<sub>6</sub> receptor subtype-specific antagonist, 5-Chloro-*N*-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046)**

There is evidence that antagonism of 5-HT<sub>6</sub> receptors (one of 14 currently observed 5-HT receptor subtypes) increases cholinergic neurotransmission. This study explores the effect of 5-HT<sub>6</sub> receptor antagonism on covert orienting of attention, a task that is known to be sensitive to manipulation of the cholinergic system.

#### **2.1.1 Introduction**

Overt orienting of attention to a target visual stimulus involves the movement of either, or both, head and eyes. In most species the purpose of overt orienting is to bring the eyes into a position of foveation to the attended target, where visual acuity is greatest. It is possible, however, to shift attention without overt movements of either the head or the eyes, such that a visual target is attended to but not foveated upon.

It has been established that orienting of attention and overt orienting to facilitate foveation are not intrinsically tied functions. In every day perception, visual acuity, enhanced when foveation to a target occurs, is highly important. However, the task described by Posner (1980) is a luminescence detection task, which does not require high acuity. Posner found that human subjects, when confronted with the task would choose to keep their eyes still, directing their attention covertly even when informed that they could move their eyes. The task involves foveating to a central point, whereupon a dimly lit cue to one side would precede a brighter light either in the same location or on the opposite side. Posner found that cues on the same side as the target light (valid) resulted in a faster response time from the subjects than those on the opposite side (invalid). He termed the difference between the response times the “validity effect” and suggested that it represented the time taken for attention to be directed from the invalid cue to the target. In a broader sense, the validity effect is believed to represent the cost of disengaging attention from one spatial location, moving to another and engaging there. Covert orienting is likely a measure solely of attentional processing, without the additional variability of the head and eye movements involved in overt orienting.

This task has applications in several fields, including neurodegenerative disorders in which attention is known to be disrupted. Furthermore, gaining insight into normal attentional function using this

task can provide a better platform from which to initiate investigations into attentional dysfunction irrespective of source.

Posner's covert orienting task has been instrumental in uncovering evidence implying cholinergic systems in the mediation of attentional orienting. Murphy and Klein (1998) demonstrated that nicotine, a cholinergic agonist, induced a decreased response time to invalidly cued targets in human subjects (casual smokers) after tobacco smoke inhalation, with the largest decrease occurring in tests immediately subsequent to smoke inhalation (i.e. when nicotine levels were highest). Witte *et al.* (1997) showed similar nicotine-induced invalid response time reduction in both non-human primate (nicotine injected subcutaneously) and human (chronic smokers inhaling tobacco smoke) subjects. In both studies nicotine induced decreases in both valid and invalid cue reaction times, with invalid cue reaction times being reduced disproportionately more than valid.

Equally, assumed cholinergic depletion has been shown to have an adverse effect on reaction times in the covert orienting task. Parasuraman *et al.* (1994) showed that Alzheimer's Disease (AD) patients demonstrate significantly larger validity effects than control subjects, resulting from increased reaction time to invalidly cued targets. Basal forebrain (BF) cholinergic system lesions in non-human primates have shown induced attentional impairments similar to those seen in AD patients (Voytko *et*

*al.*, 1994). Administration of cholinergic antagonists such as scopolamine would be expected to result in deficits analogous to those seen in AD patients and cholinergic lesioned monkeys, but although scopolamine has, using neuro-imaging techniques, been attributed hypometabolic rate-altering function, at least partially similar to that seen in AD patients (Molchan *et al.*, 1994), recent attentional studies have cast confusion upon its true function. Davidson *et al.* (1999) found that scopolamine administered to monkeys induced an overall increase in reaction times to both valid and invalidly cued targets, but with validly cued targets showing the greatest increase in reaction time. This reduces the validity effect when the expected result would be an increase as invalidly cued target reaction times increase disproportionately as in AD patients. A subsequent study by Davidson and Marrocco (2000), with scopolamine infused into the parietal cortex resulted in similar increases in both invalid and valid reaction times, confirming parietal cortex importance in primate attentional orienting, but providing no further insight into the effects of scopolamine on the validity effect.

The weight of evidence for the effect of nicotine on covert orienting in primates strongly supports a facilitatory role in disengaging attention, and so establishing the same in rodents became a logical step. The covert orienting task as described by Posner is unsuitable for rats, requiring modification (Ward and Brown, 1996; see Figure 2.1), with the effect of nicotine and scopolamine on rat covert orienting only recently being

studied. Phillips *et al.* (2000) administered nicotine or scopolamine subcutaneously, observing an overall nicotine-induced decrease in reaction times, and an overall scopolamine-induced increase in reaction times. Furthermore, consistent with the data from the AD patients (Molchan *et al.*, 1994), nicotine reduced the validity effect by disproportionately decreasing the invalidly cued target reaction times, and scopolamine increased the validity effect by disproportionately increasing the invalidly cued target reaction times. These observations further lends to the body of evidence suggesting cholinergic system involvement in attention.

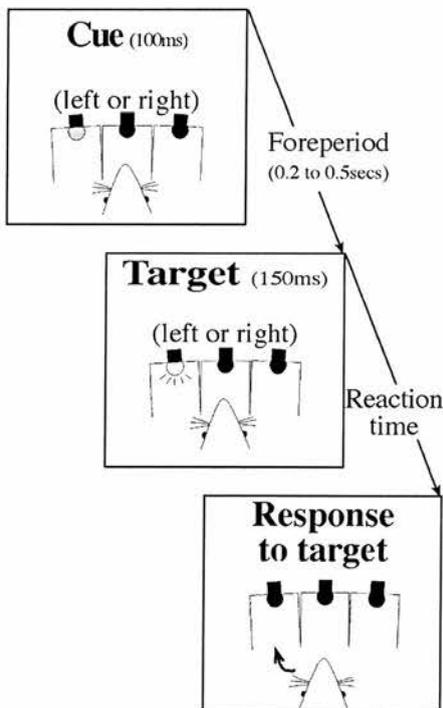


Figure 2.1. A schematic of the covert orienting paradigm in the nine-hole box. When the centre hole is lit, the rat must place its nose in the hole. A dim cue light illuminates in either the left or the right hole for 100ms. The rat must maintain its nose in the centre hole for a variable period (200-500ms) before a bright target light illuminates in either the left or the right hole for 150ms. The rat must then remove its nose from the centre hole. The reaction time is the time taken for the rat to clear its nose from the photocell in the centre hole from the onset of the target light. The movement time is the time taken for the rat to cross the beam of the photocell in the target hole from when it cleared the centre hole photocell. (redrawn from figure 1, Weese, Phillips and Brown, 1999)

It seems likely that, as nicotine decreases reaction times and validity effect in rats, so then would a serotonergic ligand with cholinergic system stimulating properties.

There is evidence that the serotonin receptor subtype 5-HT<sub>6</sub> (Kohen *et al.*, 1996, Yoshioka *et al.*, 1998) has some cholinergic modulatory properties. Studies using chronic (4 days) intracerebroventricular administration of antisense oligonucleotides (AO) selective for the 5-HT<sub>6</sub> receptor subtype have shown significant increases in stretching and yawning behaviour, as compared to the control (saline) and to similar treatment with scrambled (or missense) oligonucleotides. Atropine (a muscarinic receptor antagonist) but not haloperidol (a dopamine receptor antagonist) inhibits the increases in these behaviours. Hence it would appear that the effects of 5-HT<sub>6</sub> receptor knock-down are mediated by acetylcholine (Bourson *et al.*, 1995).

Cloning and *in situ* hybridisation histochemical procedures show high concentrations of 5-HT<sub>6</sub> receptor subtype mRNA in the raphe nuclei, hypothalamus nucleus accumbens, olfactory tubercle, dorsal striatum and areas CA1, CA2 and CA3 of the hippocampus, and frontal, entorhinal and piriform cortices (Monsma *et al.*, 1993; Ruat *et al.*, 1993; Ward *et al.*, 1995; Gérard *et al.*, 1996) with similar (save for the lack of immunoreactivity in the raphe nuclei and hypothalamus) distribution of the receptor itself (Hamon *et al.*, 1999). In the striatum, 5-HT<sub>6</sub> receptors

are expressed on the striatal GABAergic medium spiny neurons. Gérard *et al.* (1997) hypothesised that the mechanism by which 5-HT<sub>6</sub> and muscarinic cholinergic receptors interact involves GABAergic neurotransmission within the dorsal striatum.

The 5-HT<sub>6</sub> receptor subtype-specific antagonist, 4-amino-N-(2,6 bismethylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790), acutely administered systemically, resulted in dose-dependent stretching behaviour (as with the AO treatment previously) but there was no corresponding significant increase in yawning as seen in the previous study. Subjects pre-treated with scopolamine or atropine, also a muscarinic antagonist, showed reduced Ro 04-6790 induced stretching. Pre-treatment with methylatropine (a mAChR antagonist with low blood-brain barrier permeability) did not result in this reduction. These data indicate that the observed effects are centrally controlled. Furthermore, they support the hypothesis that systemic administration of a 5-HT<sub>6</sub> receptor subtype-specific antagonist results (through central nervous system action) in an increase in cholinergic neurotransmission (Bentley *et al.*, 1999).

This study used the covert orienting task designed by Posner (1980) and adapted for the rat by Ward and Brown (1996) to observe the effects of 5-HT<sub>6</sub> receptor antagonism on attention in the rat. A novel, potent and selective 5-HT<sub>6</sub> receptor antagonist, 5-Chloro-N-(4-methoxy-3-piperazin-

1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide, SB-271046, was investigated. SB-271046 has high affinity for both human ( $pK = 8.9$ ) and rat ( $pK = 9.3$ ) 5-HT<sub>6</sub> receptors, with selectivity > 200-fold compared to 55 other receptors, and has a brain penetration of 10% in the rat (Bromidge *et al.*, 1999; Routledge *et al.*, 2000). SB-271046 has been observed to have cognition enhancing effects in both the water maze task and a delayed alternation procedure, although it does not induce stretching as does administration of both 5-HT<sub>6</sub> AOs and Ro 04-6790 (SB-271046 administration (by gavage) does however potentiate physostigmine (an acetylcholinesterase inhibitor) induced yawning (Routledge *et al.*, 1999)). SB-271046 shows no effect on acquisition of the water maze task, but following repeated measures ANOVA, treated rats (10mg/kg SB-271046, the highest tested dose) do show a significant ( $n=16$ ,  $p=0.033$ ) increase in time spent in the platform quadrant of the maze (Rogers *et al.*, 1999). In the alternation task, multicomparisons revealed that treated rats (1mg/kg SB-271046) showed significant difference to the vehicle group ( $p=0.015$ ) (Rogers *et al.*, 1999). The authors hypothesise that 5-HT<sub>6</sub> receptor antagonism is involved in cognitive function, an area in which cholinergic neurotransmission has long been associated. We would hypothesise that such cognitive enhancements are mediated by the cholinergic system, and that 5-HT<sub>6</sub> antagonism enhances cholinergic activity. If 5-HT<sub>6</sub> receptor antagonism does indeed result in an increase in cholinergic neurotransmission, it would be expected that other behaviours previously shown to be

influenced by cholinergic stimulation would be similarly influenced by 5-HT<sub>6</sub> receptor antagonism. In the covert orienting task studied here, that effect would be a reduction in reaction time and decrease in the latency of attentional shifts.

## **2.1.2 Protocol**

### ***2.1.2.1 Animals***

30 male Lister hooded rats (Charles River) were used. The rats were pair-housed and maintained on a 12 hour light/dark schedule (lights on at 7am), with a diet of 15-20g of standard laboratory chow and earned 45mg pellets each day. The initial weight range was between 300g and 410g. At completion of the procedure (approx. 3 months) weight range was between 350g and 450g.

### ***2.1.2.2 Equipment***

The Nine-Hole operant box (CeNeS Ltd., Cambridge, UK) is set within a fan-ventilated, sound-proofed box. The rear of the chamber is a curved wall with nine nose-poke holes. Each hole has a photocell beam across the front to record nose-pokes and a light at the rear to either initiate the task or act as a cue or target. The covert orienting reaction time task (Brown and Robbins 1989; Ward and Brown 1996; Phillips and Brown 1999) requires only the three centre holes, and so the remaining six are closed off. At the front of the chamber, on the wall opposite the nose-poke holes, is a food hopper. The hopper is separated from the

chamber by a top-hinged perspex door with a microswitch to record when the door is opened. The door is suitably weighted to ensure that it closes after use. Food reward pellets (45mg, BioServ Inc., New Jersey, USA) are dispensed automatically into the food hopper. The chamber is lit by a single light in the centre of the ceiling, which switches off when an error is recorded.

The 5-HT<sub>6</sub> antagonist, SB-271046 (SmithKline Beecham Pharmaceuticals, Harlow, UK), is delivered in suspension within a 1% methylcellulose (Sigma Chemical Company, Missouri, USA) solution. 2l of 2% methylcellulose solution was prepared prior to the beginning of the experiment. 40g of methylcellulose was added slowly to 2l distilled water at 60°C. The vehicle solution was stirred throughout addition of the methylcellulose. Once all methylcellulose had entered suspension the heat was turned off and the solution was stirred continuously whilst cooling overnight. The resultant solution was then kept refrigerated for the duration of the experiment. Daily, throughout the experiment, the appropriate 5-HT<sub>6</sub> antagonist dose in 1% methylcellulose was achieved by adding the requisite mass of the drug to a measured volume of 2% methylcellulose solution and then adding an equal volume of distilled water.

### ***2.1.2.3 Training procedure***

The subjects were placed on the defined feeding regime 24 hours before the first training session. The initial training schedule involved habituation, whereby the rats were presented with a lit environment in which they had to push a panel door open to receive a sucrose pellet (the reinforcement reward). At the time they pressed the panel, a light above the food hopper activated to signal the availability of the reward. The sessions lasted 30 minutes, and were repeated until the rats earned approximately 100 pellets in 15 minutes (typically three to four 30 minute sessions).

The second stage of training involved uncovering the central hole of the nine-hole array and rewarding the rat for placing its nose in the hole for a minimum period when a light in the hole was on. Food was delivered to the magazine upon a successful poke. The light in the food magazine came on when the pellet was released, and at this time, the light in the central hole went off. The central hole light re-illuminated when the rat had pushed the panel over the food magazine to retrieve the reward. An incorrect action (removing the nose from the central hole too quickly) resulted in no food reward and the house light was extinguished for 1.5 seconds, during which all responses had no programmed consequence.

Once the rats were capable of maintaining a nose-poke for a delay of 300ms (after 10 days of training), they were moved onto the testing

program. The two holes either side of the central hole were uncapped, and the rat was required to place its nose in the lit central hole as before. A dim cue light activated briefly in either the left or the right hole. After a variable delay (gradually decreased over days to either of four: 200ms, 300ms, 400ms or 500ms) a bright light activated briefly in either the left or the right hole, and the rat had to poke its nose in this hole to receive a reward pellet. The cue was valid when the cue light and the target light were in the same place (50% of trials). An invalid cue was when the cue light and the target light were on opposite sides (50% of trials). Errors resulted in a 1.5 second time-out period and no food reward.

During the nose poke, the rat cannot move its nose, and so no overt orienting by head movement can be achieved. Rats do not possess a high density area of cells on the retina that would be homologous to the fovea in primates (highest to lowest cell density ratio is 5:1 (Sefton and Dreher, 1995)) so it is considered unlikely that any eye movement to orient attention would occur. No eye or head movement confirms any orienting of attention to be covert rather than overt.

Reaction time is the time between the target light activating and the rat removing its nose from the central hole. Movement time was the time to reach the target hole after the rat's nose had been fully removed from the centre hole. Rats typically worked for a maximum of 120 reward pellets a day, and took one month to achieve stable performance.

#### **2.1.2.4 Drug administration**

When performance was stable over seven days (subjects exhibited an overall validity effect), the rats received two days of vehicle (1% methylcellulose, by gavage, bi.d) followed by 8 days of the 5-HT<sub>6</sub> antagonist (by gavage, bi.d) (Rogers *et al.*, 1999). To study both chronic and acute effects concurrently, testing occurred daily during days 1 to 8 of dosing, with rats receiving the first dose of the 5-HT<sub>6</sub> antagonist two hours prior to the daily test. Rats were dosed between 9.00am and 10.30am and tested between 11.00am and 1.30pm. The second dose was given between 4.00pm and 5.00pm.

Rats were randomly assigned to three groups for the study. The control group received a vehicle only dosage; the second group received a 5-HT<sub>6</sub> antagonist dose of 1mg/kg; the third group received a 5-HT<sub>6</sub> antagonist dose of 10mg/kg.

#### **2.1.2.5 Data Analysis**

Mean reaction times and percentage of trials correct were calculated for each rat and analysed using repeat measures ANOVA (SPSS version 10.0) with validity (valid and invalid) and cue-target delay (200, 300, 400 and 500msecs) as within-subject variables and dose (0, 1 and 10mg/kg SB-271046) as between-subjects variable. The validity effect was calculated by subtracting the mean reaction times for validly cued trials from the mean reaction times for invalidly cued trials, and

analysed using repeat measure ANOVA with cue-target delay as within-subject variable and dose as between-subjects variable. Probability density distribution curves for each rat's reaction times at the 200ms cue-target delay were calculated for the factors dose and validity.

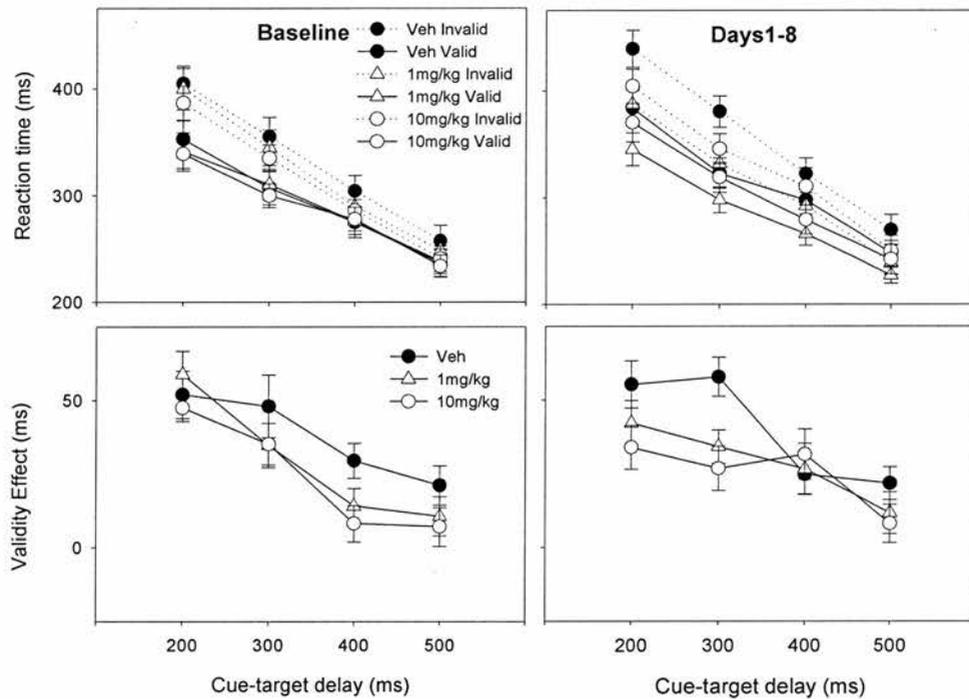


Figure 2.2 Mean  $\pm$  SEM ( $n = 10$ ) of reaction times for both validly and invalidly cued targets, and validity effects for 7 days of baseline data and 8 days of the drug trial.

### 2.1.3 Results

The baseline data show mean reaction times ( $\pm$  standard error mean,  $n = 10$ ) for 7 days of the task (Figure 2.2). Valid reaction times were significantly faster than invalid reaction times, and this effect

diminished as cue-target delay increased. Analysis of the data collected showed significant effects of validity of cue ( $F(1,27) = 174.60, p < 0.01$ ) and of cue-target delay time ( $F(3,81) = 347.99, p < 0.01$ ), and also significant simple interactions between validity of cue and cue-target delay time ( $F(3,81) = 21.14, p < 0.01$ ). These results confirm the importance of cue validity and also cue-target delay in the task. There was also observed an unpredicted, approaching significant, simple interaction between cue validity and group ( $F(2,27) = 2.77, p = 0.08$ ). This is unfortunate as it indicates a trend towards a difference in the groups at the baseline level, where a random selection should show no significant difference. Likewise, analysis of accuracy data (% trials correct) was confounded by a main effect of group in the baseline data ( $F(2,57) = 4.737, p < 0.05$ ), with those animals due to receive SB-271046 showing lower accuracy than those due to receive vehicle administration. This effect was still present after administration ( $F(2,57) = 6.017, p < 0.05$ ).

Analysis of data from days 1-3 (acute study) of drug administration showed no significant effect of dose on reaction time performance between the three test groups ( $n = 10, F(2,27) = 1.48, p > 0.05$ ), although the reaction time performance difference between groups approached significance when the acute study data was compared to the baseline data ( $F(2,27) = 2.94, p = 0.07$ ). With no main effect of dose on reaction time performance after acute administration, nor chronic, data is presented summed over all eight days of drug administration. Analysis of

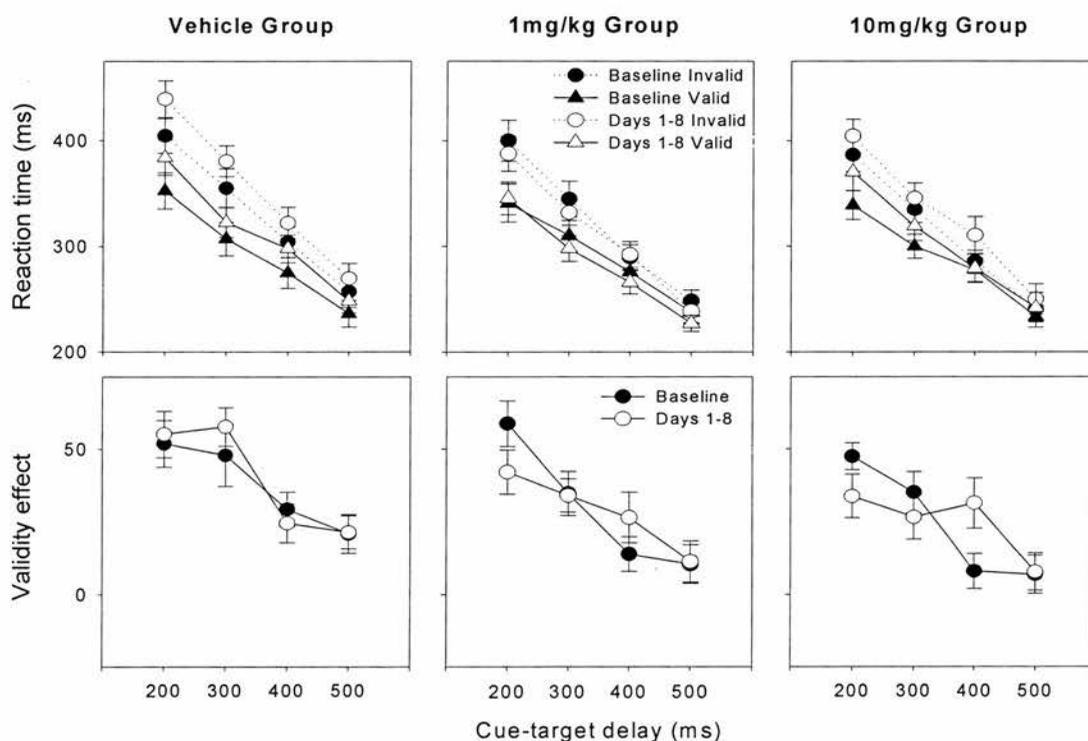
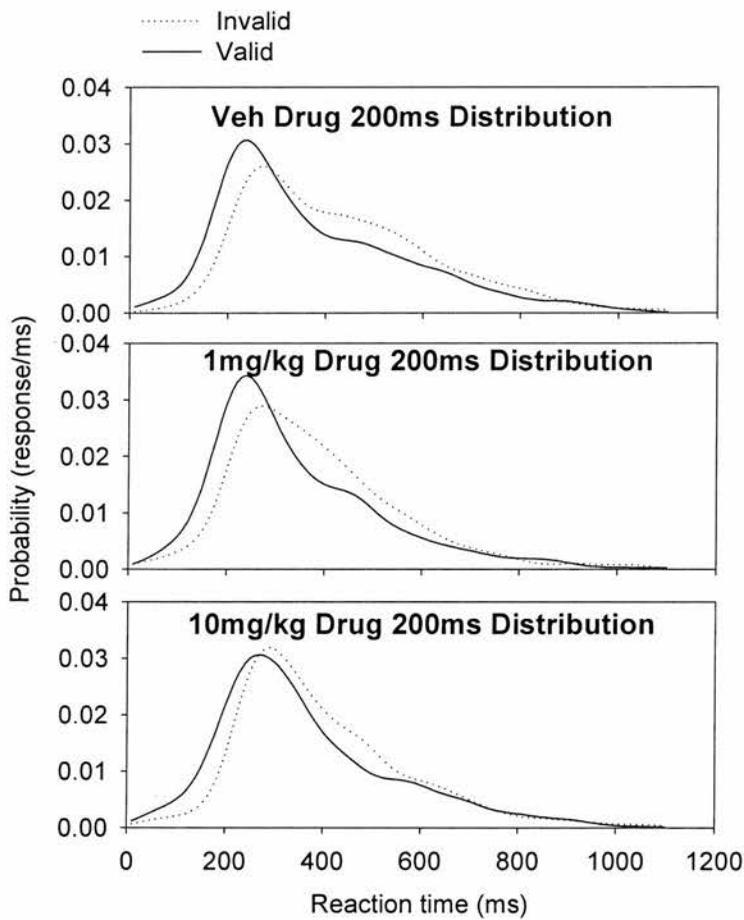


Figure 2.3 Mean  $\pm$  SEM ( $n = 10$ ) reaction times for both validly and invalidly cued targets, and validity effects for 7 days of baseline data and 8 days of drug trial observed by group. No statistically significant differences between groups were observed.

data from all eight days of drug administration (shown in Figure 2.2 and Figure 2.3, mean reaction time  $\pm$  SEM, and validity effect,  $n = 10$ ) showed no significant main effect of drug on reaction time performance between the three test groups ( $F(2,27) = 1.15, p > 0.05$ ).

There was no significant difference in reaction time performance between groups when the eight drug days were compared to the baseline data ( $F(2,27) = 1.37, p > 0.05$ ). These data confirm no chronic effect of SB-217046 administration on reaction time performance.

Figure 2.4 shows the probability density distribution curves for the three condition groups over the eight days of the drug administration, for both validly and invalidly cued targets at the 200ms cue-target delay. The curves show the probability (in responses/ms) of any one reaction time occurring at that cue-target delay period.



*Figure 2.4 Probability density distribution curves for 8 days of drug trial for each group at the 200ms delay.*

#### 2.1.4 Discussion

From the data shown in this experiment, SB-271046 is not seen to have a statistically significant effect on either covert orienting reaction times or validity effect. Our hypothesis was that, as a 5-HT<sub>6</sub> antagonist, SB-271046 would reduce reaction times to both validly and invalidly cued targets in the visuospatial covert orienting task, with a greater reduction in invalidly cued target reaction times, thus reducing the validity effect.

The baseline data presented confirms previously seen evidence for the robust nature of the covert orienting task (Brown and Robbins, 1989; Ward and Brown, 1996; Phillips and Brown, 1999; Phillips *et al.*, 2000), demonstrating both the statistically significant validity effect, and its diminishing as cue-target delay increases.

That there is a mediating effect on cholinergic neurotransmission by 5-HT<sub>6</sub> receptor activity cannot be discounted by the presented data though, despite the lack of confirmatory evidence. Other studies have provided evidence supporting such a role (Bentley *et al.*, 1999, Bourson *et al.*, 1995), and the nature of the investigation in this experiment can be attributed in part to explaining the data.

SB-271046 is a novel antagonist and remains yet to have all characteristics of its functional and behavioural effects explored. No

evidence existed affording SB-271046 either a chronic or an acute effect upon the cholinergic system, and a study that did not take a possible chronic effect into account could provide false or incomplete data. This study used three groups of ten subjects, allowing three conditions, control, low dose (1mg/kg of rat) and high dose (10mg/kg of rat) to be run simultaneously. Evidence of an acute effect would be studied on days one through three of the procedure, and that of a chronic effect, through days four through eight. Running three groups simultaneously reduced the duration for which experimentation would be required, and also removed the risk of any, so far unidentified, chronic effect interfering with data were a single group put through all three conditions. All thirty rats were trained together and then randomly distributed into the separate condition groups. This would be expected to provide three sets of normally distributed baseline data.

Analysis of the three baseline data sets (Figure 2.2) showed a difference approaching statistical significance in validity effect between each group. The baseline data for the control group shows a greater invalid reaction time than the low dose group, which shows a greater invalid reaction time than the high dose group. Before the groups received the 5-HT<sub>6</sub> antagonist, they were already showing a trend, following a pattern fitting our hypothesis based on the dose they would receive. This chance initial disparity caused interpretation of the data received at the

end of the procedure to be more complicated than would otherwise have been preferred.

Furthermore, the data shows that there is no statistically significant effect of acute administration of SB-271046. However, that the interaction between baseline reaction times and acute study data approaches a significant interaction with dose cannot be ignored, and would validate further study. These data also suggest that there is no chronic effect of administration of SB-271046 on covert orienting of attention. Other data from studies using SB-271046 lend further support to this argument (Jones, *pers comm*), although studies into the effects of acute as opposed to chronic effects of SB-271046 administration on cognition are on going. The data presented here suggest that any subsequent study would not need to proceed as far as eight days, and would permit an experimental design with only one group, but undergoing all three conditions.

The distribution curves (Figure 2.4) show that there is no difference between the three groups' reaction time performance over the eight days of the drug administration at the 200ms cue-target delay (where validity effect is largest and predicted reductions would be greatest). This further confirms that chronic SB-2712046 administration has no effect on covert orienting of attention.

In summary, the data presented provides no conclusive evidence of serotonergic mediation of cholinergic control of attention, although requirements of the experimental design may be a factor in complicating the interpretation of the results. The approaching significance of dose on acute study data relative to baseline data means the effects of SB-271046 administration on covert orienting are worth further investigation.

## **2.2 Part B: further examination of 5-HT (5-hydroxytryptamine) mediation of cholinergic activity using 5-HT<sub>6</sub> receptor subtype-specific antagonist (SB-271046)**

### **2.2.1 Introduction**

As has been observed in the first section of this chapter, evidence for mediation of cholinergic activity by SB-271046 has not been confirmed by the covert orienting task. However, it was possible that the between-subjects design had resulted in low power. Therefore an alternate design was used.

The alternate procedure, in light of no evidence suggesting a chronic effect of SB-217046 administration, removed the possibility of the initial disparity observed in the first procedure. The hypothesis remains that administration of the 5-HT<sub>6</sub> receptor-specific antagonist SB-271046 will result in a reduction in reaction times in the covert orienting task described earlier, similar to that previously observed subsequent to

nicotine administration. Furthermore, we would hypothesise that any observed effect on reaction time is mediated through serotonergic modulation of the cholinergic system.

## **2.2.2 Protocol**

### ***2.2.2.1 Animals***

26 male Lister hooded rats (Charles River) were used. The rats were maintained as previously referenced in the protocol for Part A. The initial weight range was between 220-280g. At completion of the procedure (approx. 3 months) weight range was between 350-450g.

### ***2.2.2.2 Equipment***

Equipment is as referenced in Part A.

### ***2.2.2.3 Training procedure***

Training procedure is as referenced in Part A.

### ***2.2.2.4 Drug administration***

When performance was stable over seven days, the rats received 5 days of either one of three doses of the 5-HT<sub>6</sub> antagonist SB-271046 (in 1% methylcellulose vehicle) or vehicle only (by gavage). The rats were administered with SB-271046 between the hours of 10.00am and 11.45am with testing commencing between 12.00pm and 2.30pm.

SB-271046 was administered by a pseudo-random assignment, with rats divided into sub-groups of six. Within each subgroup, no rat received the same order of dose. On any given day of the procedure a rat received either vehicle (1% methylcellulose solution), 1mg/kg, 10mg/kg or 30mg/kg SB-271046. The pseudo-random assignment of dose removed the possibility that day of administration had an effect on the rats' reaction times by ensuring that data for each dose came from all days of the procedure. The 30mg/kg dose was added to as it was considered that a possible effect in the first version of this study may have been so small as to be occluded by the baseline data differences. Addition of a higher dose could induce a larger, more observable effect.

#### ***2.2.2.5 Data analysis***

Mean reaction times and percentage of trials correct were calculated for each rat and analysed using repeat measures ANOVA with validity (valid and invalid) and foreperiod (cue-target delay; 200, 300, 400 and 500msecs) as within subject variables and with dose (0, 1, 10 and 30mg/kg SB-271046) as between-subjects variable. The validity effect was calculated by subtracting the mean reaction times for validly cued trials from the mean reaction times for invalidly cued trials, and analysed using repeat measure ANOVA with cue-target delay as within subject variable and dose as between-subjects variable.

Data collected from drug naïve subjects (the first day of SB-271046 administration) were also analysed separately, again using repeat measures ANOVA, allowing confirmation that previously administered doses were not affecting any day's observed reaction times.

### 2.2.3 Results

The baseline data show mean reaction times ( $\pm$  standard error mean,  $n = 26$ ) for 7 days of the task (Figure 2.5). Valid reaction times were significantly faster than invalid reaction times, and this effect diminished as cue-target delay increased. Analysis of the data collected showed significant effects of validity of cue ( $F(1,25) = 295.26, p < 0.01$ ) and of cue-target delay time ( $F(3,23) = 253.82, p < 0.01$ ), and also significant simple interactions between validity of cue and cue-target

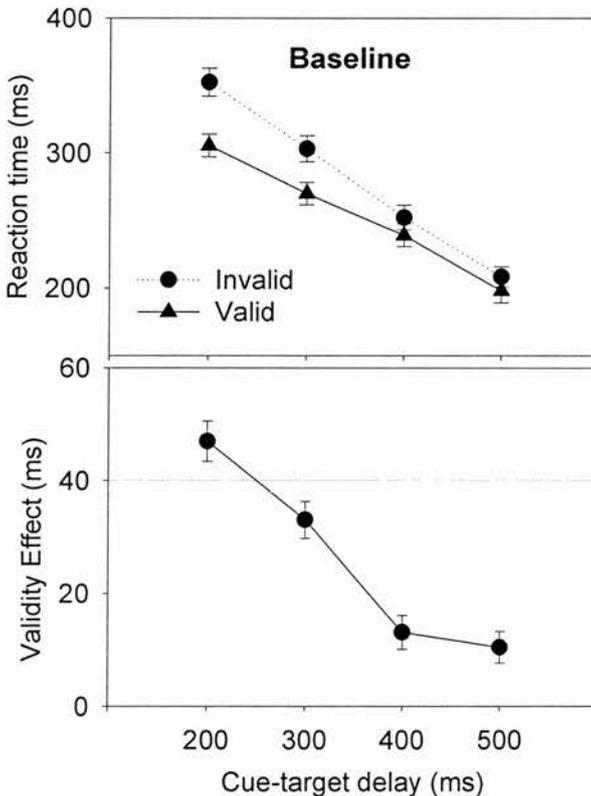


Figure 2.5 Mean  $\pm$ SEM ( $n = 26$ ) reaction times for both validly and invalidly cued targets, and validity effect for 7 days of baseline data.

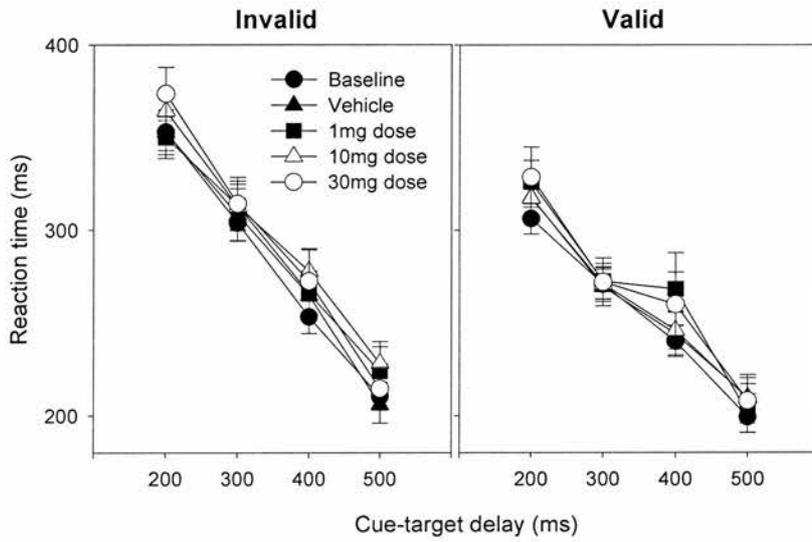


Figure 2.6 Mean  $\pm$  SEM ( $n = 26$ ) of reaction times for both validly and invalidly cued targets for baseline and drug trial data.

delay time ( $F(3,23) = 21.30, p < 0.01$ ). These results confirm the statistically robust nature of the task, comparing favourably to the data observed in Part A of this study.

Analysis of data from the drug trial showed no statistically significant effect of drug administration on either reaction times (Figure 2.6;  $F(4, 125) = 0.32, p > 0.05$ ) or validity effect (Figure 2.7;  $F(4,125) = 1.74, p > 0.05$ ).

SB-217046 was administered pseudo-randomly throughout the 5 days of drug study. By examining the data from day 1 only, it is possible to see whether there is an effect of dose on drug naïve subjects. Only vehicle, 1mg/kg and 10mg/kg doses were administered on day 1 due to

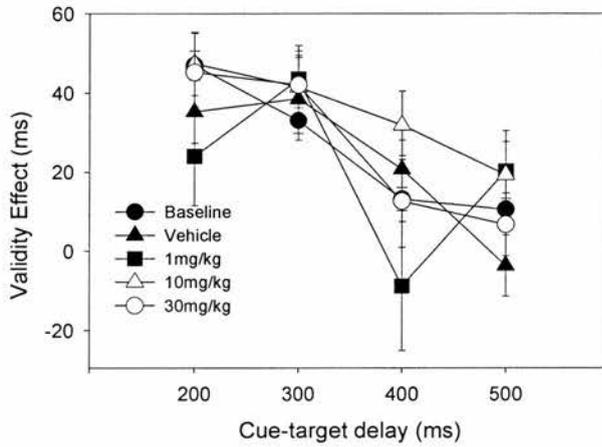


Figure 2.7 Mean  $\pm$  SEM ( $n = 26$ ) validity effects for baseline and drug trial data.

external, non-design-related constraints, so there is no data for the 30mg/kg dose. There was no statistically significant effect of SB-217046 administration on reaction time performance in drug naïve subjects (Figure 2.8;  $F(1,23) = 0.06, p > 0.05$ ).

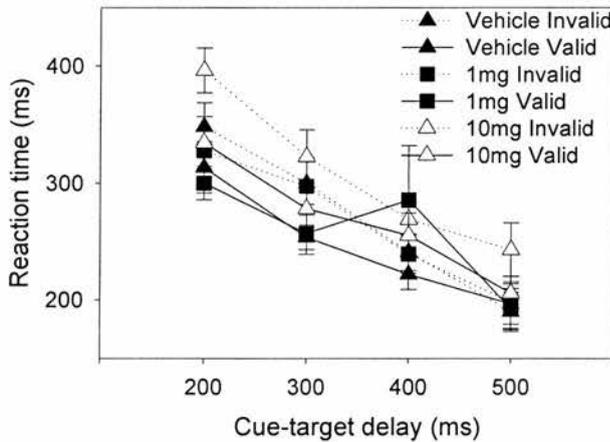


Figure 2.8 Mean  $\pm$  SEM of reaction times for both validly and invalidly cued targets for day 1 of drug trial.

Figure 2.9 shows the validity effects for the three doses of SB-271046 that were administered to drug naïve subjects. The data for the

comparison curve (baseline) for each dose was taken from the corresponding subjects receiving each dose. There was no statistically significant effect of SB-217046 on validity effect on drug naïve subjects ( $F(1,23) = 0.62, p > 0.05$ ).

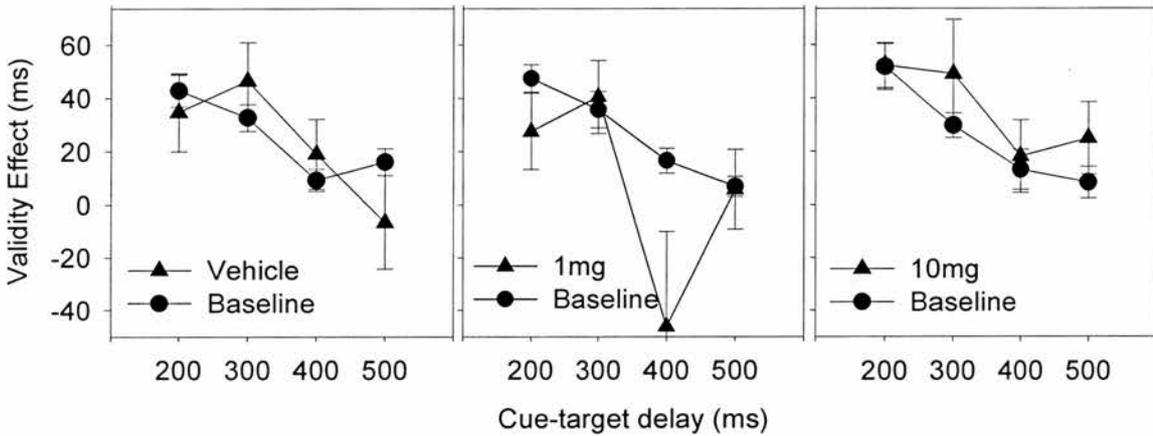


Figure 2.9 Mean  $\pm$  SEM of validity effects for 3 doses of SB-271046 in drug naïve subjects.

## 2.2.4 Discussion

There was no effect of SB-271046 administration on reaction times or validity effect in the covert orienting task. Observations suggesting that the validity effect was reduced as a function of dose of SB-271046 (Part A) are therefore likely to be the result of the baseline differences between groups.

Data from these two studies have been consistent in there being no reduction in reaction times, despite seeing such differences in previous nicotine administration studies. It is likely then that systemic administration of SB-271046 does not modulate cholinergic neurotransmission in the same fashion as systemic administration of nicotine. These data, in addition to evidence that systemic SB-271046 administration (by gavage) does not induce stretching as observed after Ro 04-6790 administration (Routledge *et al.*, 1999), suggest that 5-HT<sub>6</sub> receptor modulation of cholinergic neurotransmission does not involve brain regions associated with attentional function in this task. Furthermore, recently acquired evidence from microdialysis studies have demonstrated SB-271046 administration-induced increases in cortical glutamate (GL) and aspartate (AS), but not noradrenaline (NA), dopamine (DA) or 5-HT (Dawson *et al.*, 2000). Furthermore, no increases in any of these neurotransmitters release were observed in striatum. Administration of tetrodotoxin, a sodium channel blocker, attenuated the observed increase in release of GL and AS. The authors suggest that 5-HT<sub>6</sub> receptors mediate GL and AS release through tonic inhibition, with the antagonist, SB-217046, blocking this inhibition.

Very recent microdialysis data suggests that SB-271046 robustly increases acetylcholine release in prefrontal cortex (Jones, 2002, personal comm.), an area traditionally associated with attentional function (Sarter *et al.*, 2001). That there is no effect of SB-271046 administration in the

covert orienting task suggests that observations from previous covert orienting studies after administration of cholinergic agonists/antagonists are not founded in effects mediated by prefrontal cortex acetylcholine. It is likely then that attentional impairments/enhancements seen after systemic cholinergic manipulations are mediated by other areas receiving cholinergic input, possibly the thalamic reticular nucleus (Crabtree, 1999; Weese *et al.*, 1999).

In conclusion, the data observed in this study suggests that modulating cholinergic neurotransmission through systemic administration of a 5-HT<sub>6</sub> antagonist is not suitable for exploring cholinergic mediation of attentional function in this covert orienting task. Evidence presented so far implicates a role for the thalamic reticular nucleus (Rt), and Rt cholinergic function in covert orienting. The lack of 5-HT<sub>6</sub> receptors in Rt suggests that any potentiating effect that antagonism of 5-HT<sub>6</sub> receptors has on cholinergic function would be best explored using behavioural tasks known to exploit prefrontal cortex.

### **2.2.5 Summary of Findings**

- Rats trained in a covert orienting of attention task were administered the 5-HT<sub>6</sub> receptor-selective antagonist, SB-271046 (by gavage, bi.d)
- Administration of SB-271046 had no effect on performance in covert orienting of attention in the rat.

- It is suggested that as SB-271046 potentiates cholinergic function in PFC, then PFC ACh is unlikely to be involved in performance in covert orienting of attention.
- It is suggested that cholinergic involvement in covert orienting arises in the Rt, where there are no 5-HT<sub>6</sub> receptors.

## Chapter III

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### **3.1 Part A: does manipulation of Prolactin Releasing Peptide in Thalamic Reticular Nucleus mediate attentional function in a covert orienting task?**

The Thalamic Reticular Nucleus (Rt) is a thin lamina of GABAergic neurons surrounding the thalamus. There is evidence supporting the Rt having a role in mediating attentional function, hypothesised to act as a gateway, filtering information between cortex and thalamus. Prolactin Releasing Peptide (PrRP) is an endogenous ligand to a recently discovered receptor, GPR10, with a relatively high density of mRNA expression within Rt. This study investigates manipulation of PrRP in the Rt using an attentional task that has been shown to be sensitive to Rt manipulation.

