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The Effect of Catecholaminergic Genes on Executive Functions:

A Behavioural and fMRI Study

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A dissertation submitted for the degree of Doctor of Philosophy
to the University of Dublin, Trinity College

This research was conducted in the Department of Psychiatry and
the Trinity College Institute of Neuroscience

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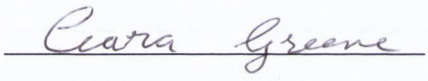

Ciara Greene

Summary

This thesis describes a study in two parts aimed at elucidating genetic links to executive functions and their neurological correlates. The catecholamines (in particular dopamine and noradrenaline) are known to be heavily involved in the modulation of executive functions, including sustained and spatial attention, response inhibition and spatial working memory. Genes involved in the synthesis and management of catecholamines were therefore considered likely targets for genetic studies of these functions.

The first study described in this thesis examined the effect of six genes, COMT, DBH, DAT1, DRD4, MAOA and 5HTT, on a battery of neuropsychological tests in a sample of 205 healthy participants. Significant effects of variants in several of these genes were observed on task performance. The main findings included the observation that the DBH gene, which controls the balance of dopamine and noradrenaline available at the synapse, exerted a significant effect on sustained and spatial attention. Possession of the variant of the gene associated with increased dopamine and reduced noradrenaline resulted in poorer sustained attention and an increase in inattention to the left side of space. The DAT1 gene, which codes for the dopamine transporter, significantly affected spatial attentional bias and reaction time during a spatial working memory task, with the 'low-dopamine' 10-repeat allele resulting in reduced left spatial inattention and faster reaction times. Surprisingly, no effect of variation in the COMT gene, involved in the degradation of dopamine, was observed on any measure.

The second study described here examined the effect of the DBH, DAT1 and COMT genes on cortical activation during performance of spatial working memory tasks. 40 participants performed two spatial working memory tasks while undergoing functional magnetic resonance imaging. Significant effects of a Val/Met polymorphism in the COMT gene were observed across the whole brain. The Met allele, which results in increased levels of prefrontal dopamine relative to the Val allele, was associated with increased blood-oxygen-level dependent signal during performance of the spatial working memory tasks, in the absence of any differences in task performance. This finding was in direct contrast with previous research on this topic, probably as a result of variations in task design. Significant effects in left hemisphere regions, including prefrontal cortex, were observed as a function of DBH genotype; carriers of the allele associated with high dopamine and low noradrenaline displayed increased activation in these regions during easy task conditions, but decreased activation during more difficult conditions. On balance, it appears likely that this result is due to the role of DBH in sustained attention and not in spatial working memory *per se*. Variation in the DAT1 gene was also found to result in differences in task-related activation in both cortical and subcortical regions. The allele associated with decreased dopamine availability at the synapse led to reduced working memory efficiency at low memory loads but improved efficiency at higher memory loads.

Overall, this thesis underscored the value of cognitive-genetic-imaging studies in the study of relationships between the various executive functions and their distinct neurochemical requirements.

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Publications arising from this thesis

Journal Articles

Greene, C.M., Bellgrove, M.A., Gill, M. & Robertson, I.H. (2008). Noradrenergic genotype predicts lapses in sustained attention. *Neuropsychologia*, epub ahead of print. doi:10.1016/j.neuropsychologia.2008.10.003.

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Greene, C.M., Bellgrove, M.A., Robertson, I.H., Gill, M., Hawi, Z.H. (2007). The influence of the dopamine beta-hydroxylase gene on spatial working memory: an fMRI study. *XV World Congress on Psychiatric Genetics Abstracts Book*, P394, pp.130.

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5HTT	5-hydroxy-tryptamine transporter
ADHD	attention-deficit hyperactivity disorder
ANT	Attention Network Task
BOLD	blood-oxygen-level dependent
CFQ	Cognitive Failures Questionnaire
COMT	catechol *O*-methyltransferase
CPT	Continuous Performance Task
DAT	dopamine transporter
DLPFC	dorsolateral prefrontal cortex
DRD4	dopamine receptor D_4
DβH	dopamine β-hydroxylase
fMRI	functional magnetic resonance imaging
HAD	Hospital Anxiety and Depression scale
MAOA	monoamine oxidase A
PCR	polymerase chain reaction
PFC	prefrontal cortex
SART	Sustained Attention to Response Task
SENSE	SENSitivity Encoding
SNP	single nucleotide polymorphism
SWM	spatial working memory
UTR	untranslated region
VNTR	variable number of tandem repeats
WCST	Wisconsin Card Sorting Test