#### **3.1.1 Introduction**

The role of the Rt in attentional function has been explored considerably since initial anatomical (Jones, 1975; Ohara and Lieberman, 1985) and electrophysiological studies (Yingling and Skinner, 1977; Skinner and Yingling, 1977). It is suggested that Rt acts as a gateway between cortex and thalamus, filtering information from thalamocortical and corticothalamic axons (Crick, 1984). The Rt receives excitatory input

from collaterals branching from both of these glutamatergic axon types, and in turn projects back to thalamus, forming inhibitory synapses. Within the Rt several sub-sectors can be identified topographically, dependent upon collaterals received from sensory and motor cortices (for review see Guillery *et al.*, 1998).

Selective attentional function relies upon the ability to select specific stimuli to be processed at the expense of others. Neurotransmission pertaining to the selected stimuli must be potentiated, with neurotransmission of corresponding unattended stimuli attenuated. If selective attention is a function of the Rt, then it must be able to filter information, enhancing processing of certain attended stimuli over others. A suggested mechanism for this in the Rt arises from inhibitory synapses to other Rt neurons, including other modality-specific areas within Rt (Jones, 1975; Cox *et al.*, 1997; Sanchez-Vives *et al.*, 1997). If neurons within Rt can inhibit other, modality specific areas, either by inhibiting input to, or neuronal activity of (or both) other Rt neurons, then processing of the relevant stimuli can occur without competition from other, irrelevant stimuli.

To complement the physiological evidence for Rt involvement in selective attention, recent behavioural studies have provided observations further suggesting this role for the Rt. Studies of c-Fos-related activity after rats were exposed to a novel complex visual environment revealed

expression in the visual area of Rt (dorsal-caudal) (Montero, 1997). This expression is attenuated by ibotenic acid lesions of visual cortical layer 6 which provides most of the innervation to visual Rt (Montero, 2000). Further studies involving functionally blind rats (amblyopic) in a novel complex somatosensory environment revealed c-Fos-related activity in the somatosensory area of Rt (Montero, 1999). It is possible, however, that such expression could be the result of exploratory behaviour, as this and any attentional factor within the task cannot be separated. Out with this possibility, such data provides evidence supporting the hypothesis that lateral inhibition within Rt permits selective attention – processing of relevant stimuli is increased at the expense of irrelevant stimuli. In the case of Montero's studies, activity within Rt is at its greatest in regions associated with processing the most valuable information. Activity in other areas is suppressed: the lateral inhibition theory suggests inhibitory connections between Rt neurons suppress processing of less valuable information, allowing faster processing of more valuable information. These data alone, however, cannot confirm such a function within Rt.

McAlonan and Brown (*unpub. obs.*) used c-Fos-related activation in rats after an attentional orienting task to provide further evidence to support the role of the Rt in selective attention. Rats were trained to either respond to, or away from, a visual cue, to allow for an observation, and hence separation of, any observed motor response. Increased c-Fos-related activity was consistently observed in visual Rt, both in subjects

trained to respond to the cue and those trained to respond away from the cue, contralateral to the attended stimulus. This suggests that Rt is involved in selective attention and not motor response, as irrespective of the subjects' response intention, activation of the visual Rt occurred only in response to the cue. Additionally, rats were classically conditioned to either a visual or an auditory stimulus (or both), and subjected to both stimuli simultaneously to observe the effects of "blocking" on attentional selectivity within Rt. Again c-Fos-related activity measurements were taken in both visual and auditory Rt (ventral caudal Rt), revealing that visual Rt was activated in rats conditioned to the visual stimulus, auditory Rt was activated in rats conditioned to the auditory stimulus, and both auditory and visual Rt were activated in rats trained to respond to both (McAlonan *et al.*, 2000).

Rt has also been observed involved in covert orienting of attention. Unilateral excitotoxic lesions (ibotenic acid) of Rt induce an increase in reaction time to validly cued targets contralateral to the lesion. There was no corresponding increase in reaction times to invalidly cued targets contralateral to the lesion, resulting in a reduction in the validity effect (Weese *et al.*, 1999). In the covert orienting task (Posner, 1980; Ward and Brown, 1996; see also Chapter II) reaction times to validly cued targets are reduced as a benefit of the subjects attention to the cue. That this was removed on the side contralateral to a Rt lesion in rats indicates that the rats received no benefit from the cue. This implicates

the Rt in attention, and makes the covert orienting task a useful test of Rt function.

The human GPR10 receptor was first cloned using polymerase chain reaction (PCR) to amplify human genome DNA in 1995 (Marchese *et al.*, 1995). Sets of primers were selected based on previously observed highly conserved regions within identified G protein-coupled receptors (GPCRs), a group of receptors of which many peptide-binding receptors are a member. hGPR10 was observed to share an amino acid identity with neuropeptide Y1 receptor subtype of 31%, with the transmembrane aspects of the receptor sharing 46% amino acid identity with neuropeptide Y1 receptor subtype.

PrRP was first isolated in 1998 as an endogenous ligand to the hGR3 receptor, specifically expressed in the human pituitary, through extraction from bovine hypothalamus tissue (Hinuma *et al.*, 1998). PrRP was so named as it was observed to potentiate prolactin release in *in vitro* rat anterior pituitary cells to a greater degree than any other peptide known to do so (vasoactive intestinal peptide, oxytocin, substance P, neurotensin, arginine-vasopressin, pituitary adenylate cyclase-activating polypeptide and galanin) . hGR3 was observed to be almost identical to GPR10 and is considered analogous to Unknown Hypothalamic Receptor-1 (UHR-1) found in the rat. These initial studies demonstrated that PrRP exists in two forms: PrRP31, so named because it is 31 amino acids in

length; and PrRP20, which is 20 amino acids in length. PrRP20 appears to be a truncated version of PrRP31, and both share an identical N-terminal portion. Both PrRP31 and PrRP20 have been identified in bovine, human and rat tissues, and the larger precursor protein to bovine PrRP (a prepropeptide) has been shown to be cleavable to form both PrRP31 and PrRP20 (Hinuma *et al.*, 1998).

Both PrRP distribution and PrRP mRNA expression have been studied extensively since its discovery. Furthermore, studies to uncover GPR10/UHR-1 localisation through mRNA expression and immunohistochemistry have provided valuable evidence for PrRP/GPR10 function within the central nervous system. Reverse transcription PCR techniques have shown PrRP mRNA expressed in both medulla oblongata and hypothalamus of rats, with higher bioactive PrRP observed in hypothalamus tissue extracts (Hinuma *et al.*, 1998). Further studies (Minami *et al.*, 1999) in rats using *in situ* hybridisation techniques have shown PrRP mRNA in the posterior region of the dorsomedial nucleus of the hypothalamus (DMH; Paxinos and Watson, 1998, label this region “ventral DMH”) and in the caudal region of the solitary tract nucleus (NTS) and in caudal ventrolateral medulla (specifically the ventrolateral intermediate reticular field (VLIRt)).

Matsumoto *et al.* (1999a) further developed an immunoassay to allow investigation of immunoreactive (ir-) PrRP in rats. This process

showed high concentrations of ir-PrRP (31 and 20) in the hypothalamus, confirming previous observations, and peripherally in the adrenal gland, although high performance liquid chromatography revealed only PrRP31 in peripheral tissues.

Immunocytochemical studies using antibodies raised to PrRP have revealed PrRP immunoreactive neurons located in the ventral DMH (Yamakawa *et al.*, 1999), demonstrating synergy between PrRP mRNA expression and PrRP localisation. Furthermore, terminals in the parastrial nucleus and the bed nucleus of the stria terminalis were heavily stained, with terminals in the paraventricular thalamic nucleus and paraventricular and ventromedial hypothalamic nuclei intermediately stained. Implications upon PrRP mediation of prolactin release were observed, as there were no observed PrRP immunoreactive neurons projecting from DMH to the external layer of the median eminence – the site of hypothalamic hormone release. This suggests that hypothalamic PrRP differs from other hypothalamic hormones in its route to affect prolactin release.

Rat brain *in situ* hybridisation studies have revealed GPR10 receptor mRNA localisation in thalamic reticular nucleus (Rt), periventricular hypothalamus, DMH, NTS, area postrema, anterior pituitary, and adrenal medulla (Roland *et al.*, 1999). There is considerable

consistency between observed PrRP immunoreactivity, PrRP mRNA expression and GPR10 mRNA expression.

PrRP function in the central nervous system is still under investigation, although several areas have been explored already. PrRP mediation of prolactin release has been under scrutiny after PrRP immunoreactivity studies failed to observe PrRP immunoreactive neurons projecting to the traditional site of hypothalamic hormone release. Matsumoto *et al.* (1999b) administered PrRP31 intravenously to male rats and to female rats exhibiting two or more consecutive oestrus cycles. Blood samples were taken 2, 5, 10 and 20 minutes after injection and plasma analysed for prolactin levels. Prolactin levels were observed to be significantly higher after 5 minutes and a PrRP dose of 50nmol/kg in females; 500nmol/kg in males. It is suggested that female rats are more sensitive to PrRP induced prolactin release because of other factors involved in the oestrus cycle.

In a third study, Matsumoto *et al.* (2000) studied c-Fos protein accumulation in the brain after intracerebroventricular (i.c.v.) PrRP31 administration. c-Fos related immunoreactivity was observed in the paraventricular nucleus (PVN). Double immunostaining for c-Fos related activity and for corticotropin releasing hormone (CRH) evidenced that c-Fos related activity was localised to CRH-positive cells in the PVN. Furthermore, synapses between PrRP neurons and CRH cell bodies were

observed in the PVN, suggesting PrRP31 has a modulatory role in CRH secretion. Subsequently rats were i.c.v. administered PrRP31 and their adrenocorticotropin (ACTH) blood plasma levels investigated. CRH mediates ACTH release, therefore any agonistic effect mediated by PrRP31 on CRH would result in an increase in blood plasma ACTH. This increase was observed in the test subjects, as well as attenuation of PrRP31 induced ACTH release after intravenous pre-treatment with  $\alpha$ -helical CRH, a potent CRH antagonist. Increased blood plasma ACTH is a factor in stress response, which implicates PrRP31 in stress.

Other central functions of PrRP and GPR10 are still under investigation. PrRP31 administered through i.c.v. significantly reduces time spent in an active state during behavioural observations, but has no effect on rats in a social interaction test (Jones *et al.*, unpublished). Evidence of GPR10 mRNA in the Rt suggests a role in either attentional function, or sleep/wake pattern control (Marks and Roffwarg, 1993; Crabtree, 1999) – two functions in which a role of the Rt has been implicated.

This study uses the covert orienting task as adapted for the rat by Ward and Brown (1996) to observe the effects of i.c.v. PrRP administration on attentional orienting. mRNA for the GPR10 receptor is expressed in the Rt relatively densely, and it is likely that the GPR10

receptor itself is as well. It is hypothesised that PrRP in Rt will stimulate Rt neurons, and that this effect will potentiate processing of relevant stimuli, and thus attentional function in the covert orienting task, via corticothalamic collaterals and the Rt's projections to thalamus and subsequent feedback from thalamocortical collaterals.

### **3.1.2 Protocol**

#### ***3.1.2.1 Animals***

As a preliminary experiment, 2 male Lister hooded rats (Charles River) were used. The rats were pair-housed until cannulae implantation and maintained on a 12 hour light/dark schedule (lights on at 7am), with a diet of 15-20g of standard laboratory chow and earned 45mg pellets each day. The initial weight range was between 300-350g. At completion of the procedure weight range was between 340-480g.

#### ***3.1.2.2 Equipment***

The Nine-Hole operant box, food pellets and covert orienting requirements are as referenced in Chapter II Part A.

The injection and guide cannulae were supplied by SmithKline Beecham Pharmaceuticals, Harlow, UK. When inserted into the guide cannulae, the injection cannulae projected 1mm beyond the guide cannulae. The injection cannulae were attached to tubing using two-part

epoxy glue to ensure stability and an airtight seal and allowed to dry for 24 hours. Cannulae were secured to the skull using screws and Simplex Rapid (Austenal Dental Products Ltd, The Crystal Centre, Harrow, England). Simplex Rapid is a powder that requires mixing with methyl methacrylate (Wright Cottrell and Company, Dundee, Scotland) to produce an acrylic solution.

Anaesthesia is administered via a Narkovet 2 (North American Dräger) with Halothane vapour mix controlled by a Halothan vapor 19.1 (Drägerwerk AG, Lübeck).

The Prolactin Releasing Peptide (SmithKline Beecham Pharmaceuticals, Harlow, UK) was initially stored as a solid at  $-40^{\circ}\text{C}$ . For the microinjections the PrRP was dissolved in sterile saline (Baxter Healthcare Ltd, Norfolk, England) producing concentrations of  $2\mu\text{g}/\mu\text{l}$  and  $6\mu\text{g}/\mu\text{l}$  and stored in the freezer at  $-40^{\circ}\text{C}$ .

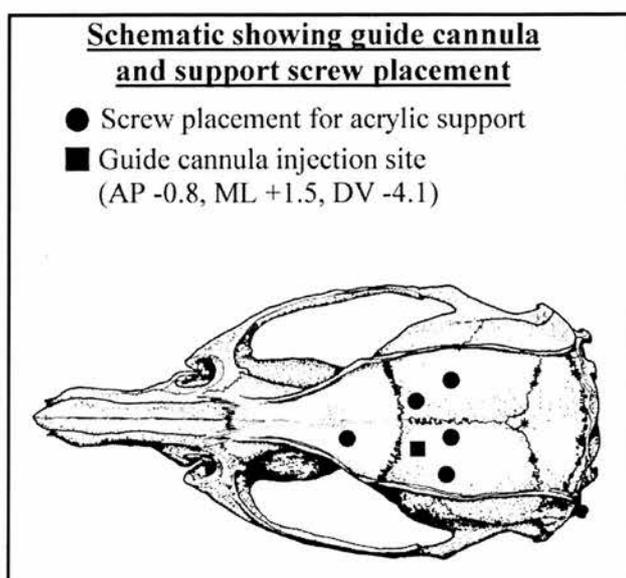
Microinjections were administered to the rats using  $10\mu\text{l}$  Hamilton microlite 700 series syringes (Aldrich Chemical Company, Milwaukee, WI, USA) and an sp200i syringe pump (World Precision Instruments, Stevenage, UK).

### ***3.1.2.3 Training procedure***

Training procedure is as referenced in Chapter II Part A.

#### ***3.1.2.4 Surgery***

When performance was stable the rats were unilaterally implanted with guide cannulae into the left lateral ventricle. The rats were anaesthetised with a halothane (Rhodia Ltd, Avonmouth, Bristol, England), nitrous oxide and oxygen mix, initially with halothane concentration at 4%, nitrous oxide at 0.8l/min and oxygen at 0.4l/min. Once anaesthetised, rats were mounted on the stereotaxic frame, and halothane concentration was reduced to 1.8% to maintain anaesthesia. The guide cannulae were implanted using stereotaxic co-ordinates (Paxinos and Watson, 1986); level skull -3.3mm, AP -0.8mm, ML 1.5mm (from bregma), DV -4.1mm (from skull surface at insertion site). Five screws were attached to the skull to provide support for acrylic, applied to the skull to stabilise the guide cannulae. Figure 3.1 shows the locations of the screws in relation to the implanted cannula.



*Figure 3.1 Schematic of the surface of the rat's skull, showing placement for the guide cannula, the guide cannula support frame, and screw placement for acrylic support. Adapted from Paxinos and Watson, 1986.*

The skull surface was dried before application of the acrylic to ensure strong bonding and to minimise the possibility of infection. The stereotaxic frame supporting the guide cannula was removed when the acrylic was dry. The halothane concentration was reduced to 1% and the wound was sutured using 4/0 coated vicryl sutures (Ethicon, Edinburgh, UK) and Intervet P.E.P. powder (Cyanamid Ltd, Hampshire, UK) was administered to further reduce the chance of infection. Sterile caps were secured to the guide cannulae. Rats were removed from the stereotaxic frame and left to recover in a warm environment. Upon recovery, rats were returned to the holding room with free access to food and water for

the next 24 hours. Rats were weighed daily to monitor recovery. Caps were changed daily with sterile replacements.

#### ***3.1.2.5 Drug administration***

Three days after surgery rats were returned to the covert orienting task and baseline data were recorded. When performance was stable over 6 days, rats received microinjections of 5 $\mu$ l sterile saline. A 10 $\mu$ l Hamilton syringe was filled with alcohol, and the tubing was flushed through with sterile saline. The Hamilton syringe was attached to the tubing via a 30gauge needle, with the end of the tubing pinched to minimise air-bubble formation in the tubing. The plunger on the syringe was fully depressed, then withdrawn by 0.2 $\mu$ l to create a small air-bubble at the injection cannula end. The injection cannula was then placed into sterile saline and the plunger of the syringe was drawn back to take about 8 $\mu$ l sterile saline into the tubing. The air-bubble at the injection cannula end of the tubing was marked to allow monitoring of injection progress. The sp200i pump depressed the syringe plunger at a rate of 3 $\mu$ l/min, with full injection of 5 $\mu$ l sterile saline taking 100 seconds. The injection cannula was left in place for a further 90 second infusion period. The injection cannula was then removed from the guide cannula, which was sealed with a sterile cap. The rat was then placed back in its holding cage for 20 minutes before being placed in the nine-hole operant box and the task begun.

Rats received injections of 5µl sterile saline on days 1 and 2 of the procedure. On day 3 rats received injections of 5µl of 2µg/µl PrRP in sterile saline (10µg PrRP). On day 4 rats received an injection of 5µl sterile saline, with 5µl 2µg/µl PrRP again on day 5. Rats received sterile saline injections again on day 6. On day 7 rats received 5µl 6µg/µl PrRP in sterile saline (30µg PrRP), with saline again on day 8 and 6µg/µl PrRP on day 9. On each day, rats underwent the covert orienting task 20 minutes after injection. Data were collected throughout the procedure and analysed daily. From day 10 to day 16 rats were administered with 5µl sterile saline, and data recorded from the task was monitored until rats' reaction times had returned to baseline level.

### ***3.1.2.6 Histology***

Rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB; Disodium hydrogen orthophosphate and Sodium dihydrogen orthophosphate in distilled water) after anaesthesia with 0.8ml Dolethal (Univet, Bicester, Oxfordshire, UK). Brains were stored overnight at 4°C in 20% sucrose solution, then cut to 50µm sections on a microtome (Jung Histoslide 2000, Reichert-Jung, Cambridge Instruments GmbH) into 0.1M phosphate buffer saline (0.9%) (PBS). Sections were stained with cresyl violet to confirm guide cannulae placement using the following protocol. Sections were mounted onto pre-treated glass slides then stored overnight in a formalin bath. Sections were then de-fatted with xylene, and re-hydrated with ethanol, then 50% ethanol solution, then distilled

water. Sections were immersed in cresyl violet solution (cresyl fast violet acetate soluted in distilled water and glacial acetic acid, pH adjusted to 3.5 with sodium acetate) for 2 minutes then washed in running water for 5 minutes. Sections were subsequently dehydrated in 50% ethanol solution, ethanol and finally xylene. Coverslips were applied with DPX mountant (BDH Laboratory Supplies, Poole, UK).

### **3.1.2.7 Data analysis**

Mean reaction times were calculated for each rat and analysed using repeat measures ANOVA (SPSS version 10.0) with validity (valid and invalid) and cue-target delay (200, 300, 400 and 500msecs) as within subject variables, and dose (0, 10 and 30 $\mu$ g/5 $\mu$ l) as between-subjects variable. The validity effect was calculated by subtracting the mean reaction times for validly cued trials from the mean reaction times for invalidly cued trials, and analysed using repeat measure ANOVA with cue-target delay as within subject variable and dose as between-subjects variable. Probability density distribution curves for each rat's reaction times at the 200ms cue-target delay were calculated for the factors dose and validity.

Time to completion of task was recorded each day to monitor for any reduction in time spent active as has been previously observed (Jones *et al.*, unpublished observations). These data were analysed using repeat measures ANOVA with dose as a within subjects variable.

### 3.1.3 Results

The baseline data show mean reaction times ( $\pm$  standard error mean,  $n = 2$ ) for 6 days of the task (Figure 3.2). There is no significant difference between reaction times to invalidly or validly cued targets ( $F(1,1) = 23.945$ ,  $p > 0.05$ ), and there are insufficient degrees of freedom for a repeat measures ANOVA to analyse the effect of cue-target delay. Figure 3.3 shows the reaction times to validly and invalidly cued targets for the saline vehicle only, the  $10\mu\text{g}/5\mu\text{l}$  dose and for the  $30\mu\text{g}/5\mu\text{l}$  dose, each in relation to the baseline data. There is no significant effect of saline vehicle on reaction times ( $F(1,2) = 13.67$ ,  $p > 0.05$ ), nor is there a significant effect on validity effect ( $F(1,2) = 0.368$ ,  $p > 0.05$ ). There is no

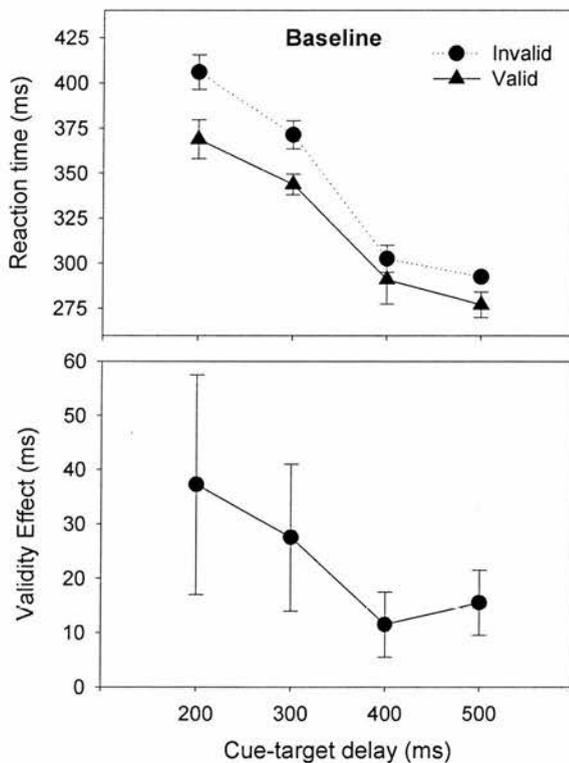


Figure 3.2 Mean  $\pm$  SEM ( $n = 2$ ) reaction times for both validly and invalidly cued targets, and validity effects for 6 days of baseline data.

significant effect of PrRP administration on reaction times ( $F(2,3) = 1.761, p > 0.05$ ), nor on validity effect ( $F(2,3) = 0.274, p > 0.05$ ). Figure 3.4 shows reaction times to invalidly and validly cued targets for the three days after the administration of the  $30\mu\text{g}/5\mu\text{l}$  PrRP dose. Although there was no statistically significant effect of PrRP administration on reaction times or validity effect, the observed reaction times and validity effect at the  $30\mu\text{g}/5\mu\text{l}$  dose suggests that PrRP administration's effect on reaction times cannot be dismissed. There is an observed, but not statistically significant reduction in reaction times to both validly and invalidly cued targets, and a similar reduction in validity effect. The reduction in

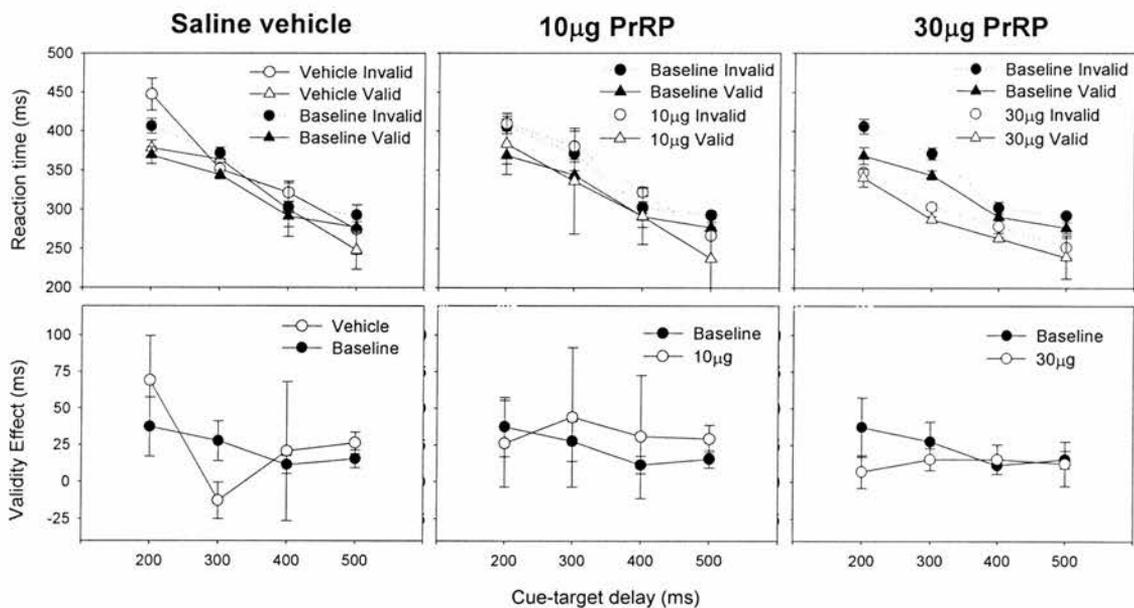


Figure 3.3 Mean  $\pm$  SEM ( $n = 2$ ) reaction times for both validly and invalidly cued targets, and validity effects for 6 days of baseline data and 2 days of each PrRP dose administration. No statistically significant effect of PrRP administration was observed.

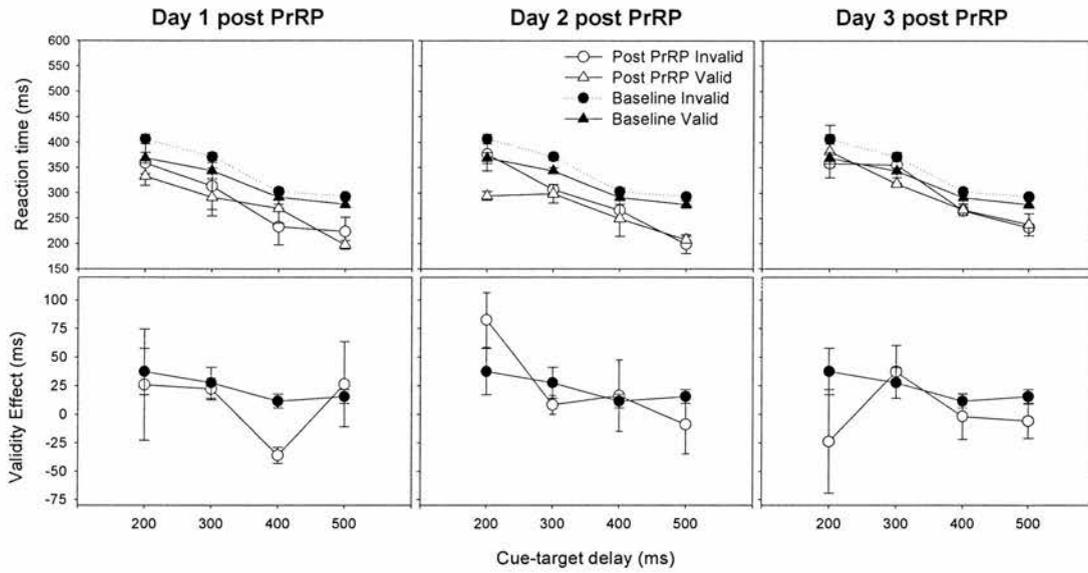
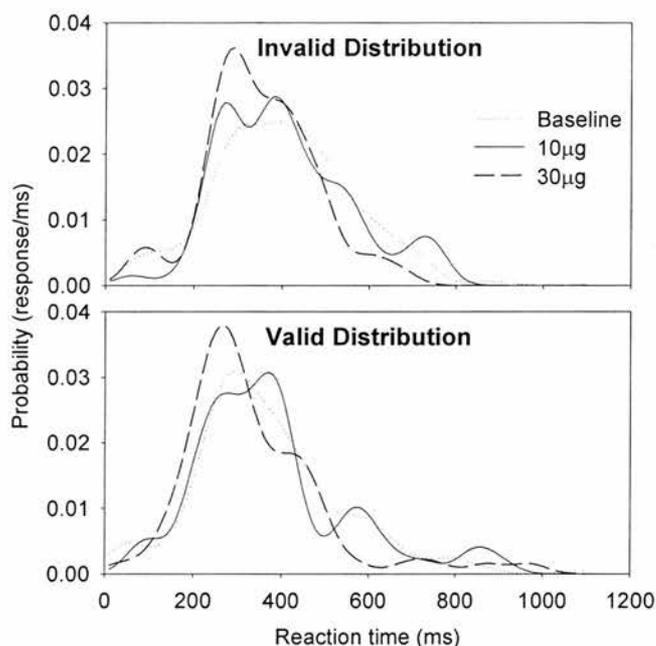


Figure 3.4 Mean  $\pm$  SEM ( $n = 2$ ) reaction times for both validly and invalidly cued targets, and validity effects for 3 days post final 30 $\mu$ g PrRP dose administration.

reaction times is evident on the two days after final administration of the 30 $\mu$ g/5 $\mu$ l dose. Reaction times appear to return to baseline level on the third day after the final administration of the 30 $\mu$ g/5 $\mu$ l dose. Although this reduction is not statistically significant, the data were analysed to provide figures of distribution density probability at the 200ms cue-target delay period where differences between valid and invalid, PrRP administration and baseline data would be expected to differ most.

Figure 3.5 shows data from both rats used in the study, separating the invalidly cued targets from the valid, for baseline data, the 10 $\mu$ g/5 $\mu$ l and the 30 $\mu$ g/5 $\mu$ l PrRP dose. The curves show the probability of any one reaction time occurring during the course of the trial. There appears to be an increase in the probability of a faster reaction time to invalidly cued



*Figure 3.5 Probability density distribution curves for invalidly and validly cued targets at the 200ms cue-target delay period, for 6 days of baseline data and 2 days each of 2 doses of PrRP ( $n = 2$ ).*

trials for both doses of PrRP administration, and to validly cued trials at the 30µg/5µl PrRP dose. These data can be further split so that each individual subject can be studied. That there was no statistically significant effect of PrRP administration on reactions times would indicate that there may be some between subjects disparity that is not evidenced by observing the data when subjects are combined.

Figure 3.6 presents data as in Figure 3.5, but for subject 00/494 only. This figure shows that there is an increased probability of a faster reaction time at the 30µg/5µl PrRP dose to validly cued targets.

Figure 3.7 shows the data for subject 00/519. This data shows an increased probability of a faster reaction time to invalidly cued targets at the 30µg/5µl PrRP dose, with a slightly increased chance of faster

reaction time after the 10 $\mu$ g/5 $\mu$ l PrRP dose. There is only a slightly increased probability of faster reaction time to validly cued targets after the 30 $\mu$ g/5 $\mu$ l PrRP dose.

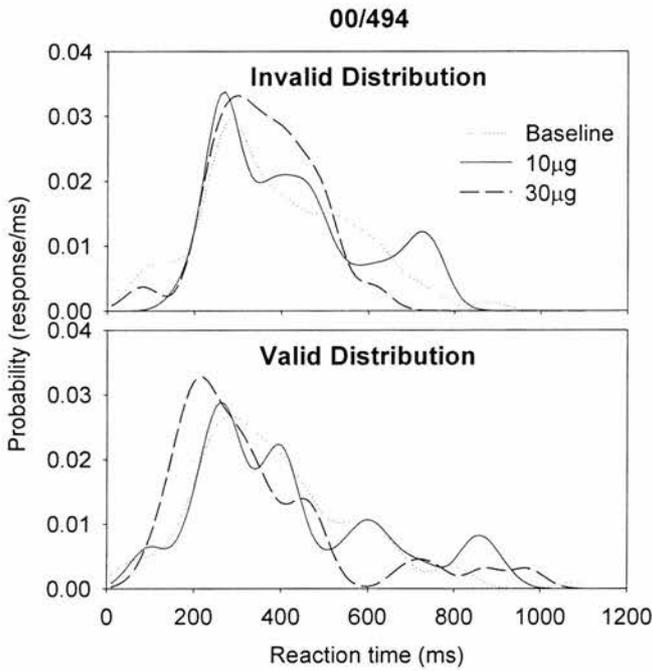


Figure 3.6 Probability density distribution curves for rat 00/494 for invalidly and validly cued targets at the 200ms cue-target delay period, for 6 days of baseline data and 2 days each of 2 doses of PrRP.

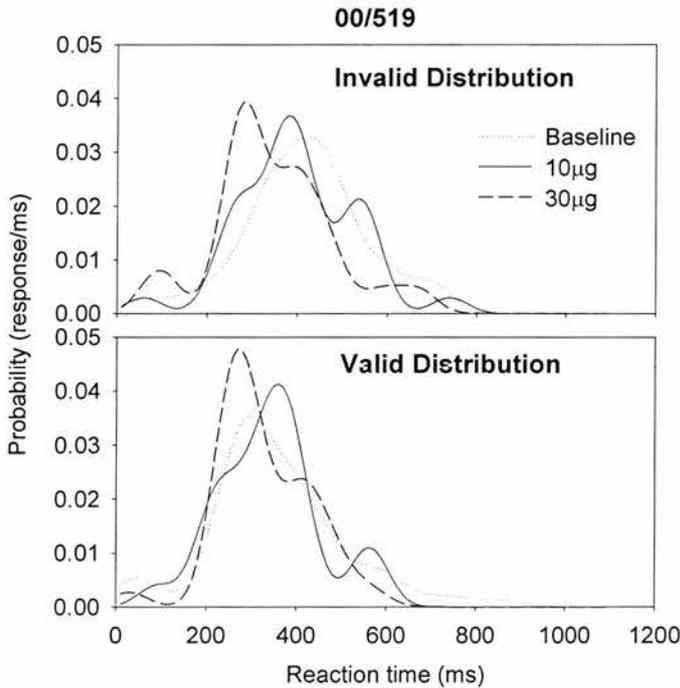


Figure 3.7 Probability density distribution curves for rat 00/519 for invalidly and validly cued targets at the 200ms cue-target delay period, for 6 days of baseline data and 2 days each of 2 doses of PrRP.

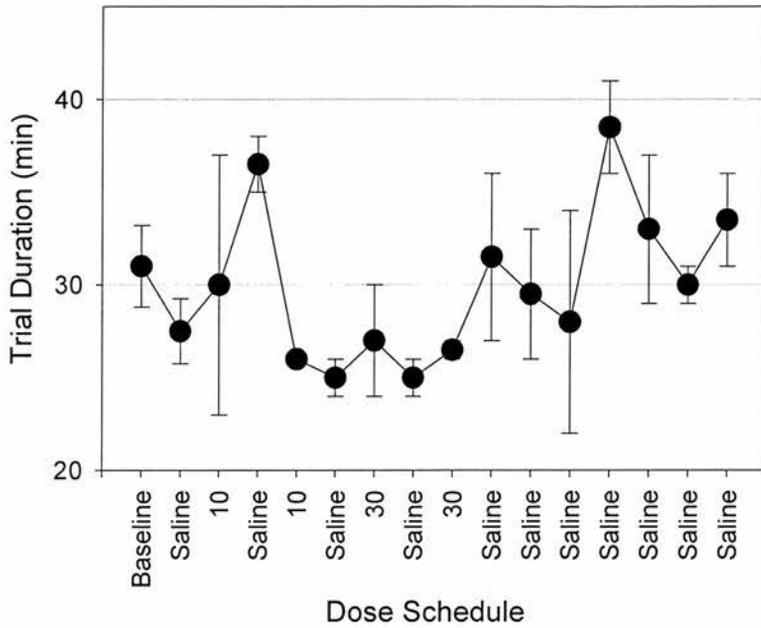


Figure 3.8 Mean  $\pm$  SEM ( $n = 2$ ) times to trial completion for baseline, saline vehicle dose, and 2 doses of PrRP.

Times taken to complete the task are presented in Figure 3.8. This shows the mean time to complete the task, with means for 6 days of baseline and the initial 2 days of saline vehicle data, then following the daily dosing schedule. There is no statistically significant evidence in this data that rats showed reduced activity during the covert orienting task ( $F(2,2) = 0.726, p > 0.05$ ). The statistical analysis also rules out that there was a PrRP-induced reduction in time to complete the task

### 3.1.4 Discussion

From the data analysed in this study, it can be seen that there is no statistically significant effect of PrRP administration on reaction times in the covert orienting task. Although there is a lack of statistical evidence to

support a role for PrRP in attentional function, these data alone are not enough to suggest that PrRP has no role in attentional function.

This study used only two animals, and so it would be expected that there would be a high degree of variability in the observations. Despite the low number of subjects however, the baseline data for the study is consistent with observations previously made from this covert orienting task. The baseline reaction times show low standard error, as do the reaction times observed after the 30 $\mu$ g/5 $\mu$ l PrRP dose. Variability is high (although lowest after the 30 $\mu$ g/5 $\mu$ l PrRP dose) in all measures of validity effect and also in reaction times after the 10 $\mu$ g/5 $\mu$ l PrRP dose. The low variability and relatively low variability in validity effect after the 30 $\mu$ g/5 $\mu$ l PrRP dose warranted analysis beyond ANOVA however, as despite statistical evidence, there appeared to be an effect of PrRP administration, reducing reaction times, with the invalid reaction time reduced disproportionately more than the valid (thereby also reducing the validity effect). The distribution analyses provide data on the probability of any one reaction time occurring during the task, and the 200ms cue-target delay was selected for analysis as this would have the highest observed difference between reaction times to valid and invalidly cued targets. Subsequently, these would be most sensitive to any changes in attentional function.

Figure 3.5 shows that there is a minor increase in probability of a faster reaction time to both valid and invalidly cued targets after the 30µg/5µl PrRP dose at the 200ms cue-target delay. Figures 3.6 and 3.7 show the same data, but with each rat in the study analysed separately. It is revealed in these figures that the observations previously seen as a reduction in reaction times to both valid and validly cued targets is instead a large increase in the probability of a faster reaction time to a validly cued target in case 00/494. Case 00/519 shows an increased probability of a faster reaction time to invalidly cued targets. This would suggest that the reductions in reaction times observed were indeed not a result of PrRP administration, but are instead random noise.

The data are further clouded however by observations of reaction times taken on the three days immediately after the last administration of the 30µg/5µl PrRP dose. The reduction in reaction times observed after the 30µg/5µl PrRP dose appears to continue for at least one, and perhaps two days. No statistical analyses were performed on these data as there was no statistical significance of PrRP administration. However, that the reaction times, at least on the day after last administration of the 30µg/5µl PrRP dose are observed to be similar to those on the days of the 30µg/5µl PrRP dose suggests that any replication would provide valuable confirmation – either of an effect of PrRP, or otherwise.

The observations of the times to completion of trial suggest that any PrRP-induced reduction in activity (Jones *et al.*, unpublished) is compensated for by motivation in the task. Figure 3.8 appears to show a decrease in time to complete the task after administration of PrRP, however this is not supported by statistical evidence, and so must be attributed to chance. It is possible that an unidentified factor of day of administration affected time to complete the task in a fashion that is not taken into account in the analysis.

## **3.2 Part B: does manipulation of Prolactin Releasing Peptide in Thalamic Reticular Nucleus mediate attentional function in a covert orienting task?**

### **3.2.1 Introduction**

The first section of this chapter details observations made after i.c.v. administration of PrRP to two rats. The data were inconclusive, however, and it was considered necessary to replicate the procedure. To minimise the effects of the day of administration having any non-specific effect on reaction times, PrRP was administered in a pseudo-random fashion, with three days of saline only injection between each dose of PrRP. This would remove the possibility of any wash-out period as possibly observed in the initial study having an impact upon reaction times measured on subsequent dose days.

### **3.2.2 Protocol**

#### ***3.2.2.1 Animals***

7 male Lister hooded rats (Charles River) were used. The rats were pair-housed until cannulae implantation and maintained on a 12 hour light/dark schedule (lights on at 7am), with a diet of 15-20g of standard laboratory chow and earned 45mg pellets each day. The initial weight range was between 300-350g. At completion of the procedure weight range was between 350-480g.

#### ***3.2.2.2 Equipment***

Equipment is as referenced in Part A.

#### ***3.2.2.3 Training Procedure***

Training procedure is as referenced in Part A.

#### ***3.2.2.4 Surgery***

Surgery is as referenced in Part A.

#### ***3.2.2.5 Drug Administration***

9 days after surgery rats were returned to the covert orienting task and baseline data were recorded. One subject died during recovery. Rats performed the task on five consecutive days, with the only day that all six rats completed the task recorded as baseline data. Time restriction prevented further baseline data being obtained. On the sixth day each rat

received a dose of PrRP in sterile saline (2 each received a dose of 10µg/5µl, 30µg/5µl or 56µg/5µl) by the method previously described in Part A. On each day 20 minutes after administration each rat was placed in the 9-hole operant box, and the covert orienting task commenced. For the following three days, rats received 5µl injections of sterile saline. On the next day rats again received a dose of PrRP in sterile saline (2 each received a dose they had not previously had). The following three days rats received 5µl sterile saline, and then on the final day rats received the dose of PrRP that they had not already received. Data were collected throughout the procedure, and analysed daily.

#### ***3.2.2.6 Histology***

Rats were perfused 2 hours after completion of the covert orienting task on the day of their last PrRP dose. Perfusion and cresyl violet staining was as referenced in Part A. Brains were also analysed for c-Fos-related activity using the following protocol. Sections were washed 5 times for three minutes in 0.1M PBS, then placed on a stirrer for 1 hour in blocking solution (0.1M PBS, 20% normal goat serum, 0.1% triton). Sections were washed as previously in 0.1M PBS, then incubated in anti-c-Fos (Oncogene Research Products, Calbiochem, CN Biosciences, Nottingham, UK) (1:20,000) in antibody diluting solution (ADS; 0.1M PBS, 1% normal goat serum, 0.1% triton) at 4°C for 2 nights. Subsequently sections were washed in 0.1M PBS as before, then incubated on a stirrer in vector IgG solution (anti-rabbit IgG at 5µl/ml

ADS) for 1 hour. After washing in 0.1M PBS again, sections were incubated on a stirrer in Vectastain ABC complex (Vector Laboratories Ltd, Peterborough, UK) (reagents A and B at 10 $\mu$ l/ml ADS) for a further hour. Sections were then washed in 0.1M PBS again, and finally immersed in Sigma Fast 3,3'-Diaminodenzidine tablets (DAB; Sigma Chemical Company, St Louis, MO, USA) in distilled water until reasonable colour was developed (up to 10 minutes). Sections were washed again in 0.1M PBS and then mounted on glass slides. Sections were de-fatted in xylene and cover slips were applied as under cresyl violet protocol. Two rats were removed from the sample as the guide cannula had failed to penetrate the lateral ventricle.

#### ***3.2.2.7 Data Analysis***

Data analysis was as referenced in Part A.

#### **3.2.3 Results**

The baseline data show mean reaction times ( $\pm$  standard error mean,  $n = 4$ ) for 1 day of the task (Figure 3.9). There is no significant difference between reaction times to invalidly or validly cued targets ( $F(1,3) = 0.79$ ,  $p > 0.05$ ), nor between cue-target delays ( $F(3,1) = 94.067$ ,  $p > 0.05$ ).

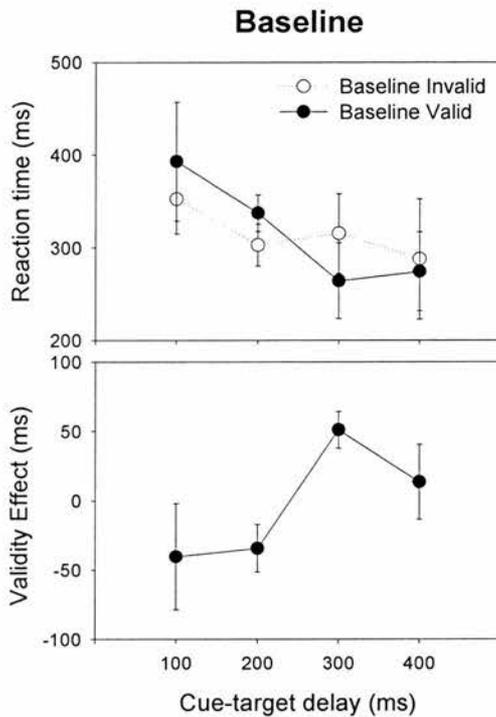


Figure 3.9 Mean  $\pm$  SEM ( $n = 4$ ) reaction times to both validly and invalidly cued targets, and validity effects for 1 day of baseline data.

Figure 3.10 shows the reaction times to validly and invalidly cued targets for the  $10\mu\text{g}/5\mu\text{l}$  dose, the  $30\mu\text{g}/5\mu\text{l}$  and for the  $56\mu\text{g}/5\mu\text{l}$  PrRP dose, each in relation to the baseline data. There is no significant effect of PrRP administration on reaction times in the covert orienting task ( $F(3,12) = 0.13, p > 0.05$ ). There is a significant effect of validity of cue ( $F(1,12) = 9.719, p < 0.05$ ) and of cue-target delay ( $F(3,10) = 42.352, p < 0.05$ ). As there is no significant effect of either validity or cue-target delay in the baseline data, but there is in all data collected, the data from PrRP administration days was analysed separately. Using  $10\mu\text{g}/5\mu\text{l}$ ,  $30\mu\text{g}/5\mu\text{l}$  and  $56\mu\text{g}/5\mu\text{l}$  PrRP doses as within subjects variables, there was no significant effect of PrRP administration on reaction times ( $F(3,9) = 0.064, p > 0.05$ ). There was a significant effect of validity of cue ( $F(1,12) = 9.719, p < 0.05$ ) and cue-target delay ( $F(3,10) = 42.352, p < 0.05$ ). As

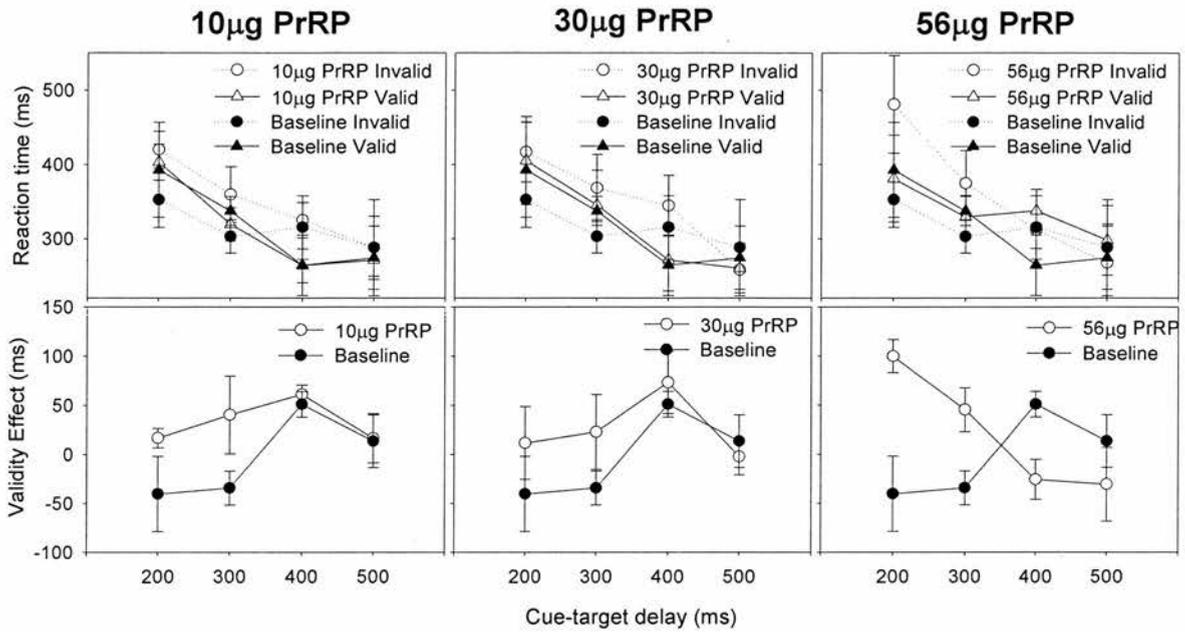
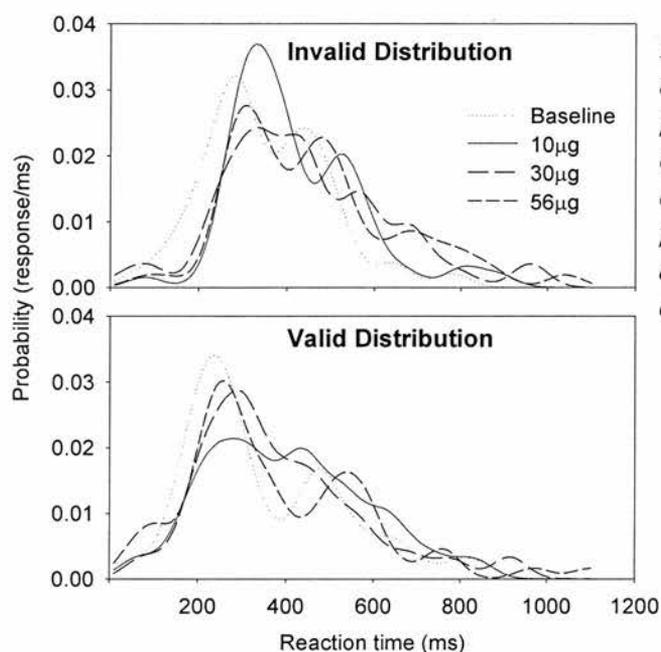


Figure 3.10 Mean  $\pm$  SEM ( $n = 4$ ) reaction times for both validly and invalidly cued targets, and validity effects for 1 day of baseline data and each PrRP dose administration. No statistically significant effect of PrRP administration was observed.

there is no significant effect of either validity or cue-target delay in the baseline data, but there is in all data collected, the data from PrRP administration days was analysed separately. Using 10µg/5µl, 30µg/5µl and 56µg/5µl PrRP doses as within subjects variables, there was no significant effect of PrRP administration on reaction times ( $F(3,9) = 0.064, p > 0.05$ ). There was a significant effect of validity of cue ( $F(1,12) = 9.719, p < 0.05$ ) and cue-target delay ( $F(3,10) = 42.352, p < 0.05$ ).

Probability distribution curves for reaction times at the 200ms cue-target delay period for both validly and invalidly cued targets for each dose of PrRP were produced. Figure 3.11 shows the data for the four rats separated into validly and invalidly cued targets. For both validly and



*Figure 3.11 Probability density distribution curves for invalidly and validly cued targets at the 200ms cue-target delay period, for 1 day of baseline data and 1 day each of 3 doses of PrRP (n = 4).*

invalidly cued targets there is decreased probability of faster reaction times after all doses of PrRP. There are at least two peaks of probability of reaction time response for each dose at both valid and invalid, creating variability as observed in the statistical analysis of reaction times.

Task duration was recorded as previously in order to observe possible effects of PrRP administration on activity levels. This was hampered by several rats failing to complete the task on saline vehicle days before the second PrRP dose. Figure 3.12 shows a general reduction in time taken to complete the task as the experiment progressed. This is likely due, as well as the subjects failing to complete the task during baseline recordings (resulting in only one day of baseline data collected), to rats having free access to food for several days after cannula

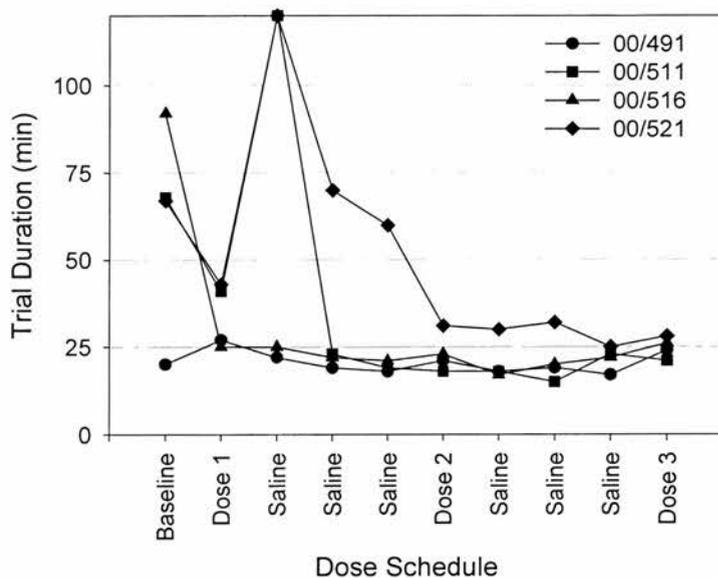


Figure 3.12 Individual rats ( $n = 4$ ) times to completion of task for baseline data, saline vehicle data and 3 doses of PrRP.

implantation surgery. Temporal constraints forced acceleration in returning rats to the task for baseline data recording.

### 3.2.3.1 Histological Observations

As previously mentioned, of the six rats used in the procedure, two were removed after histological analysis due to guide cannulae not penetrating the lateral ventricles.

Analysis of c-Fos-related activity in the Rt indicated that there was none discernable. Other regions where c-Fos-related activity has been observed before following PrRP administration showed clearly labeled

neuronal cell bodies – various hypothalamic structures – confirming that the labeling protocol was successful.

### **3.2.4 Discussion**

From the data observed in this procedure there are no statistically significant effects of i.c.v. PrRP administration on reaction times in the covert orienting task. However, it is clear that the data observed in this second procedure is different from that observed during the initial study. The baseline data were inadequate due to time restraints on the procedure and reluctance on the subjects' part to complete the task after surgery. This was not an inability to perform the task, more an apparent satiation as the subjects neared the completion of the task, most likely caused by rats returning to the covert orienting task too soon after removal of post-surgery free access to food. It is therefore unlikely, despite apparently consistent superficial observations of validity effect during this time, that the rats had achieved stability in their task performance. For this reason, the data observed after PrRP administration were analysed both with the baseline data and without. It is clear however, even without the suggested distortion induced by baseline variability, that there is no observable effect of PrRP administration on reaction time performance in this task.

Out with the subjects' ability to perform the task consistently, there are other factors that may have influenced the observations. PrRP has been observed to induce increased ACTH in blood plasma in rats, an

endocrine function that is implicated in stress response (Matsumoto *et al.*, 2000). There are currently no data on effects of stress on covert orienting, although Radulovic *et al.* (1999) explored the role of CRH in a context/tone-dependant fear conditioning paradigm in mice. The authors concluded that CRH has a modulatory role on learning and not attention, and further found that although a 20pmol dose was sufficient to induce learning modulation, 100pmol was required before a stress response was observed. In the Matsumoto *et al.* (2000) study a dose of 20mmol PrRP was sufficient to induce C-Fos related activity in CRH-positive parvocellular PVN, with a dose of 10mmol required to significantly increase blood plasma ACTH. This increase was significantly attenuated by intravenous (i.v.) administration of 2mg  $\alpha$ -helical CRH 15 minutes prior to PrRP administration. It is therefore feasible that stress has an impact upon performance in the covert orienting task, and therefore in both the initial study, and this study, rats were possibly affected by some form of stress response.

Observations of individual rats' reaction times did not clarify the data either. None of the data from the four rats analysed suggested that PrRP administration had any effect on reaction times in the task. A factor in the stress response after both chronic administration (10 days; non-food deprived rats) of CRH (Buwalda *et al.*, 1998) and acute administration (food deprived and non-food deprived rats) of the CRH-related peptide, urocortin (has high affinity for CRH receptors; Currie *et al.*, 2001) is

reduced appetite and hence reduced weight gain. That no increase in time to complete the task, and hence consume 120 pellets was observed in rats performing the task suggests that any effect on appetite had no effect on task performance.

It is possible then, that observations from the initial study were chance, despite the consistency over days. It is equally possible that any effect of PrRP administration on reaction times in the covert orienting task is masked by an unmeasured stress response induced by PrRP. However, this stress response is as likely to have manifested in the initial study as in this study, so any disparity between the two studies cannot be wholly attributed to it. The non-significant reduction in reaction times and validity effect observed in the initial study are as likely to be an artifact of any stress response as are the results observed in this study. Any replication of this study would require co-administration of  $\alpha$ -helical CRH, or another CRH antagonist, in order to minimise stress response induced by PrRP. Further understanding of PrRP administration effects on CRH and ACTH levels, combined with  $\alpha$ -helical CRH administration to create a dose response curve would be necessary to ensure correct dose administration to negate PrRP induced increased CRH levels only.

Experimental data revealed after completion of these studies have demonstrated GPR10 receptor localisation within both human and rat brain. Previous *in situ* hybridisation studies (Roland *et al.*, 1999) showed

a relatively dense distribution of GPR10 mRNA in the Rt, although until recently, localisation of the receptor itself was unexplored. Immunohistochemistry studies based on antibodies raised in rabbit against 1 of 3 tested peptide sequences from the rat GPR10 sequence have shown that there is general synergy between observed central GPR10 mRNA and localisation of the receptor itself (Jones *et al.*, unpublished). Disparity is, however, observed between the levels of GPR10 mRNA in Rt and the distribution of the receptor. The authors describe “moderate levels of GPR10” present in Rt as opposed to “high expression of GPR10 mRNA”.

Evidence of a role of PrRP in Rt comes from a study on modulations of sleep patterns in rats after PrRP administration. 0.1nmol PrRP administered centrally significantly increase rapid eye movement (REM) sleep, whereas a 1.0nmol PrRP dose significantly increases both REM sleep and non-REM sleep (Zhang *et al.*, 2000). Rt is implicated in control of sleep/wake patterns, with recording taken from identified Rt neurons demonstrating differing firing patterns according to the sleep/awake state of the unanaesthetised freely-moving rat (Marks and Roffwarg, 1993).

The mediating effect PrRP/GPR10 on sleep/wake patterns, and the role of Rt in such would suggest a role for PrRP/GPR10 in sensory awareness during the wake period. The data presented here neither confirm nor fully deny this possibility, and it is suggested that further

investigation involving monitoring stress/CRH/ACTH levels would be of value.

### **3.2.5 Summary of Findings**

- Rats trained in the covert orienting of attention task were administered PrRP (i.c.v.).
- There was no effect of PrRP administration on performance in covert orienting of attention in the rat.
- It is suggested that new data, revealing differential distribution patterns between the GPR10 receptor and its mRNA (specifically that the GPR10 receptor is not distributed in the Rt as densely as the presence of mRNA in the Rt would suggest), imply that no effect of PrRP administration on covert orienting of attention would be expected.

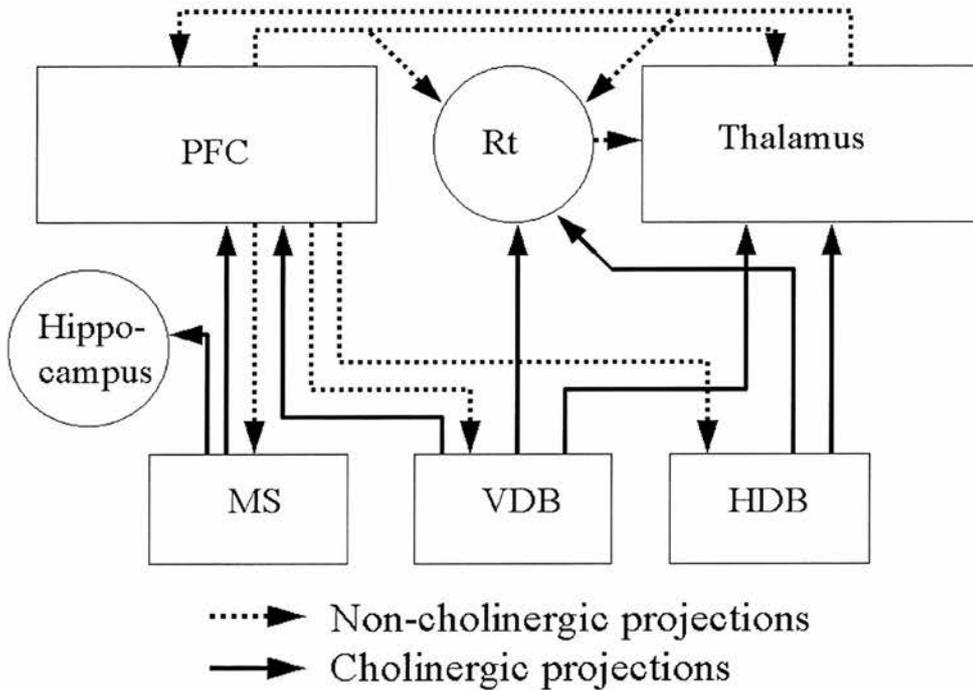
## Chapter IV

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### **Do 192-IgG-saporin lesions of basal forebrain or rostral thalamic reticular nucleus mediate attentional function in a set-shifting task?**

The basal forebrain (BF) consists of several identified nuclei, including the cholinergic Nucleus Basalis of Meynert, vertical/horizontal limb of the Diagonal Band and Medial Septum and the GABAergic Substantia Innominata. These nuclei have projections to cortex, hippocampus, amygdala, hypothalamus, thalamus and thalamic reticular nucleus (Rt), and receive afferents from cortex, locus coeruleus, dorsal and media raphe nuclei, mesopontine tegmentum, midbrain and pontine reticular formation, ventral tegmental area, substantia nigra pars compacta and the tubero mammillary hypothalamic nucleus (see Figures 4.1 and 4.2).

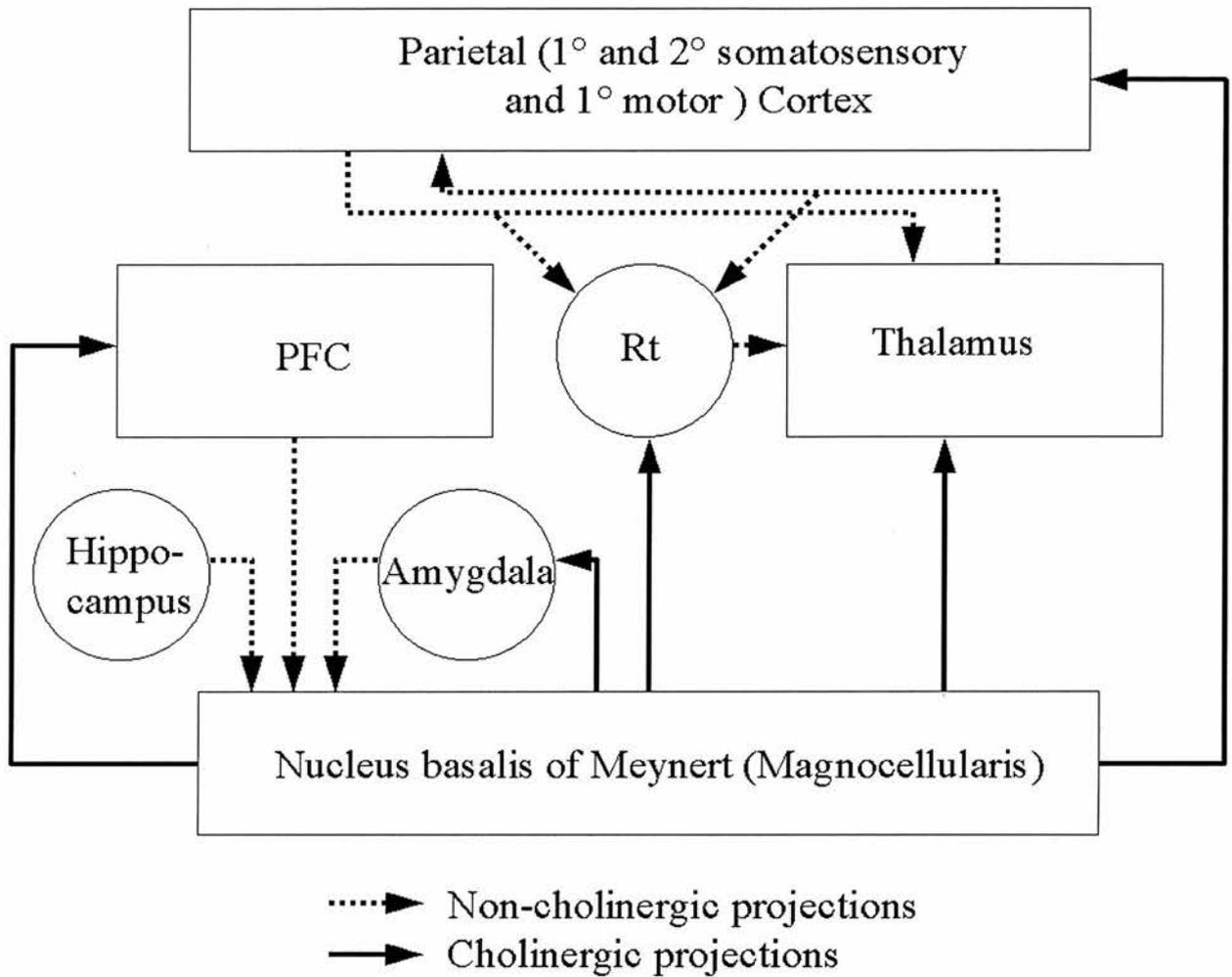
Previous manipulations of BF have shown involvement in attentional function in rats. Manipulations of the cholinergic projections of the BF have been implicated in attentional function, most recently in a set-shifting task in non-human primates (see Introduction for review). This investigation looks at the effects of BF cholinergic depletion on set-shifting in rats.



*Figure 4.1 Projections to and from basal forebrain nuclei; medial septum (MS), vertical limb of the diagonal band of Broca (VDB) and horizontal limb of the diagonal band of Broca (HDB). These include prefrontal cortex (PFC), thalamic reticular nucleus (Rt), various other thalamic nuclei (see Introduction) and hippocampus.*

#### 4.1 Introduction

The basal forebrain (BF) is a collection of neurons located in the ventral portion of the rat brain. The anterior boundary of BF is marked by the olfactory tubercle and the nucleus accumbens, with lateral boundaries marked by the amygdala and the piriform cortex. At its caudal extent, BF is bounded by the internal capsule, stria terminalis and caudate putamen (Paxinos and Watson, 1996).



*Figure 4.2 Projections to and from the Nucleus Basalis of Meynert (Magnocellularis) (nbM). These include the prefrontal cortex (PFC), thalamic reticular nucleus (Rt), various other thalamic nuclei (see Introduction), hippocampus, amygdala, and parietal (primary and secondary somatosensory) cortex.*

Within BF, several identifiable colonies of neurons make up defined nuclei. These are the predominantly cholinergic neurons of the Medial Septum (MS) and horizontal and vertical limbs of the Diagonal Band (H/VDB), which collectively make up the septal complex, and the Nucleus Basalis of Meynert (Nucleus Basalis Magnocellularis in rats (nbM)). The substantia innominata (SI) is a dispersed collection of

GABAergic/cholinergic neurons, interdigitated with nbM, so named due to initial lack of information about its function and neuronal make-up. Other nuclei are also sometimes considered part of BF, although are not always included in analysis of BF function during studies, mostly due to their projection pathways. The magnocellular preoptic nucleus (MCPO) is laterally adjacent to the HDB, and consists of mainly cholinergic cells similar in appearance to those of the HDB (Woolf, 1991; Semba, 2000). The VDB and HDB are bordered by the ventral pallidum laterally and dorsally respectively. Neither of these two nuclei have been reported as bearing cholinergic projections to cortical areas or thalamic areas considered involved in attention.

#### ***4.1.1 Manipulations of BF cholinergic function with 192-IgG-saporin***

There is considerable evidence that the BF cholinergic system is involved in attentional function. Recent manipulations of the BF cholinergic system have used the selective immunotoxin 192-IgG-saporin to destroy cholinergic neurons and thus their projections to cortex and thalamus, either by intracerebroventricular (i.c.v.) administration, or by intraparenchymal administration in either the terminal regions of the BF projection neurons or the nuclei within the BF itself (Bergersweeney *et al.*, 1994; Bushnell *et al.*, 1998; McGaughy and Sarter, 1998; McGaughy *et al.*, 2002). Histological observations after i.c.v. administration show a loss of cholinergic innervation to cortex and hippocampus corresponding with the terminal regions of BF cholinergic neuron efferents in the MS,

V/HDB and nbM/SI. Likewise, the MS, V/HDB and nbM/SI show extensive loss of cholinergic neurons (Bergersweeney *et al.*, 1994). Damage can be seen in most areas receiving cholinergic projections from BF, with the exception of the amygdala (Heckers *et al.*, 1994), which receives afferents from BF not possessing the rNGF receptor. Intact nbM/SI cholinergic neurons after either i.c.v. or intraparenchymal administration of 192-IgG-saporin are likely those with projections to amygdala. Numerous studies have shown cholinergic neuronal depletion in both nbM and SI after administration of 192-IgG-saporin to either terminal regions of BF projection neurons or the nbM/SI cell bodies themselves (Bergersweeney *et al.*, 1994; Bushnell *et al.*, 1998; McGaughy and Sarter, 1998; McGaughy *et al.*, 2002; for review see Wrenn and Wiley, 1998).

192-IgG-saporin is a conjugate of the ribosome-inactivating protein, saporin, with the monoclonal antibody 192-IgG which targets the p75 nerve growth factor receptor (rNGF) (Wiley *et al.*, 1991). The 192-IgG-saporin conjugate is predominantly selective for the cholinergic neurons of the BF due to the rNGF receptor being found almost exclusively on these neurons. Immunocytochemical and *in situ* hybridisation studies have shown that rNGF is found in the terminal regions of the magnocellular cholinergic neurons of the BF, and in regions containing those neuronal cell bodies (MS, V/HDB and nbM) and that mRNA for rNGF has been observed only in terminal regions (cortex

and hippocampus). This suggests that rNGF is synthesised in the terminal regions of the BF cholinergic projection neurons and retrogradely transported down the projecting axons to the cell bodies in the BF (Korsching *et al.*, 1985).

192-IgG-saporin, when administered in to the central nervous system, binds to the rNGF receptor via the monoclonal antibody 192-IgG, then, upon transportation into the cell body breaks apart, allowing the saporin to inactivate the cells' ribosomes, destroying the cell. Unlike excitotoxins, the immunotoxin saporin does not immediately kill the neurons it targets, so it is necessary to wait for the full effect before behavioural observations can be taken.

#### ***4.1.2 BF lesions and attention in rats***

Two weeks after unilateral intraparenchymal administration of 192-IgG-saporin into the SI, rats are reported as being impaired in a cued target detection task (Bushnell *et al.*, 1998). This operant task required the rat to initiate a trial by pressing the food hopper panel then respond to a target illumination after presentation of a cue. The cue was either valid or invalid as previously described in the Posner covert orienting of attention task. The target appeared a variable period of time after the onset of the cue, and a response to the target (not within 100ms of target onset to remove anticipatory errors) resulted in reinforcement. At two weeks post infusion responses to the targets presented contralateral to the 192-IgG-

saporin infusion were impaired; a reduction in accuracy and longer response latencies were noted. After 10, and up to 22 weeks (when testing ended), rats were observed to respond with greater speed (shorter response latencies), but less accuracy to the contralateral targets. Accuracy for contralateral targets also decreased as a function of cue-target delay period, whereas accuracy to ipsilateral targets increased as cue-target delay increased. Subjects treated with 192-IgG-saporin bilaterally that underwent the same task (Chiba *et al.*, 1999) were observed to be impaired in responding to targets that had been cued invalidly. This reflects an increase in the attentional “cost” of the invalid cue.

Selective lesions of the nbM have also been shown to impair performance in the 5-choice serial reaction time task (5CSRT), an operant task that requires the rat to respond to a spatially unpredictable illuminated signal by poking its nose in the lit hole, ignoring other available holes. Rats that showed reduction in choline acetyltransferase (ChAT) positive cells (indicative of loss of cholinergic neurons) in nbM were impaired in accuracy in the task, which was not seen in rats with reduction in VDB. Subjects administered a low dose of 192-IgG-saporin were less impaired than those receiving a high dose, although they were impaired in accuracy if the rate of stimulus presentation was increased. The 192-IgG-saporin-induced impairment was ameliorated by increasing the duration of the stimulus presentation. Furthermore, microdialysis

techniques were used to monitor acetylcholine (ACh) efflux. Efflux in prelimbic cortex was reduced in 192-IgG-saporin treated rats compared to controls, both before and during performance of the 5CSRT (McGaughy *et al.*, 2002). The 5CSRT has previously been shown to elicit an increase in mPFC ACh efflux (Passetti *et al.*, 2000).

*In vivo* microdialysis analysis of frontoparietal cortical ACh efflux in rats undergoing an operant task taxing sustained attention indicates an increase in ACh levels during the task (Himmelheber *et al.*, 2000). The task presented the rat with two levers and reinforced a left lever press after an illumination signal, and the right lever press following no signal (the start of a trial was signaled by extension of both levers). Cortical ACh increased during the task, with a reduction at the end of the task. During the task, a visual distracter was presented (flashing houselight for 12 minutes) resulting in a response bias to the left lever for the first 6 minutes and a corresponding reduction in cortical ACh efflux as attentional demand was reduced. During the last 6 minutes of the task, performance recovered and, correspondingly, ACh efflux also increased. Further *in vivo* microdialysis studies, looking at a low-demand version of the sustained attentional task, also resulted in increases in cortical ACh efflux, but in the case of this task 192-IgG-saporin administration intraparenchymally into nbM did not result in an impairment in the task. Furthermore, ACh efflux increased in both high and low-demand tasks when the subjects were placed in the operant box prior to initiation of the

task. This suggests that cortical ACh release is also linked to anticipatory and/or contextual elements involved in operant performance and resultant attentional processing, and furthermore, that this release is not from the BF projection. (Himmelheber *et al.*, 2001). Using the same task (McGaughy *et al.*, 1999) showed that performance was enhanced by systemic administration of the cholinergic (nicotinic) agonist ABT-418. Furthermore, rats that had received injections of 192-IgG-saporin into nbM did not show any increase in performance after administration of ABT-418. This further demonstrates that although the cholinergic neurons of the nbM are involved in attentional function, the specific nature of this involvement is still not understood fully. Other behavioural data after non-192-IgG-saporin lesions to the rat BF are available, but without the selectivity of the 192-IgG-saporin, it is possible that the data are contaminated by destruction of non-cholinergic cells within the BF regions targeted.

#### ***4.1.3 BF lesions and attention in primates***

There is also evidence supporting a role for BF in attention from studies involving primates with BF lesions. Although there is less data available for lesions selective for cholinergic neurons in primate BF (due to the only recent development of a saporin conjugate selective for primate p75 receptors), there is evidence from non-selective excitotoxin studies (for review see Introduction).

In particular, primates with N-methyl-D-aspartate (NMDA) lesions of BF are impaired in an attentional set-shifting task. The task requires the subject to discriminate a complex visual stimulus on a monitor based on dimensional properties of the stimulus. Marmosets with BF lesions are impaired in their ability to ignore previously reinforced stimuli (a reversal) (Roberts *et al.*, 1992).

Marmosets treated with the excitotoxin, quinolinic acid, in prefrontal cortex also show impairments on this set shifting task. Lateral prefrontal cortex lesioned marmosets show impairment in shifting attention from one stimulus to another when the novel stimulus is of a different dimension to the currently attended stimulus. Orbital prefrontal cortex lesioned marmosets were impaired in their ability to reverse between two stimuli in a similar fashion to those with BF lesions (Dias *et al.*, 1996).

#### ***4.1.4 The rat attentional set-shifting task***

The primate attentional set shifting task, or ID/ED task, has recently been adapted for rats (Birrell and Brown, 2000). As primates have been shown to be impaired in attentional shifting after prefrontal cortex lesions, it was appropriate that a comparison should be attempted in rats. The rat attentional set shifting task is analogous to the primate task in that it tests the rats ability to learn the reinforcement value of novel stimuli, discriminating them from other stimuli within that dimension

(simple discrimination; SD); to be able to discriminate stimuli of one dimension in the presence of a second, irrelevant dimension (compound discrimination; CD); to be able to ignore previously rewarded stimuli in favour of previously unrewarded stimuli of the same dimension (reversal); to learn novel stimuli of the same dimension as the previously rewarded dimension in the presence of an irrelevant dimension (intra-dimensional shift; ID); to learn a strategy to solve reversals; to shift attention from a previously rewarded dimension to the previously unrewarded dimension in the presence of novel stimuli (extra-dimensional shift; ED). The primate attentional set-shifting task presents the subjects with a monitor upon which are shown visual stimuli in the form of shapes and/or lines. The primates must visually discriminate the lines from the shapes and from each other and must learn the rules necessary to solve the task. The rat attentional set shifting task presents the subjects with a choice of two bowls to dig in for a reward. The bowls are distinguishable by up to three dimensions; odour and/or digging medium and/or bowl texture. Differing odours/digging mediums/textures are termed exemplars and require the rat to discriminate between and within a dimension.

Rats with ibotenic acid-induced prelimbic cortex lesions demonstrate a deficit in ability to perform the ED shift in a similar fashion to marmosets with quinolinic acid-induced lateral frontal cortex lesions (Birrell and Brown, 2000). These data suggest that not only does the rodent mPFC fulfill a function analogous to the primate lateral frontal

cortex, but that the rat attentional set shifting task is suitable for looking at attentional function in rats and manipulations of cholinergic innervation of cortex.

#### **4.1.5 Rt and attention**

The complexity of BF interaction with cortex is compounded by BF projections to Rt and subsequent modulation of thalamo-cortical/cortico-thalamic projections by Rt. That Rt is involved in attentional function has already been discussed (see Chapter III), and evidence indicates that this involvement stems from cholinergic mediation. However, any manipulation of cholinergic neurons in a BF nucleus that has projections to Rt for the intent of studying effects on attention should also consider the implications of Rt function on observations.

Rt receives its cholinergic input from two sources: BF and also the brainstem nuclei; pedunculo-pontine tegmental nucleus (PPTg) and laterodorsal tegmental nucleus (LDTg) (Spreafico *et al.*, 1993; Kolmac and Mitrofanis, 1998). Projections synapsing with Rt neurons from BF bear the rNGF receptor that 192-IgG-saporin targets, with those from the brainstem not bearing the receptor. This means that selective lesioning of BF cholinergic neurons will not only denervate cortex of ACh, but also denervate Rt.

Evidence as to the topography of cholinergic projections from BF to Rt is inconclusive. Several studies have observed cholinergic projections from BF to the rostral pole, dorso- and ventro-rostral tip of the Rt (Hallanger *et al.*, 1987; Chen and Bentivoglio, 1993; Oda *et al.*, 1998; Kolmac and Mitrofanis, 1999), with innervation from PPTg and LDTg synapsing with caudal Rt (Hallanger *et al.*, 1987; Spreafico *et al.*, 1993). There is some evidence to suggest that BF cholinergic neurons project throughout the rostro-caudal extent of the Rt, although it is considered that by far the majority of rostral Rt cholinergic afferents arise in BF and caudal cholinergic afferents arise in PPTg; caudal projections from BF to Rt are mostly GABAergic (Asanuma and Porter, 1990). However, there is evidence to suggest that the p75 rNGF receptor is present throughout Rt, both anatomical (Pioro and Cuello, 1990) and electrophysiological: nerve growth factor increases burst size and intraburst frequency in auditory Rt neurons (Villa *et al.*, 1996).

The aim of this study is to administer 192-IgG-saporin intraparenchymally into either the nbM or Rt of rats and observe their performance on the attentional set-shifting task. It is hypothesised that rats with nbM lesions, and hence reduced cholinergic innervation to both cortex and Rt, and impaired attentional function, will show reduced performance in aspects of the task, most likely in either reversal performance or in the ED shift. It is further hypothesised that infusion of 192-IgG-saporin into Rt will lesion cholinergic BF neurons with

projections to Rt, allowing distinctions to be made between observed impairments in the attentional set-shifting task after denervation of cortex and Rt and denervation of Rt alone. The nbM receives projections from mPFC and in turn sends cholinergic efferents to Rt. In reducing cholinergic input to cortex through nbM lesions, it is necessary to establish whether other terminal regions of those neurons influence the attentional set-shifting task.

## **4.2 Protocol**

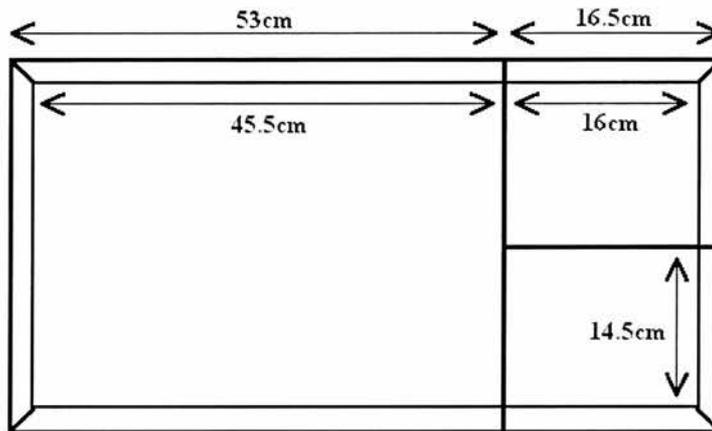
### ***4.2.1 Animals***

55 male Lister hooded rats (Charles River) were used. The rats were pair-housed until surgery and maintained on a 12 hour light/dark schedule (lights on at 7am), with a diet of 15-20g of standard laboratory chow each day, reduced on habituation day and testing/task acquisition days according to consumption during completion of the task. The initial weight range was between 400-550g. At completion of the procedure weight range was between 450-600g.

### ***4.2.2 Equipment***

192-IgG-saporin (Advanced Targeting Systems, San Diego, CA, USA) is administered in Dulbecco's saline. The stock volume of Dulbecco's saline consists of 20 $\mu$ l monovalent solution A (0.0132g CaCl<sub>2</sub>·2H<sub>2</sub>O + 0.010g MgCl<sub>2</sub>·6H<sub>2</sub>O in 100ml distilled H<sub>2</sub>O) mixed into 20ml divalent solution B (0.04g KCl + 0.04g KH<sub>2</sub>PO<sub>4</sub> + 1.6g NaCl + 0.23g

$\text{Na}_2\text{HPO}_4$  in 200ml distilled  $\text{H}_2\text{O}$ ). The pH of the Dulbecco's saline is adjusted to between 6.9 and 7.4.



*Figure 4.3 the attentional set shifting apparatus with the dimensions of the box*

The set-shifting apparatus is constructed from two home cages (opaque, white plastic), held together with clasps (Figure 4.3). The box is, in total, 69.5cm long (top; bottom 61.5cm), 40.5cm wide (top; bottom 31.5cm) and 18.5cm deep. At one end, the box is compartmentalised such that there are two sections of equal size (length: 16.5cm top, 16cm bottom; width: 17cm top, 14.5cm bottom) that can be sealed off from the main chamber of the box, either individually or together, by transparent, perspex partitions which slot in to wooden runners screwed into the walls of the box. The main chamber of the box has a hinged transparent perspex lid, as do each of the two smaller compartments. The lids are secured

when closed by ball catches. The floor of the box contains sawdust of the same type as in the rats' homecages.

Ceramic bowls are 4.5cm deep (external; 4cm internal) and 8.5cm in diameter (external; 6.5cm internal). During testing they are filled with either sawdust or an appropriate medium.

#### ***4.2.3 Set-shifting training procedure***

Between 10 and 14 days after surgery the subjects with bilateral lesions received habituation and training in the set-shifting task. Habituation involved a ceramic bowl identical to those used in the task being placed in the rat's home cage, filled with sawdust, and with several Honey Loops (Kellogg Company) in the bottom. Rats were then left in their home cage to investigate the bowl, with a reduction in their daily lab chow according to the weight of Honey Loops eaten. The following day, rats were removed from their homecages and placed in the set-shifting apparatus. Task acquisition involves rats presented with two bowls filled with sawdust, a Honey Loop reward in the bottom of each, placed within the closed off compartments of the set-shifting apparatus. The partition was removed and a timer started to give the rat 10 minutes (or as long as it needs if this is less) to uncover and eat both of the Honey Loop rewards. The rat is required to uncover a total of 12 rewards to complete this stage of the training. If the rat does not uncover both of the rewards within 10 minutes, then both bowls are rebaited and the timer started again.

Once the rat has achieved this level of training, the final stage of task acquisition involves the rat being exposed to two simple discriminations. The first simple discrimination (SD) involves two bowls filled with sawdust, one scented with the herb mint, and one scented with the herb oregano. The bowl containing the oregano scented sawdust is baited with a reward (half a Honey Loop) and the two bowls are placed in the set-shifting apparatus compartments. Placement of bowls in the set-shifting apparatus throughout the task is determined by random order displayed on the data recording sheets (Figure 4.4). The partition is lifted and the timer is started. The rat has up to 10 minutes to uncover the reward from the baited bowl. If the rat digs in the incorrect bowl, the time is recorded on the data sheet and the trial is marked as incorrect. The rat is then allowed to dig in the correct bowl to recover the reward. If the rat digs in the correct bowl, the time is recorded, and the trial marked as correct. Whether the rat only experienced one bowl before digging or both is also recorded. The rat is allowed to dig in the incorrect bowl for the first four trials, and thereafter if it digs incorrectly, the compartment containing the baited bowl is closed off with a partition. The rat must dig in the baited bowl on six consecutive occasions to have reached criterion, including those during the initial four exploratory trials. The probability of the rat having randomly got those six trials correct consecutively is beneath 5%, and thus it can be safely assumed that after six consecutive correct digs, the rat has learned the discrimination and identifies the smell of oregano with the reward.

Rat#	Date						
Trial	Left		Right	Correct	1st/2nd	31+	1st/2nd
1	<b>O10</b>		O9				
2	O9		<b>O10</b>				
3	O9		<b>O10</b>				
4	<b>O10</b>		O9				
5	O9		<b>O10</b>				
6	<b>O10</b>		O9				
7	<b>O10</b>		O9				
8	O9		<b>O10</b>				

*Figure 4.4 A sample of a data sheet from a simple discrimination. The exemplar in bold text is the rewarded stimulus.*

The second simple discrimination exposes the rat to the second dimension that it will experience during the set-shifting task. One bowl is filled with confetti and another is filled with small bits of polystyrene. The bowl containing confetti baited with the reward, and the same procedure as in the first simple discrimination is employed. The rat is again allowed four exploratory trials whereupon it is allowed to dig in the correct bowl if the first bowl that it digs in is incorrect. Again the rat must dig in the correct bowl on six consecutive occasions to be considered to have achieved set.

Upon completion of training, the rat is returned to its homecage. The rat's chow is reduced according to the amount of Honey Loops eaten.

#### ***4.2.4 The attentional set-shifting task***

The following day the rat undergoes the set-shifting task. There are seven discriminations in total for the rat to learn; a SD containing only one differing dimension; a compound discrimination (CD) containing two differing dimensions, with correct and incorrect exemplars remaining the same as they were in the simple discrimination, but with another, irrelevant dimension added (containing two differing exemplars); a reversal (REV1), where the exemplars remain the same as in the CD, but the correct and incorrect exemplars are reversed; an intra-dimensional shift (ID) where the exemplars of both the relevant and irrelevant dimensions are changed (total change scenario), but the new correct exemplar is of the same dimension as the previous one; a second reversal (REV2), where the rules of the first reversal are applied to the ID shift; an extra-dimensional shift (ED), another total change scenario, where the correct exemplar is now chosen from the previously irrelevant dimension; a third reversal (REV3), again following the rules of the previous two. Each of these will singularly be referred to as “shifts”. Criteria for having learned a discrimination are the same as during training SDs.

Exemplars are paired together in three groups out with the four exemplars used in the training. Table 4.1 shows the exemplars used in the task, their designation on the data sheets and the pairings used during the task.

*Table 4.1 the exemplars used in the attentional set shifting task and their designation for the purpose of pairings, dimension and data sheet recording*

<b>Dimension</b>	<b>Pairing 1</b>	<b>Pairing 2</b>	<b>Pairing 3</b>
Medium	Coarse Tea (M1)	Pebbles (M3)	Coarse Sawdust (M5)
	Fine Tea (M2)	Beads (M4)	Fine Sawdust (M6)
Odour	Cinnamon (O1)	Thyme (O3)	Nutmeg (O5)
	Cumin (O2)	Paprika (O4)	Cloves (O6)

Thus coarse and fine tea are always paired with cinnamon and cumin, pebbles and wooden beads with thyme and paprika and coarse and fine sawdust with nutmeg and cloves. During any individual trial all four exemplars within the pair are present. Thus if cinnamon is rewarded, then the rat, within that shift, will be exposed to a mix of either cinnamon/coarse tea and cumin/fine tea, or cinnamon/fine tea and cumin/coarse tea; the same exemplar is never in both bowls at the same time.

The rules for the discriminations during the task itself are the same as those in the simple discriminations undergone during training. The rat, during each shift, has the opportunity to dig in the correct bowl if it digs in the incorrect bowl first. Thereafter, should the rat dig in the incorrect bowl, the partition to the compartment in which the correct bowl is placed is lowered. The rat is given the opportunity to fully explore the incorrect

bowl during this time. Recordings are made of the time when the rat first digs, whether it experiences one or both bowls before it digs, and whether it digs in the correct bowl or not. The rat is given 10 minutes during each trial to dig in a bowl. Should the rat not dig after the 10 minutes has elapsed, the partitions are lowered, separating the rat from the bowls. The trial is marked as a fail. In the unlikely occurrence of the rat failing three trials within any one shift, the task is aborted. Likewise, the rat is permitted to take up to 60 trials to learn the discrimination within each shift. Should the rat fail to achieve set within this limit, the task is aborted. Table 4.2 shows an example of a completed task.

#### ***4.2.5 Counter-balancing of task***

12 unoperated control rats were put through the habituation, training and attentional set-shifting task as described above. Counter-balancing of order of exposure to pairings within the task and of ED shift between odour to medium and medium to odour ensured that there was no bias towards any of the exemplars or either dimension. Table 4.3 summarises the counter-balancing, showing the pairing order and ED shift type for the 12 unoperated control rats. Within each pair of rats there was further counter-balancing, as one rat was rewarded by attending to the even numbered exemplar (e.g. M2, reversing to M1), and the other rewarded by attending to the odd number exemplar (e.g. O1, reversing to O2).