Chapter 1

General Introduction

1.1 Introduction to executive functions

'Executive function' is a general term applied to those functions performed at least in part by the prefrontal cortex which guide and manage the various independent cognitive processes undertaken in the brain so as to achieve a desired result. These include the ability to maintain attention on the task at hand, to select the appropriate response from among a multitude of options while inhibiting inappropriate responses and to retain and manipulate task-relevant information in working memory. The role of neurotransmitters in the brain is central to understanding the interactions of these processes. The catecholamines (in particular dopamine and noradrenaline) play a crucial part in prefrontal cognition (Diamond, Briand, Fossella, & Gehlbach, 2004; Muller, von Cramon, & Pollmann, 1998; Parasuraman, Greenwood, Kumar, & Fossella, 2005; Simon, Scatton, & Moal, 1980), and the genetic regulation of these neurotransmitters has been the subject of much investigation.

1.1.1 Attention – spatial and sustained

The ability to allocate cognitive resources as needed is crucial to our survival in the world. One of the best established theories of attention has conceptualised it as consisting of three subsystems, known as the alerting, orienting and executive control (or conflict) networks (Posner & Peterson, 1990). The executive control network, thought to be involved in resolving conflict between various competing responses (Fan, McCandliss, Sommer, Raz, & Posner, 2002), has been found to primarily, although not exclusively, activate anterior cingulate and dorsolateral prefrontal areas (E.E. Smith,

Jonides, Marshuetz, & Koeppe, 1998), while the processes of the orienting network, which directs attention and selects appropriate sensory input, is strongly linked to regions of the parietal cortices (Corbetta, Kincade, Ollinger, McAvoy, & Shulman, 2000). The alerting network, which controls arousal and vigilance, is proposed to activate right dorsolateral prefrontal and right parietal regions (J.T. Coull, Frith, Frackowiack, & Grasby, 1996), as well as midbrain regions such as the locus coeruleus. It is this hypothetical partitioning of attention into systems for spatial attention or orienting on the one hand, and non-spatial attention or alerting on the other that is of particular relevance to this thesis.

Spatial attention includes both the ability to consciously focus attention on a particular location in space and the more automatic allocation of attention to particular spatial locations. A right hemisphere dominance for certain types of spatial processing has been observed in normal populations, with evidence that the right hemisphere allocates attention to both left and right hemispace, while the left hemisphere allocates attention only to right hemispace (Heilman, Bowers, Valenstein, & Watson, 1986; Mesulam, 1981; Weintraub & Mesulam, 1987).

This dominance of the right hemisphere for spatial processing leads to the phenomenon of 'pseudoneglect', whereby healthy individuals tend to pay slightly more attention to the left visual field and relatively less attention to the right (Bowers & Heilman, 1980). In contrast, individuals with right brain lesions or with disorders of the frontostriatal system frequently exhibit signs of inattention to the left side of space, or 'left neglect'

(Bradshaw, 2001). Some evidence of left neglect is also seen in children with attention-deficit hyperactivity disorder (ADHD`; Sheppard, Bradshaw, Mattingley, & Lee, 1999; Voeller & Heilman, 1998). The suggestion has been made that this is linked with cortical dopamine levels as administration of methylphenidate (Ritalin) appears to ameliorate the deficit (Nigg, Swanson, & Hinshaw, 1997; Sheppard *et al.*, 1999).

Lateral biases in spatial attention can be assessed using tasks such as the line bisection task, in which participants are asked to mark the midpoint of a horizontal line (Schenkenberg, Bradford, & Ajax, 1980), or the Landmark Task (Harvey, Milner, & Roberts, 1995) in which participants are required to indicate which side of a pre-bisected line is shorter. Healthy participants tend to bisect lines to the left of the midpoint and to perceive the right side of pre-bisected lines as being shorter, while patients with left neglect consistently display the opposite trend (Bowers & Heilman, 1980; Harvey, Milner, & Roberts, 1995). Similar patterns of response are observed using the Greyscales task (J. B. Mattingley, Bradshaw, Nettleton, & Bradshaw, 1994) in which participants examine two shaded rectangles, one of which is shaded darker on the right side, while the other is shaded darker on the left side. Normal participants, who pay more attention to the left side of space, tend to perceive the line which is darker on the left end to be darker overall. Patients exhibiting symptoms of left neglect demonstrate the opposite pattern of response.

Non-spatial attention, on the other hand, includes elements of sustained attention, alerting and arousal which enable one to maintain alertness in the absence of exogenous

cues or support (I.H. Robertson, Manly, Andrade, Baddeley, & Yiend, 1997; Sturm *et al.*, 1999). Sustained attention is often operationalised as the ability to focus for long periods of time on a monotonous task and is assessed using continuous performance tasks (e.g. T.E. Goldberg *et al.*, 2003; Klee & Garfinkel, 1983; Loo *et al.*, 2003) which require participants to respond to a rare target in a stream of stimuli. It has been argued however (I.H. Robertson *et al.*, 1997) that the occurrence of a rare target can in itself have an exogenous alerting effect, and that a more sensitive measure of sustained attention can be in the context of routine responding, with the requirement to *withhold* response to a rare target while responding to all other stimuli. In accordance with these criteria, the Sustained Attention to Response Task (SART) was developed, where participants respond to presentation of a series of numbers, but withhold their response to presentation of the number 3. The SART has been shown to be a reliable measure of sustained attention and alertness in both healthy subjects (R. C. K. Chan, 2001; Cheyne, Carriere, & Smilek, 2006; Manly, Robertson, Galloway, & Hawkins, 1999; I.H. Robertson *et al.*, 1997; Smallwood *et al.*, 2004) and clinical populations, including individuals with ADHD (Johnson *et al.*, 2007; O'Connell, Bellgrove, Dockree, & Robertson, 2004), traumatic brain injury (R. C. Chan, 2005; R. C. K. Chan, 2001; McAvinue, O'Keeffe, McMackin, & Robertson, 2005; I.H. Robertson *et al.*, 1997) and narcolepsy (Fronczek, Middelkoop, van Dijk, & Lammers, 2006).

fMRI and PET studies have identified many brain regions, both cortical and subcortical, which are activated during tasks tapping sustained attention and non-spatial alerting. Paus and Zatorre (1997) have parsed these activations into two interacting

networks; sustained attention proper appears to be regulated by cortical regions, most notably right frontal and inferior parietal cortex. The fact that normal deactivation of these regions with time on task does not occur confirms the crucial role of these regions in sustained attention (J. T. Coull, Frackowiak, & Frith, 1998). In conjunction with this, a subcortical network encompassing the thalamus and striatum appears to be involved in arousal (Tomas Paus & Zatorre, 1997). Interestingly, this arousal network also includes the anterior cingulate, a region in the frontal cortex which has been implicated in a variety of functions, including cognitive control and error processing and may play a role in coordinating the activities of disparate brain regions (Botvinick, Nystrom, Fissell, Carter, & Cohen, 1999; T. Paus, 2001).

The role of the right prefrontal cortex in sustained attention has been confirmed in a number of studies (e.g. Fassbender *et al.*, 2004; Pardo, Fox, & Raichle, 1991; Wilkins, Shallice, & McCarthy, 1987). Activation patterns in the parietal cortex are slightly more complex; while the intraparietal sulcus displays activation related to both spatial and non-spatial forms of attention, the superior parietal cortex is activated during spatial attention alone (J. T. Coull & Frith, 1998). Sustained attention may be viewed as requiring contributions from both the alerting and executive control attention networks. The alerting network is activated involuntarily in response to a stimulus which grabs attention, such as a rare target which must be responded to. Executive control on the other hand voluntarily directs attention towards a stimulus at the expense of other more interesting but irrelevant stimuli. The involvement of both of these networks in

sustained attention provides an explanation for the activation of a fronto-parietal network.