Table 4.2 an example of a possible order of exemplar exposure in the attentional set-shifting task. The exemplars in bold text are rewarded. During each trial within a discrimination rats could be presented with either of the two pairing options

Discrimination	Exemplars (correct in bold)
SD	<b>Cinnamon</b> /sawdust and cumin/sawdust
CD	<b>Cinnamon</b> /fine tea and cumin/coarse tea Or <b>Cinnamon</b> /coarse tea and cumin/fine tea
REV1	<b>Cumin</b> /fine tea and cinnamon/coarse tea Or <b>Cumin</b> /coarse tea and cinnamon/fine tea
ID	<b>Thyme</b> /pebbles and paprika/beads Or <b>Thyme</b> /beads and paprika/pebbles
REV2	<b>Paprika</b> /pebbles and thyme/beads Or <b>Paprika</b> /beads and thyme/pebbles
ED	Nutmeg/ <b>coarse sawdust</b> and cloves/fine sawdust Or Cloves/ <b>coarse sawdust</b> and nutmeg/fine sawdust
REV3	Nutmeg/ <b>fine sawdust</b> and cloves/coarse sawdust Or Cloves/ <b>fine sawdust</b> and nutmeg/coarse sawdust

*Table 4.3 Counter balancing within the unlesioned, control subjects.*

No. Rats	Pairing order	ED shift
2	1 ⇒ 2 ⇒ 3	Odour ⇒ Medium
2	1 ⇒ 2 ⇒ 3	Medium ⇒ Odour
2	2 ⇒ 3 ⇒ 1	Odour ⇒ Medium
2	2 ⇒ 3 ⇒ 1	Medium ⇒ Odour
2	3 ⇒ 1 ⇒ 2	Odour ⇒ Medium
2	3 ⇒ 1 ⇒ 2	Medium ⇒ Odour

Rats receiving bilateral injections of 192-IgG-saporin in Dulbecco's saline underwent habituation, training and the attentional set-shifting task as described above. Efforts were made to ensure counter-balancing occurred during testing although evidence suggests that full counter-balancing within this attentional set-shifting task is unnecessary (there is no significant difference between odour to medium shifts and medium to odour shifts) (Birrell and Brown, 2000) and data can only be analysed from those subjects with successful bilateral lesions.

#### **4.2.6 Histology**

Operated rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB; Disodium hydrogen orthophosphate and Sodium dihydrogen orthophosphate in distilled water) after anaesthesia with 0.8ml Dolethal (Univet, Bicester, Oxfordshire, UK). Brains were stored overnight at 4°C in 20% sucrose solution, then cut to 50µm sections from bregma +1.5 to -4.5 on a microtome (Jung Histoslide 2000, Reichert-Jung, Cambridge Instruments GmbH) into 0.1M phosphate buffer saline (0.9%) (PBS). Sections were stained with cresyl violet and for vesicular acetylcholine transporter protein (VACHT; to allow visualisation of cholinergic neurons), parvalbumin (to allow visualisation of GABAergic Rt neurons), acetylcholinesterase (AChE) and rat nerve growth factor (rNGF).

For cresyl violet, sections were mounted onto pre-treated glass slides then stored overnight in a formalin bath. Sections were then defatted with xylene, and re-hydrated with ethanol, then 50% ethanol solution, then distilled water. Sections were immersed in cresyl violet solution (cresyl fast violet acetate soluted in distilled water and glacial acetic acid, pH adjusted to 3.5 with sodium acetate) for 2 minutes then washed in running water for 5 minutes. Sections were subsequently dehydrated in 50% ethanol solution, ethanol and finally xylene. Coverslips were applied with DPX mountant (BDH Laboratory Supplies, Poole, UK).

For VACHT, sections were washed 5 times for three minutes in 0.1M PBS, then placed on a stirrer for 1 hour in blocking solution (0.1M PBS, 20% normal goat serum, 0.1% triton). Sections were washed as previously in 0.1M PBS, then incubated in anti-VACHT (Phoenix Pharmaceuticals, Inc., CA, USA) (1:4000) in antibody diluting solution (ADS; 0.1M PBS, 1% normal goat serum, 0.1% triton) at 4°C for 1 night. Subsequently sections were washed in 0.1M PBS as before, then incubated on a stirrer in vector IgG solution (anti-rabbit IgG at 5µl/ml ADS) (Vector Laboratories Ltd, Peterborough, UK) for 1 hour. After washing in 0.1M PBS again, sections were incubated on a stirrer in Vectastain ABC complex (Vector Laboratories Ltd, Peterborough, UK) (reagents A and B at 10µl/ml ADS) for a further hour. Sections were then washed in 0.1M PBS again, and finally immersed in Sigma Fast 3,3'-Diaminodenzidine tablets (DAB; Sigma Chemical Company, St Louis, MO, USA) in distilled water until reasonable colour was developed (up to 10 minutes). Sections were washed again in 0.1M PBS and then mounted on glass slides. Sections were de-fatted in xylene and cover slips were applied as under cresyl violet protocol.

For parvalbumin, sections were treated as for VACHT except that sections were incubated in anti-parvalbumin (Sigma-Aldrich) (1:8000) for 1 night in ADS rather than anti-VACHT and sections were incubated in vector IgG solution (anti-mouse IgG at 5µl/ml ADS) rather than in anti-rabbit IgG.

For rNGF, sections were treated as for VACHT except that sections were incubated in anti-rNGF (Oncogene Research Products, Calbiochem-Nocbiochem, International) (1:5000) for 3 nights in ADS and sections were incubated in vector IgG solution (anti-mouse IgG at 5 $\mu$ l/ml ADS).

For acetylcholinesterase, sections were mounted onto pre-treated glass slides then stored overnight at 4°C then overnight again at 37°C in 300ml incubation medium (300ml stock incubation solution (750ml distilled water + 500mg copper sulphate + 750mg glycine + 74ml 0.2M acetic acid, buffered to pH 5.0 with 1M NaOH) + 230mg acetylthiocholine iodide + 10mg ethopropazine). Sections were washed 4 times for 3 minutes each in distilled water then immersed in sulphide solution (300ml distilled water + 1.5ml ammonium sulphide, buffered to pH 7.5 with glacial acetic acid) until stain is developed (1-2 minutes). Sections are washed in distilled water as before and dehydrated, de-fatted and cover-slipped as in cresyl violet stain procedure.

Sections were analysed under light microscope at magnifications X10 and X40. Images from the sections were displayed on a monitor taken from a camera (Sony CCD) mounted on the microscope. Images were also relayed to computer and captured using a digital camera (Pixera).

During cell counting VACHT/rNGF immunoreactive neurons in the nbM/SI were counted as one due to the difficulty in separating the two, essentially interspersed nuclei. Likewise, when counts of MS and VDB were taken the nuclei were treated as one, as well as with HDB and MCPO.

#### **4.2.7 Surgery**

##### **4.2.7.1 Unilateral rostral Rt lesions**

At initiation of the procedure, the subjects (n = 15) were administered 192-IgG-saporin under anaesthesia. The rats were anaesthetised with a halothane (Rhodia Ltd, Avonmouth, Bristol, England), nitrous oxide and oxygen mix, initially with halothane concentration at 4%, nitrous oxide at 0.8l/min and oxygen at 0.4l/min. Once anaesthetised, rats were mounted on the stereotaxic frame, and halothane concentration was reduced to 1.8% to maintain anaesthesia. 192-IgG-saporin was administered using a 0.5µl Hamilton syringe (Aldrich Chemical Company, Milwaukee, WI, USA) with a 30 gauge needle attached, at stereotaxic co-ordinates (Paxinos and Watson, 1986); level skull -3.3mm, AP - 1.4mm, ML - 2.0mm (from bregma); DV - 6.6mm (from skull surface at injection site). 192-IgG-saporin was administered over the course of 3 minutes, with the needle left *in situ* for a further 3 minutes after administration. Rats were either administered 192-IgG-saporin unilaterally at a dose of 0.3µl of 0.5µg/µl Dulbecco's

saline (n = 3), 0.65 $\mu$ g/ $\mu$ l (n = 8) or 0.8 $\mu$ g/ $\mu$ l (n = 4). Upon completion of administration, Halothane concentration was reduced to 1% and head wounds were sealed using wound clips and PEP powder was administered to reduce risk of infection. Rats were then removed from the stereotaxic frame and returned to their home cages, single housed, and left to recover in a warm environment. Upon recovery, rats were returned to the holding room with free access to food and water for the next 24 hours. Rats were weighed daily to monitor recovery.

#### **4.2.7.2 Bilateral rostral Rt lesions**

Subjects (n = 16) received the same treatment as above, with injections of 0.3 $\mu$ l of 0.65 $\mu$ g/ $\mu$ l 192-IgG-saporin at the following co-ordinates: level skull –3.3mm, AP – 1.4mm, ML  $\pm$  2.0mm (from bregma); DV – 6.6mm (from skull surface at injection site). 2 subjects received similar injections but at co-ordinates; AP – 1.5mm.

#### **4.2.7.3 Bilateral nbM lesions**

Subjects (n = 12) received the same treatment as above but were injected with 192-IgG-saporin bilaterally at a dose of 0.25 $\mu$ g/ $\mu$ l at stereotaxic co-ordinates; level skull –3.3mm, AP –0.75mm, ML  $\pm$ 3.3mm,  $\pm$ 2.3mm (from bregma), DV –8.1mm and –7.8mm respectively (from skull surface at injection site) (n = 2; 0.2 $\mu$ l per site); and level skull – 3.3mm, AP –0.7mm, ML  $\pm$ 2.9mm, DV –6.7mm (from dura) (n = 10; 0.5 $\mu$ l per site). The needle was left *in situ* for 3 minutes then 192-IgG-

saporin was administered rapidly to create a bolus, with the needle left *in situ* for a further 3 minutes after administration. The dose was considerably reduced from that administered into Rt both to reduce necrotic damage at injection site as observed in sections of Rt lesioned subjects, and to permit comparison with data from other 192-IgG-saporin nbM lesioned rats (Baxter *et al.*, 1995; McGaughy *et al.*, 1999; McGaughy *et al.*, 2000; McGaughy *et al.*, 2002).

#### **4.2.7.4 Control (unoperated)**

Subjects (n = 12) received no treatment prior to performing the set-shifting task.

#### **4.2.8 Data analysis**

Data (time to first dig (latency to dig), whether only one or both bowls were investigated prior to first dig and correct or incorrect dig) were collected on set-shifting data sheets. Correct/incorrect data for trials to criterion were entered into Sigmaplot (version 5.0) and analysed in SPSS (version 10.0). Trials to criterion were analysed using repeat measures ANOVA with discrimination as a within-subjects variable and presence of lesion as a between-subjects variable.

Dig latency data were entered into Sigmaplot and categorised by whether only one or both bowls were experienced prior to onset of digging. Data from the last 5 (correct) trials in each shift were analysed

using repeat measures ANOVA with dig (whether one or two bowls were encountered prior to initiating digging) as within-subjects variables and treatment as a between-subjects variable. Only the last 5 correct trials were sampled in this analysis as all would represent a rat making a correct decision, and there would be no contamination of neophobic reaction to exemplars. A subject learning the discrimination in 6 trials might have a dig latency on the first trial that is affected by not having experienced the exemplars before. Data from a subject learning the discrimination in more than 6 trials would not have the 1<sup>st</sup> trial included were the first 6 trials to be analysed. It was therefore decided that all 1<sup>st</sup> trial data would be excluded from data analysis, and so only the last 5 trials were included for all subjects. Furthermore, discrimination could not be included in the analysis as a within-subjects variable as several data points were missing due to some subjects by chance encountering the correct (or incorrect) bowl first on six consecutive trials within a discrimination in which they dug correctly, thereby achieving criterion. In these discriminations no latency data is available for the alternate digging scenario.

## 4.3 Results

### 4.3.1 *Thalamic reticular nucleus lesions*

Subjects administered with 192-IgG-saporin unilaterally into Rt received one of three doses, 0.5 $\mu$ g/ $\mu$ l, 0.65 $\mu$ g/ $\mu$ l and 0.8 $\mu$ g/ $\mu$ l (see Table 4.4). Analysis of sections stained for both VAcHT and rNGF show an ipsilateral loss of, presumably cholinergic, neurons bearing the p75 receptor in nbM/SI at all doses. In all cases loss of neurons extends into the HDB/MCPO (see Table 4.5) and in some cases into the VDB and medial septum (insufficient sections were collected to collate data). Loss of cholinergic neurons in nbM proximal to the injection site is almost complete, with, in some cases, neurons remaining intact towards the caudal extent of the nbM/SI (Figure 4.5 and 4.6). Loss of BF neuronal staining in VAcHT stained sections corresponds to that in rNGF stained sections (0.65 $\mu$ g/ $\mu$ l nbM: 87%, range 80-100%; HDB: 27%, range 22-100%), confirming previous co-localisation of the p75 rNGF receptor on BF cholinergic neurons. There appears to be a difference of approximately 10 cells per count difference (rising to 20 at the caudal nbM/SI) between VAcHT labeled cells and rNGF labeled cells both before and after administration of 192-IgG-saporin (Figure 4.5 and 4.6). There are more VAcHT labeled cells than rNGF labeled cells in the nbM/SI, possibly correlating to the nbM cholinergic projections to amygdala that are known not to bear the p75 rNGF receptor.

Table 4.4 shows number of rats undergoing injection of 192-IgG-saporin into rostral Rt, which of those were observed to have lesions, which of those completed the attentional set-shifting task and number of rats that completed the attentional set-shifting task after treatment that failed to induce a bilateral cholinergic lesion.

<b>Treatment</b>	<b>Treatment n</b>	<b>Successful lesion n</b>	<b>Lesion n completing task</b>	<b>Error lesion n completing task</b>
0.50µg/µl unilateral in Rt	3	1	N/A	N/A
0.65µg/µl unilateral in Rt	8	5	N/A	N/A
0.80µg/µl unilateral in Rt	4	1	N/A	N/A
0.65µg/µl bilateral in Rt	18	7	2	10

Table 4.5 Mean  $\pm$  SEM percentage cell loss of VACHT stained cells in the nbM/SI and HDB/MCPO after administration of 192-IgG-saporin to rostral Rt. Data for % loss HDB/MCPO are only shown for unilaterally administered subjects where % loss was calculated from cells in the unlesioned contralateral side. % loss for bilaterally administered subjects are calculated from contralateral sides of unilaterally lesioned subjects.

192-IgG-saporin dose	% cell loss nbM/SI	% cell loss HDB/MCPO
0.50 $\mu$ g/ $\mu$ l unilateral in Rt (n = 1)	73%	48%
0.65 $\mu$ g/ $\mu$ l unilateral in Rt (n = 5)	71% $\pm$ 4 (69-91%)	65% $\pm$ 9
0.80 $\mu$ g/ $\mu$ l unilateral in Rt (n = 1)	82%	89%
0.65 $\mu$ g/ $\mu$ l bilateral in Rt (n = 7)	70% $\pm$ 2	Insufficient sections to count

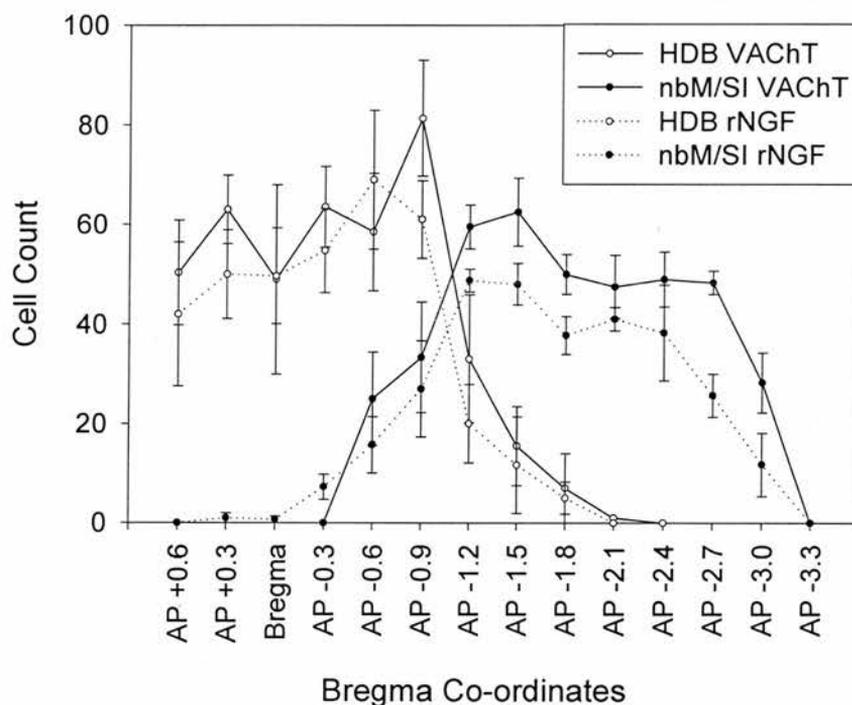


Figure 4.5 Mean  $\pm$  SEM (n = 5) cell counts for unlesioned basal forebrain nuclei stained for VACHT and rNGF. HDB and MCPO are counted together, as are nbM and SI because there are no clear borders between the nuclei in the observed sections. There is a consistently lower count of rNGF immunoreactive neurons in the nbM/SI complex as compared to the VACHT immunoreactive neurons in the same subjects

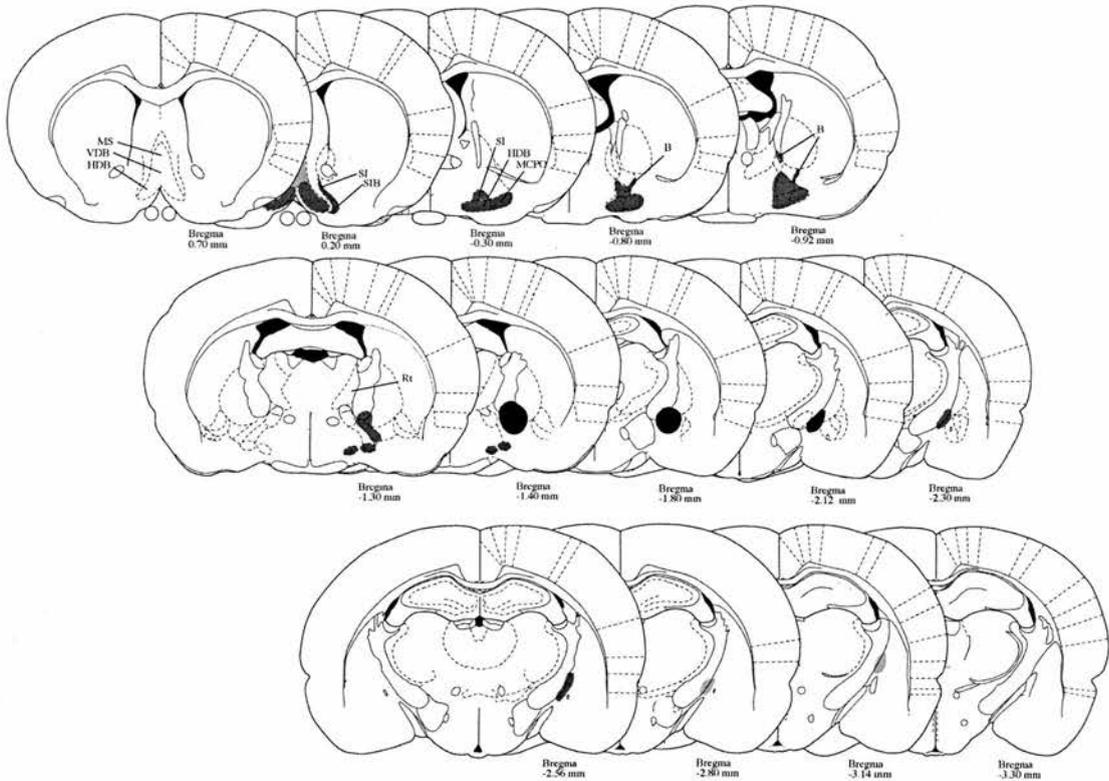
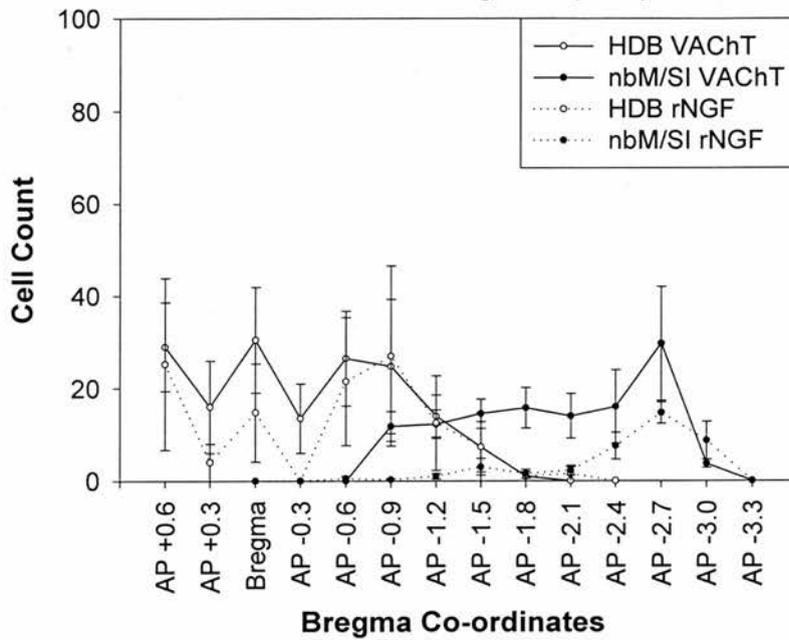


Figure 4.6 Mean  $\pm$  SEM cell counts ( $n = 4$ ) for BF of subjects administered  $0.65\mu\text{g}/\mu\text{l}$  192-IgG-saporin into rostral Rt: VAcHT and rNGF. There are consistently less rNGF immunoreactive cells counted in nbM/SI than VAcHT immunoreactive cells. This difference is in the order of 10-20 cells throughout the extent of the nbM/SI. There is a notable rise in cell count for both VAcHT and rNGF immunoreactive cells at the caudal end of the nbM/SI. Above is a schematic of largest (light grey), smallest (black) and average (grey) spread of cell loss in the lesioned subjects (adapted from Paxinos and Watson, 1996).

VACht staining in the Rt is reduced throughout the rostro-caudal extent of the nucleus. Thalamic nuclei proximal to the Rt that receive their cholinergic input from brainstem nuclei that do not bear the p75 receptor appear undamaged.

There is a reduction in staining of the cortex ipsilateral to the lesion. The nbM efferents to cortex terminate in somatosensory and motor cortex, with those from V/HDB and MS terminating in mPFC; MS also projects to hippocampus. Subjects where the loss of BF cholinergic neurons extends into MS show reduction in VACht staining in hippocampus. These observations are duplicated in the AChE stained sections.

Figures 4.7-4.15 show VACht stained coronal sections at magnification X4 (Figures 4.7-4.10) and X2.5 (Figure 4.11-4.13) and rNGF stained coronal sections at magnification X10 (Figures 4.14-4.15) of both unlesioned and lesioned brains (after 192-IgG-saporin into rostral Rt) alongside schematics of the rat brain adapted from Paxinos and Watson (1998).

Sections stained for parvalbumin showed minimal loss of, presumably GABAergic, neurons in the Rt around the injection site. It is presumed that this is non-selective damage induced by a potentially too

high dose or physical damage inflicted during the injection. Immediately beyond any visible damage of this type, Rt neurons remain intact.

Data were only successfully collected from 1 subject from each of the 0.5 $\mu$ g/ $\mu$ l and 0.8 $\mu$ g/ $\mu$ l 192-IgG-saporin administered subject groups, and 5 subjects from the 0.65 $\mu$ g/ $\mu$ l group. It is considered that this is caused by movement of Rt away from the injection needle during initial insertion of the needle. Nearly all of the “misses” were rostral to the Rt. Data do not indicate any loss of BF cholinergic neurons after injection of 192-IgG-saporin rostral to the Rt. Subjects administered 192-IgG-saporin at stereotaxic co-ordinates AP -1.5 in order to combat this were all found to have injection sites caudal to the rostral portion of the Rt, which due to its shape, meant that they entered the ventral anterior thalamic nucleus. The ventral anterior thalamic nucleus does not receive cholinergic input from BF (Semba, 2000) so would not be expected to be damaged by 192-IgG-saporin other than any physical mechanical damage caused by the injection.

Observations of sections from bilaterally administered subjects indicate the same pattern of effects as seen in unilaterally injected rats. Of 18 subjects injected bilaterally, 7 exhibited correct placement of needle with corresponding bilateral losses of BF cholinergic neurons (> 50%) and reductions in VAcHT staining in the terminal regions of those neurons' projection fields. Of those 7, the mean loss of nbM/SI cells was

70% (see Table 4.5). Control data for unlesioned subjects were collected from the unlesioned side of unilaterally administered subjects (n = 5). Although there is a small chance of a reduced cell count due to contralateral effects of the 192-IgG-saporin administration into Rt, there is little evidence to suggest that the few contralateral projections from BF to Rt are collaterals of projections to ipsilateral Rt (Chen and Bentivoglio, 1993).

Of the 10 bilaterally injected subjects that completed the set-shifting task, it was concluded that 5 had no lesion because the 192-IgG-saporin injected showed no selective lesion effect. Histological analysis showed injection sites in rostral Rt, but with no corresponding cholinergic depletion, suggesting that the conjugate had broken down. The remaining 5, along with all successful bilaterally induced lesions, received a different batch of 192-IgG-saporin, but only showed unilateral lesions due to injection sites rostral of Rt.

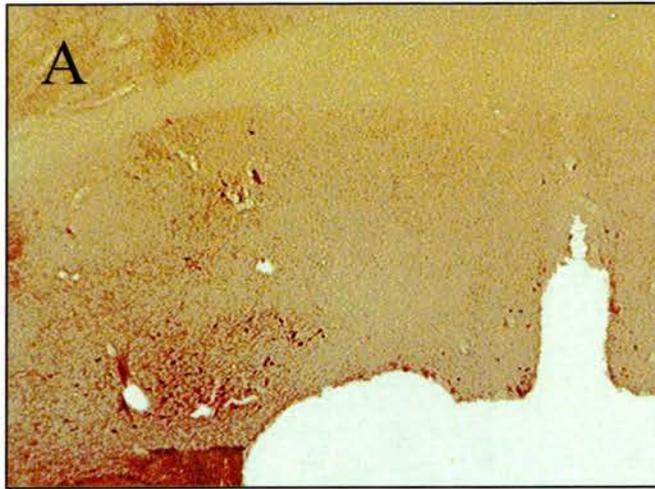
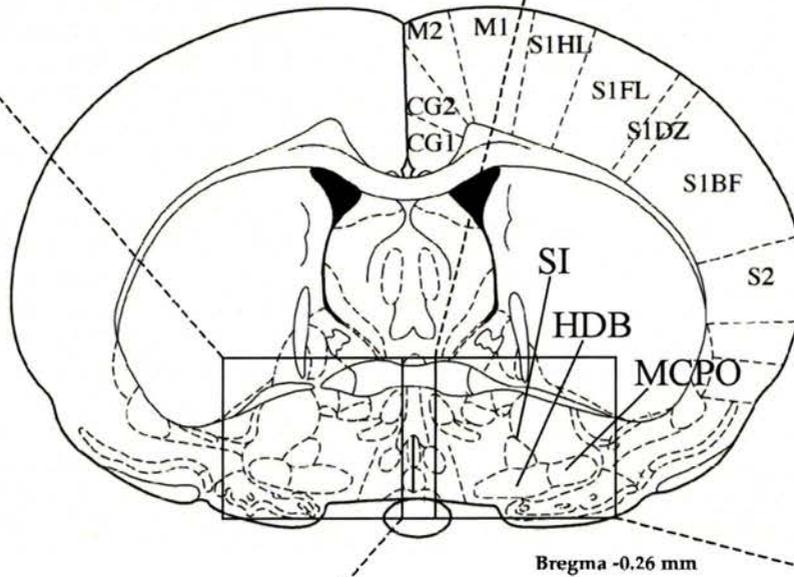


Figure 4.7 Photomicrograph (magnification X4) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -0.26). Sections show vesicular acetylcholine transporter protein (VAcHT) stained cholinergic neurons of the horizontal limb of the diagonal band (HDB), magnocellular preoptic nucleus (MCPO), substantia innominata (SI)...



...and nucleus basalis magnocellularis (nbM) in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



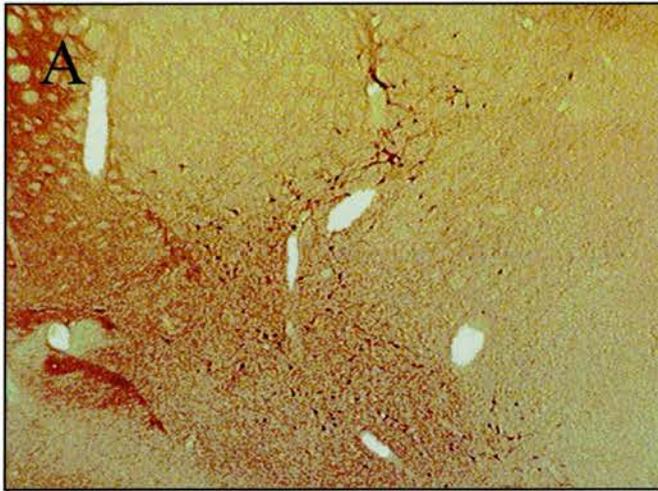
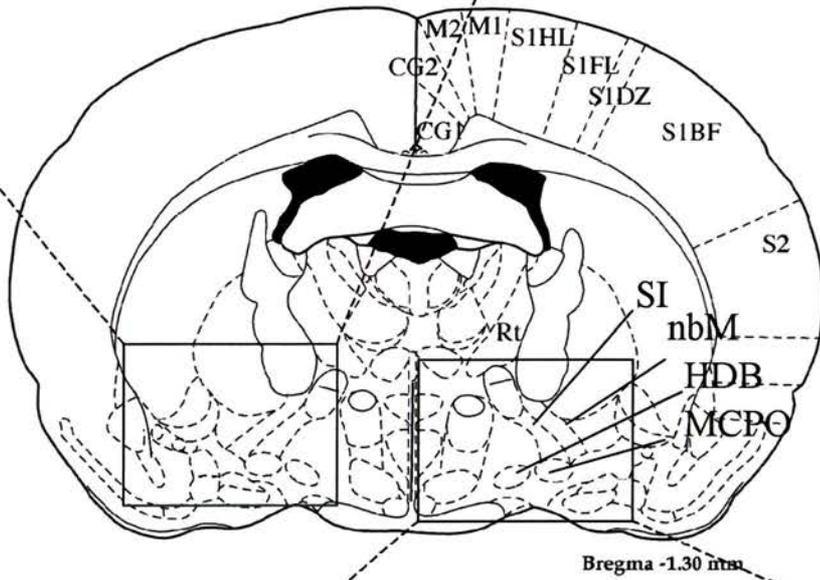


Figure 4.8 Photomicrograph (magnification X4) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -1.30). Sections show VAcHt stained cholinergic neurons of the SI, nbM, HDB and MCPO in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



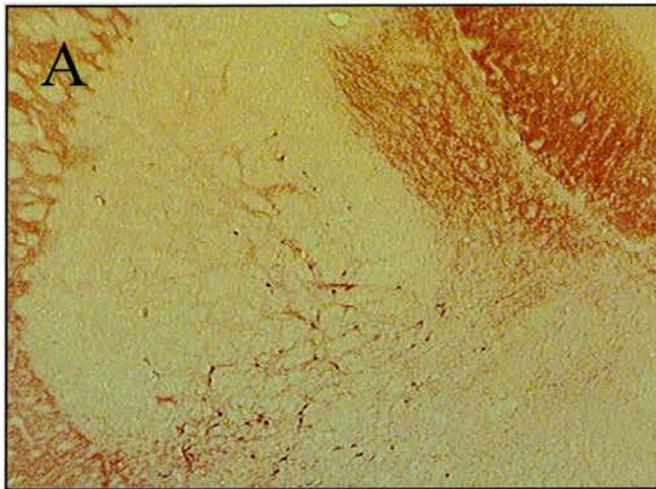
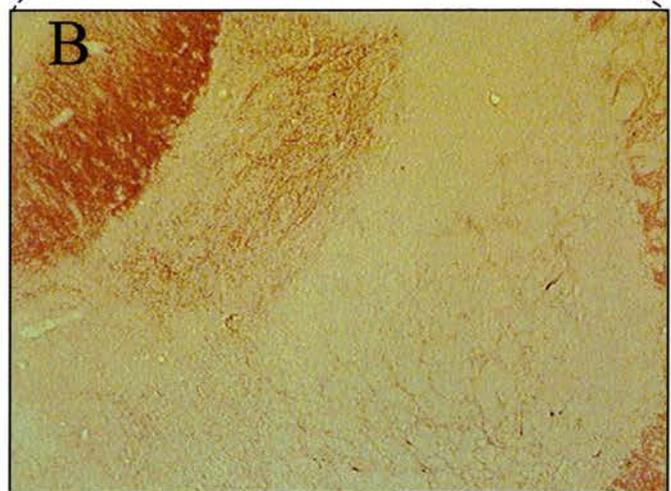
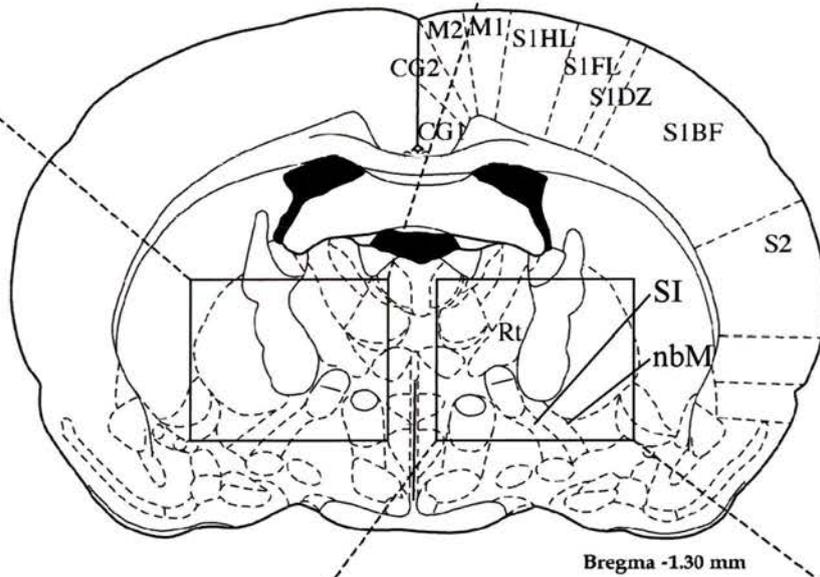


Figure 4.9 Photomicrograph (magnification X4) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -1.30). Sections show VAcHT stained cholinergic neurons of the SI and nbM and terminals in Rt of a typical brain (Section A) and a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



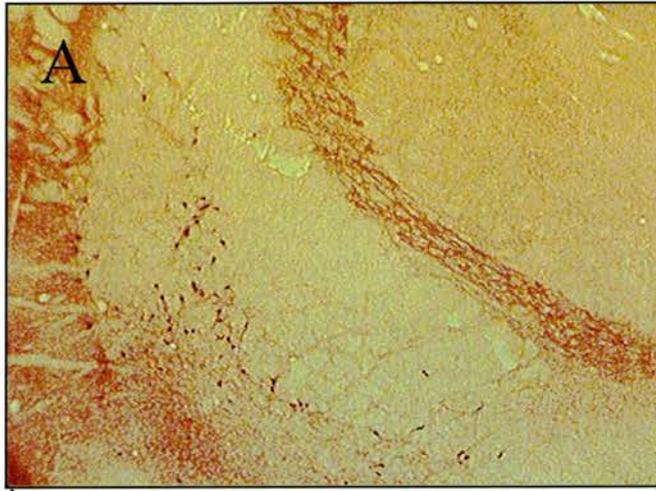
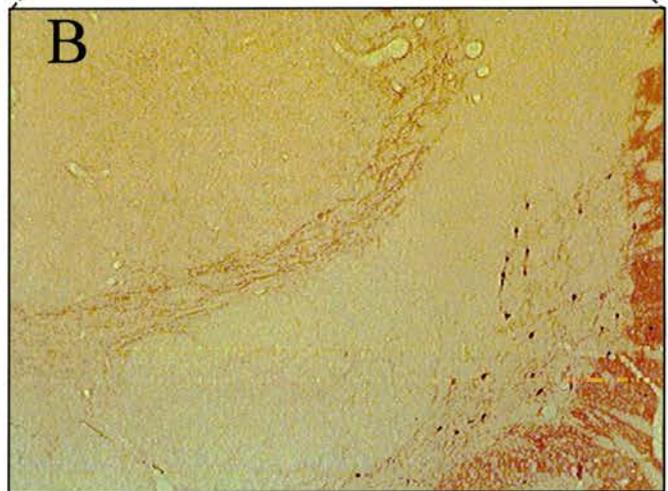
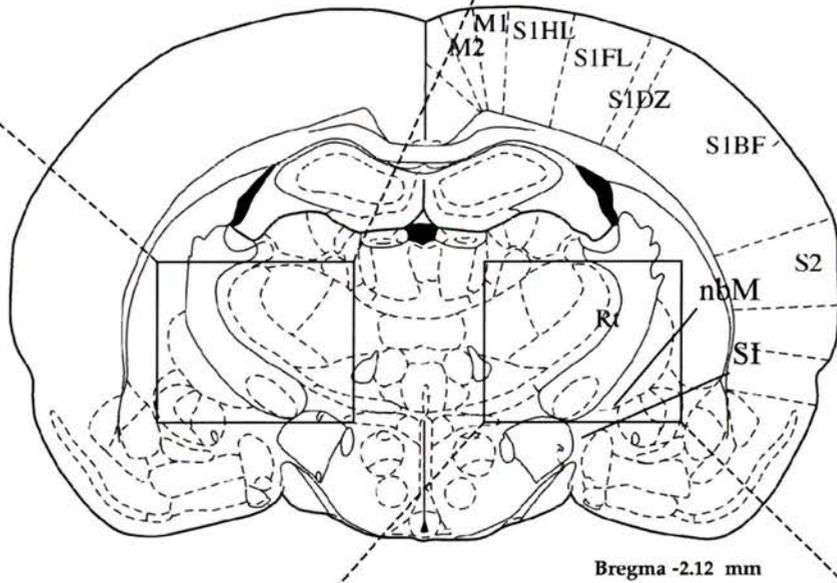


Figure 4.10 Photomicrograph (magnification X4) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -2.12). Sections show VAcHT stained cholinergic neurons of the SI and nbM and terminals in Rt of a typical brain (Section A) and a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



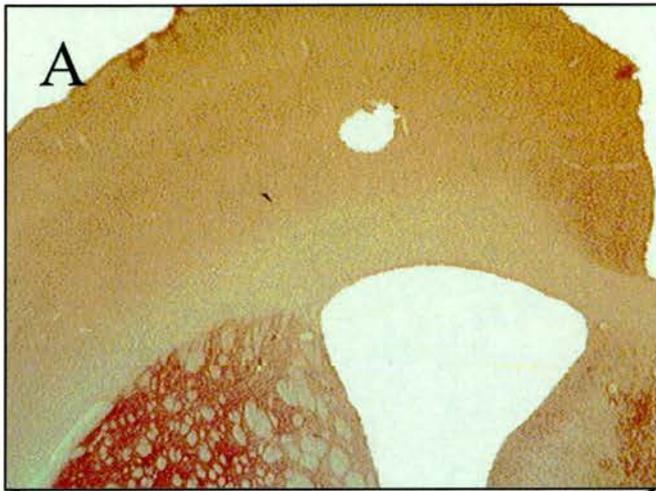


Figure 4.11 Photomicrograph (magnification X2.5) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -0.26). Sections show VAcHT stained cholinergic terminals in cortex of a typical brain (Section A) and a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)

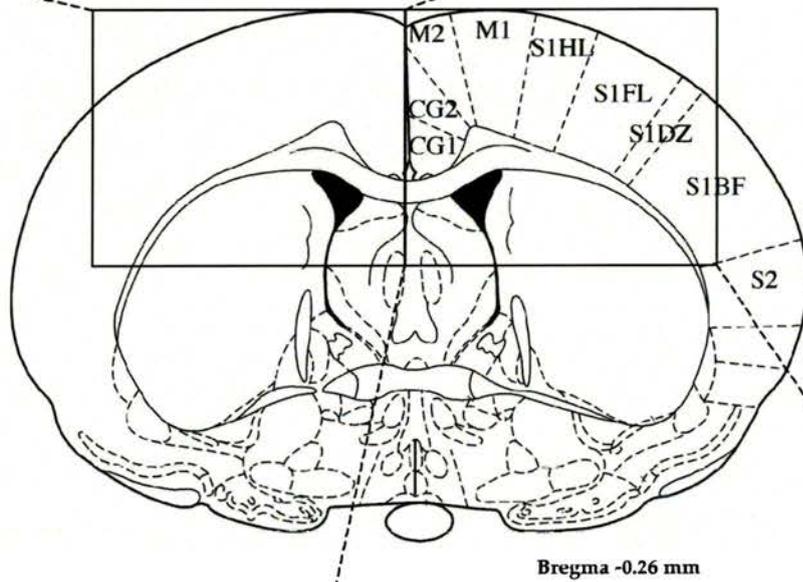
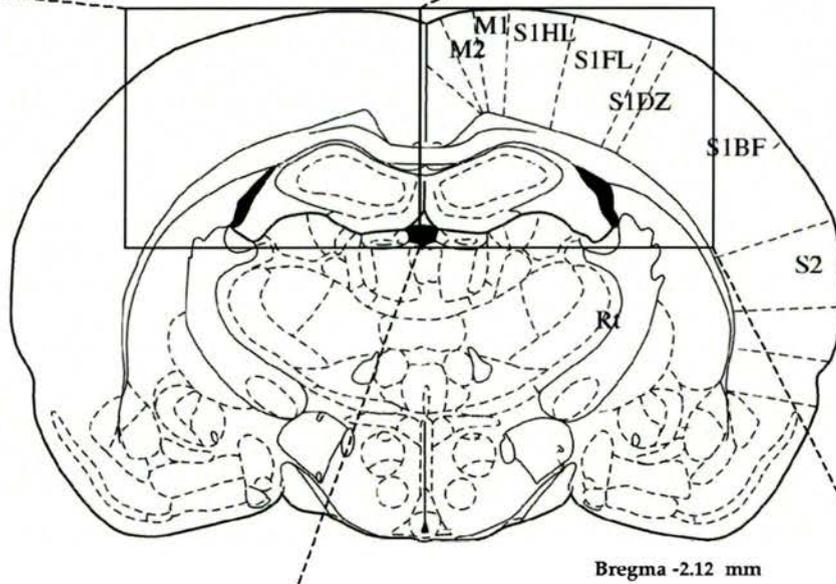




Figure 4.12 Photomicrograph (magnification X2.5) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -2.12). Sections show VAcHT stained cholinergic terminals in cortex of a typical brain (Section A) and a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



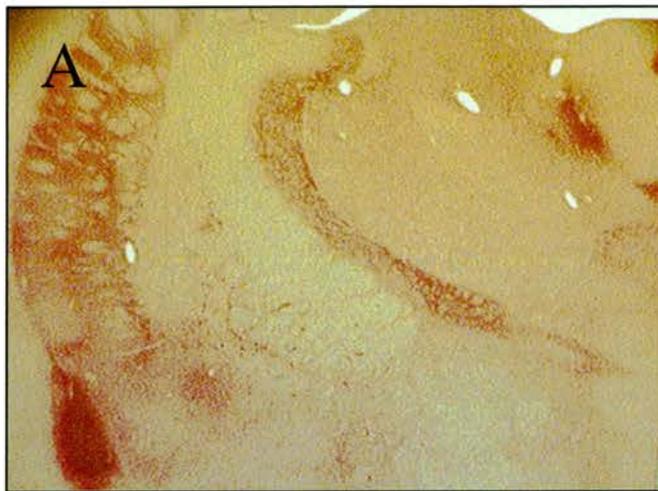
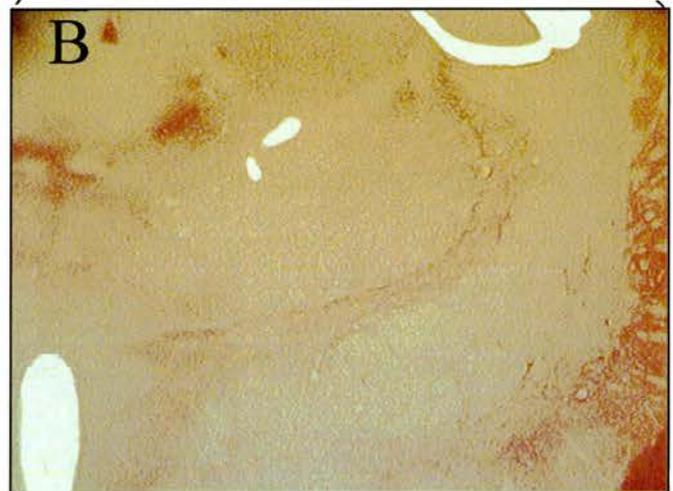
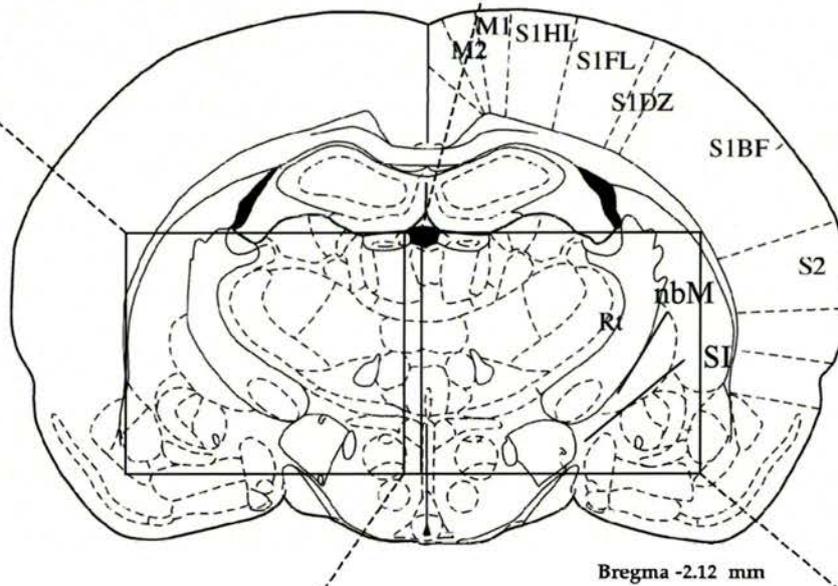