As with the process of sustained attention, regions of both prefrontal and parietal cortex have been implicated in spatial attention. It is important to note however that the term 'spatial attention' covers both the semi-automatic process of orienting towards a stimulus, and the voluntary allocation of attentional resources to one spatial location to the exclusion of another. Studies have shown that prefrontal cortex (particularly dorsolateral prefrontal cortex, DLPFC) is activated only during tasks or conditions that require goal-directed choices to be made between competing stimuli (e.g. Chen, Wei, & Zhou, 2006; Krueger, Fischer, Heinecke, & Hagendorf, 2007), while spatial orienting is controlled by right-lateralised parietal regions including the right superior parietal cortex and intraparietal sulcus (Corbetta *et al.*, 2000; Corbetta, Miezin, Shulman, & Petersen, 1993; Fan, McCandliss, Fossella, Flombaum, & Posner, 2005; Vandenberghe *et al.*, 1996). This involvement of dorsolateral prefrontal areas in the executive component of spatial attention accords well with the involvement of the PFC in sustained attention described above; while parietal regions may be involved in attending to a stimulus or location, other, possibly more arousing but task-irrelevant, stimuli must be repressed in favour of the possibly boring task-relevant stimulus. A repetitive transcranial magnetic stimulation (rTMS) study has provided support for this, demonstrating that the DLPFC plays a role in inhibiting task-irrelevant information; specifically, right DLPFC was found to reduce the impact of task-irrelevant spatial

information while left DLPFC controlled task-irrelevant verbal information (Sandrini, Rossini, & Miniussi, 2008).

The action of the catecholamines (particularly dopamine and noradrenaline) is a critical factor in performance of tasks requiring sustained attention and alerting. One of the most obvious indications of the role of dopamine in attentional processes is the efficacy of the dopaminergic drug methylphenidate in reducing sustained attention deficits in children with ADHD (e.g. Hood, Baird, Rankin, & Isaacs, 2005; Katherine A. Johnson *et al.*, 2008; Levy & Hobbes, 1988). Amphetamine, a catecholaminergic agonist whose primary action is to release dopamine into the synaptic cleft and prevent reuptake of dopamine from the synapse via the dopamine transporter (Sulzer, Sonders, Poulsen, & Galli, 2005), has been shown to improve sustained attention in healthy human controls (Mackworth, 1965; Silber, Croft, Papafotiou, & Stough, 2006) and rats (Bizarro, Patel, Murtagh, & Stolerman, 2004; Grilly, 2000). Administration of amphetamine also ameliorates sustained attention deficits in people with ADHD (Oades, 1987; Sostek, Buchsbaum, & Rapoport, 1980; Spencer *et al.*, 2001) and in animal models of the disorder (Chudasama, Nathwani, & Robbins, 2005; Sagvolden & Xu, 2008).

The five-choice serial reaction time task (5CSRTT) is a common test of attention in animals, requiring elements of both spatial and sustained attention. Response accuracy on the task appears to be improved by the administration of dopamine agonists (see Chudasama & Robbins, 2004 for a review). Some involvement of dopamine in spatial attention is suggested by the finding that dopamine agonists such as bromocriptine and

methylphenidate reduce unilateral neglect in rats with cortical lesions (Fleet, Valenstein, Watson, & Heilman, 1987) and in children with ADHD (Nigg, Swanson, & Hinshaw, 1997; Sheppard *et al.*, 1999). Dopamine also appears to be important in attentional shifting; the dopamine antagonist haloperidol impaired performance on an attentional search task where participants had to shift attention to a number of different locations, but not on a focused attention task (J. T. Coull, Sahakian *et al.*, 1995).

Noradrenergic projections from the locus coeruleus innervate a wide variety of cortical and sub-cortical brain regions, and may play a role in regulating the balance between focused and diffuse attention (Aston-Jones, Rajkowski, & Cohen, 1999). Specifically, noradrenaline may enhance attention by reducing the influence of distracters on behaviour, thereby increasing an organism's ability to focus on a task (Robbins, 1984). The α-2-adrenoreceptor antagonist idazoxan increases noradrenaline release. Administration of the drug effectively narrows the focus of spatial attention, decreasing reaction times to stimuli presented in the same spatial location, but not in different locations (A. P. Smith, Wilson, Glue, & Nutt, 1992).

Noradrenaline activity appears to interact with an organism's underlying level of arousal in its effects on attention. An alerting cue presented just prior to a stimulus usually has the effect of decreasing reaction times to the stimulus (Fan *et al.*, 2002). Clonidine and guanfacine, α-2-adrenoreceptor agonists which decrease noradrenergic cell firing and release, attenuated the effect of a warning cue on reaction times in a selective attention task in monkeys (Witte & Marrocco, 1997). This complements the

work of Carli *et al.* (1983) who reported that rats with lesions to the dorsal noradrenergic ascending bundle were impaired in attention relative to normal rats when exposed to highly arousing white noise, implying that noradrenaline activity is critical to attention during periods of high arousal. Clonidine has also been shown to impair sustained attention in human subjects (J. T. Coull, Middleton, Robbins, & Sahakian, 1995; A. Smith & Nutt, 1996), however this impairment is reduced when white noise is presented during the task, again demonstrating an interaction between noradrenaline and arousal levels in the control of attention.

The distinction between spatial and sustained attention described above is in some ways an artificial one; considerable evidence has emerged from the neuropsychology literature supporting an overlap between these processes, both functionally and anatomically. Both processes rely heavily on a right-lateralised fronto-parietal network, and patients with fronto-parietal lesions frequently display deficits both in spatial awareness on the contralateral side to the injury (Vallar & Perani, 1986) and in the ability to independently sustain attention (Wilkins, Shallice, & McCarthy, 1987). Sustained attention capacity has also been shown to exert a modulatory influence on spatial attention (I. H. Robertson, Mattingley, Rorden, & Driver, 1998). Participants who perform poorly in tests of sustained attention allocate spatial attention atypically compared with participants who have relatively better sustained attention (M.A. Bellgrove, Dockree, Aimola, & Robertson, 2004), and the presentation of an auditory alerting signal ameliorated unilateral neglect in patients with right hemispheric lesions (I. H. Robertson *et al.*, 1998). These data support Posner and Peterson's (1990)

hypothesis that under certain circumstances the parietal spatial attention system can be activated by the sustained attention system, via either cortical or subcortical mechanisms. Recent research has also indicated that the neurochemical substrates of spatial and non-spatial attention may overlap more than previously thought. Recent data suggest that the control of spatial attention, particularly between the hemi-fields, may also rely on dopaminergic mechanisms (M. A. Bellgrove, Chambers, Johnson, Daibhis *et al.*, 2007; M. A. Bellgrove, Hawi, Kirley, Fitzgerald *et al.*, 2005), and studies of sustained attention have demonstrated a role for noradrenergic, dopaminergic and cholinergic systems (S. R. Chamberlain *et al.*, 2006; J. T. Coull, 1998; Everitt & Robbins, 1997).

1.1.2 Response Inhibition

Closely associated with the process of sustained and selective attention is the ability to inhibit a prepotent response when appropriate. The tests used to examine the two processes are often very similar, frequently involving vigilance tests which require participants to withhold their response to a rare target. Response inhibition may be considered a measure of executive control, as it requires the conscious and goal-directed override of a largely automated process. It is also frequently thought of as a measure of behavioural impulsivity (S. R. Chamberlain & Sahakian, 2007).

A number of fMRI studies (e.g. Garavan, Ross, & Stein, 1999; Liddle, Kiehl, & Smith, 2001; e.g. Rubia, Smith, Brammer, & Taylor, 2003) and an rTMS study (Chambers *et al.*, 2006) have indicated that right inferior frontal cortex is necessary for response inhibition (e.g. Chambers *et al.*, 2006; Garavan, Ross, & Stein, 1999; Liddle, Kiehl, &

Smith, 2001; e.g. Rubia *et al.*, 2003). Crucially, many of these studies used an event-related design which allowed them to selectively examine activations related to response inhibition while excluding activity related to error awareness or processing. Several studies have demonstrated activation of the anterior cingulate during response inhibition, however it appears more likely that the anterior cingulate is involved in monitoring performance and assessing the likelihood of errors than in inhibiting responses per se (Carter *et al.*, 1998; Chevrier, Noseworthy, & Schachar, 2007). A network of other brain regions including the inferior parietal lobe (Garavan, Ross, & Stein, 1999; Menon, Adleman, White, Glover, & Reiss, 2001) and striatum (Casey *et al.*, 1997) have also been implicated.