Figure 4.13 Photomicrograph (magnification X2.5) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -2.12). Sections show VAcHT stained cholinergic neurons of the SI and nbM and terminals in Rt of a typical brain (Section A) and a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



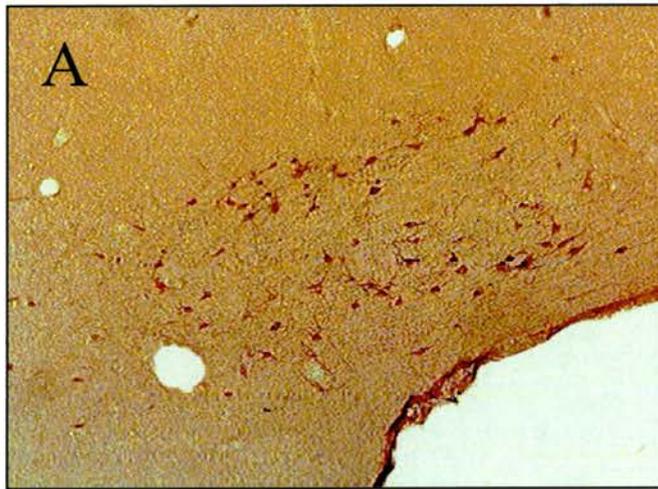
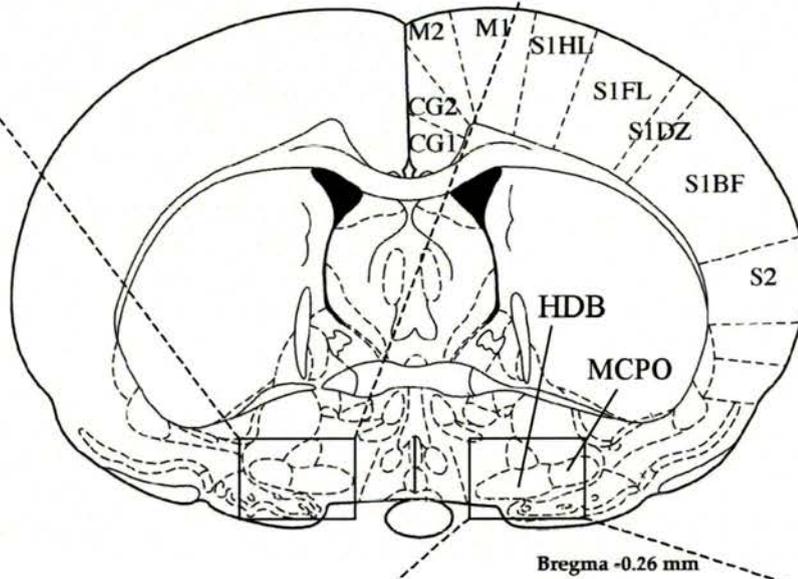


Figure 4.14 Photomicrograph (magnification X10) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -0.26). Sections show rat nerve growth factor (rNGF) stained cholinergic neurons of the HDB and MCPO in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



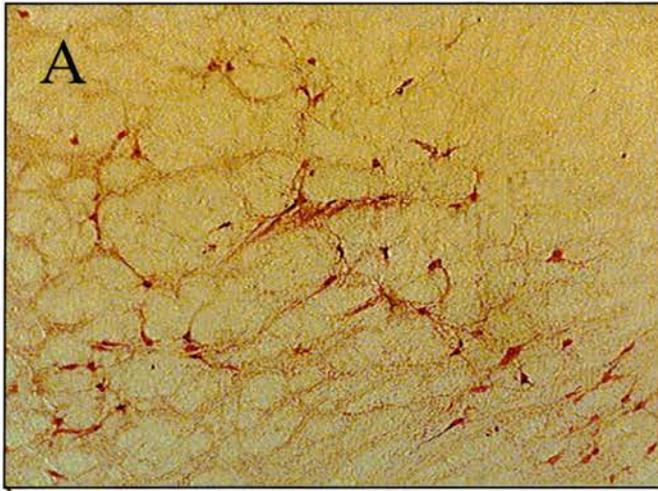
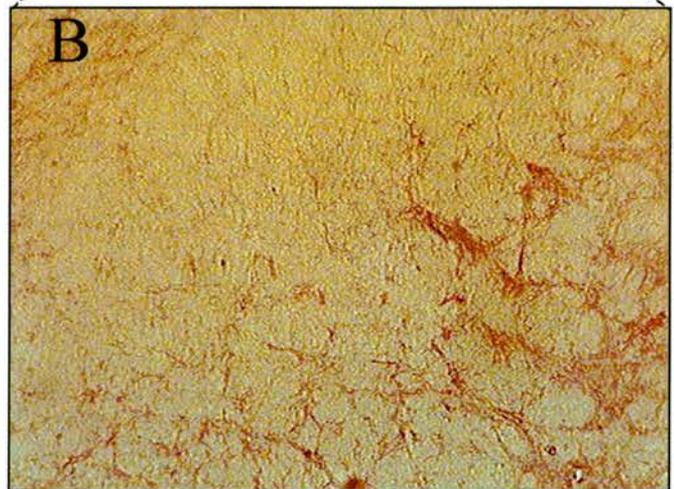
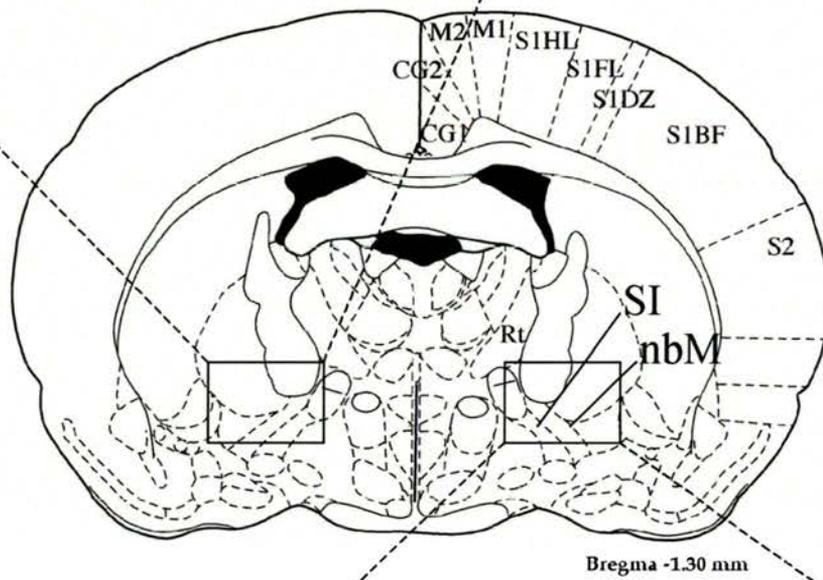


Figure 4.15 Photomicrograph (magnification X10) and schematic of coronal rat brain section (stereotaxic coordinates: Bregma -1.30). Sections show rNGF stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into rostral Rt (Section B)



#### ***4.3.2 Set-shifting after bilateral rostral Rt lesions***

As noted in table 4.4, only two of the bilateral Rt lesioned subjects completed the attentional set-shifting task. Performance in both trials to criterion and dig latency in these two subjects did not appear to differ from those of the control subjects used in the BF lesion study, and with two subjects, it was considered that results of statistical analyses would be too weak from which to draw conclusions.

That of the 7 subjects with successful bilateral lesions, only 2 completed the task suggests however that there is an effect of the lesion upon ability to perform the task. It is unlikely an effect of satiety, as subjects showed no consistent loss of weight after surgery. Cessation occurred at varied discriminations within the task schedule (REV1, REV2 and ED) suggesting that there was no one specific performance deficit that could be related to observed impairments from other lesion studies. Furthermore, there was no consistency with exemplars (pairings 1 (M1/M2/O1/O2) and 3 (M5/M5/O5/O6)), suggesting that no pairing had become more difficult or more aversive as a result of the surgery. The 2 subjects completing the task did not have the smallest lesions either, with loss of VACHT stained nbM neurons of 66% and 67% (compared to the overall mean of  $70\pm 2\%$ ). Possibilities will be considered in light of BF lesioned subjects' performance later in this chapter.

### 4.3.3 Basal forebrain lesions

Table 4.6 shows number of rats undergoing injection of 192-IgG-saporin into nbM, which of those were observed to have lesions, which of those completed the attentional set-shifting task and number of rats that completed the attentional set-shifting task after treatment that failed to induce a bilateral cholinergic lesion.

Treatment	Treatment n	Successful lesion n	Lesion n completing task
None	12	N/A	12
0.25µg/µl bilateral in nbM (4 injection sites: 0.2µl/site)	2	2	2
0.25µg/µl bilateral in nbM (2 injection sites: 0.5µl/site)	10	10	8

Table 4.7 Mean  $\pm$  SEM percentage cell loss of VAcHt stained cells in the nbM/SI after administration of 192-IgG-saporin to nbM. % loss for bilaterally administered subjects is calculated from contralateral sides of unilaterally lesioned subjects.

192-IgG-saporin dose	% cell loss nbM/SI
None (n = 5)	0%
0.25µg/µl bilateral in nbM (n = 12)	77% $\pm$ 2 (60-83%)

Subjects administered with 192-IgG-saporin bilaterally into nbM received either 0.5µl or 0.2µl of 0.25µg/µl in one site or two sites per side respectively. Initial investigations compared the two site per side (n = 2) method with the one site per side method (n = 2). There was observed no

difference between the two methods during analysis of histology, so subsequent administrations of 192-IgG-saporin utilised the one site per side method (n = 8).

All subjects administered 192-IgG-saporin were observed in VACHT and rNGF stained sections to exhibit a loss of, presumably cholinergic, nbM/SI neurons (mean 77%; range 60-83% (n = 12 (with 24 counts due to bilateral lesions))). In some of the subjects this neuronal loss extended into the HDB/MCPO (Figure 4.18), although most severely in the regions of HDB/MCPO nearest the injection site (stereotaxic coordinates, AP -0.6 through to -2.1 where the MCPO ends). In some subjects loss of BF neurons was less evident at the caudal end of the nbM/SI. The same control data as used in the bilateral Rt lesions were used to establish percentage loss. All subjects showed reduced VACHT staining in Rt in a similar fashion to that observed in Rt lesioned animals.

Figures 4.19-4.28 show VACHT stained coronal sections at magnification X10 (Figures 4.19-4.25) and X4 (Figure 4.26-4.28) of both unlesioned and lesioned brains (after 192-IgG-saporin into nbM) alongside schematics of the rat brain adapted from Paxinos and Watson (1998).

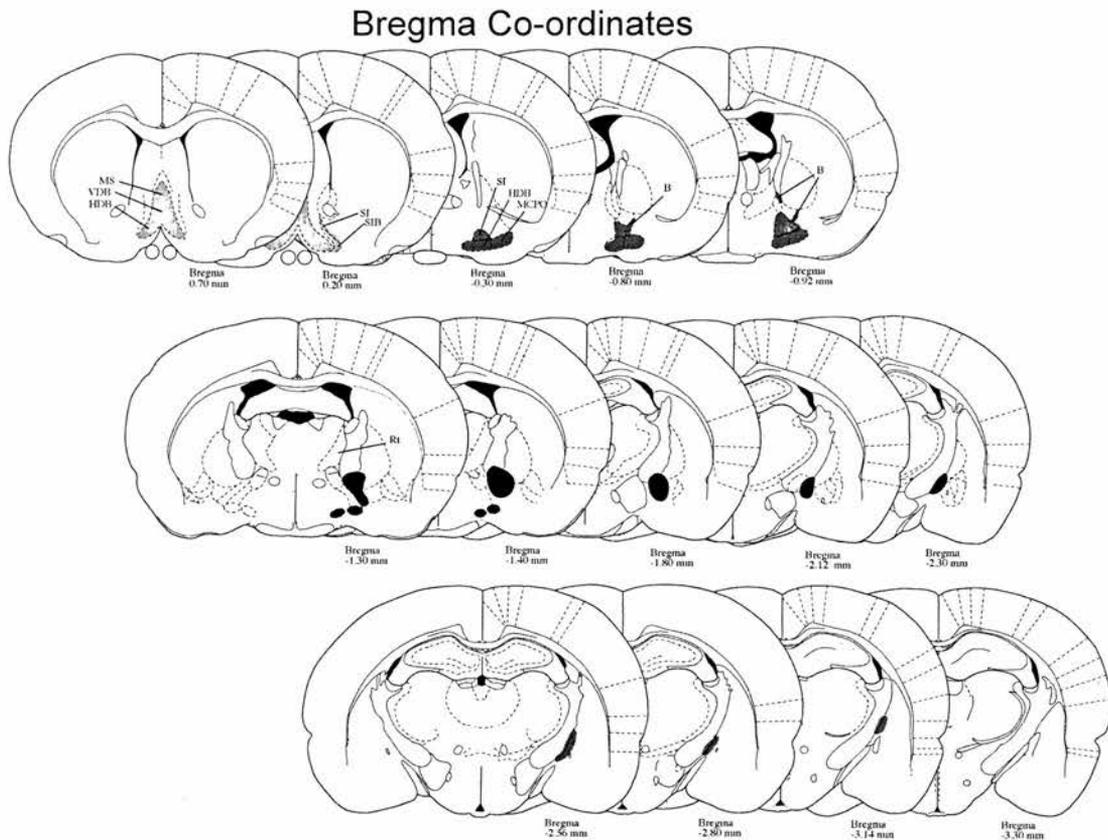
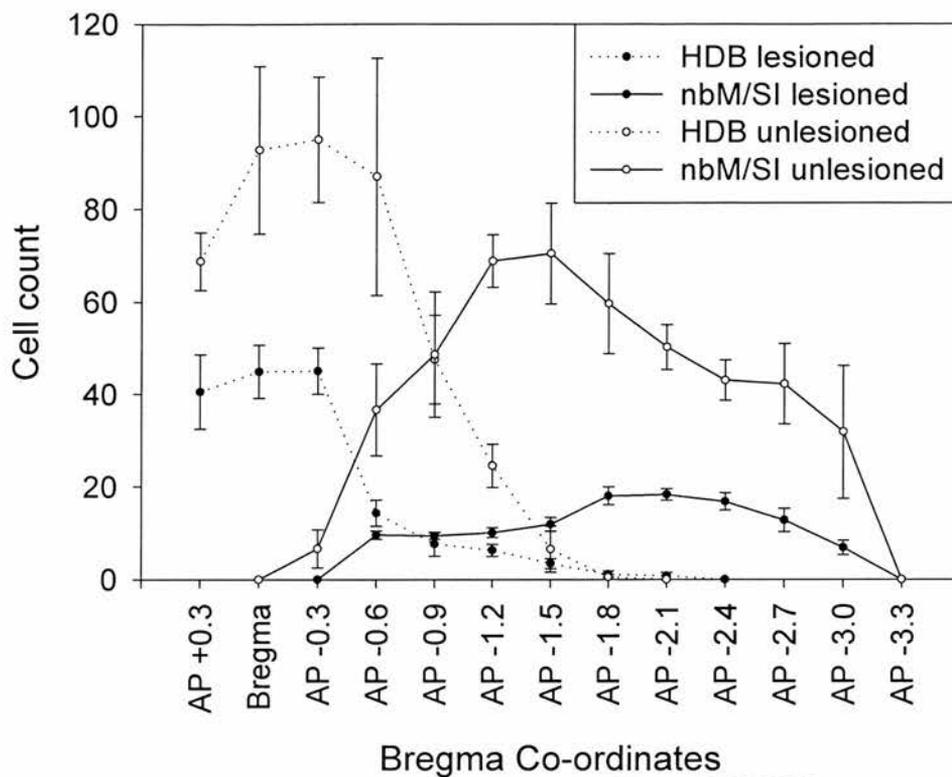
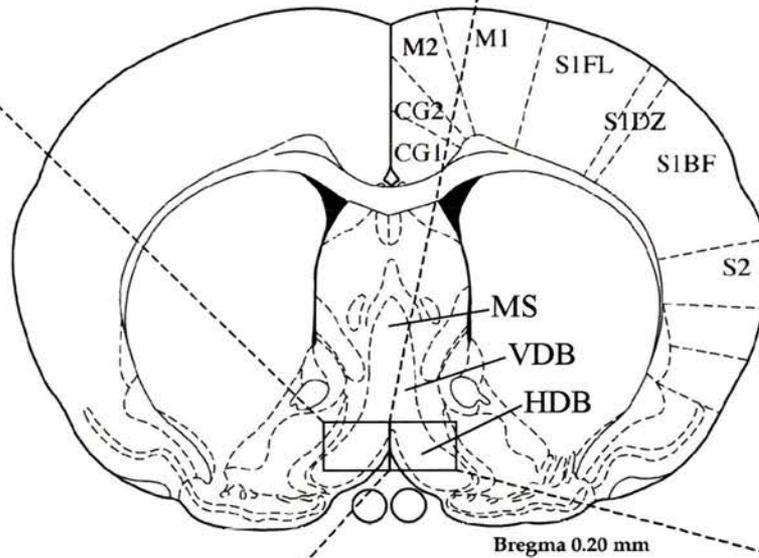


Figure 4.18 Mean  $\pm$  SEM cell counts (VAcHT) for unlesioned BF ( $n = 5$ ) and 192-IgG-saporin administered BF ( $n = 12$ ). Injection point was Bregma  $-0.7$ . Cell count was reduced in both HDB and nbM/SI, with largest reduction between Bregma  $-0.3$  and  $-1.8$ . Below is a schematic showing largest (light grey), smallest (black) and average (grey) spread of cell loss after 192-IgG-saporin administration in nbM.



Figure 4.17 Photomicrographs (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma +0.20). Sections show vesicular acetylcholine transporter protein (VAcHT) stained cholinergic neurons of the horizontal limb of the diagonal band of Broca (HDB) in a typical brain (Section A)...



And in a typical brain after administration of 192-IgG-saporin into nucleus basalis Magnocellularis (nbM) (Section B). Cortical regions are cingulate area 1 (CG1); cingulate area 2 (CG2); 2° motor cortex (M2); 1° motor cortex (M1); 1° somatosensory, forelimb region (S1FL); 1° somatosensory, dysgranular region (S1DZ); 1° somatosensory, barrel field (S1BF); 2° somatosensory (S2)





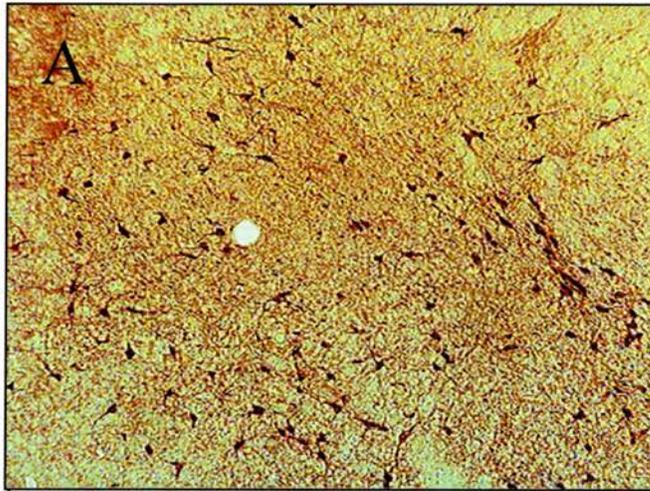
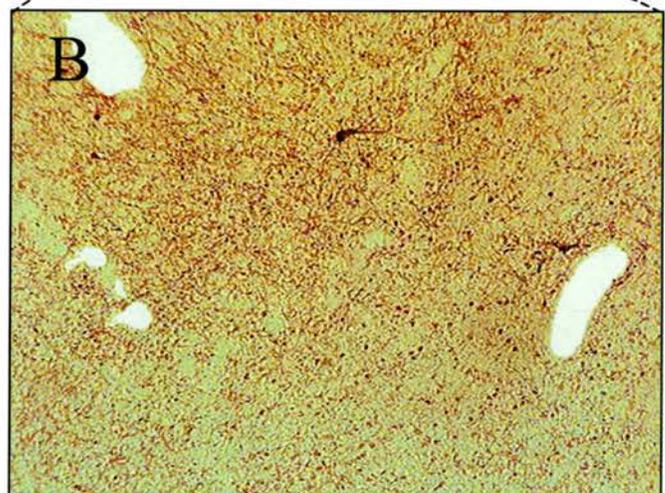
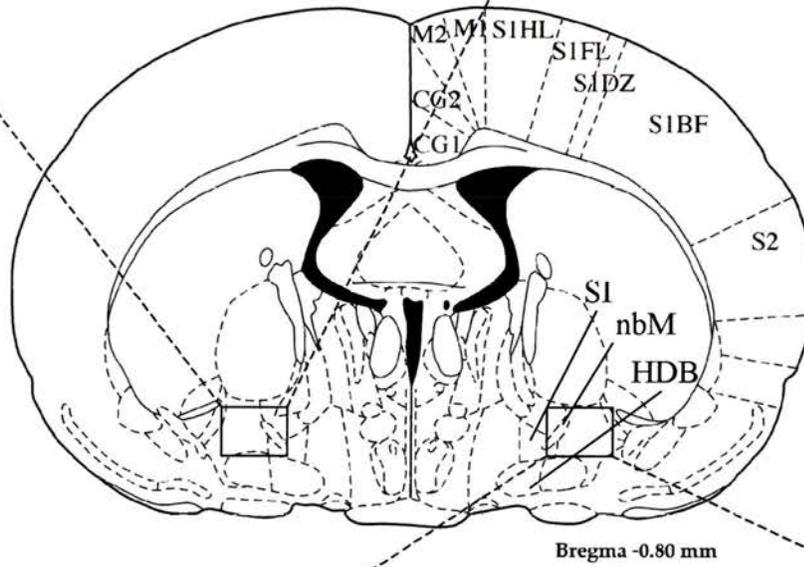


Figure 4.19 Photomicrograph (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -0.80). Sections show VAcHt stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



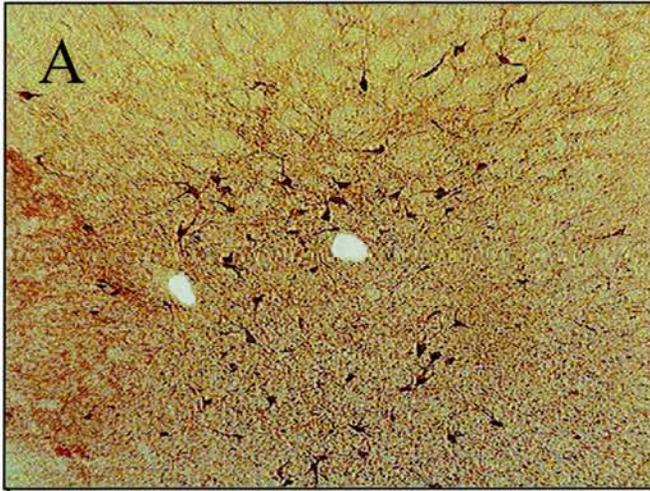
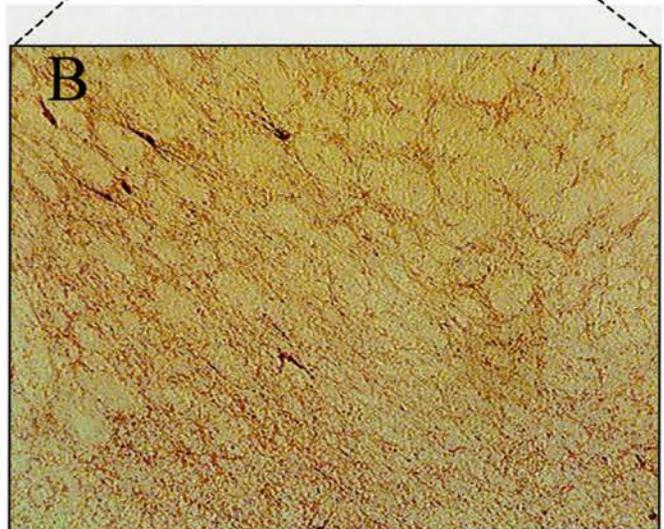
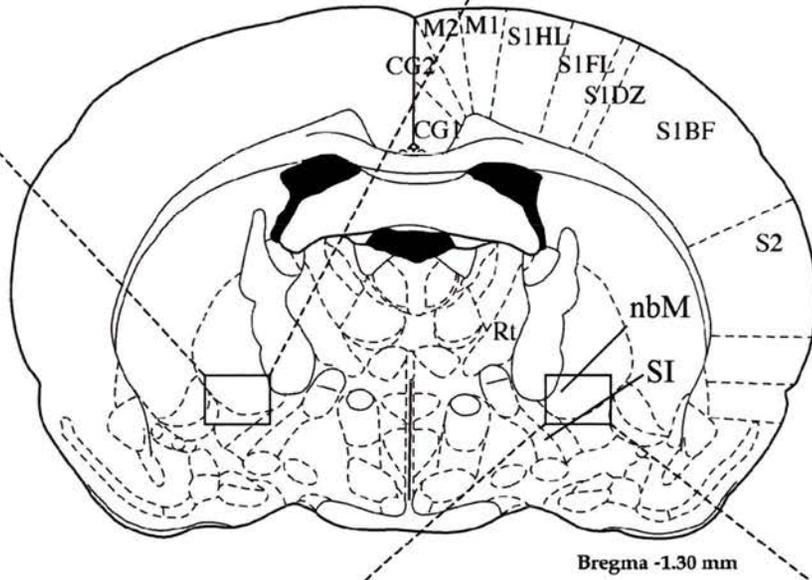


Figure 4.20 Photomicrograph (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -1.30). Sections show VAcHt stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



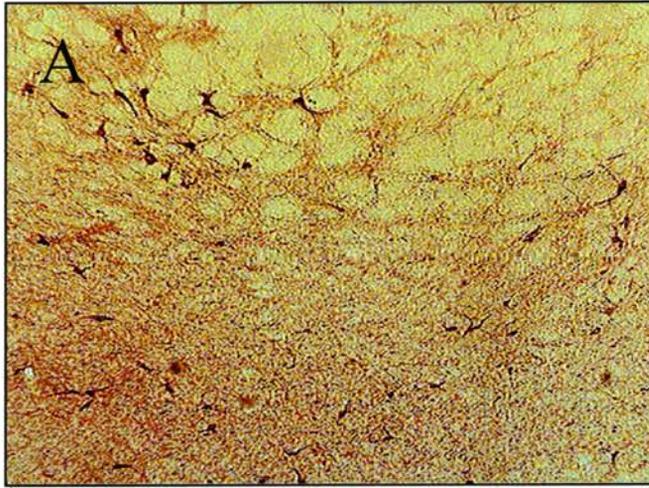
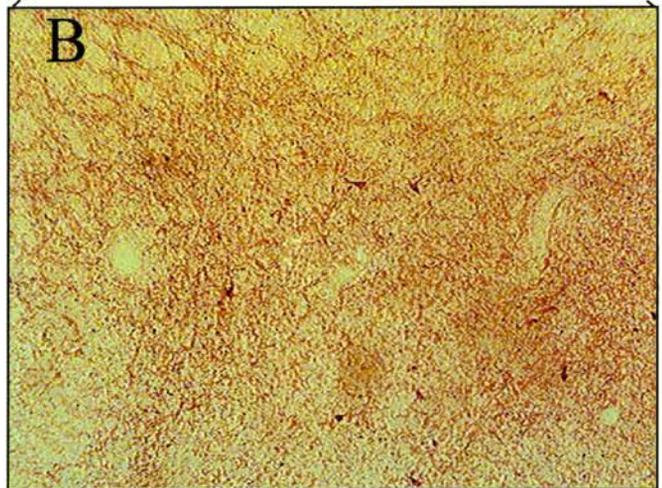
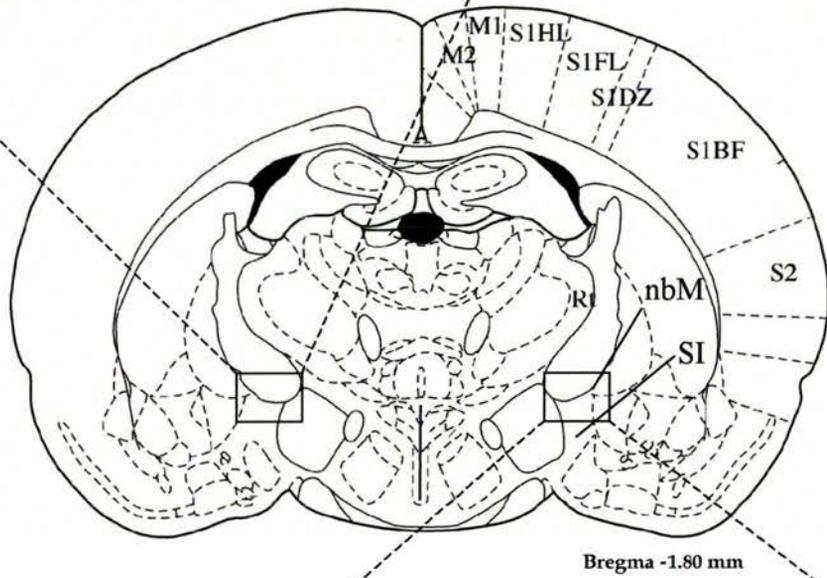


Figure 4.21 Photomicrograph (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -1.80). Sections show VAcHT stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



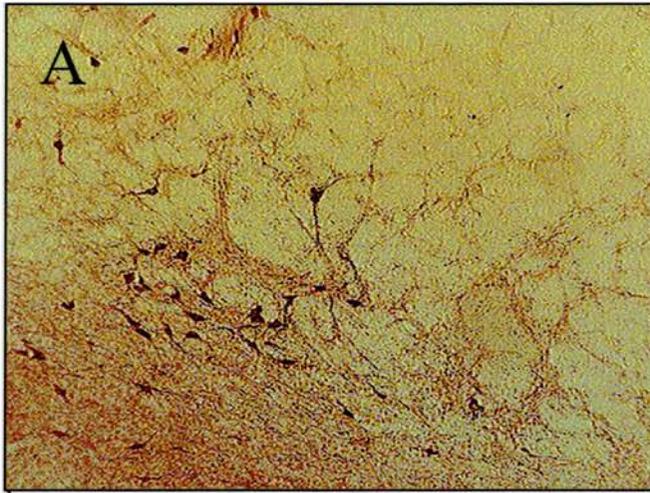
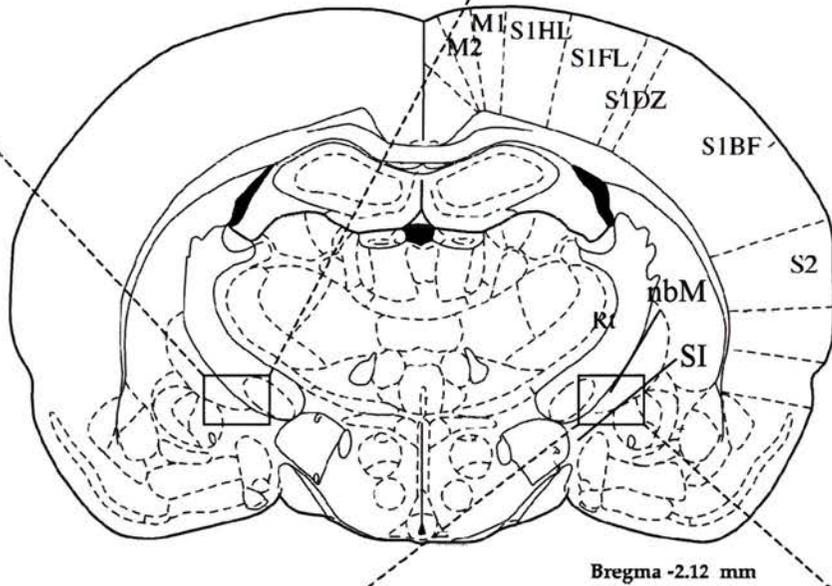


Figure 4.22 Photomicrograph (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -2.12). Sections show VAcHT stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



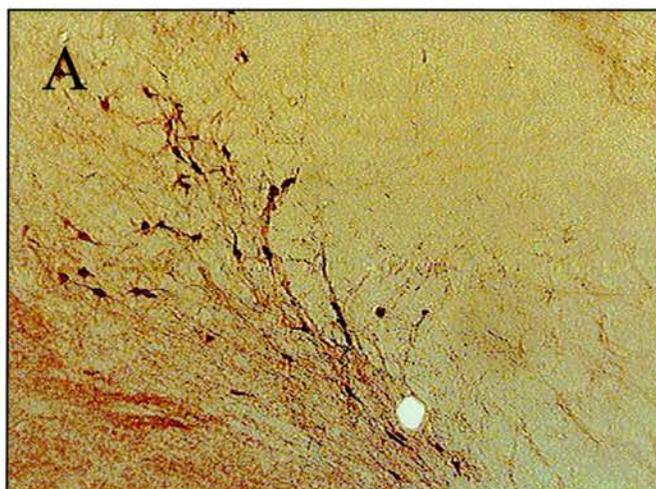
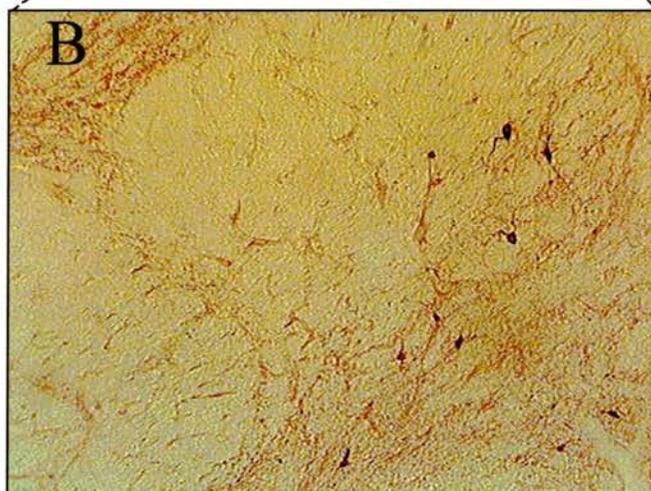
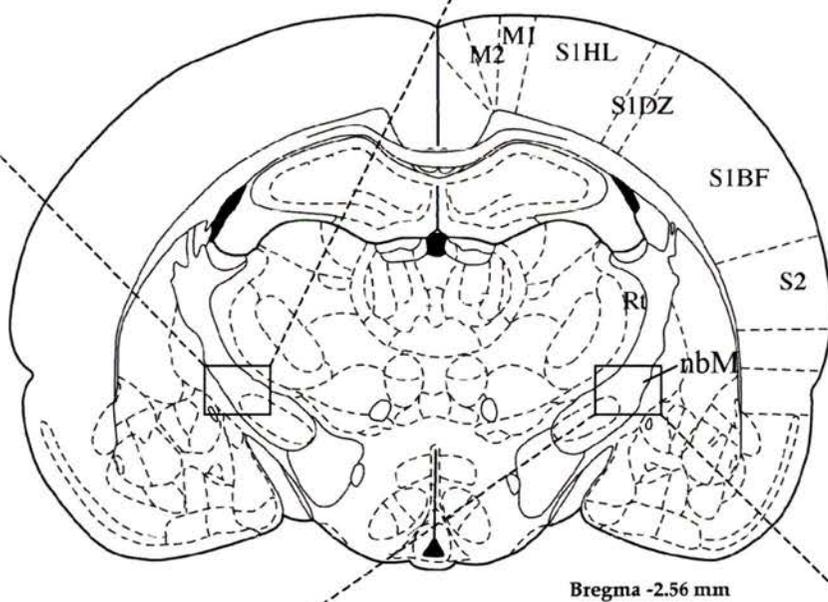


Figure 4.23 Photomicrograph (magnification X10) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -2.56). Sections show VAcHt stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



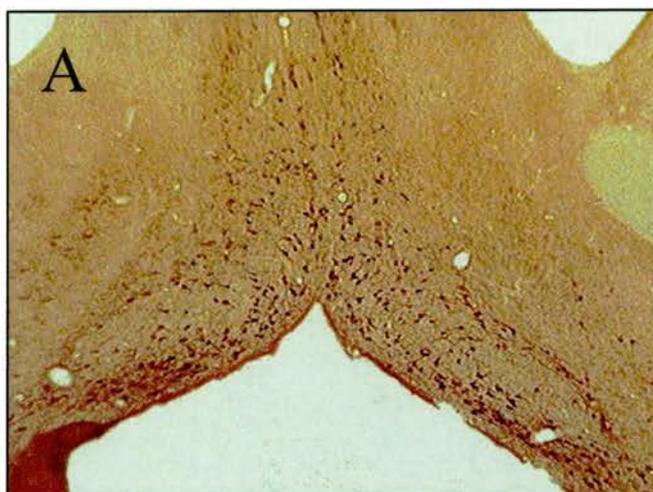
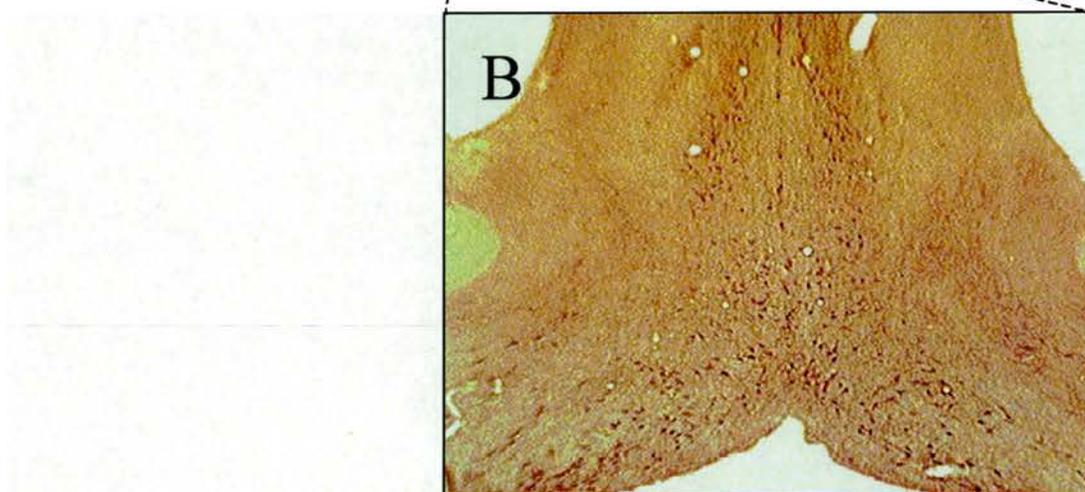
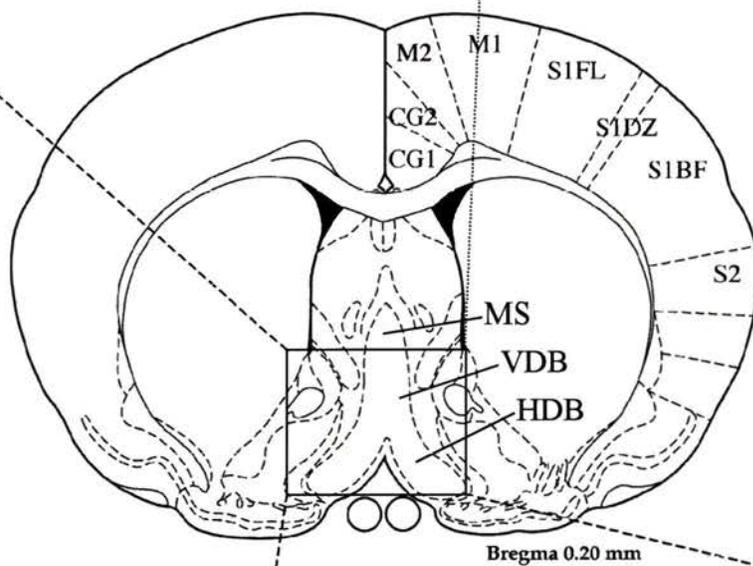


Figure 4.24 Photomicrograph (magnification X4) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma +0.20). Sections show VAcHt stained cholinergic neurons of the HDB, VDB and MS in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



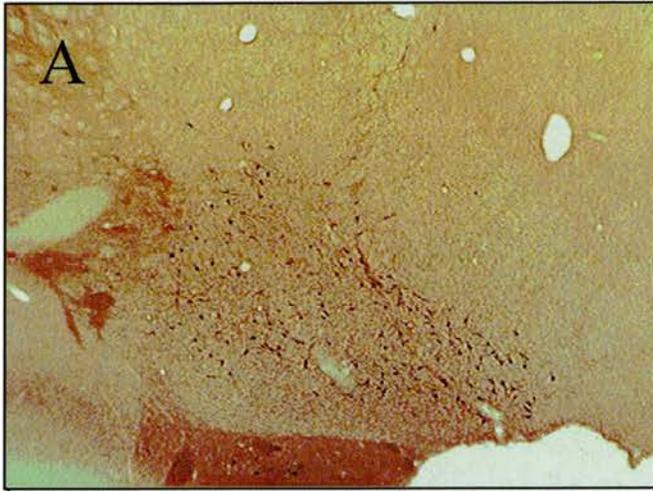
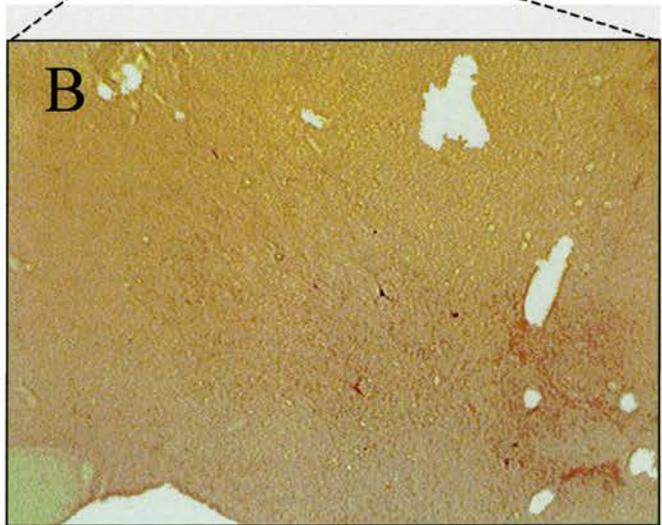
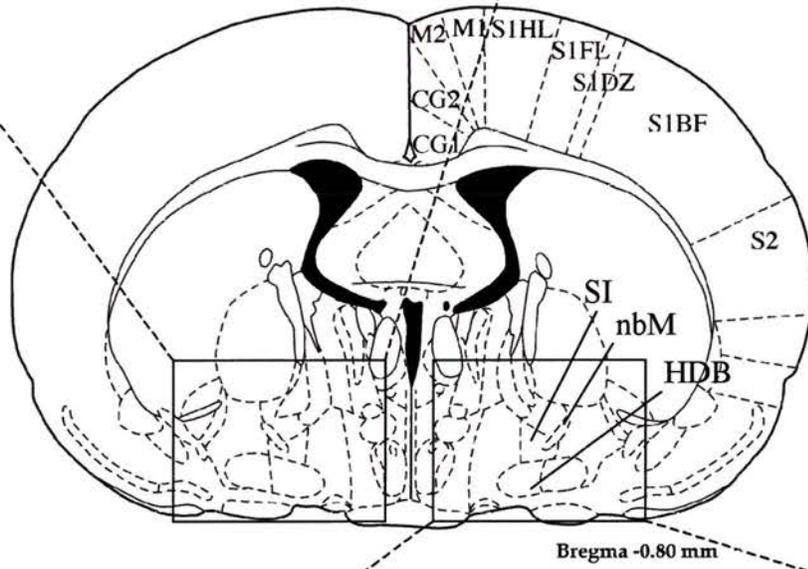


Figure 4.25 Photomicrograph (magnification X4) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -0.80). Sections show VAcHt stained cholinergic neurons of the SI, nbM, HDB and MCPO in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



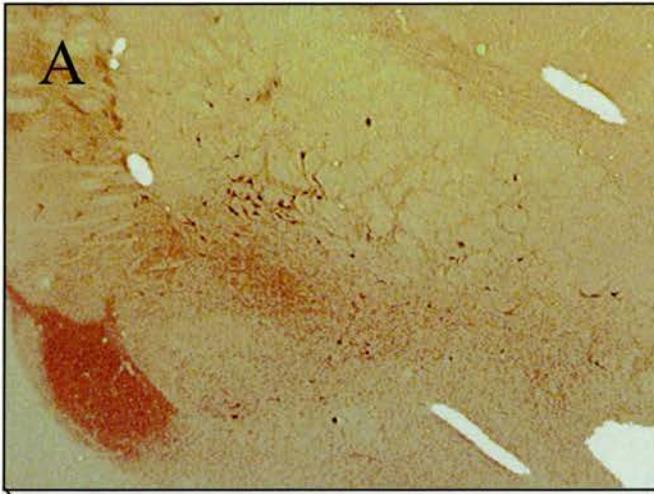
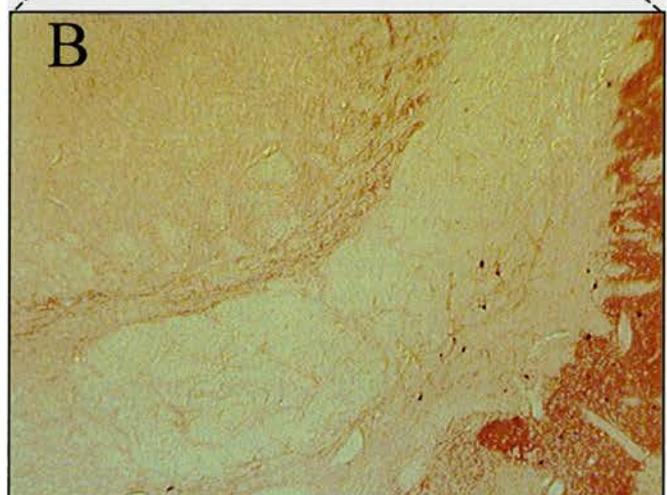
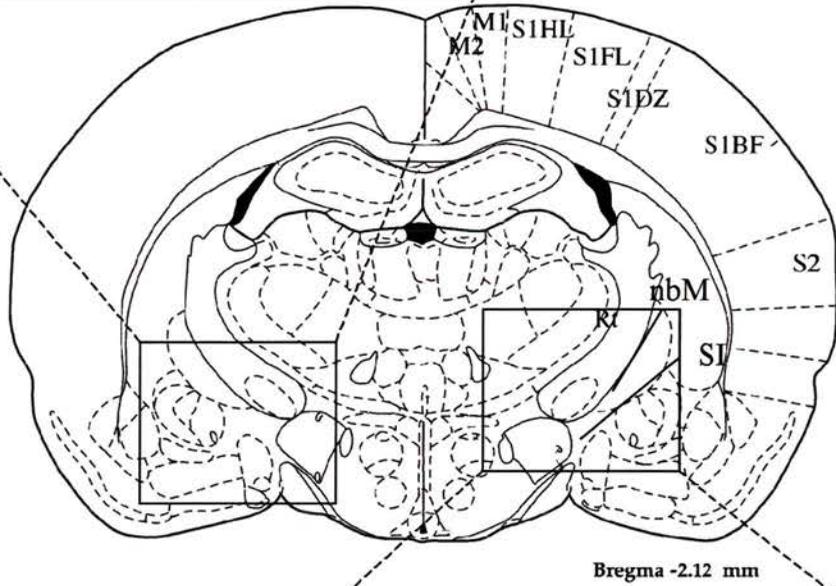


Figure 4.26 Photomicrograph (magnification X4) and schematic of coronal rat brain section, (stereotaxic coordinates: Bregma -2.12). Sections show VAcHT stained cholinergic neurons of the SI and nbM in a typical brain (Section A) and in a typical brain after administration of 192-IgG-saporin into nbM (Section B)



#### 4.3.4 Set-shifting after BF lesions

Ten of the twelve lesioned subjects completed the task. No significant effect of lesion was observed on trials to criterion ( $F(1,20) = 1.610, p > 0.05$ ) (Figure 4.29). There is a main effect of discrimination ( $F(6,15) = 10.152, p < 0.001$ ), with *post hoc* simple comparison indicating a significant difference between the ID and the ED shifts ( $F(1,20) = 15.277, p < 0.01$ ). It is therefore concluded that nbM lesions do not affect ability to form and shift attentional sets.