A body of research indicates a critical role for noradrenaline, but not dopamine, in response inhibition. Catecholamine agonists including modafinil, methylphenidate and the selective noradrenaline reuptake inhibitors atomoxetine and desipramine improve response inhibition (S. R. Chamberlain *et al.*, 2006; Overtoom *et al.*, 2003; Turner *et al.*, 2003), but neither dopamine agonists (Overtoom *et al.*, 2003) nor antagonists (Eagle, Tufft, Goodchild, & Robbins, 2007) have any effect on performance. Serotonin activity has also been implicated in behavioural impulsivity (Pattij & Vanderschuren, 2008), however a number of studies of pharmacological agents which either increased or decreased serotonin levels found no effect of the neurotransmitter on response inhibition (S. R. Chamberlain *et al.*, 2006; Samuel R. Chamberlain *et al.*, 2007; Clark *et al.*, 2005).

1.1.3 Spatial Working Memory

One of the most common theories of working memory underpinning most cognitive neuroscience studies is Baddeley's conceptualisation of working memory as consisting of a central executive which oversees the activities of two 'slave' subsystems, a phonological loop which holds verbal or auditory information, and a visuospatial sketchpad which retains visual and spatial information (Baddeley, 1992). The visuospatial sketchpad can be further broken down into systems for spatial and object memory. Discussion of working memory in this thesis will be restricted to spatial working memory, which may be defined as the ability to hold spatial locations in short-term memory, and to manipulate that information as required.

The central executive in Baddeley's theory is thought to be responsible for resolving conflicts between sensory inputs, and directing attention as appropriate. This fits well with research suggesting that the processes of spatial attention and spatial working memory are closely related. Attention has been shown to be a crucial element in remembering spatial locations (Awh, Anllo-Vento, & Hillyard, 2000), and it is hypothesised that spatial locations are held in working memory as a result of a shift in spatially selective attention to the relevant location (Awh & Jonides, 2001; Awh, Smith, & Jonides, 1995; Smyth & Scholey, 1994). This 'attention-based rehearsal' would necessarily involve the alerting, orienting and executive attention networks described above. In addition, many of the same cortical areas such as the intraparietal sulcus, precentral sulcus and middle temporal gyrus are involved in both processes (Awh & Jonides, 1998; Awh, Smith, & Jonides, 1995; LaBar, Gitelman, Parrish, &

Mesulam, 1999; Mayer *et al.*, 2007). Working memory is known to be dopamine-dependent (Muller, von Cramon, & Pollmann, 1998; Parasuraman *et al.*, 2005) as are the functions of the executive control network (Diamond & Goldman-Rakic, 1989; Simon, Scatton, & Moal, 1980) and both are thought to be localised in the right prefrontal cortex, further tying the process to the attention networks described above.

A number of cortical areas have been implicated in spatial working memory. Most neuroimaging studies support the general hypothesis of a fronto-parietal circuit subserving the processes of spatial working memory, while many have focused on the role of the prefrontal cortex. Studies of spatial working memory generally show activation in bilateral superior parietal cortex, inferior parietal cortex, intraparietal sulcus, superior frontal cortex and extrastriate cortex, as well as variable activation in ventrolateral and dorsolateral prefrontal cortex (Carlson *et al.*, 1998; D'Esposito *et al.*, 1998; Diwadkar, Carpenter, & Just, 2000; J. Jonides *et al.*, 1993; McCarthy *et al.*, 1994; Owen, Evans, & Petrides, 1996; E. E. Smith, Jonides, & Koeppe, 1996). While activity in parietal and frontal cortices is generally considered crucial to memory processes, Awh and Jonides (1998) note that activation in extrastriate cortex seems more likely to reflect enhanced visual responses during the memory conditions of these tasks than any other process.

The different roles of the parietal and prefrontal cortices in spatial working memory will be discussed in detail below, but the question of hemispheric involvement in spatial working memory requires some comment here. In the studies discussed above,

while some activation was seen in homologous areas in the left hemisphere, most show a greater role for the right hemisphere in spatial working memory. This is in line with evidence regarding spatial selective attention (Corbetta *et al.*, 1993; Weintraub & Mesulam, 1987). Some studies (e.g. S.M. Courtney, Ungerleider, Keil, & Haxby, 1996) did however find a higher than usual proportion of bilateral activity, and Baker *et al.* (1996) found that neural activity in the DLPFC occurred bilaterally during a spatial delayed response task while activation specific to a spatial location task was restricted to the right inferior and dorsolateral PFC.

The parietal cortex is reliably activated during working memory (e.g. Diwadkar, Carpenter, & Just, 2000; John Jonides *et al.*, 1998; LaBar *et al.*, 1999) and appears to form a network with the prefrontal cortex that is crucial for the performance of spatial working memory tasks. While dorsolateral prefrontal cortex may be responsible for executive processes involved in working memory, it appears likely that parietal regions are involved in the lower order storage of mnemonic information (Bradley R. Postle *et al.*, 2006) or in visual object localisation (see Haxby *et al.*, 1991). Coactivation of DLPFC and parietal cortex was observed using fMRI in working memory tasks involving dynamic tracking of spatial locations (McCarthy *et al.*, 1994) and encoding of location information (Diwadkar, Carpenter, & Just, 2000). Visuospatial attention processes similarly activate a network comprised of regions in the superior frontal sulcus and the intraparietal sulcus (Corbetta, Kincade, & Shulman, 2002).

The role of the prefrontal cortex in spatial working memory has been the subject of some debate. Dorsolateral prefrontal cortex (DLPFC) has been traditionally associated with spatial working memory (P.S. Goldman-Rakic, 1988, , 1995), and this has been supported by empirical research (Awh & Jonides, 1998; Ungerleider, Courtney, & Haxby, 1998). It has however been suggested that information to be stored in working memory initially only requires activation of the ventrolateral PFC. DLPFC would then only be recruited when monitoring or manipulation of this information is required (Petrides, 1994). Evidence from human lesion studies (e.g. D'Esposito & Postle, 1999) indicates that lateral PFC does not make a necessary contribution to simple working memory storage, however lateral PFC (especially DLPFC) is probably necessary for the manipulation of information.

A considerable body of research has emerged in support of this, indicating that activation in DLPFC occurs only when some manipulation of spatial information over and above storage is required (McCarthy *et al.*, 1994; Owen, Evans, & Petrides, 1996). For example, DLPFC activation was seen during performance of a spatial n-back task (E. E. Smith, Jonides, & Koeppe, 1996) which requires constant updates of locations held in working memory, but not in normal storage/maintenance tasks such as that used by Jonides *et al.* (1993) or Courtney *et al.* (1996). In both of these studies, activation was restricted to premotor and ventrolateral prefrontal cortex. Load-dependent activation of bilateral DLPFC has been shown during performance of both spatial (Carlson *et al.*, 1998; Jansma, Ramsey, Coppola, & Kahn, 2000) and non-spatial (T. S. Braver *et al.*, 2001) n-back tasks, and attention switching tasks (Kübler, Murphy,

Kaufman, Stein, & Garavan, 2003). These results confirm the importance of DLPFC when information must be manipulated or when distracting stimuli are present, and it has even been suggested that the processes undertaken by DLPFC in working memory may actually be non-mnemonic (Collette *et al.*, 1999; Lebedev, 2004). Strong support for this theory comes from a rTMS study showing that application of rTMS to the DLPFC disrupted manipulation but not maintenance of information in working memory, while rTMS of superior parietal cortex disrupted both manipulation and retention of the same information (Bradley R. Postle *et al.*, 2006).

A number of studies (Cohen *et al.*, 1997; S. M. Courtney, Ungerleider, Keil, & Haxby, 1997; B. R. Postle, Berger, & D'Esposito, 1999; B. R. Postle & D'Esposito, 1999; Zarahn, Aguirre, & D'Esposito, 1999) have however shown activation of DLPFC in the active maintenance of spatial information over a delay. The activation has been shown to be greater when the information held in working memory was also manipulated (D'Esposito, Postle, & Rypma, 2000), however the finding of any DLPFC activation in WM maintenance trials differs from the reports described above. An explanation of this may be found in the fact that DLPFC recruitment increases with increasing memory load even when no manipulation of information held in working memory is required (Manoach *et al.*, 1997; Rypma, Prabhakaran, Desmond, Glover, & Gabrieli, 1999). Similarly, Diwadkar *et al.* (2000) found that cortical activation in DLPFC and parietal cortex increased as a function of the number of object locations to be maintained and the dimensionality of the display. In accordance with this, Rypma *et al.* (1999) suggest that the DLPFC circuits used during manipulation of information held in working

memory may be recruited during maintenance tasks where subjects must actively maintain information loads that approach or exceed short-term memory capacity. One fMRI study demonstrated an effect of increased memory load only in right DLPFC, and only during the encoding period of the task (Rypma & D'Esposito, 1999), suggesting that load-sensitive processes in DLPFC contribute to encoding processes but not to the maintenance of information *per se* (D'Esposito, Postle, & Rypma, 2000).