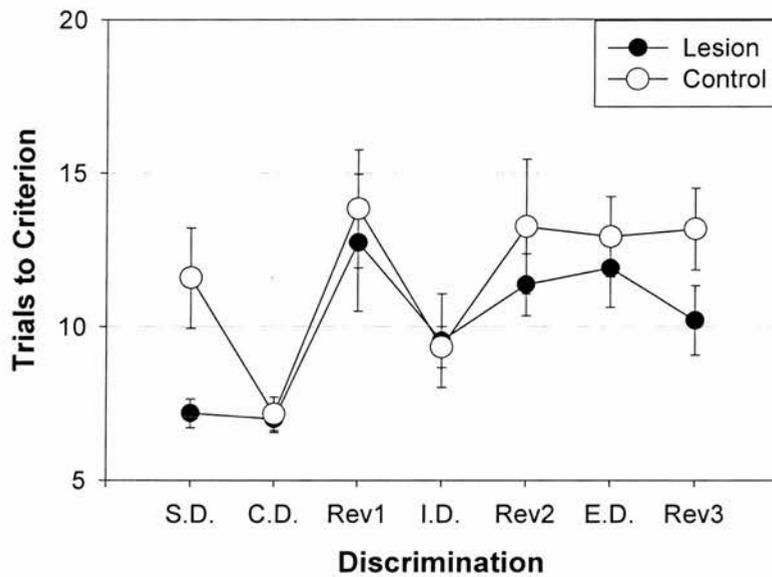


Figure 4.29 Mean  $\pm$  SEM trials to criterion for 192-IgG-saporin bilateral nbM lesioned rats ( $n = 10$ ) and unoperated controls ( $n = 12$ ). There was no significant effect of lesion on performance.

However, in analysis of latency to dig, there is a main effect of lesion on performance ( $F(1,20) = 16.066, p < 0.005$ ) (Figure 4.30). There is also a main effect of dig choice ( $F(1,20) = 118.139, p < 0.001$ ) and an

interaction between dig choice and group ( $F(1,20) = 17.143, p < 0.005$ ), indicating that the lesioned subjects take significantly longer to initiate digging, and that this effect is greater if they require investigation of both bowls prior to digging.

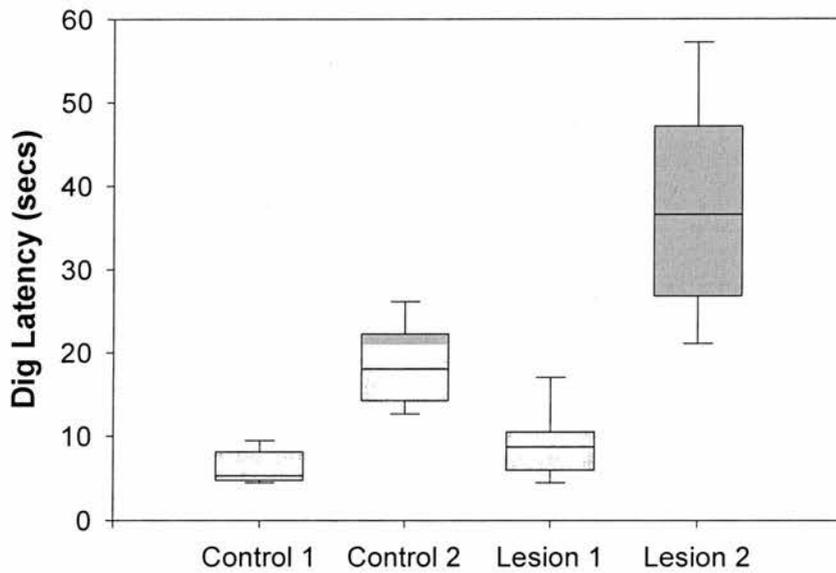


Figure 30 Box plot showing median  $\pm$  quartile and 10<sup>th</sup>/90<sup>th</sup> percentile latency to dig for 192-IgG-saporin bilateral nbM lesioned rats ( $n = 10$ ) and unoperated controls ( $n = 12$ ) after investigation of one bowl (\*1) or both (\*2). Rats with lesions took longer to initiate digging if they required investigation of both bowls prior to digging than those without lesions.

## 4.4 Conclusions

### 4.4.1 Rt lesions and set-shifting

To summarise the results, it has been observed that administration of 192-IgG-saporin into rostral Rt leads to loss of BF cholinergic neurons

and their projections to Rt and corresponding cortical regions at all rostro-caudal levels. No specific effect of administration of 192-IgG-saporin into rostral Rt was observed on performance in the attentional set-shifting task.

It should be noted from subjects with lesions of Rt that the loss of cholinergic innervation of Rt is throughout its rostro-caudal extent. It would have been predicted from previous data that cholinergic projections to Rt would be depleted dorso-rostrally/caudally and ventro-rostrally. Sections analysed from subjects presented here show depletion of VAcHT staining even in ventro-caudal Rt, where cholinergic projections from BF were thought to be minimal, and brainstem cholinergic projections in the majority. It is clear from other intact thalamic nuclei that receive their cholinergic projections from brainstem, that there is no depletion in non-p75 rNGF-bearing cholinergic neurons. As concentration of 192-IgG-saporin increases, the chances of decreased selectivity also rise. That other nuclei receiving cholinergic projections are intact adjacent to Rt suggests that rather than loss of selectivity being the cause of depletion of cholinergic innervation to ventro-caudal Rt, instead there are more cholinergic projections from BF to ventro-caudal Rt than had previously been thought. This corresponds with data suggesting that rNGF has a modulatory effect on auditory Rt, which is located in the ventro-caudal region of Rt (Villa *et al.*, 1996). That the majority of observations so far

published suggest that BF cholinergic projections to Rt are to rostral Rt is likely based on tracing studies after injection into rostral Rt only.

It is clear from the data presented that administering 192-IgG-saporin into rostral Rt is somewhat difficult, as of the 18 subjects given bilateral injections, only 7 had bilateral lesions, and of those 7 only 2 completed the task. It is likely that the rostral region of the Rt, being narrow, may also move slightly during insertion of the needle prior to administration. That the needle track marks on the “misses” were consistently forward of the Rt, other than those where the AP stereotaxic co-ordinates were adjusted from  $-1.4$  to  $-1.5$  (where the track marks indicated that the injection had been caudal to the rostral Rt), and considering that the rostral Rt is more than 0.1mm deep at the stereotaxic co-ordinates used, is more likely that the Rt was pushed either forward or back by the needle, than the “misses” are due to errors in practice. Recent advances in surgical techniques would suggest using pulled pipette tips for such injections in the future.

There is, therefore, less data from bilaterally Rt lesioned subjects' attentional set-shifting than would be preferable. With an “n” of 2 it is harder to state that Rt lesions have no effect on performance in the attentional set-shifting task. Certainly of the subjects that completed the task, no observations were made of any effect of the lesion. Interestingly, the 5 subjects that received accurately placed injections, but showed no

cholinergic lesion, were impaired in dig latency during the task. Likewise were those 5 subjects with unilateral lesions due to misplacement. This may suggest that the lesioned subjects might also be impaired, but that the low number of subjects failed to show it. The only observable neuronal damage in the subjects receiving the ineffective 192-IgG-saporin was in the Rt immediately surrounding the injection site - no cholinergic depletion was recorded. It is likely that the conjugate of the 192-IgG and the saporin broke down and that the damage observed is caused by saporin in the localised area of the injection. These data compare with the nbM lesioned subjects dig latency data, and may, with further study and replication, provide evidence that the latency impairment arises in the Rt and is a result of loss of/inhibition of GABAergic projections from Rt to thalamus.

However, that 5 out of the 7 subjects with bilateral lesions did not complete the task suggests that there was an overall effect of the lesions on the rats' ability, or motivation, to perform the task. As stated previously, Rt receives cholinergic p75 rNGF receptor bearing projections from V/HDB, nbM and possibly the SI. In the Rt lesions observed all of these projections were reduced, making it difficult to establish a cause for non-completion of the task. In contrast, bilateral lesions of the nbM are far easier to induce, and result in less damage to other BF cholinergic nuclei, and thus their cortical projection fields in the case of axon collaterals. Of the 12 subjects administered 192-IgG-saporin bilaterally

into nbM, 10 completed the task, suggesting that the cause of the Rt lesioned subjects' reluctance to complete the task likely involves V or HDB and cholinergic projections from those nuclei to either cortex or Rt. That V/HDB send cholinergic projections mainly to rostro-ventral Rt and mPFC, and nbM sends cholinergic projections to rostro/caudo-dorsal Rt (Kolmac and Mitrofanis, 1999) and somatosensory and motor cortices (Basterville et al., 1993) suggests that the behaviour resulting in failure to complete the set-shifting task is mediated in either mPFC or rostro-ventral Rt. That non-selective lesions of mPFC failed to show similar reluctance to complete the task (Birrell and Brown, 2000) directs the probable cause to rostro-ventral Rt. As it looks like it is Rt's role to gate information between cortex and thalamus, then it is possible that Rt's inhibitory effect on the anterior thalamic nuclei may result in the observed behaviour. Rostral Rt projects topographically to anterior thalamic nuclei (Gonzaloruz and Lieberman, 1995), an area of the thalamus involved in both limbic function and spatial memory (with projections to hippocampus) (Mitchell *et al.*, 2002). There is some evidence suggesting that the medial dorsal thalamic nucleus may have a motivational role in task performance (McAlonan *et al.*, 1993), but it is also not possible to absolutely define reasons for failure to complete the task. Furthermore, the medial dorsal thalamic nucleus receives projections from the mPFC, and that mPFC excitotoxic lesions failed to induce the observed reluctance to complete the task complicates any hypothesis suggesting that medial dorsal thalamic nucleus is involved in apparent motivational

deficits seen here. Likewise, that there is no consistency with regard to when during the task, or with which exemplars, the rats ceased to dig for reward suggests that no single process is involved in the observed behaviour. It could be argued that the criteria for ruling a subject to have failed the task (three 10 minute trials where the subject fails to dig) are too harsh. Indeed, were deficits to result from learned inhibition rather than perseveration, it might be expected that subjects would not dig at all during several trials. However, those bilateral Rt lesioned subjects that failed the task at the REV2 stage or ED stage, show unimpaired REV1 performance. This suggests that dropping those subjects has not contributed to any failure to find a specific deficit in any one discrimination, or overall – all the data collected up to the point the subject failed the task followed control performance levels. Future work looking at the effects 192-IgG-saporin lesions of H/VDB on attentional set-shifting may shed more light on the matter.

#### ***4.4.2 nbM lesions and set-shifting***

Administration of 192-IgG-saporin into the nbM of the BF results in a loss of cholinergic projection neurons in nbM and corresponding cortical terminal fields. Loss of cholinergic neurons in nbM has no effect on the rats' ability to form and shift attentional set. Loss of nbM cholinergic neurons leads to an increase in the time taken for a rat to initiate digging which is proportionately greater if it does not dig when it first encounters a bowl.

Lesions of nbM, unlike those of Rt, result mainly in losses to nbM/SI cholinergic neurons and their projection terminal fields in the Rt and the cortex. Observations suggest that nbM/SI is intact in its more caudal regions after 192-IgG-saporin administration. These neurons are likely the nbM neurons that project to amygdala; the only nbM cholinergic neurons not to bear the p75 rNGF receptor. Other than these, nbM/SI cholinergic neurons are heavily depleted, leading to cholinergic denervation of somatosensory cortex and motor cortex (Parietal cortex) as well as Rt. That there is no effect of nbM lesions on the subjects' ability to form and shift set indicates that brain regions associated with those functions are not impaired. However, the fact that dig latency is affected by such lesions suggests that dig latency is an important factor in the rat attentional set-shifting task. The rat task as developed by Birrell and Brown (2000) is unique in that it is the only ED/ID task to not present both of the sets of stimuli at the same time. In primate ED/ID tasks the stimuli are introduced to the subject at the same time by means of a monitor. Obviously the subject must take time to look at both of the stimuli, but they are both within the subjects field of vision at initiation of the trial. The rat is required to investigate the first bowl it encounters then move onto the second; it cannot rapidly shift its attention from one to the other. The dig latency measured in the rat attentional set-shifting task therefore has no correlation with any measurements taken in any other ED/ID tasks.

It is clear from the data presented here that subjects with bilateral cholinergic nbM lesions have an increased overall dig latency which is proportionately greater if the bowl they initially investigate is not, at that point, perceived to be correct. The question that must be answered though, is why? It has already been stated that rats with nbM lesions are impaired in sustained attention tasks, and that acetylcholine release in mPFC is increased during a sustained attentional task. It is therefore likely that subjects with bilateral cholinergic nbM lesions would be impaired in any sustained attentional aspect of the set-shifting task. However, the data from subjects with presumed localised saporin lesions of rostral Rt also show increased dig latency. Rt projects only to thalamus, and there was no observed cholinergic depletion that could affect mPFC or other cortical areas. General lack of information about ACh efflux in Rt during behaviour means that it is difficult to conclude whether a loss of cholinergic innervation to Rt, or indeed of GABAergic neurons in Rt, would have an effect on sustained attention.

Behaviour observed suggests that rather than investigate the second bowl relatively quickly after the first bowl is perceived to be incorrect, the subjects are more likely to investigate the holding area of the set-shifting apparatus for a while before returning their attention to the task. It could be argued that this constitutes a reduction in sustained attention and that despite this their performance in forming attentional set

is unimpaired. It is also possible that this increase in dig latency arises through other psychological processes, or a combination. Although it is likely that sustained attention is involved, it is also probable that the cause of the increase stems from a decision making component that is impaired by reduced capacity for sustained attention. There does not appear to be any motor function deficit involved in the increased dig latency, as observations during the task suggest rats' motor function was intact. The subjects had no difficulty initiating movement, and did not appear to be moving more slowly than normal. Other than investigating the holding area of the task apparatus, they would also groom themselves and chew on the wooden runners for the perspex barriers that separated the holding area from the bowl containing chambers. In future versions of the attentional set-shifting task, video recordings will be used to monitor rat behaviour during the task, permitting later analysis of behaviour that may explain such results as increased dig latency.

#### **4.5 Summary of Findings**

- Rats underwent an attentional set-shifting task after the selective immunotoxin, 192-IgG-saporin, was administered bilaterally into either nbM or rostral Rt.
- Histological observations support the idea that cholinergic projections from BF to cortex send collaterals to multiple regions of Rt

- There was no effect of 192-IgG-saporin lesion of nbM on the subjects' ability to form or shift attentional set.
- Data from Rt lesions were inconclusive due to the low number of successful bilateral lesions, and even lower number of subjects completing the attentional set-shifting task
- 192-IgG-saporin lesions of nbM resulted in an increase in latency to initiate digging in the task, specifically if the subject refrains from digging at the first bowl that it encounters
- It is suggested that this increase in latency to dig represents an impairment in sustained attention (as has previously been observed after 192-IgG-saporin lesions of nbM), or in processes involved in decision making.

## Chapter V

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### **Do i.c.v. 5,7-dihydroxytryptamine (serotonergic) lesions affect attentional set shifting in the rat?**

It has already been observed that central acetylcholine is involved in attentional function. Evidence has also already been presented that several of the serotonin (5-HT) receptors subtypes modulate central cholinergic function. Furthermore, there is evidence to suggest that administration of the serotonergic antagonist SB-271046 potentiates performance in an attentional set-shifting task for rats (Hatcher *et al.*, 2002). Serotonergic receptors are spread throughout the brain, although only a few of the 14 subtypes modulate cholinergic function.

The neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) injected intracerebroventricularly (i.c.v.) selectively depletes forebrain 5-HT levels (Duncan *et al.*, 1992). This study investigates the effects of 5,7-DHT (i.c.v.) on central acetylcholine levels and performance in the rat attentional set-shifting task.

## 5.1 Introduction

There is not yet a clear understanding of the role of central 5-HT on attention. Evidence presented in Chapter II suggests that although 5-HT has a modulatory effect on central cholinergic function, the 5-HT<sub>6</sub> receptor subtype-specific antagonist, SB-271046, has only recently been shown to modulate acetylcholine (ACh) levels in prefrontal cortex (Jones, 2002, *pers comm*). Dawson *et al.* (2000) reported increased extracellular glutamate levels in frontal cortex, and then further investigated levels of 5-HT, noradrenaline (NE), dopamine (DA) and glutamate in frontal cortex (cannula implant at stereotaxic co-ordinates from bregma (Paxinos and Watson, 1986): AP 3.5, ML – 3.2, DV –1.5 (from skull surface); targeting primary motor cortex) and hippocampus, as well as striatum and nucleus accumbens. A 10mg/kg injection of SB-271046 leads to a significant increase in extracellular glutamate levels in frontal cortex and dorsal hippocampus. No changes were observed in 5-HT, NE or DA levels in either cortex or hippocampus. Atropine, a cholinergic (muscarinic) antagonist did not attenuate the observed glutamate level increases in either cortex or hippocampus, indicating that these increases are not modulated by acetylcholine (Dawson *et al.*, 2001). There are suggestions that as there are no 5-HT<sub>6</sub> receptors in cortex, GABAergic ( $\gamma$ -aminobutyric acid) interneurons are involved in either the cortical glutamate increases, or both cortical and hippocampal increases. The presence of 5-HT<sub>6</sub> receptors on the GABAergic interneurons has been considered for some time (Gérard *et al.*, 1997), although more recently

(Woolley *et al.*, 2000) co-localisation has been observed in multiple brain regions. It is thought by some that this may also account for results suggesting a functional interaction between 5-HT<sub>6</sub> receptors and acetylcholine (Dawson *et al.*, 2001).

Data from microdialysis studies using the 5-HT<sub>6</sub> receptor-specific antagonist Ro 04-6790 show a “modest (50%) increase in ACh outflow” that does reach statistical significance. By comparison, the atypical antipsychotics, olanzapine and clozapine, both of which antagonise with high affinity, although are not selective for, the 5-HT<sub>6</sub> receptor (Meltzer, 1994; Bymaster *et al.*, 2001) induce a 1500% and 500% increase in extracellular hippocampal ACh levels respectively (Shirazi-Southall *et al.*, 2002). Olanzapine also antagonises the 5-HT<sub>3</sub> receptor, although not with as high an affinity as it does the 5-HT<sub>6</sub> receptor (Bymaster *et al.*, 2001) and the 5-HT<sub>3</sub> receptor is found at highest density in the rat hippocampus (Laporte *et al.*, 1992). 5-HT<sub>3</sub> mRNA distribution studies have shown not only high correlation between mRNA density and binding site distribution (Tecott *et al.*, 1993), but also that mRNA is present in GABAergic interneurons in the hippocampus. 5-HT<sub>3</sub> receptor-like immunoreactivity has been observed on these GABAergic interneurons (Morales and Bloom, 1997), with 90% of 5-HT<sub>3</sub> receptor-bearing neurons being GABAergic (Morales *et al.*, 1996), in a similar fashion to theories of 5-HT<sub>6</sub> receptor distribution. This is thought to be the likely the reason for observations of olanzapine-induced ACh level increases in hippocampus, despite contradictory earlier reports suggesting that

stimulating 5-HT<sub>3</sub> receptors in hippocampus induces ACh release (Consolo *et al.*, 1994) (see Table 5.1 for 5-HT receptor subtype that modulate ACh and their distribution).

Likewise, 5-HT<sub>3</sub> receptor-specific agonists have been observed to attenuate ACh release in cortex (Barnes *et al.*, 1989), and it is also considered that GABAergic neurons bearing the 5-HT<sub>3</sub> receptor are responsible for this (Ramirez *et al.*, 1996), as GABA antagonists potentiate ACh release after (5-HT<sub>3</sub> receptor-specific antagonist) ondansetron-induced release has been attenuated by 2-methyl-5-HT.

Ondansetron has also been administered to marmosets performing an object discrimination task, where scopolamine-induced task acquisition impairments were reversed after administration of the 5-HT<sub>3</sub> receptor-specific antagonist (Carey *et al.*, 1992).

Other 5-HT receptor subtypes also mediate cholinergic function. 5-HT<sub>1A</sub> receptors are found on about 25% of the cholinergic neurons in the septal complex in the forebrain (Kia *et al.*, 1996), as well as in the hippocampus. The 5-HT<sub>1A/1B</sub> agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Hjorth *et al.*, 1982; Middlemiss and Fozard, 1983) induces increases in extracellular ACh levels in both cortex and hippocampus, although the cortical increase appears mediated by 5-HT<sub>1B</sub> receptors, as it is attenuated by combination 5-HT<sub>1A/1B</sub> antagonists,

but not by 5-HT<sub>1A</sub> receptor-specific antagonist WAY-100635 (Izumi *et al.*, 1994; Consolo *et al.*, 1996); 8-OH-DPAT has a low affinity for 5-HT<sub>1B</sub> and 5-HT<sub>7</sub> receptors. It is, however, not obvious as to the mechanism behind these increases in ACh levels, as although the septal cholinergic neurons do project to both cortex and hippocampus, they are reported as inhibitory (Van Den Hooff and Galvan, 1992), and furthermore, the increases are thought to be mediated by post-synaptic 5-HT<sub>1A</sub> receptors (Consolo *et al.*, 1996).

The 5-HT<sub>1B</sub> receptor has both an inhibitory and an excitatory effect on cholinergic function. As has been noted, it appears that 5-HT<sub>1A/1B/7</sub> agonist, 8-OHDPAT, potentiates ACh release in hippocampus. However, CP 93129, another 5-HT<sub>1B</sub> agonist (species-specific to rat), inhibits ACh release in hippocampus (Cassel *et al.*, 1995). It is suggested that any observed facilitatory effect of 5-HT<sub>1B</sub> agonists is due to an indirect route. Thus, although the joint 5-HT<sub>1A/1B/7</sub> agonist 8-OH-DPAT potentiates ACh release in hippocampus through an action that can be attenuated by 5-HT<sub>1B</sub> antagonists, pindolol and propandolol, it is unlikely that this is a direct action of the agonist on the 5-HT<sub>1B</sub> receptors in the hippocampus. Furthermore, locally administered CP 93129 is also observed to induce increases in ACh levels in cortex (Consolo *et al.*, 1996), although again this is considered to be through an indirect route.

The 5-HT<sub>4</sub> receptor, also located in the hippocampus (Vilaró *et al.*, 1996), is reported as modulating ACh release in cortex. Agonists (BIMU 1 and BIMU 8) administered intracerebroventricularly potentiate cholinergic release in cortex, whilst simultaneously administered antagonists (GR 125487 and GR 113808) attenuate this effect. The antagonists on their own do not reduce cortical ACh levels (Consolo *et al.*, 1994a). There is also some behavioural evidence linking 5-HT<sub>4</sub> receptors to cognition, and it is believed that the cholinergic modulation by 5-HT<sub>4</sub> is the mechanism behind this (Fontana *et al.*, 1997).

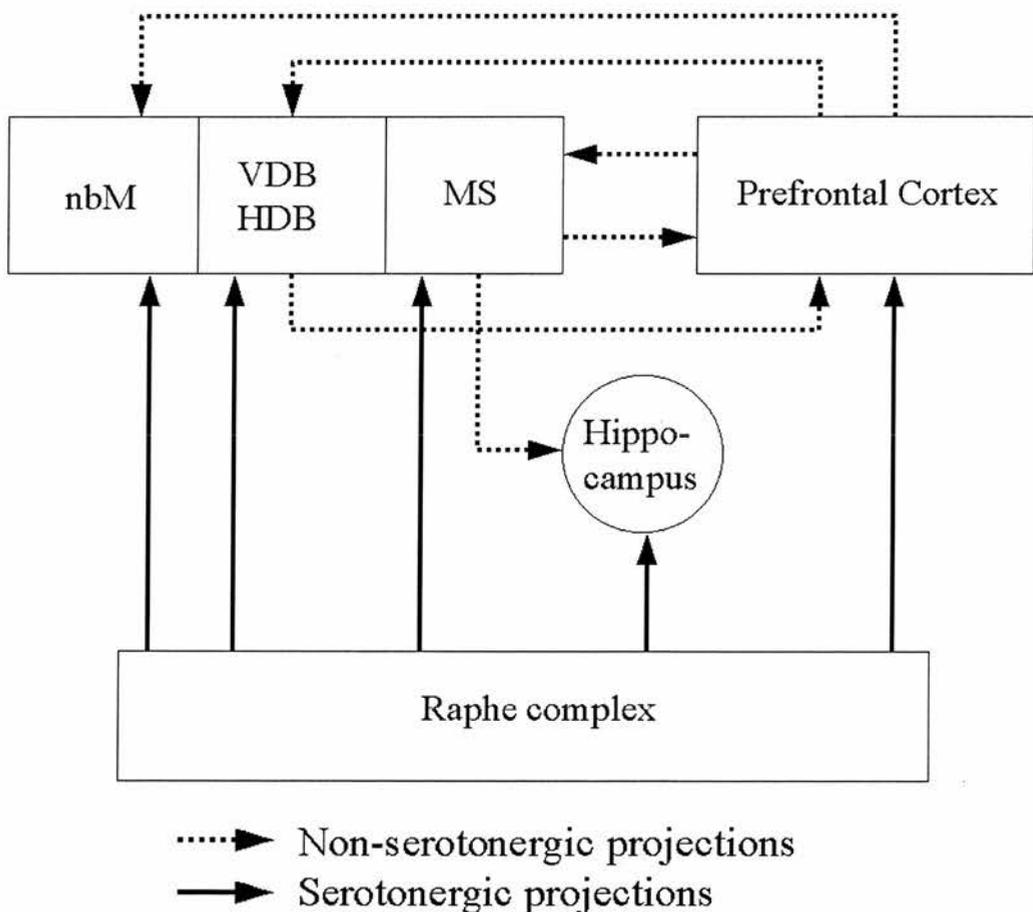


Figure 5.1 Projections from the Raphe complex to the basal forebrain; medial septum (MS), vertical and horizontal limb of the diagonal band of Broca (VDB and HDB) and nucleus basalis of Meynert (Magnocellularis) (nbM); hippocampus and prefrontal cortex.

Table 5.1 Distribution of 5-HT receptor subtypes that modulate ACh activity.

5-HT receptor subtype	Distribution	Pre- or post-synaptic	Reference
5-HT <sub>1A</sub>	Medial prefrontal cortex	Post-synaptic	Miquel <i>et al.</i> , 1991; Kia <i>et al.</i> , 1996;
	Hippocampus	Post-synaptic	Izumi <i>et al.</i> , 1994;
	Septum	Post-synaptic	Consolo <i>et al.</i> , 1996,
	Dorsal/medial raphe nucleus	Pre-synaptic	Weissmann-Nanopoulos <i>et al.</i> , 1985
5-HT <sub>1B</sub>	Frontoparietal cortex	Post-synaptic	Bruinvels <i>et al.</i> , 1993;
	Hippocampus	Post-synaptic	Amara <i>et al.</i> , 2001;
	Anterior raphe nucleus	Pre-synaptic	Doucet <i>et al.</i> , 1995
5-HT <sub>2A</sub>	Cortex	Post-synaptic	Willins <i>et al.</i> , 1997;
	Hippocampus	Post-synaptic	Pazos <i>et al.</i> , 1987
5-HT <sub>2C</sub>	Medial prefrontal cortex	Post-synaptic	Palacios <i>et al.</i> , 1991;
	Hippocampus	Post-synaptic	
5-HT <sub>3</sub>	Hippocampus	Post-synaptic	Laporte <i>et al.</i> , 1992
5-HT <sub>4</sub>	Septum	Post-synaptic	Waeber <i>et al.</i> , 1994
	Hippocampus	Post-synaptic	
5-HT <sub>6</sub>	Cortex	Post-synaptic	Gérard <i>et al.</i> , 1996,
	Hippocampus	Post-synaptic	'97
5-HT <sub>7</sub>	Cortex	Post-synaptic	Neumaier <i>et al.</i> , 2001
	Hippocampus	Post-synaptic	

It is evident then that 5-HT in the CNS modulates cholinergic function through various receptor subtypes, and by various mechanisms, not all of which are understood. Serotonergic lesions using 5,7-dihydroxytryptamine (5,7-DHT) deplete serotonin levels in the forebrain, effectively antagonising any process mediated by post-synaptic 5-HT receptors (see Figure 5.1 for serotonergic projections to forebrain). Animals are pre-treated with noradrenergic and dopaminergic uptake blockers to prevent destruction of those systems, so limiting depletion to 5-HT only.

Ward *et al.* (1999) used this procedure to investigate the effects of 5-HT depletion on task acquisition and performance in a conditional visual discrimination task, requiring rats to press a lever for reward, left if a visual stimulus was presented at high frequency, right if the visual stimulus was presented at low frequency. One group of subjects were administered (i.c.v.) 5,7-DHT prior to task acquisition, a second group were administered the neurotoxin after acquisition, allowing both groups to then be tested as control parameters were manipulated to investigate the selective effects of 5-HT depletion on differing elements of the task.

Lesioned subjects were observed to have a reduction of 5-HT in dorso-lateral prefrontal cortex of 79-86%, and in hippocampus of 83-85%. Subjects with lesions tested on task acquisition were facilitated in learning the task according to errors made and also in sessions to

criterion. There were observed no differences between lesioned subjects and sham-controls with regard to latency to respond. During various manipulations of the task parameters, only in one was there a difference between lesioned and unlesioned subjects: altered rate of stimulus presentation frequency. Lesioned subjects were not impaired by increasing the frequency of stimulus presentation. Furthermore, infusion of the 5-HT<sub>1A</sub> agonist, 8-OH-DPAT, directly into the serotonergic dorsal raphe nucleus (DRN) resulted in an improvement of unlesioned subjects performance in response to increase in stimulus presentation frequency.

It is clear that depletion of 5-HT in forebrain potentiates early learning of this task, and attenuates disruption created by increased frequency of stimulus presentation. That administration of a 5-HT<sub>1A</sub> receptor agonist into DRN also attenuates this disruption in unlesioned subjects suggests that that particular behaviour is mediated by ascending projections from DRN bearing the 5-HT<sub>1A</sub> receptor. Stimulating the 5-HT<sub>1A</sub> receptor is known to reduce forebrain 5-HT levels (Fletcher *et al.*, 1993), likely by receptors located pre-synaptically in the raphe nuclei functioning as autoreceptors.

Several studies have monitored the effects of either combined serotonergic/cholinergic lesions, or serotonergic/cholinergic lesions on their own, on levels of ACh and 5-HT in the brain (Dekker *et al.*, 1993; Murtha and Pappas, 1994; Wirth *et al.*, 2000; Lehmann *et al.*, 2000).

However, although levels of 5-HT have been measured in cortex and hippocampus after 5,7-DHT lesions, and ACh levels have likewise been measured after 192-IgG-saporin/ibotenic acid lesions of nbM, there is very little evidence as to the effects of 5,7-DHT lesions on cortical or hippocampal ACh levels without simultaneous nbM lesions. It has been observed that the serotonin-releasing agent p-chloroamphetamine induces a 160% increase in extracellular ACh levels in hippocampus, and this effect is potentiated by 5,7-DHT lesions (Nilsson *et al.*, 1992). Behavioural data from combination lesion studies suggests that motor deficits elicited by ACh lesions only are not affected by combination with serotonergic lesions, although diurnal and nocturnal hyperactivity induced by serotonergic lesions alone is attenuated by combination with ACh lesions. Furthermore, in the combination lesions there is observed impairment in a T-maze alternation test, the water-maze working memory test and the radial-maze (Lehmann *et al.*, 2000).

It is evident then that serotonergic-cholinergic interaction is important in cognition, although the effects of forebrain 5-HT depletion on attention have only been explored to a limited degree. Rats with 5,7-DHT lesions are impaired in the 5 choice serial reaction time task (5CSRT); the number of anticipatory errors made increases, although performance in other areas is unaffected. This suggests an impulsivity role for 5-HT in the forebrain, although it is likely that this caused by an interaction between 5-HT and dopamine as administration of the D-1

receptor antagonist, SCH 23390, attenuates the lesion-induced increase in anticipatory errors (Harrison *et al.*, 1997). Interestingly, administration of the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT (which as previously noted reduces forebrain 5-HT), in rats undergoing the 5CSRT results in significantly reduced choice accuracy, increased errors of omission, latencies to respond correctly and to collect food reward as well as increased anticipatory errors. These impairments are attenuated completely by administration of the 5-HT<sub>1A</sub> antagonist WAY 100635 five minutes prior to 8-OH-DPAT administration, although WAY 100635 has no effect on 5CSRT performance on its own. Combination administration of 8-OH-DPAT with 5,7-DHT blocks the 8-OH-DPAT induced impairment in choice accuracy and reduces the 8-OH-DPAT induced increased latency to collect reward. WAY 100635 injected in to DRN blocks completely the 8-OH-DPAT induced increase in latency to collect reward. As Harrison *et al.* (1997) observed, 5,7-DHT administration results in an increase in anticipatory errors. 8-OH-DPAT does the same, but in combination, the increase is less marked (Carli and Samanin, 2000). It is likely that pre-synaptic 5-HT<sub>1A</sub> receptors and post-synaptic 5-HT<sub>1A</sub> receptors have differing functions in this task as evidenced by the differing effects observed here.

Contradictory evidence as to the effects of 5,7-DHT on performance in the 5CSRT also exists however. Ruotsalainen *et al.* (2000) found that 5,7-DHT mildly reduces choice accuracy, and that p-

chloroamphetamine (used here for its neurotoxic effects, which are more selective than 5,7-DHT, targeting the ascending projections of the DRN, and leaving the median raphe nucleus relatively intact) administration results in increased impulsivity. They also noted that administration of scopolamine (mAChR antagonist), but not mecamylamine (nAChR antagonist), induces impaired choice accuracy, but that this was not potentiated by the presence of serotonergic lesions. Scopolamine also increases impulsivity, whilst mecamylamine decreases impulsivity. It appears from these data, however, that serotonergic involvement in attention is related to response control, and that cholinergic involvement is related to choice accuracy. These data provide no support for an interaction between 5-HT and ACh systems in any one aspect of attentional function.

However, most attentional tasks, whilst being able to be broken down into their constituent behavioural elements, do contain elements where serotonergic function is important. Recent data suggest that administration of the 5-HT<sub>6</sub> receptor-specific antagonist SB-271046 in rats performing the attentional set-shifting task (Birrell and Brown, 2000) are able to reverse with significantly fewer trials to criterion required at the first reversal stage (Hatcher *et al.*, 2002). It is also noted that after administration of SB-271046 there is no longer a significant difference between the ID and the ED shift (as there is in the controls (vehicle administered)), although this does not translate to a significant difference

between the ED shift in the SB-271046 administered subjects and the ED shift in the controls. It appears that SB-271046 facilitates cognitive flexibility in the attentional set-shifting task, allowing the subjects to adjust more quickly, in an opposite fashion to the cognitive rigidity (increased difficulty to reverse) reported after N-methyl-D-aspartate lesions of BF in marmosets (Roberts *et al.*, 1992). It has been previously noted that 5-HT<sub>3</sub> receptors also mediate attentional set-shifting, although this effect was shown to be by modulation of cholinergic function rather than a direct serotonergic effect (Carey *et al.*, 1992).

New evidence for 5,7-DHT-induced impairments in attentional function has arisen recently (Laidlaw *et al.*, unpublished observations) in a simple reaction time task in which rats are cued for availability of reward. The rats are trained to nose-poke in an operant chamber then to respond to an auditory signal by pressing a perspex panel occluding a food hopper. The number of trials the rat must perform before a food reward is obtained is signaled by a cue. A bright cue indicates that the food reward will be obtained on the current trial (rewarded trial). A dimly illuminated cue indicates that the rat must perform two trials for reward. No cue indicates that the rat must perform three trials to reward. Trials to reward are randomised during the task. Laidlaw *et al.* administered 8-OH-DPAT (subcutaneous) on its own, 5,7-DHT lesion on its own, then a combination of lesion and 8-OH-DPAT, lesion and WAY 100635, and lesion and citalopram (a selective serotonin reuptake inhibitor). They also

tested the effect of the lesion when the cues were removed altogether and when the cue meaning was reversed.

Preliminary analysis of these data suggest that 8-OH-DPAT leads to an increase in reaction time on all trials, a reduction in the percentage of correct trials and in the percentage of anticipatory errors and an increase in percentage of late errors. 5,7-DHT lesioned subjects also show a particular increase in late errors on rewarded trials as well as an increase in movement time on rewarded trials. The combination of 5,7-DHT lesion with 8-OH-DPAT administration also resulted in a reduction in percentage correct. WAY 100635 restored the performance of lesioned rats to the level of that of controls. Lesioned rats given citalopram had reduced movement times at rewarded trials and two trials to reward, an effect not observed in controls given citalopram. Removal of cues affects both sham and lesioned animals in the same fashion, whereas reversal of cues seems to result in less impairment in the lesioned animals. Thus, preliminary conclusions are that reduction of forebrain 5-HT either through the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT or 5,7-DHT lesion impair attentional function in this task, most likely mediated by both pre-synaptic (lesion and low dose 8-OH-DPAT) and post-synaptic 5-HT<sub>1A</sub> receptors (high dose 8-OH-DPAT).

The attentional set-shifting task tests a subject's ability to form and shift attentional set, as well as perseveration to previously correct

stimuli. The attentional set-shift task may also measure sustained attention. In this study, the same rats investigated by Laidlaw *et al.* were put through the rat attentional set-shifting task as described by Birrell and Brown (2000) with the hypothesis that 5,7-DHT lesions would improve task acquisition during the training period, and reduce the reversal impairment seen in control subjects. This hypothesis is based on the evidence that selective antagonism of the 5-HT<sub>6</sub> receptor improves reversal performance. As other selective serotonergic antagonists on their own do not reduce PFC ACh (although they do attenuate agonist induced increases in ACh), this will also allow observation of the effects of 5,7-DHT lesions (antagonism of all post-synaptic 5-HT receptors) on PFC ACh levels. It would be hypothesised that an observed increase in PFC ACh would correlate with improvement in reversal performance. If a change in PFC ACh is not observed, and there is an improvement in performance in the task, then the suggestion would be that increased PFC ACh is not responsible for improvements in reversal performance seen after SB-271046 administration.

## **5.2 Protocol**

### **5.2.1 Animals**

18 male Lister hooded rats (Charles River) were used. The rats were pair-housed until surgery and maintained on a 12 hour light/dark schedule (lights on at 7am), with a diet of 15-20g of standard laboratory chow each day, reduced on testing, training and habituation days

according to consumption during completion of the task. The initial weight range was between 330-500g. At completion of the procedure weight range was between 400-550g.

### ***5.2.2 Equipment***

See Chapter IV for equipment used in the attentional set-shifting task.

### ***5.2.3 Surgery***

At the initiation of the procedure, the subjects were administered 5,7-DHT under anaesthesia. 30 minutes prior to anaesthesia, rats were given a 25mg/kg injection (intraperitoneal) of the noradrenergic uptake inhibitor Desipramine (25mg/ml solution). The rats were then anaesthetised with a halothane (Rhodia Ltd, Avonmouth, Bristol, England), nitrous oxide and oxygen mix, initially with halothane concentration at 4%, nitrous oxide at 0.8l/min and oxygen at 0.4l/min. Once anaesthetised, rats were mounted on the stereotaxic frame, and halothane concentration was reduced to 1.8% to maintain anaesthesia. Body temperature was maintained with a heated blanket and monitored with an anal probe throughout the procedure.

5,7-DHT was administered into the right ventricle using a 100µl Hamilton syringe (Aldrich Chemical Company, Milwaukee, WI, USA) with a 30 gauge needle attached, at stereotaxic co-ordinates (Paxinos and Watson, 1986); level skull -3.3mm, AP - 0.92mm, ML - 1.5mm (from

bregma); DV – 3.5mm (from dura at injection site). 5,7-DHT was administered over the course of 2 minutes, with the needle left *in situ* for a further 3 minutes after administration. Rats were administered 5,7-DHT unilaterally at a dose of 150µg in 20µl of 1mg/ml ascorbic acid (n = 9) or were given an injection of 20µl of 1mg/ml ascorbic acid only (n = 9). Upon completion of administration, Halothane concentration was reduced to 1%, head wounds were sealed using wound clips and PEP powder was administered to reduce risk of infection. Finally, before being removed from the stereotaxic frame and returned to their homecages, rats were then given a 0.05ml injection (subcutaneous) of the anti-inflammatory, carprofen (Rimadyl: Pfizer, Kent, UK). Upon return to their home cages rats were single housed, and left to recover in a warm environment. Upon recovery, rats were returned to the holding room with free access to food and water for the next 24 hours. Rats were weighed daily to monitor recovery.

#### ***5.2.4 Training procedure***

Between 25 and 40 days after surgery, following testing in the reaction time task detailed above, the subjects received training in the set-shifting task. See Chapter IV for habituation and training procedure.

#### ***5.2.5 The attentional set-shifting task***

The following day the rat undergoes the set-shifting task. See Chapter IV for details of the attentional set-shifting task.

### 5.2.6 Counter-balancing of task

Testing was conducted with the experimenter blind to the group assignment. See Table 5.2 for discrimination orders.

*Table 5.2 Pairing orders for discriminations in the attentional set-shifting task for subjects completing the task after presence of lesion confirmed by histological analysis.*

<b>Pairing order</b>	<b>ED shift</b>	<b>No. Sham/Lesion</b>
1 ⇒ 2 ⇒ 3	Odour ⇒ Medium	2 Sham, 0 Lesions
1 ⇒ 2 ⇒ 3	Medium ⇒ Odour	0 Sham, 2 Lesions
2 ⇒ 3 ⇒ 1	Odour ⇒ Medium	2 Sham, 0 Lesions
2 ⇒ 3 ⇒ 1	Medium ⇒ Odour	2 Sham, 1 Lesion
3 ⇒ 1 ⇒ 2	Odour ⇒ Medium	1 Sham, 1 Lesion
3 ⇒ 1 ⇒ 2	Medium ⇒ Odour	0 Sham, 1 Lesion

### 5.2.7 Histology

Upon completion of the task, all operated rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB; Disodium hydrogen orthophosphate and Sodium dihydrogen orthophosphate in distilled water) after anaesthesia with 0.8ml Dolethal (Univet, Bicester, Oxfordshire, UK). Brains were stored overnight at 4°C in 20% sucrose solution, then cut to 50µm sections in the horizontal plane on a microtome (Jung Histoslide 2000, Reichert-Jung, Cambridge Instruments GmbH) into 0.1M phosphate buffer saline (0.9%) (PBS). Sections were stained for acetylcholinesterase and for serotonin.

For serotonin sections were washed 5 times for three minutes in 0.1M PBS, then placed on a stirrer for 1 hour in blocking solution (0.1M PBS, 20% normal goat serum, 0.1% triton). Sections were washed as previously in 0.1M PBS, then incubated in anti-serotonin (Sigma-Aldrich) (1:5000) in antibody diluting solution (ADS; 0.1M PBS, 1% normal goat serum, 0.1% triton) at 4°C for 1 night. Subsequently sections were washed in 0.1M PBS as before, then incubated on a stirrer in vector IgG solution (anti-rabbit IgG at 5µl/ml ADS) (Vector Laboratories Ltd, Peterborough, UK) for 1 hour. After washing in 0.1M PBS again, sections were incubated on a stirrer in Vectastain ABC complex (Vector Laboratories Ltd, Peterborough, UK) (reagents A and B at 10µl/ml ADS) for a further hour. Sections were then washed in 0.1M PBS again, and finally immersed in Sigma Fast 3,3'-Diaminodenzidine tablets (DAB; Sigma Chemical Company, St Louis, MO, USA) in distilled water until reasonable colour was developed (up to 10 minutes). Sections were washed again in 0.1M PBS and then mounted on glass slides. Sections were de-fatted in xylene and cover slips were applied as under cresyl violet protocol described in Chapter IV.

For acetylcholinesterase sections were mounted onto pre-treated glass slides then stored overnight at 4°C then overnight again at 37°C in 300ml incubation medium (300ml stock incubation solution (750ml distilled water + 500mg copper sulphate + 750mg glycine + 74ml 0.2M acetic acid, buffered to pH 5.0 with 1M NaOH) + 230mg

acetylthiocholine iodide + 10mg ethopropazine). Sections were washed 4 times for 3 minutes each in distilled water then immersed in sulphide solution (300ml distilled water + 1.5ml ammonium sulphide, buffered to pH 7.5 with glacial acetic acid) until stain is developed (1-2 minutes). Sections are washed in distilled water as before and dehydrated in 50% ethanol solution then ethanol and finally de-fatted in xylene. Coverslips were applied with DPX mountant (BDH Laboratory Supplies, Poole, UK).

Sections were analysed under light microscope at magnifications X10 and X4. Images from the sections were displayed on a monitor taken from a camera (Sony CCD) mounted on the microscope. Images were also relayed to computer and captured using a digital camera (Pixera).

MRN and DRN serotonergic cells were counted at magnification X10 on 11 serotonin stained sections from stereotaxic co-ordinates interaural 1.18mm to 4.18mm.

### ***5.2.8 Data analysis***

Data (time to first dig, whether only one or both bowls were investigated prior to first dig and correct or incorrect dig) were collected on set-shifting data sheets. Correct/incorrect data for trials to criterion were entered into Sigmaplot (version 5.0) and analysed in SPSS (version 10.0). Trials to criterion were analysed using repeat measures ANOVA

with discrimination as a within-subjects variable and treatment (5,7-DHT lesion against control) as a between-subjects variable. As it has been observed that 5,7-DHT lesions increase errors in certain behavioural tasks, number of errors to criterion was also analysed using repeat measures ANOVA with discrimination as a within-subjects variable and treatment group (5,7-DHT lesion or control) as a between-subjects variable.

For the Latency to first dig, data were taken from the last 5 trials of each discrimination and entered into Sigmaplot, categorised by whether only one or both bowls were sampled prior to onset of digging. Latency to dig data were analysed using repeat measures ANOVA with dig choice as within-subjects variable and treatment (5,7-DHT lesion or control) as a between-subjects variable. There were insufficient data points to include discrimination as a within subjects variable due to subjects only digging in the first bowl they encountered during several discriminations, or the second bowl in some cases.

It has also been noted that task acquisition in some tasks is enhanced by 5,7-DHT lesions, so the data for trials to criterion and errors to criterion for the simple discriminations from the training day were also analysed using repeat measures ANOVA with discrimination as a within-subjects variable and treatment (5,7-DHT lesion or control) as a between-subjects variable. Likewise, training day dig latency data were analysed

using repeat measures ANOVA as defined for the main task. However, in order to investigate the potential for differences in acquisition of task, data were analysed from the first 6 trials of a discrimination (some of which will be incorrect responses) and the last 5 trials (all correct but without a possible “first encounter”). As has been previously described, there is a chance that a “first encounter” may skew results, with a subject taking considerably longer to dig than in any subsequent trials due to unfamiliarity with the exemplars. Data were not available for a full analysis of only the first trials as all of the subjects with lesions dug on encountering the first bowl during the second training simple discrimination.

### **5.3 Results**

#### ***5.3.1 Histology***

Six of the nine subjects administered i.c.v. 5,7-DHT were observed in serotonin stained sections to exhibit a loss of, presumably serotonergic, DRN is 50.76% (range 45.72-76.91%; n = 6). There was no significant reduction of DRN/MRN + MRN neurons (stereotaxic coordinates interaural 2.66-1.18), with mean loss of neurons at 15.04% (range 2.42-21.83%; n = 6). Figure 5.2 shows mean cell count in DRN and MRN in sham lesioned subjects and 5,7-DHT lesioned subjects.

AChE stained sections showed no observable alteration in AChE. It cannot, however, be concluded from this that no change in release of acetylcholine in either cortex, hippocampus, Rt or BF resulted from 5,7-DHT lesions. As already commented, there are no published data on the effects of only 5,7-DHT lesions on ACh levels, therefore it is impossible to compare these observations with previous data. It can be concluded that any alterations in ACh levels as a result of 5,7-DHT lesions are not recordable by AChE stained sections.

Figures 5.3-5.7 show serotonin stained horizontal sections at magnification X10 and figures 5.8-5.11 show AChE stained horizontal sections at magnification X4 of both unlesioned and lesioned brains (i.c.v. 5,7-DHT) alongside schematics of the rat brain adapted from Paxinos and Watson (1998).

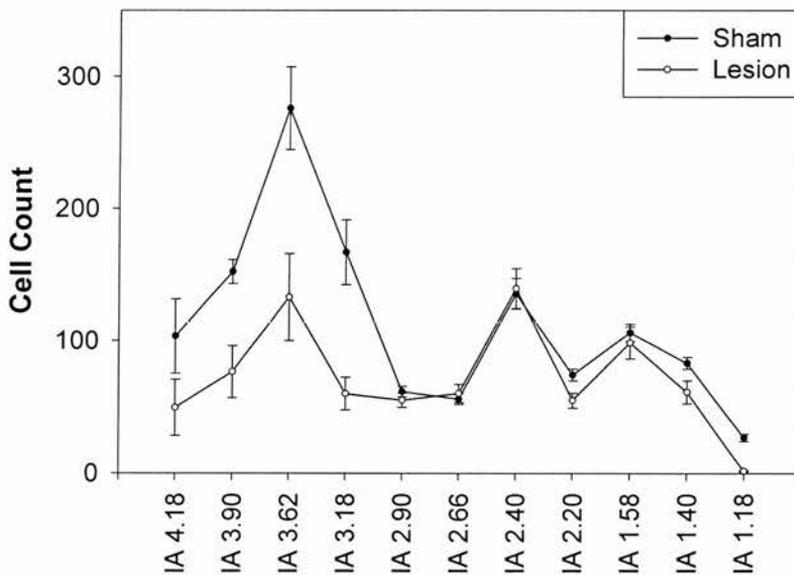
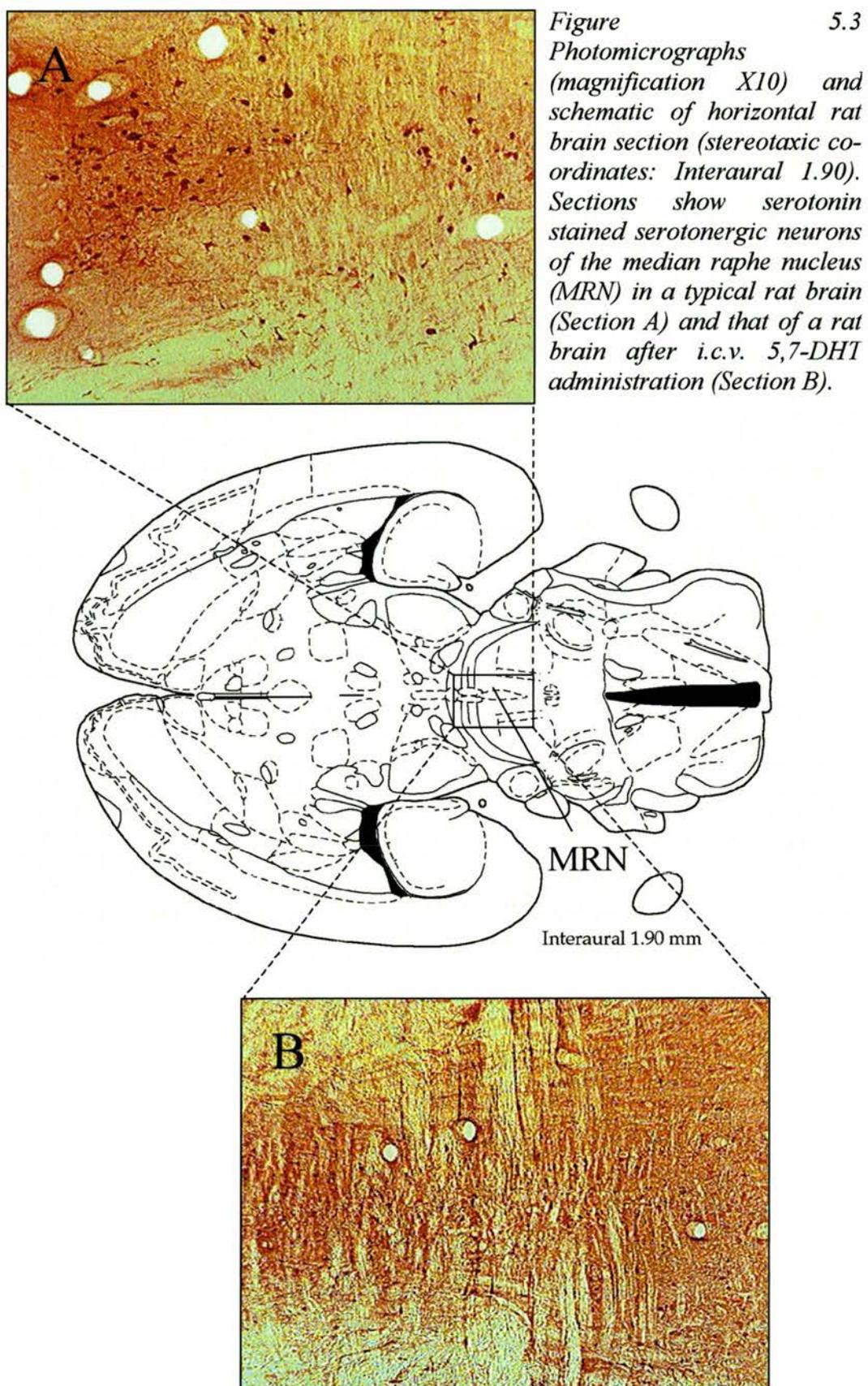
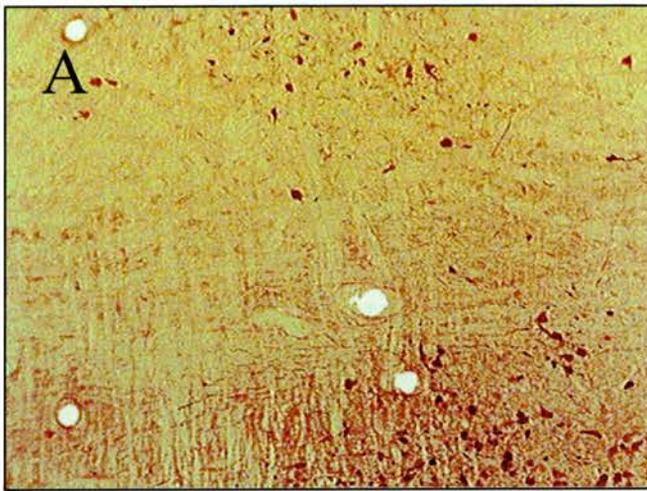
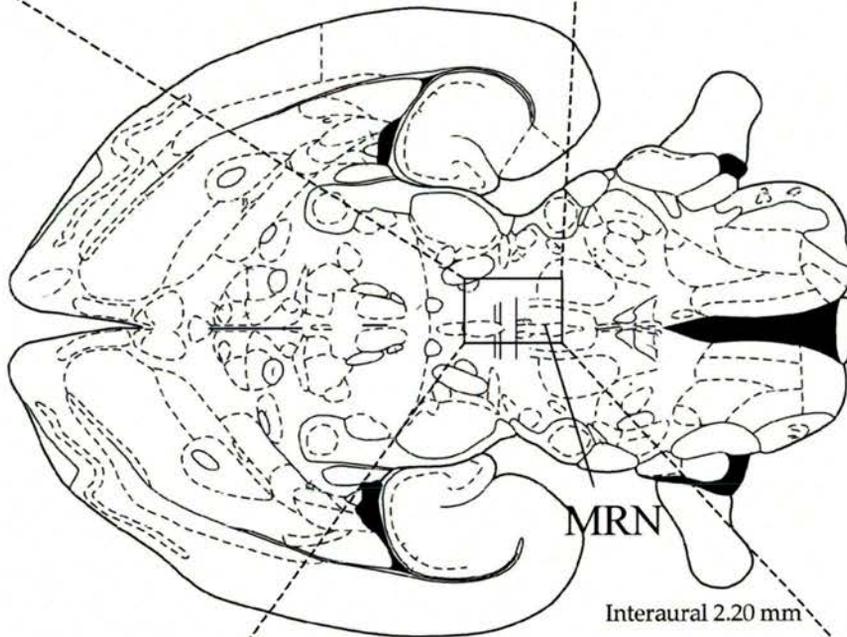


Figure 5.2 Mean  $\pm$  SEM cell counts (serotonin) for D/MRN sham ( $n = 9$ ) and i.c.v. 5,7-DHT administered subjects ( $n = 6$ ). D/MRN. DRN extends between interaural 4.18 and 2.40. MRN extends between interaural 2.66 and 1.18. There is a large reduced cell count in DRN but not in MRN.





*Figure* 5.4  
*Photomicrographs*  
 (magnification X10) and  
 schematic of horizontal rat  
 brain section (stereotaxic co-  
 ordinates: Interaural 2.20).  
 Sections show serotonin  
 stained serotonergic neurons  
 of the MRN in a typical rat  
 brain (Section A) and that of  
 a rat brain after i.c.v. 5,7-  
 DHT administration (Section  
 B).



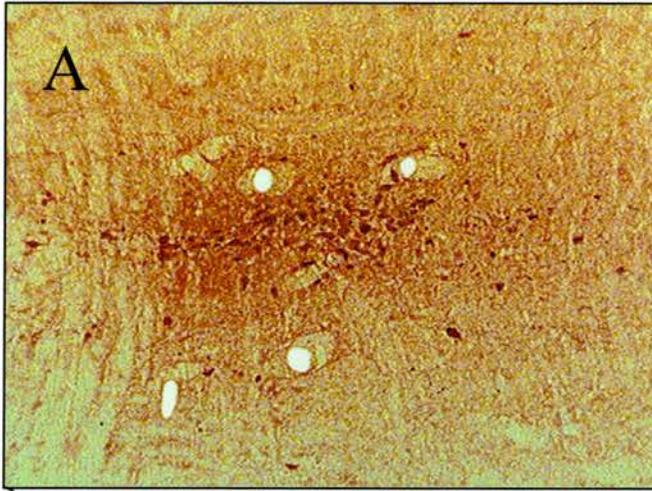
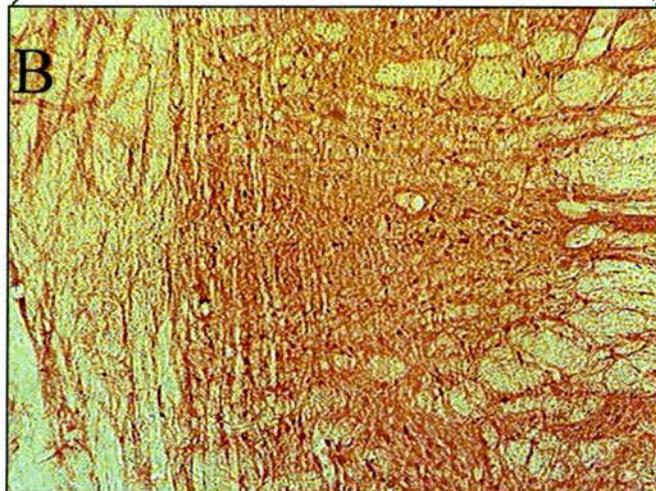
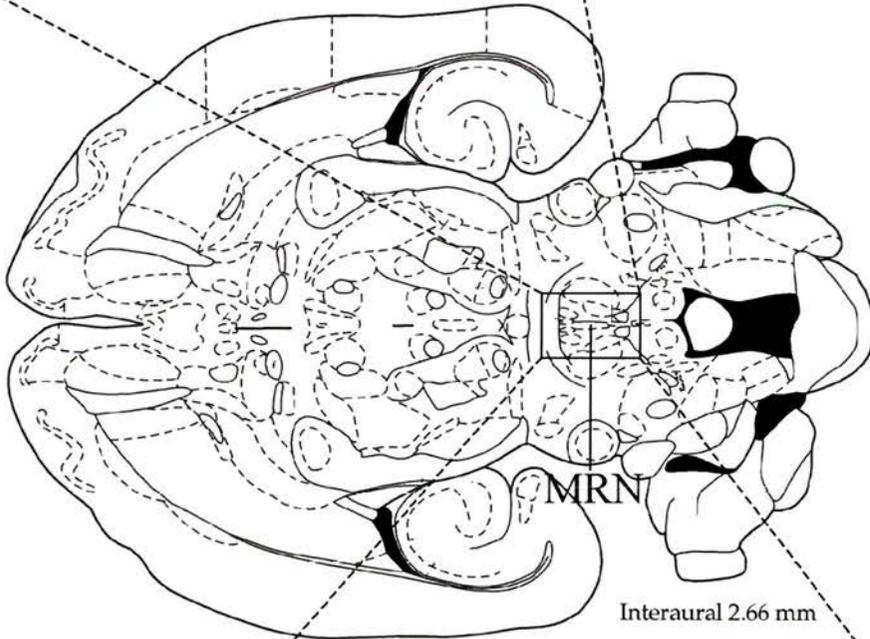


Figure 5.5  
Photomicrographs (magnification X10) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 2.66). Sections show serotonin stained serotonergic neurons of the MRN in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).



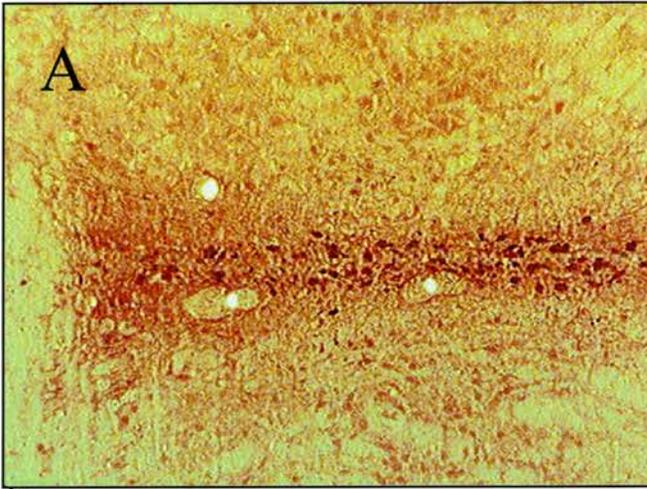
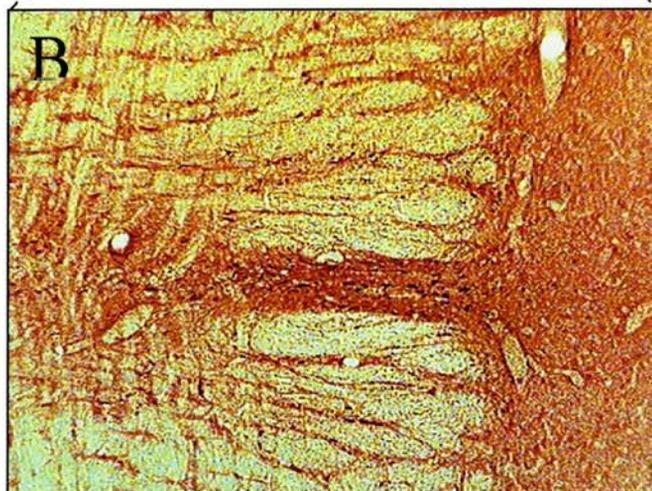
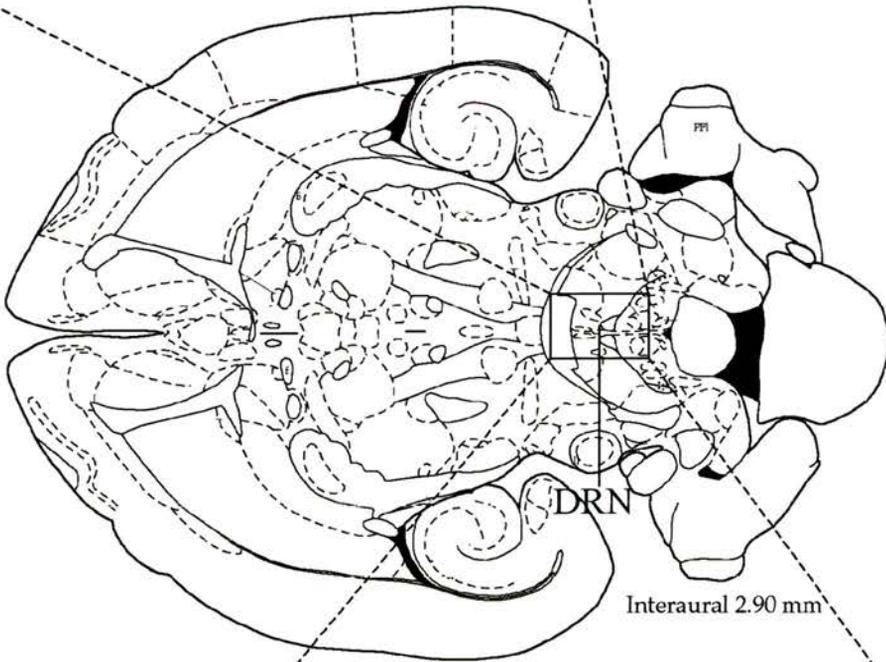


Figure 5.6  
Photomicrographs (magnification X10) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 2.90). Sections show serotonin stained serotonergic neurons of the DRN in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).



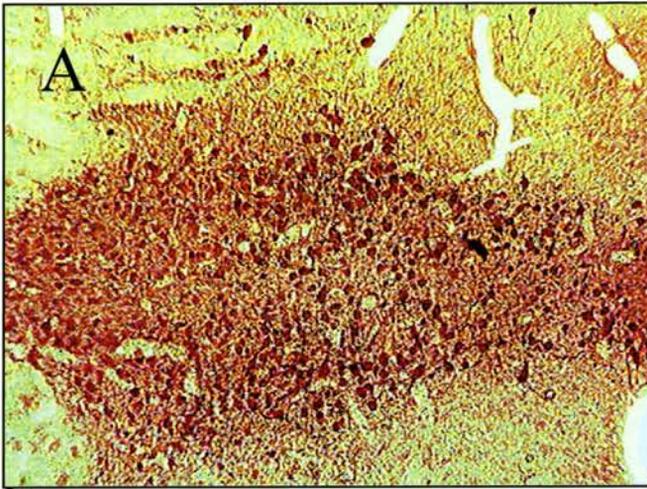
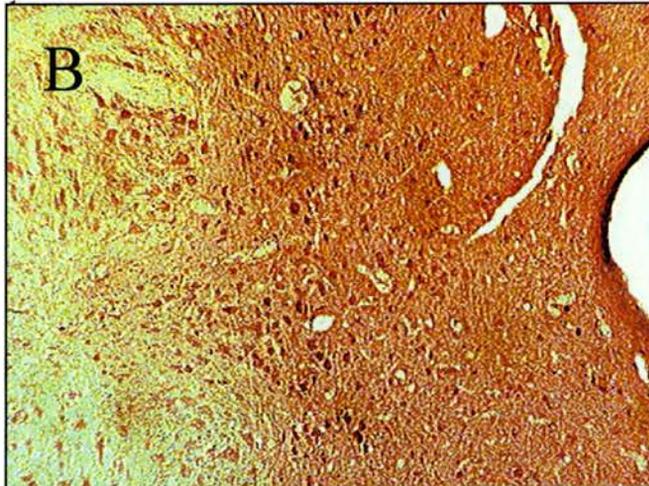
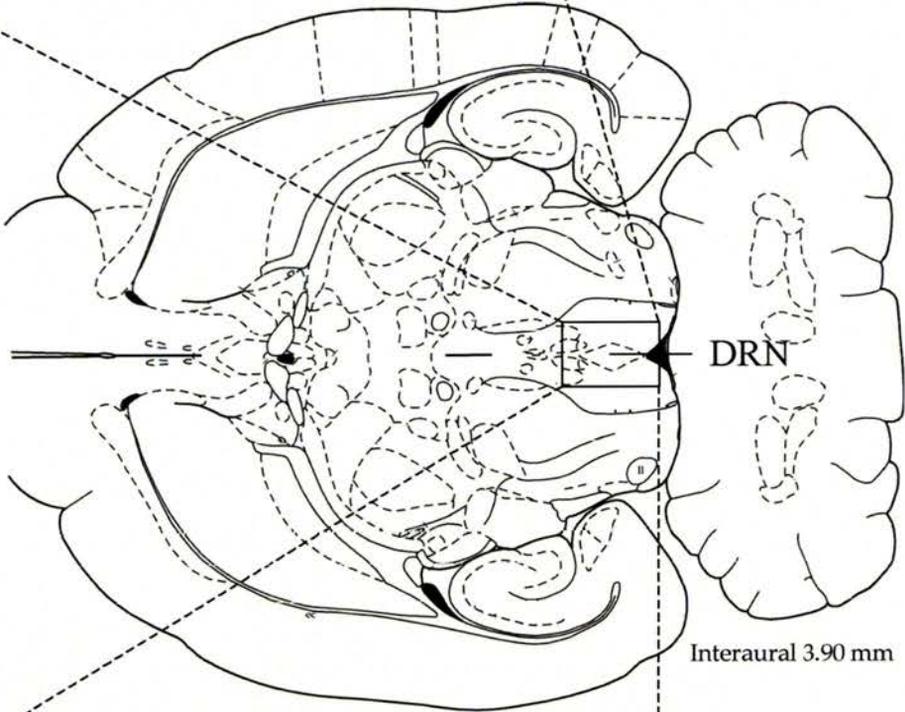


Figure 5.7  
 Photomicrographs (magnification X10) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 3.90). Sections show serotonin stained serotonergic neurons of the DRN in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).



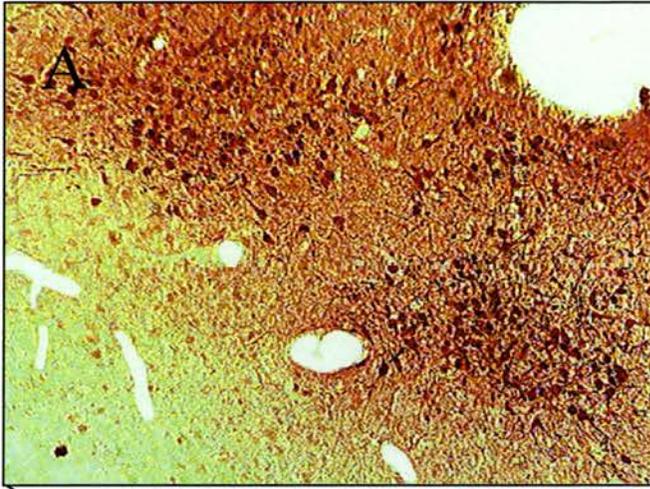


Figure 5.8  
Photomicrographs (magnification X10) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 4.18). Sections show serotonin stained serotonergic neurons of the DRN in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).

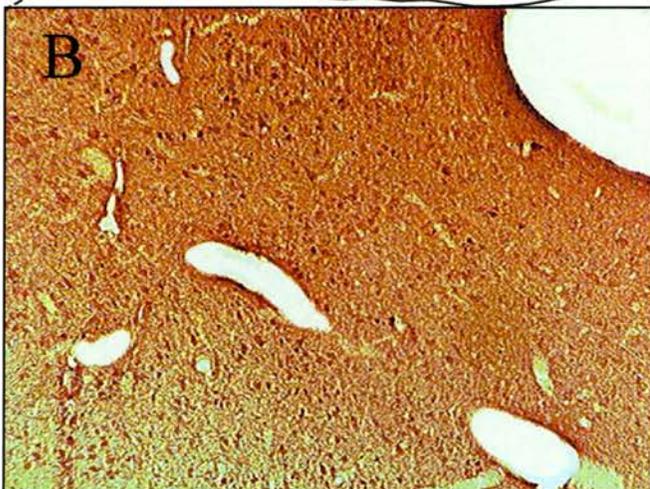
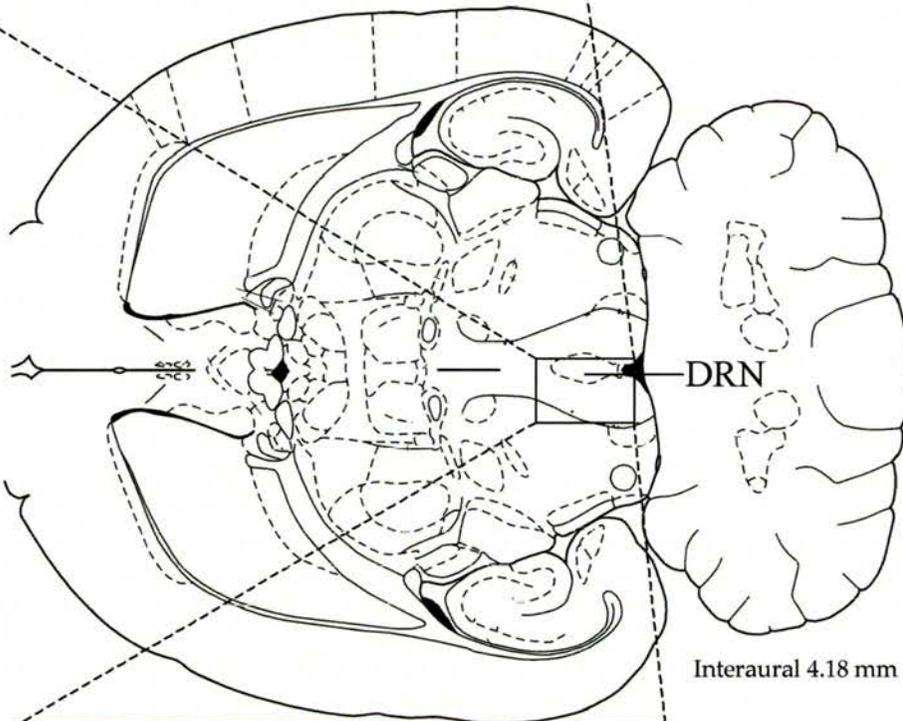




Figure 5.9  
 Photomicrographs (magnification X4) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 2.90). Sections show acetylcholinesterase (AChE) stained tissue of the forebrain in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).

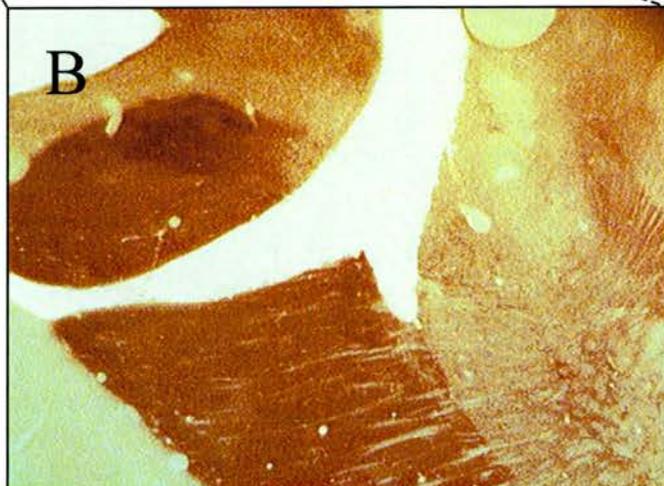
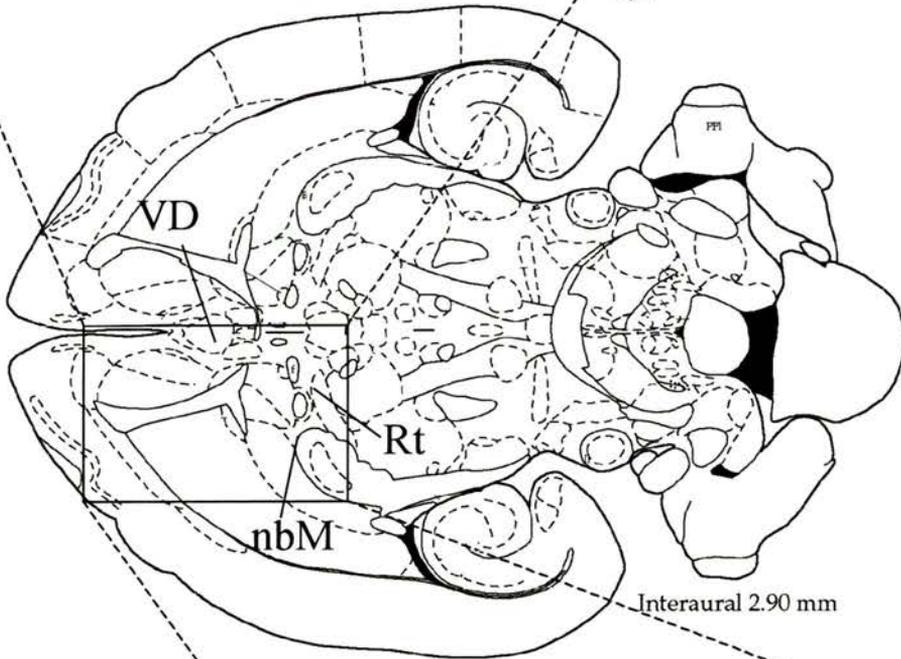




Figure 5.10  
 Photomicrographs (magnification X4) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 3.90). Sections show AChE stained tissue of the forebrain in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).

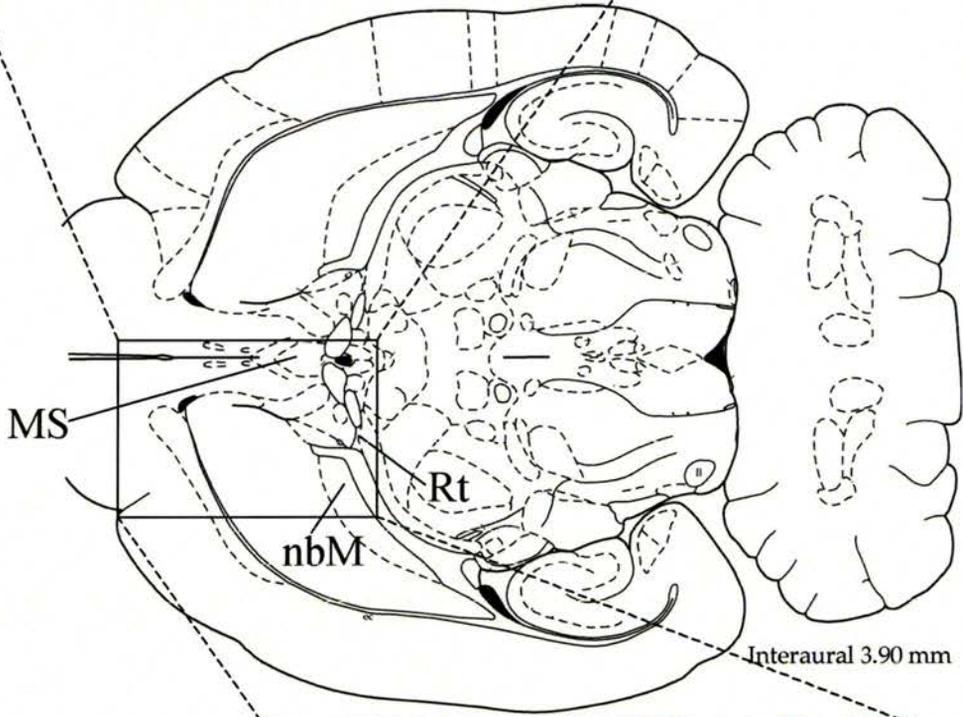




Figure 5.11  
Photomicrographs  
(magnification X4) and  
schematic of horizontal rat  
brain section (stereotaxic co-  
ordinates: Interaural 4.90).  
Sections show AChE stained  
tissue of the forebrain in a  
typical rat brain (Section A)  
and that of a rat brain after  
i.c.v. 5,7-DHT administration  
(Section B).

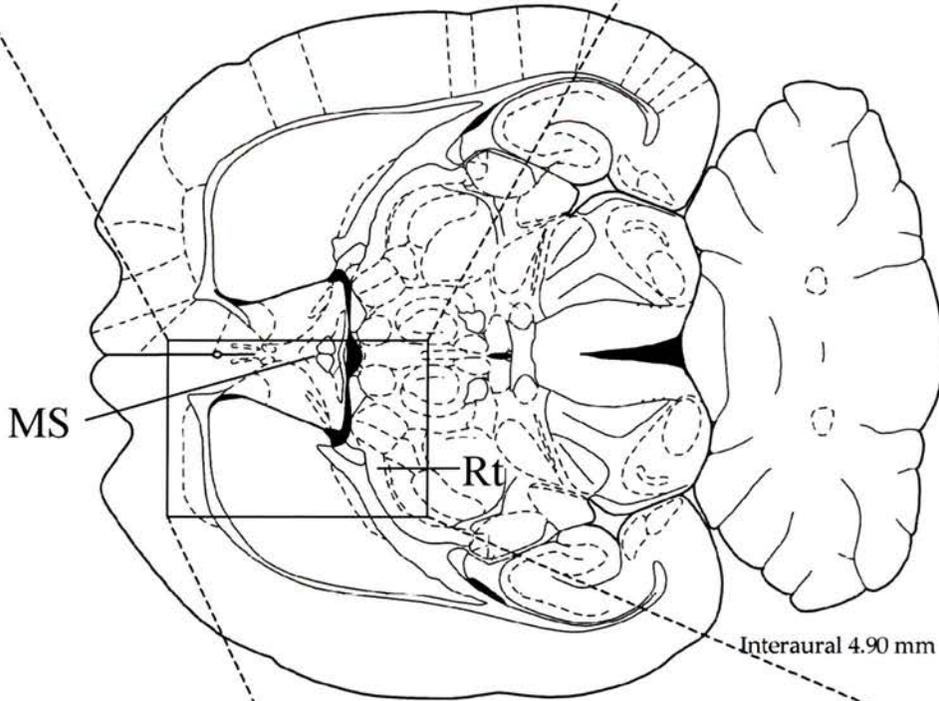
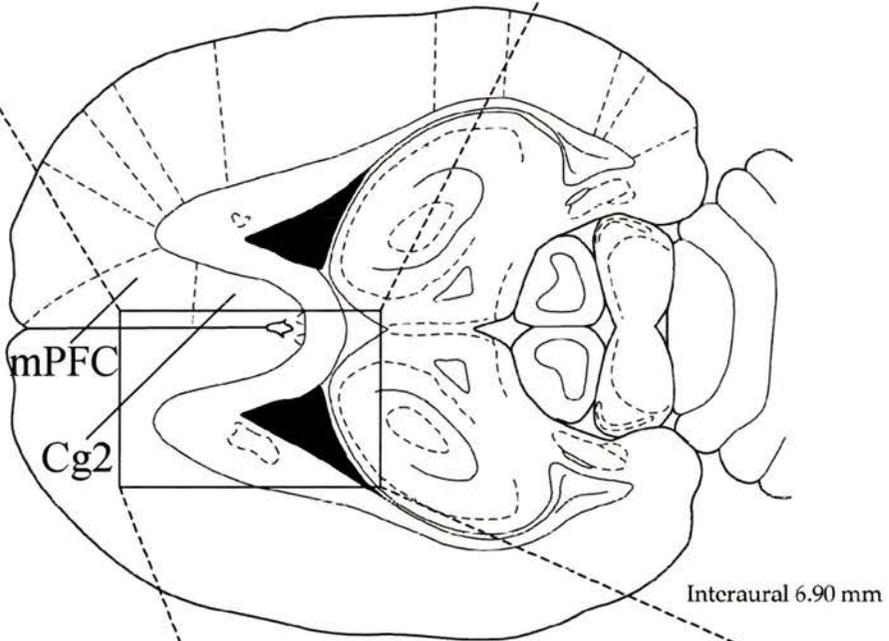




Figure 5.12  
Photomicrographs (magnification X4) and schematic of horizontal rat brain section (stereotaxic coordinates: Interaural 6.90). Sections show AChE stained tissue of the forebrain in a typical rat brain (Section A) and that of a rat brain after i.c.v. 5,7-DHT administration (Section B).



### 5.3.2 Set-shifting

There was no significant effect of 5,7-DHT lesion on performance in the training simple discriminations ( $F(1,15) = 0.361, p > 0.05$ ), indicating that 5,7-DHT lesions do not affect performance in task acquisition of the attentional set-shifting task (Figure 5.12). There is no effect of 5,7-DHT lesion on performance in errors to criterion ( $F(1,15) = 0.058, p > 0.05$ ).

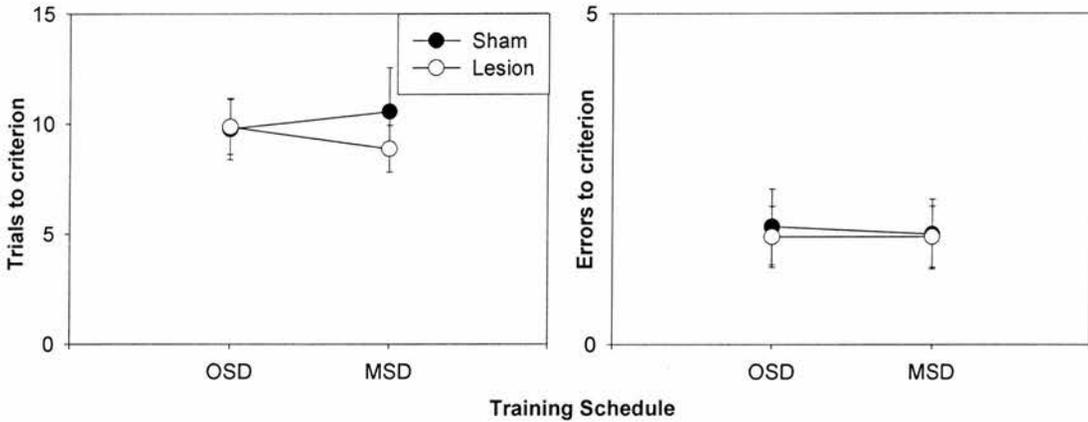


Figure 5.12 Mean  $\pm$  SEM trials and errors to criterion for lesioned ( $n = 6$ ) and unlesioned ( $n = 9$ ) subjects during training in the attentional set-shifting task. OSD is odour simple discrimination; MSD is medium simple discrimination. There is no significant effect of 5,7-DHT lesion on trials or errors to criterion.

There was no significant effect of 5,7-DHT lesion on dig latency in data taken from the first 6 trials of the training SDs ( $F(1,15) = 1.608, p > 0.05$ ) or the final 5 trials ( $F(1,15) = 1.250, p > 0.05$ ) (Figure 5.13).

There was a main effect of dig choice as in previous data for both the first six ( $F(1,15) = 33.547, p < 0.001$ ) and the final 5 trials ( $F(1,15) = 104.191, p < 0.001$ ). Although not available for statistical analysis due to insufficient data points, the data for the training SDs was plotted according to discrimination (Figure 5.14). There appears to be a decrease in dig latency for subjects requiring to experience the second bowl prior to initiating digging during the first six trials, but not during the last five

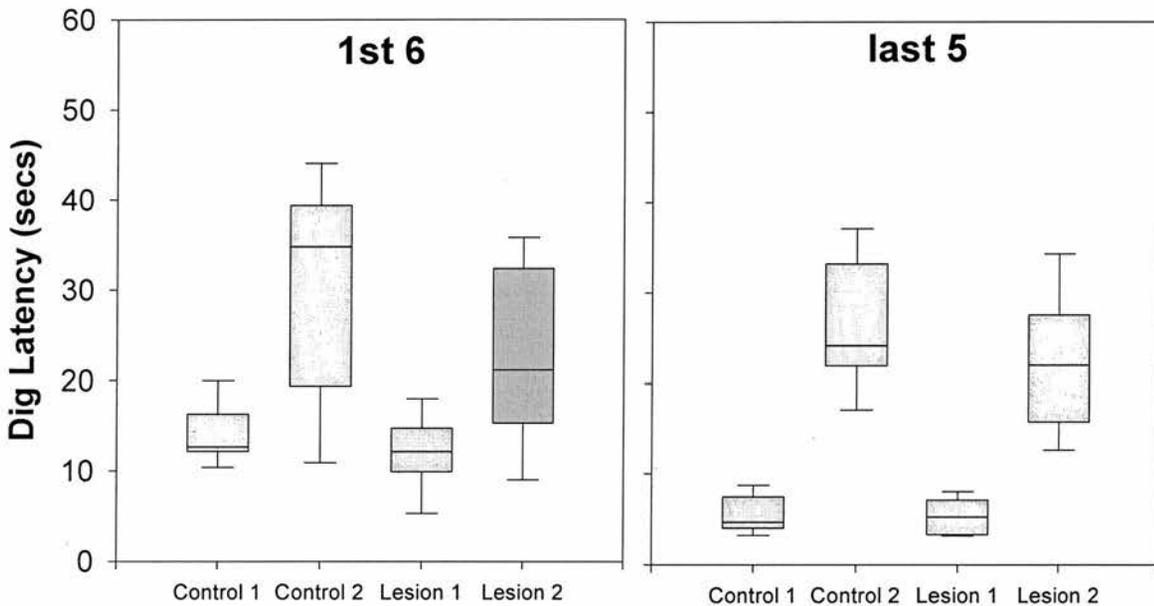


Figure 5.13 Box plot showing median  $\pm$  quartile and 10<sup>th</sup>/90<sup>th</sup> percentile latency to dig for lesioned ( $n = 6$ ) and sham lesioned ( $n = 9$ ) subjects for the first six, and last five, trials of the two training simple discriminations. Data are split by whether the rat experienced both bowls prior to initiating digging (\*2) or just one (\*1). There is no significant effect of lesion on dig latency, although there is a main effect of dig choice in both sets of data.

of the second discrimination.

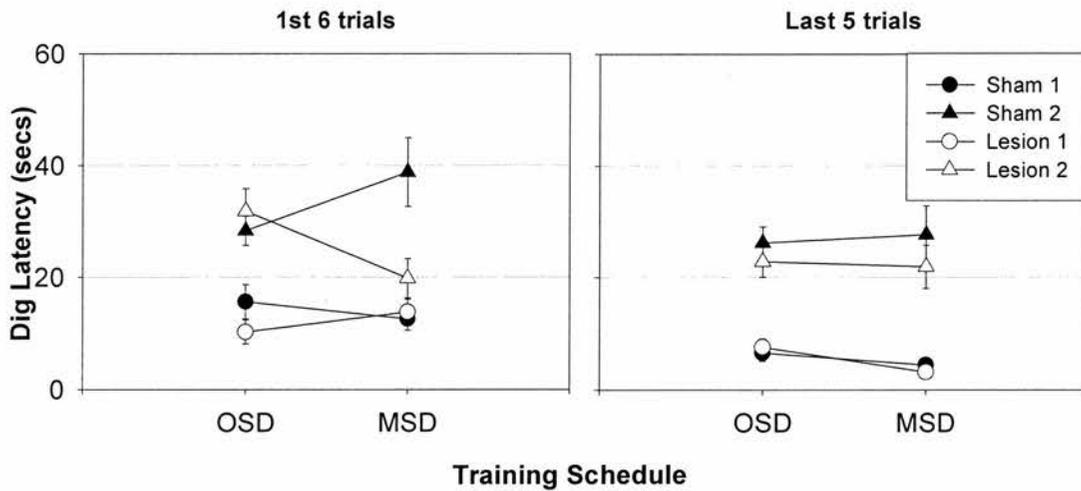


Figure 5.14 Mean  $\pm$  SEM dig latencies for training simple discriminations for sham ( $n = 9$ ) and lesion ( $n = 6$ ) subjects. Data are split by whether the rat experienced both bowls prior to initiating digging (\*2) or just one (\*1). There appears to be a reduction in latency to dig for subjects requiring to investigate the second bowl prior to initiating digging during the second SD. Missing data points means that there is no statistical evidence to support this. O/MSD are odour/medium training simple discrimination respectively.

Five of the six successfully lesioned subjects completed the task. There was no significant effect of 5,7-DHT lesion on either the number of trials to reach criterion ( $F(1, 10) = 1.011, p > 0.05$ ) (Figure 5.15) or the number of errors made ( $F(1, 10) = 1.525, p > 0.05$ ). There was a main effect of discrimination ( $F(6, 60) = 2.878, p < 0.05$ ), with *post hoc* simple comparison showing an approaching-significant difference between ID and ED shifts ( $F(1, 10) = 4.879, p = 0.052$ ). There was, however, no interaction between discrimination and treatment group ( $F(6, 60) = 0.577, p > 0.05$ ).

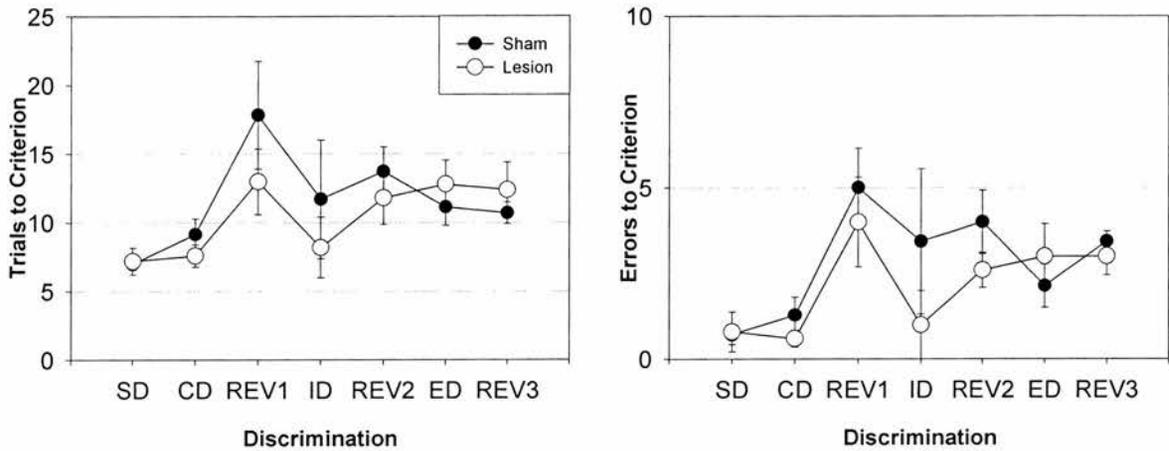


Figure 5.15 Mean  $\pm$  SEM trials and errors to criterion for lesioned ( $n = 5$ ) and sham lesioned ( $n = 7$ ) subjects in the attentional set-shifting task. There is no significant effect of lesion on trials or errors to criterion, although there is a main effect of discrimination.

There is no significant effect of 5,7-DHT lesion on performance in latency to dig ( $F(1,10) = 0.033, p > 0.05$ ) (Figure 5.16). There is a main effect of dig choice ( $F(1,10) = 33.477, p < 0.001$ ).

These data suggest that although there is no effect of 5,7-DHT lesion on set formation and shifting on either acquisition of the attentional set-shifting task, or the task itself, nor in errors made during set formation and shifting, there may be a slight effect on the latency to dig during task acquisition, although perhaps most notably on the first few trials of the second training SD. It is difficult to interpret these data however, as they include both correct and incorrect trials. The statistical evidence cannot support that the 5,7-DHT lesioned subjects were facilitated in initiating digging during the second SD of training.

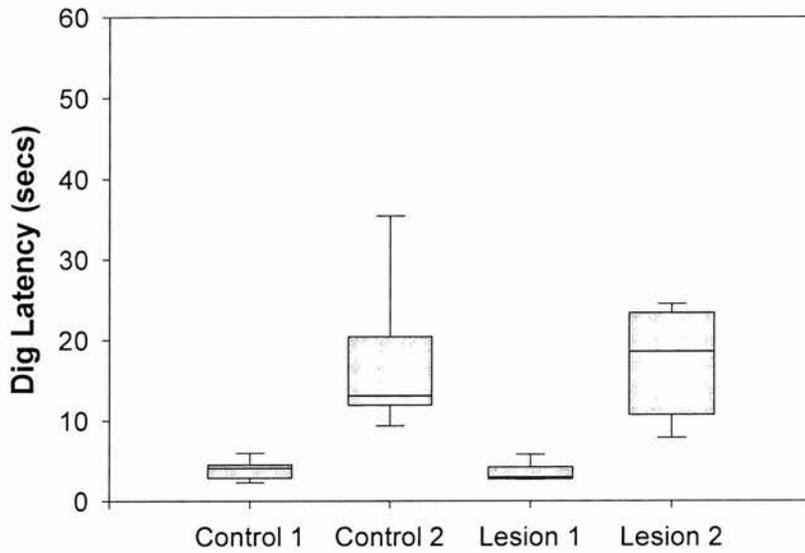


Figure 5.16 Box plot showing median  $\pm$  quartile and 10<sup>th</sup>/90<sup>th</sup> percentile latency to dig for lesioned ( $n = 5$ ) and sham lesioned ( $n = 7$ ) subjects for the last five trials of each discrimination in the attentional set-shifting task. Data are split by whether the rat experienced both bowls prior to initiating digging or just one. There is no significant effect of lesion on dig latency, although there is a main effect of dig choice.

#### 5.4 Conclusion

The data presented here support the conclusion that there is no effect of a 5,7-DHT lesion on performance in either trials to criterion or latency to dig at any stage of an attentional set-shifting task. There is minor evidence supporting a facilitatory role of 5,7-DHT lesions on latency to dig during the training process, which may be a result of a cognitive enhancement effect, speeding up processing of information, allowing faster decision making after initial exposure to the task. It is also

possible that these observed effects are due to cognitive flexibility, permitting the subjects to adapt from digging in both bowls during initial training process, to selecting one during the two simple discriminations, more quickly. However, that this effect does not translate into either a significant effect or a reduction in trials to criterion during the training process suggests that any cognitive enhancement, or increase in flexibility, is very minor. Furthermore, that the data from the testing day does not demonstrate any significant difference from that observed in controls, either in trials to criterion or latency to dig, suggests that the effect is so small that it is lost during testing by non-lesioned subjects achieving that pattern of behaviour late within the second training simple discrimination, or early during the test procedure itself.

It was hypothesised that subjects with 5,7-DHT lesions would show a cognitive flexibility similar to that observed after 5-HT<sub>6</sub> receptor-specific antagonist, SB-271046, administration. It is clear from the data presented here that any cognitive flexibility induced by antagonising post-synaptic 5-HT<sub>6</sub> receptors is masked by a variety of other effects. As has already been reported above, several of the 5-HT receptor subtypes are observed to mediate cholinergic function, not just 5-HT<sub>6</sub>. It could be hypothesised that 5-HT<sub>6</sub> antagonists induce cognitive flexibility by potentiating cholinergic function in the mPFC as has recently been reported from microdialysis studies. It has also been noted that lesions (although not selective for any one neuronal type) of mPFC result in an

increase in cognitive rigidity, increasing trials to criterion at the ED discrimination stage of this task (Birrell and Brown, 2000). In the rat attentional set-shifting task it has been observed that there is a correlation between increase in trials to criterion at the REV1 discrimination stage and increase at the ED stage (V J Brown, unpublished observations). Furthermore, Hatcher *et al.* (2002) provides evidence that the correlation may also work in reverse: a reduction in trials to criterion at the REV1 stage correlates with a reduction in the ED stage. Evidence from data observed thus far suggests that whenever the performance at the REV1 discrimination stage is altered, there is a corresponding increase/decrease at the ED stage. This is not the case for changes in performance at the ED discrimination however, as Birrell and Brown (2000) demonstrate in their data after ibotenic acid lesions of mPFC: trials to criterion at the ED discrimination stage increase, with no corresponding change in the performance at the REV1 stage of the task.

It is clear from data presented after the lesion study that mPFC is important in attentional set-shifting (Birrell and Brown, 2000). It is also possible that there is involvement of mPFC in the performance of subjects after SB-271046 administration (Hatcher *et al.*, 2002). Thus, that there are no observed effects on the attentional set-shifting task after 5,7-DHT lesions suggests that other mechanisms are negating any benefit that antagonising post-synaptic cortical 5-HT<sub>6</sub> receptors may have on the task. This is also corroborated by the histological evidence that

acetylcholinesterase levels are not altered in forebrain after 5,7-DHT lesions. Given that serotonergic projections from the raphe complex terminate throughout the forebrain, it is a possibility that 5-HT post-synaptic receptors other than 5-HT<sub>6</sub> are also affecting cortical acetylcholine after the 5,7-DHT lesions. As discussed previously, 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>4</sub> all mediate cortical acetylcholine levels. Both stimulating and antagonising 5-HT<sub>1A</sub> receptors with 8-OH-DPAT and WAY 100635 respectively leads to increases in mPFC acetylcholine, as does stimulating 5-HT<sub>2A/C</sub> receptors with DOI (1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride). Administration of WAY 100635 inhibits 8-OH-DPAT induced increases in mPFC acetylcholine, as administering M100907, a 5-HT<sub>2A/2C</sub> antagonist inhibits DOI induced acetylcholine increases (Ichikawa *et al.*, 2002a). Furthermore, these data suggest that dopamine is also involved in maintaining cortical acetylcholine levels. It seems likely that a compensatory effect has resulted in cortical acetylcholine levels after i.c.v. 5,7-DHT administration being maintained at baseline level.

Various nuclei of the basal forebrain receive serotonergic projections (Vertes *et al.*, 1999; Gasbarri *et al.*, 1999), and as has been previously reported, MS, VDB and HDB all send cholinergic projections to mPFC. Thus depletion of serotonergic projections to basal forebrain may well have a knock-on effect in cortex. It has been reported in *in vitro* studies that 5-HT administered into the MS and H/VDB excites “GABA-

type” neurons, which in turn excite cholinergic and other non-cholinergic neurons in the nuclei of BF. These effects are attenuated by M100907 and by ICS 205-930, a 5-HT<sub>3</sub>/5-HT<sub>4</sub> antagonist, suggesting that the effects are mediated by several of the 5-HT receptor subtypes (Alreja, 1996). It has, however, also been observed that 5-HT inhibits neurons within the MS and V/HDB. This could be the result of two types of synapse between serotonergic neurons and BF neurons. Neurons within the MS and V/HDB receive input from serotonergic projections in two distinct types: synapses with soma and proximal dendrites (thought to be inhibitory), and synapses with distal dendrites of unknown neuron type (thought to be excitatory) (Milner and Veznedaroglu, 1993). It is possible that these distal dendrites belong to the “GABA-type” neurons as described by Alreja (1996), and that these neurons have excitatory synapses with neurons within BF.

With the complexity of serotonergic input to the forebrain, and the likelihood of indirect routes also affecting cortical acetylcholine levels, it is very difficult to accurately predict the effects of depleted forebrain 5-HT on acetylcholine levels. The data presented here suggest that there is no overall change of forebrain acetylcholine after 5,7-DHT administration.

There is, as yet, however, no confirmation that mPFC acetylcholine levels are responsible for the observations that have been

made after non-selective mPFC lesion and administration of SB-271046. It would be necessary to target mPFC acetylcholine directly in order to confirm the theory. This could be done using the selective immunotoxin, 192-IgG-saporin, injecting either into those areas of BF that project to mPFC, or into mPFC itself. As has already been commented in Chapter IV, if mPFC cholinergic function is responsible for the deficits observed after non-selective mPFC lesions (Birrell and Brown, 2000), then depletion of cholinergic innervation of the area should demonstrate this fact. Likewise, such data would also go some of the way to validating the hypothesis that increases in mPFC acetylcholine after SB-271046 administration are responsible for the increased cognitive flexibility resulting in reduced trials to criterion at the REV1 discrimination stage of the attentional set-shifting task.

However, it should be noted that most evidence points towards either the mPFC or the thalamic reticular nucleus as areas most significantly involved in attention. Yet the thalamic reticular nucleus has not been observed to be involved in attentional set-shifting thus far, and so the most obvious place to direct investigations to is mPFC.

## **5.5 Summary of Findings**

- Rats underwent the attentional set-shifting task after serotonergic lesions were induced by administering 5,7-DHT into a lateral ventricle.
- There was no effect of 5,7-DHT-induced lesions on performance in the attentional set-shifting task
- It is suggested that the antagonist action at the 5-HT<sub>6</sub> receptor caused by loss of serotonergic input, and resulting in an increase in prefrontal ACh (SB-271046 administration improves performance in the attentional set-shifting task), was masked by the effects of a reduction of serotonergic innervation of other 5-HT receptor subtypes with varying roles in the modulation of cholinergic function.

## Discussion

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This thesis has looked at the involvement of the central cholinergic system and specifically the modulation of attention by manipulations of the cholinergic system. In Chapters II and III, the approach taken was to use methods of systemic manipulation of cholinergic function, with subsequent studies aimed at targeting specific cholinergic systems. Before discussing the larger implications of the observations recorded from these studies, it is necessary to summarise the results. Furthermore, this final discussion will also explore possible future applications of such results, and also what the immediate next steps should be to gain further insight into what has been observed here.

### 6.1 Summary of Results

#### 6.1.1 Chapter II

- Rats trained in a covert orienting of attention task were administered the 5-HT<sub>6</sub> receptor-selective antagonist, SB-271046 (by gavage, bi.d)
- Administration of SB-271046 had no effect on performance in covert orienting of attention in the rat.
- It is suggested that as SB-271046 potentiates cholinergic function in PFC, then PFC ACh is unlikely to be involved in performance in covert orienting of attention.

- It is suggested that cholinergic involvement in covert orienting arises in the Rt, where there are no 5-HT<sub>6</sub> receptors.

The experiment reported in Chapter II looked at manipulation of cholinergic function through administration of an orally available selective antagonist to the serotonergic receptor subtype, 5-HT<sub>6</sub>. Previous data suggest that antagonism of the 5-HT<sub>6</sub> receptor in rats leads to an agonist effect on the cholinergic system (Bourson *et al.*, 1995; Bentley *et al.*, 1999), with an increase in medial prefrontal cortex (mPFC) acetylcholine (ACh) levels (Jones, 2002, *pers comm*). Behavioural effects induced by antagonising the 5-HT<sub>6</sub> receptor are attenuated by muscarinic cholinergic antagonists (Bentley *et al.*, 1999). It has also previously been reported that the cholinergic agonist, nicotine, administered systemically, mediates attentional function in an adaptation of Posner's covert orienting task (Ward and Brown, 1996; Phillips *et al.*, 2000). It was hypothesised that a 5-HT<sub>6</sub> antagonist, SB-271046, by stimulating the cholinergic system, would mediate attentional function in the covert orienting task in a similar fashion to nicotine. This study was performed, and replicated with a slightly altered protocol designed to minimise uncontrollable variables, and there was no recorded effect of 5-HT<sub>6</sub> antagonism on covert orienting in the rat. It is concluded that although 5-HT<sub>6</sub> antagonism may potentiate cholinergic function, levels of ACh in areas vital to covert orienting performance are not affected by administration of SB-271046.

If an increase in mPFC ACh levels does not modulate attentional function in the covert orienting task, then presumably another brain region with cholinergic projections must be involved. There are already data suggesting that the thalamic reticular nucleus (Rt) is important in attentional function (Montero, 1997; Montero, 1999). Excitotoxic lesions of Rt result in impairment in the covert orienting task (Weese *et al.*, 1999), and c-Fos activity has been recorded in areas of Rt after a conditioned blocking study (McAlonan *et al.*, 2000). The Rt receives cholinergic projections from basal forebrain, a region that also sends cholinergic projections to mPFC, and also receives collaterals from thalamo-cortical and cortico-thalamic projections (Crick, 1984; Semba, 2000).

### ***6.1.2 Chapter III***

- Rats trained in the covert orienting of attention task were administered PrRP (i.c.v.).
- There was no effect of PrRP administration on performance in covert orienting of attention in the rat.
- It is suggested that new data, revealing differential distribution patterns between the GPR10 receptor and its mRNA (specifically that the GPR10 receptor is not distributed in the Rt as densely as the presence of mRNA in the Rt would suggest), imply that no effect of PrRP administration on covert orienting of attention would be expected.

At the time of this study, there was strong evidence to suggest that there was a high density of the GPR10 receptor in rat Rt. Localisation studies had demonstrated a high density of GPR10 mRNA in Rt (Roland *et al.*, 1999), although there were no data available on the expression of the receptor itself. It was hypothesised that Prolactin releasing peptide (PrRP), the endogenous ligand to the GPR10 receptor, would have a modulating effect on Rt function, and thus also on attentional function. PrRP was therefore administered through permanent injection cannula into the left lateral ventricle of rats trained in the covert orienting task.

There was no observed effect of PrRP administration on performance in the covert orienting task, suggesting that PrRP does not modulate Rt's involvement in covert orienting. Later studies demonstrated that although there were GPR10 receptors present in the Rt, the proportion of GPR10 receptors in relation to GPR10 mRNA was lower in Rt compared to other brain regions where GPR10 mRNA is located and the GPR10 receptor is expressed (Jones *et al.*, unpublished observations). This would suggest that PrRP does not modulate attentional function via Rt.

### ***6.1.3 Chapter IV***

- Rats underwent an attentional set-shifting task after the selective immunotoxin, 192-IgG-saporin, was administered bilaterally into either nbM or rostral Rt.

- Histological observations support the idea that cholinergic projections from BF to cortex send collaterals to multiple regions of Rt
- There was no effect of 192-IgG-saporin lesion of nbM on the subjects ability to form or shift attentional set.
- Data from Rt lesions were inconclusive due to the low number of successful bilateral lesions, and even lower number of subjects completing the attentional set-shifting task
- 192-IgG-saporin lesions of nbM resulted in an increase in latency to initiate digging in the task, specifically if the subject refrains from digging at the first bowl that it encounters.
- It is suggested that this increase in latency to dig represents an impairment in sustained attention (as has previously been observed after 192-IgG-saporin lesions of nbM), or in processes involved in decision making.

In order to further understand the role of ACh in Rt's modulation of attentional function it is necessary to manipulate selectively cholinergic innervation of Rt. The excitotoxic lesions of Rt previously reported were not selective for ACh terminals projecting to Rt, instead destroying the GABAergic Rt neurons. Use of the selective immunotoxin, 192-IgG-saporin, which destroys neurons bearing the p75 rat nerve growth factor (rNGF) receptor (Wiley *et al.*, 1991), would permit a lesion only affecting the cholinergic neurons of the basal forebrain (BF) (almost the only

neurons bearing the rNGF receptor in the rat brain (Korsching *et al.*, 1985)).

An attempt was made to selectively lesion the cholinergic innervation to rostral Rt by injecting 192-IgG-saporin unilaterally into rostral Rt. Although caudal Rt receives cholinergic innervation, this was observed to be only from the pedunculopontine and laterodorsal tegmental nuclei (neither of which bear the rNGF receptor) (Spreafico *et al.*, 1993; Kolmac and Mitrofanis, 1998). Evidence suggests involvement of mPFC, BF, Rt, visual cortex and thalamus in selective visual attention (see Introduction and Chapter IV for projections schematic), and although pedunculopontine and laterodorsal tegmental nuclei clearly influence Rt, thalamus and BF, the data presented here focus on BF and its projections rather than travelling further downstream.

It was observed that injection of 192-IgG-saporin into rostral Rt selectively lesioned rNGF receptor bearing neurons. However, it is clear from the recorded data that cholinergic innervation of rostral Rt from BF was not the only cholinergic projection affected by the lesion. There was observed loss of cholinergic innervation to both cortex and hippocampus as well as Rt, and loss of staining of cholinergic neurons throughout BF and throughout the rostro-caudal extent of Rt. These data suggest that projections from BF to Rt may be axon collaterals of BF to cortex projections, and that furthermore, there are projections from BF to Rt

throughout its rostro-caudal extent, and not simply to rostral Rt as previously reported.

192-IgG-saporin lesions of rostral Rt cannot be considered to be a viable method of selectively manipulating cholinergic modulation of Rt function. However, these lesions do provide a means of lesioning BF cholinergic innervation of both cortex and Rt. Therefore a behavioural task was looked for that could be used to explore the implications of this manipulation.

The rat attentional set-shifting task is an adaptation of the CANTAB ID/ED attentional set-shifting task (see Roberts *et al.*, 1988), itself an analogue of the Wisconsin Card Sort Test (Milner, 1964). The ID/ED task has been used to explore the effects of various brain lesions, including non-selective damage to BF in marmoset monkeys, and the version for the rat has been used to examine the role of the rat prefrontal cortex (Birrell and Brown, 2000; McAlonan and Brown, 2002). It was hypothesised that cholinergic function was involved in attentional set-shifting, and that ACh levels in either Rt or mPFC were a source of mediation of attentional function in this task.

There was no conclusive effect of bilateral 192-IgG-saporin lesions on performance in the rat attentional set-shifting task, although it is noted that the number of subjects used in this procedure was low due to

difficulty in creating a successful bilateral lesion, and a low number of successfully lesioned subjects completing the task.

Given the difficulties encountered, the procedure was altered, and 192-IgG-saporin was injected directly into the nucleus basalis Magnocellularis (Meynert) of BF in a subsequent study (Chapter III). The nbM projects both to Rt and cortex, and is a large structure. A greater proportion of subjects had successful bilateral lesions after this procedure than after injection into rostral Rt, and a greater proportion also completed the task.

There was no observed effect of bilateral 192-IgG-saporin lesions of nbM on ability to form, and shift attentional set, although it was observed that subjects with bilateral lesions took longer to initiate digging for reward during the task, particularly if they required to investigate both stimuli prior to making a decision. This did not appear to be a motor deficit, as subjects moved normally about the task apparatus during the trials. It is concluded that a psychological process involved in making the decision to initiate digging is impaired after 192-IgG-saporin lesions to nbM, and that this could be related to decision making, or a deficit in sustained attention as has previously been reported after such lesions (McGaughy *et al.*, 1999; Himmelheber *et al.*, 2000; Himmelheber *et al.*, 2001).

#### 6.1.4 Chapter V

- Rats underwent the attentional set-shifting task after serotonergic lesions were induced by administering 5,7-DHT into a lateral ventricle.
- There was no effect of 5,7-DHT-induced lesions on performance in the attentional set-shifting task.
- It is suggested that the antagonist action at the 5-HT<sub>6</sub> receptor caused by loss of serotonergic input, and resulting in an increase in prefrontal ACh (SB-271046 administration improves performance in the attentional set-shifting task), was masked by the effects of a reduction of serotonergic innervation of other 5-HT receptor subtypes with varying roles in the modulation of cholinergic function.

Administration of the 5-HT<sub>6</sub> antagonist, SB-271046, does mediate performance in the attentional set-shifting task however. Subjects require fewer trials in the first reversal shift to learn the discrimination after SB-271046 than do controls. SB-271046 administration is observed to lead to an increase in mPFC ACh levels, and non-selective lesions of mPFC are observed to impair attentional set-shifting (Hatcher *et al.*, 2002). From this result, it was hypothesised that rats with depletion of forebrain serotonin would show similar performance in the attentional set-shifting task as did the rats administered SB-271046. Rats received 5,7-dihydroxytryptamine (5,7-DHT) into their right lateral ventricles, leading to a lesion in the serotonergic dorsal raphe nucleus (DRN), and leaving

the serotonergic median raphe nucleus mostly intact. Acetylcholinesterase stained brain sections showed no change in cortical, hippocampal or BF ACh levels, and the subjects' performance in the set-shifting task did not differ from sham-lesioned controls. Evidence suggests that forebrain serotonin receptors have varying effects on forebrain ACh levels, and that lack of alteration in forebrain ACh levels is a result of the widespread effect of the 5,7-DHT lesion on forebrain serotonin levels.

This thesis has explored the interactions between the cholinergic system of the BF and its projections to thalamus, including Rt, and cortex. In summarising the data here presented, it is also necessary to elaborate on future directions that might further elucidate the neuronal mechanisms underlying attention. It is clear from data presented previously that mPFC is involved in selective attention. It is also clear that Rt is involved in selective attention. However, as this thesis has stated, the mechanisms underlying selective attention are many. It would appear that increased ACh in mPFC does not improve performance in the covert orienting task, yet that systemically stimulating the cholinergic system does. It could be hypothesised that ACh levels in Rt are important in the modulation of covert orienting. In order to study this it would be necessary to refine the methods for making selective manipulations of the cholinergic innervation of Rt. Intraparenchymal injection of 192-IgG-saporin into rostral Rt via Hamilton syringe has been shown not to denervate cholinergic input to Rt sufficiently selectively, and the reasons why

already discussed. A refinement of the lesioning method that might now be considered would be to use drawn pipettes to inject 192-IgG-saporin into rostral Rt for more precise targeting, allowing smaller, more specific lesions.

Likewise, it would be possible to administer 192-IgG-saporin into differing BF regions to observe the behavioural effects of more specific lesions. Injections were made into nbM, but also noted that in some cases both HDB and MCPO ventral to nbM were also lesioned. HDB does not project to parietal cortex, unlike nbM, so it would be possible to lesion cholinergic input to mPFC and Rt without denervating BF cholinergic input to other areas of cortex by selectively lesioning HDB. Likewise, VDB also projects to mPFC and Rt without sending projections to parietal cortex. Should lesions of HDB cause damage to nbM, thus denervating nbM parietal cortex input, there is the option of trying to selectively lesion VDB only.

192-IgG-saporin could also be injected into other terminal fields of BF cholinergic projections. It has already been shown that injections into Rt destroy cholinergic projections from BF to Rt as well as cholinergic projections to hippocampus and mPFC. Therefore it would be expected that injections of 192-IgG-saporin into mPFC would show similar results, depleting cholinergic input to Rt. This remains to be demonstrated. This approach may, however, permit comparison of

selective lesions of various terminal areas of BF projections, which may share some inputs (via collateralisation) in addition to other, independent inputs from BF. A recent study by McGaughy *et al.* (2002) demonstrated no effect on set-shifting, using the same task as used here, after injection of 192-IgG-saporin into infralimbic/prelimbic areas of rat medial prefrontal cortex. Impairment in reversal learning was observed after orbital frontal cortex lesions with 192-IgG-saporin, but unlike subjects with excitotoxic lesions of the same region (McAlonan and Brown, 2002), the impairment was only observed when odour was the attended to dimension.

That 192-IgG-saporin is the most selective agent currently available for lesioning BF cholinergic neurons suggests that, certainly in the near future, this will be the tool of choice for such manipulations. Use of these techniques may allow for specific psychological processes in attentional function to be linked to specific regions or nuclei within the CNS. Cholinergic function is important in selective attention, with the cholinergic BF and its projections having a significant mediatory role. It's clear that mPFC and Rt are both involved in attentional function, although the specific role of ACh in these functions is yet to be realised.

## 6.2 Future directions

### 6.2.1 Clozapine

One avenue of immediate future study involves further attempting to understand how cognitive enhancers function in the CNS. Several commercially available antipsychotic drugs are also described as cognitive enhancers, but the mechanism of action is not necessarily known. The specificity of receptor action on these compounds is often diverse, with an overall facilitatory effect on cognitive function. The antipsychotic clozapine has a strong affinity to 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptor subtypes (Roth *et al.*, 1994), as well as to D4 dopaminergic receptors, M1 mAChRs and  $\alpha$ 1 adrenergic receptors. Clozapine also has weak affinity for the D2 dopaminergic receptor. Most evidence suggests that the pharmacological effects of clozapine on behaviour arise from antagonist action on the 5-HT<sub>2C</sub> and D4 receptors and weak antagonist action on D2 receptors (Meltzer, 1994). However, recent data indicating that the selective 5-HT<sub>6</sub> antagonist, SB-271046, induces an increase in mPFC ACh levels, as well as improving performance in attentional set-shifting in rats (Hatcher *et al.*, 2002) suggests that perhaps clozapine's 5-HT<sub>6</sub> antagonist properties (Bymaster *et al.*, 2001) may also be involved in observed cognitive improvement. Clozapine is reported as having facilitatory effects on attention, verbal fluency and some executive functions in schizophrenic human subjects, although data on improvements in spatial and working memory are

inconclusive (Meltzer and McGurk, 1999; Lee *et al.*, 1999). Like SB-271046, clozapine induces an increase in mPFC ACh levels (Ichikawa *et al.*, 2002b), although as WAY 100635 does not attenuate the increase (but does attenuate clozapine-induced DA level increases), it is unlikely that the mechanism involves the 5-HT<sub>1A</sub> receptor or clozapine-induced DA level increases (Ichikawa *et al.*, 2002a).

Likewise, the antipsychotic, olanzapine, also has affinity for several receptor types, including antagonist properties at both 5-HT<sub>3</sub> and 5-HT<sub>6</sub> receptors (Bymaster *et al.*, 2001). Olanzapine is reported as improving verbal learning and memory and executive function, but not attention or working memory, in schizophrenic human subjects (Meltzer and McGurk, 1999). The WCST is used regularly in studies of schizophrenia to measure cognitive dysfunction (for review see Everett *et al.*, 2001), making the rat attentional set-shifting task an appropriate model for observing animal performance after administration of antipsychotics used for treating schizophrenia.

Clozapine and olanzapine are administered to patients with cognitive dysfunction however, so any study designed to compare an animal model with human data would need to induce an impairment that could be attenuated by treatment with either drug. It has been observed that there is a correlation between trials to criterion performance in the REV1 and ED discrimination stages in rat attentional set-shifting. Worse

performance at the ED discrimination (i.e. increased trials to criterion) correlates with worse performance at the REV1 discrimination. It is also noted that the 5-HT<sub>6</sub> antagonist, SB-271046, administered to rats subsequently performing the attentional set-shifting task, leads to a significant decrease in trials to criterion at the REV1 discrimination, as well as a non-significant reduction in trials to criterion at the ED discrimination. Furthermore, it has already been reported that excitotoxic lesions of rat prelimbic cortex lead to an impaired performance in the ED discrimination (Birrell and Brown, 2000).

It could be hypothesised that administration of clozapine or olanzapine, with 5-HT<sub>6</sub> antagonist properties would improve performance at the REV1 discrimination. However, to simulate a cognitive impairment, the protocol of the set-shifting task would be altered such that a “false ED shift” was introduced between the CD and the REV1 discrimination. By this, it would ensure that on the last three trials of the CD discrimination, the bowl pairing remained the same. Thus, for example, in a discrimination where cinnamon and cumin were paired with fine and coarse tea, with cinnamon as the rewarded exemplar, on the last three trials cinnamon and fine tea may be the rewarded pair, with cumin and coarse tea unrewarded. Then on the first three trials of the reversal fine tea and cumin would be the rewarded pair, with cinnamon and coarse tea unrewarded. In this fashion, although as far as the rewarded dimension is concerned, the protocol would be as standard, there would be a run of

six trials where there was consistent reward in the same exemplar of the irrelevant dimension (see Table 6.1). It would be predicted that this would increase the difficulty of the reversal, thus increasing trials to criterion, and also increase the difficulty of the ED discrimination. The subject, when faced with the ED discrimination, having already experienced the “false ED shift”, may perseverate more on the previously relevant dimension.

*Table 6.1 Exemplars rewarded in the CD and REV1 with medium as relevant dimension and odour as irrelevant dimension. Although M1 is consistently rewarded in the CD discrimination, and M2 is consistently rewarded in the REV1 discrimination, O2 is also rewarded six times consecutively.*

	<i>M1</i>	<i>M2</i>	<i>O1</i>	<i>O2</i>
<b>CD</b>	✓		✓	
	✓			✓
	✓		✓	
	✓			✓
	✓			✓
	✓			✓
<b>REV1</b>		✓		✓
		✓		✓
		✓		✓
		✓	✓	✓
		✓		✓
		✓	✓	✓

**“False ED”**

It would be hypothesised that administration of a cognitive enhancer such as SB-271046, clozapine or olanzapine would have an effect on this increase in trials to criterion at the REV1 and ED discriminations. That SB-271046 is described as increasing “cognitive flexibility” (Hatcher *et al.*, 2002) suggests that any cognition enhancing properties of the atypical antipsychotics such as clozapine and olanzapine mediated by their interaction with 5-HT<sub>6</sub> receptors would have a similar effect. How cognitive flexibility would affect any observed decrease in performance in control subjects is, however, debatable. There are currently no data available on the effects on performance of treating already impaired subjects with a cognitive enhancer. It is equally arguable that increasing cognitive flexibility would increase trials to criterion in the REV1 discrimination as that it would decrease them. If antagonising the 5-HT<sub>6</sub> receptor facilitates the ED discrimination (Hatcher *et al.*, observed a statistically insignificant reduction in trials to criterion at the ED discrimination after administration of SB-271046), then it may result in an increase in trials to criterion at the REV1 discrimination after the “false ED”. Or the facilitation effect may be with regard to the REV1 discrimination itself, with trials to criterion reduced as the subject is improved in attending to the dimension and learning the REV1 more quickly.

Thus it would be hypothesised that antagonising the 5-HT<sub>6</sub> receptor will result in change in performance at the REV1 and/or ED

discrimination, and that introducing the “false ED” within the CD and REV1 discrimination will result in a decrease in performance at the REV1 and ED discriminations. A study involving 24 subjects is currently planned. Eight will be put through the control procedure of the task, and sixteen will receive one of two doses of the atypical antipsychotic clozapine before performing the task. Subjects will be perfused after completion of the task, either 1 hour 45 minutes after completion of the REV1 discrimination or 1 hour 45 minutes after completion of the ED discrimination. This will permit data to be collected for histological analysis of activity of the immediate early gene c-Fos. Previous data demonstrate that acute administration of clozapine in rats induces an increase in c-Fos activity in the corticolimbothalamic circuit (Cochran *et al.*, 2002); specifically, prelimbic area of mPFC and the mediodorsal thalamic nucleus. Conversely, the same data indicate a decrease in local cerebral glucose metabolism in the prelimbic area, mediodorsal nucleus and Rt. Cochran *et al.* suggest that it is only certain populations of neurons within mPFC that are activated by clozapine, such as inhibitory GABAergic interneurons, which in turn inhibit pyramidal neurons, reducing neuronal activity. That clozapine has affinity for a variety of receptors means that it is unclear whether these specific effects would modulate performance in the attentional set-shifting task. These data will allow for comparison with subjects that have performed the attentional set-shifting task after clozapine administration. It must, however, be taken into account that these data are from single dose administrations of

clozapine, whereas our observations will come after chronic (10 day) exposure.

### ***6.2.2 Alternate set-shifting manipulations***

Within the set-shifting task, several psychological functions are investigated. It has already been commented that set-shifting performance, and thus impairment, is recorded at the ED discrimination stage of the task. Likewise, reversal learning is recorded at the REV1 stage primarily (this is where deficits have been observed in previous investigations). Performance at the ED and REV1 discriminations is impaired after excitotoxic lesions of the prelimbic area of mPFC and the orbital frontal cortex respectively. The psychological mechanisms which cause performance impairment are not fully defined however. The degree to which perseveration (either to a given stimulus in the case of REV1 impairments, or to a dimension in the case of ED impairments) rather than learned irrelevance is the cause of the performance deficit is currently unclear. The proposal would be to manipulate the set-shifting task in one of two ways to examine the nature of observed deficits.

Two dimensions, odour and digging medium, have been used in the set-shifting task as described in this thesis. A third dimension, bowl texture, has been previously shown to be discernible by rats in this task. The proposal would be to investigate the effects of adding a third dimension at the ED discrimination stage to investigate the effects of

learned irrelevance vs. perseveration at this discrimination. Two options available at the ED discrimination are either a) remove the previously rewarded dimension, and introduce a novel dimension which would be unrewarded (removing opportunity for perseveration), or b) remove the previously unrewarded dimension and introduce a novel rewarded dimension (removing the opportunity for learned irrelevance). An example of the task would is presented in Table 6.2.

*Table 6.2 shows an example of the order of exemplars in the attentional set-shifting task in a test of learned irrelevance vs. perseveration as a mechanism behind deficits in ED performance after excitotoxic lesions of prelimbic area of mPFC.*

<b>Discrimination</b>	<b>Exemplars (correct in bold)</b>
<b>SD</b>	<b>O1</b> , O2
<b>CD</b>	<b>O1</b> , O2, M1, M2
<b>REV1</b>	<b>O2</b> , O1, M1, M2
<b>ID</b>	<b>O3</b> , O4, M3, M4
<b>REV2</b>	<b>O4</b> , O3, M3, M4
<b>ED</b>	Either a) <b>M5</b> , M6, T5, T6 or b) <b>T5</b> , T6, O5, O6
<b>REV3</b>	Either a) <b>M6</b> , M5, T5, T6 or b) <b>T6</b> , T5, O5, O6

Subjects would be previously habituated to all three dimensions on the day prior to testing, as they currently are to the two dimensions.

Controls, as well as subjects with prelimbic area lesions would undergo the task. If ED discrimination deficits in prelimbic lesioned rats are caused by one of perseveration or learned irrelevance, then it would be hypothesised that there would be differences in performance at the ED discrimination between version a) and version b) of the task.

To investigate learned irrelevance vs. perseveration as mechanisms underlying deficits in reversal learning, it would be necessary to introduce alternate stimuli at the REV1 stage of the task. In a similar fashion to the above protocol, two variants of the task would be needed to compare performance; one permitting no learned irrelevance, and one permitting no perseveration. To remove learned irrelevance (version c), the previously unrewarded exemplar from the rewarded dimension would be replaced with a novel exemplar that would be rewarded. To remove perseveration (version d), the previously rewarded exemplar from the rewarded dimension would be replaced with a novel exemplar that would be unrewarded. Table 6.3 shows possible exemplar orders for this version of the attentional set-shifting task.

Table 6.3 shows an example of the order of exemplars in the attentional set-shifting task in a test of learned irrelevance vs. perseveration as a mechanism behind deficits in REV1 performance after excitotoxic lesions of orbital frontal cortex.

<b>Discrimination</b>	<b>Exemplars (correct in bold)</b>
<b>SD</b>	<b>O1</b> , O2
<b>CD</b>	<b>O1</b> , O2, M1, M2
<b>REV1</b>	Either c) <b>O7</b> , O1, M1, M2 or d) <b>O2</b> , O7, M1, M2
<b>ID</b>	<b>O3</b> , O4, M3, M4
<b>REV2</b>	<b>O4</b> , O3, M3, M4
<b>ED</b>	<b>M5</b> , M6, O5, O6
<b>REV3</b>	<b>M6</b> , M5, O5, O6

Differences would be expected in performance after excitotoxic lesions of orbital frontal cortex depending on whether observed deficits in performance in the REV1 discrimination are mediated by learned irrelevance or perseveration.

### 6.2.3 Excitotoxic lesions of basal forebrain

Further investigation of the effects of BF manipulations in attentional set-shifting are intended. Selective lesions of nbM using 192-IgG-saporin have been made and no effects on set-shifting trials to criterion performance have been observed. It has been previously reported that excitotoxic lesions of nbM in monkeys induces deficits in reversal learning in the ID/ED task (Roberts *et al.*, 1992) and also that excitotoxic

lesions of orbital frontal cortex (which receives projections from nbM) induce a similar deficit (Dias *et al.*, 1996). It can be concluded that it is not loss of cholinergic modulation from nbM that induces this deficit. The intention is, therefore, to replicate the monkey data and lesion nbM of the rat with the excitotoxin, ibotenic acid. If a REV1 deficit is observed as seen in orbital frontal lesioned rats, then it can be concluded that the deficit is caused by destruction of non-cholinergic projections (most likely GABAergic, the other identified neurotransmitter system that projects from nbM to prefrontal cortex) from nbM to prefrontal cortex. If this is the case, then this will demonstrate greater functional homology between rodent and monkey.

#### **6.2.4 Modafinil**

Recent data have demonstrated the effects of administration of the “cognitive enhancer”, modafinil (Turner *et al.*, 2003) on healthy human subjects in a number of neuropsychological tests. Performance improvement was observed in tests of digit span, visual pattern recognition memory, spatial planning and stop-signal reaction time. There was no observed effect of modafinil administration on the ID/ED task however. This may be that healthy human subjects already demonstrate optimal performance in the task however. Modafinil could be administered to rats with excitotoxic lesions of either prelimbic area or orbital frontal cortex (or nbM if expected impairment is observed). It is clear that subjects with these lesions, although impaired, are still capable

of forming and shifting set. If subjects are solving the discriminations, taxing alternate neural mechanisms, then it is possible that modafinil, or other potential cognitive enhancers could attenuate lesion-induced deficits. Identifying alternate neural mechanisms is difficult though. Some insight can be gained through the study of immediate early genes, such as c-Fos, although in a task as complicated as the attentional set-shifting task this may prove hard to interpret. The intention is to analyse brain sections for c-Fos staining after clozapine administration, comparing data from the REV1 discrimination with that from the ED discrimination by varying the time that the subjects are sacrificed. However, whether anything can be concluded from such data remains to be seen, and even then may only be a guide to other manipulations rather than providing any conclusive evidence itself.

### **6.3 Conclusion**

Data have been presented from a series of studies investigating the modulation of ACh, and the effects of such modulations on attention. It has been observed that selective 5-HT<sub>6</sub> antagonist-induced potentiation of cholinergic function has no effect on covert orienting; i.c.v. PrRP administration has no effect on covert orienting either, and given recent distribution studies, this result would have been expected. Lesions of nbM with 192-IgG-saporin do not affect attentional set formation and shifting, although they do affect latency to initiate a digging response in the task. Subjects with 192-IgG-saporin Rt lesions show a non-specific impairment

in the attentional set-shifting task which requires further investigation. Finally, subjects with 5,7-DHT serotonergic lesions also show no difference in attentional set-shifting to controls.

It is evident that ACh is involved in attentional function, and that cholinergic innervation of various regions of the brain, including mPFC and Rt, has an important role in the modulation of selective attention. However, neither cholinergic modulation of mPFC, nor Rt, can alone account for all aspects attentional function. The interaction between ACh and other neurotransmitters in a variety of brain regions all combine together to mediate the psychological process of attention.

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