As with attention, the various processes of working memory are connected with the action of the catecholamines. Of these, dopamine appears to be the most critical for working memory performance (Muller, von Cramon, & Pollmann, 1998). Among the earliest evidence of this was a study by Brozoski *et al.* (1979) showing that impairments in visuospatial working memory were found after dopamine depletion, but not after noradrenaline or serotonin depletion. The indirect dopamine agonist methylphenidate enhances spatial working memory on novel tasks but impairs performance on familiar tasks (Elliott *et al.*, 1997), implying an interaction between dopamine activity and arousal. The role of dopamine in spatial working memory has been investigated in a large number of studies using various dopamine agonists and antagonists. Many of these target either D_1 or D_2 type receptors.

The PFC of primates, including humans, contains many D_1 receptors and very few D_2 receptors (P. S. Goldman-Rakic, Lidow, & Gallager, 1990). The introduction of D_1 antagonists to monkey PFC induces poorer performance in working memory tasks (A. F. Arnsten, Cai, Murphy, & Goldman-Rakic, 1994; Sawaguchi & Goldman-Rakic,

1991). In human subjects, the introduction of pergolide, a mixed D_1/D_2 receptor agonist, improved performance on a working memory test while introduction of the D_2 receptor agonist bromocriptine had no effect (Muller, von Cramon, & Pollmann, 1998). In addition, working memory deficits in schizophrenia patients are associated with alteration of dopamine D_1 receptor transmission in DLPFC (Abi-Dargham *et al.*, 2002). These results are taken to indicate a critical role in working memory for D_1 receptors.

D_2 type receptors are considerably more common in the striatum than the PFC, and a number of studies have produced conflicting results with regard to their involvement in working memory. Rodent and monkey studies have shown that D_1 receptors are more involved in working memory than D_2 receptors (Sawaguchi, Matsumura, & Kubota, 1988), and some studies (e.g. Muller, von Cramon, & Pollmann, 1998) show that D_2 agonists such as bromocriptine have no effect on working memory. Bromocriptine has however been shown to enhance spatial working memory in other studies (Monica Luciana & Collins, 1997; Mehta, Swainson, Ogilvie, Sahakian, & Robbins, 2001) while the D_2 antagonist haloperidol impairs it (Monica Luciana & Collins, 1997). It has been suggested that discrepancies in results between different studies may reflect the fact that different tasks require different optimal levels of dopamine receptor activation (Zahrt, Taylor, Mathew, & Arnsten, 1997). The D_2 antagonist sulpiride has also been seen to lead to dose-dependent impairments on a range of cognitive tasks, including spatial working memory (Mehta, Sahakian, McKenna, & Robbins, 1999). As D_2 receptors are known to be plentiful in the striatum, and the pattern of effects seen in this study was very similar to that seen in Parkinson's Disease, it has been suggested that

the effects of the D_2 antagonist on cognitive function are striatal rather than prefrontal. A study by the same group found that sulpiride acted to impair performance in a delayed response task without distraction, but conversely, to protect against minimal levels of task-irrelevant distraction (Mehta, Manes, Magnolfi, Sahakian, & Robbins, 2004). The suppression of D_2 receptors occasioned by sulpiride may therefore act to improve attentional focussing, resulting in participants being unaffected by low levels of distraction, and simultaneously to impair their ability to switch attention between tasks.

A large body of research has suggested that the relationship between dopamine availability and cognitive performance may not be a linear one. Rather, several studies have provided evidence that levels of extracellular dopamine within a particular range are required for optimal frontal cognition, including spatial working memory. Murphy *et al.* (1996), in a study of the effects of dopamine agonists on spatial working memory in rats, found that there was "a critical range of dopamine turnover", whereby too much or too little dopamine led to impairments in the rats' ability to perform the task. The fact that noradrenergic and glycine/NMDA antagonists mitigated both the increase in dopamine produced by the DA agonist and the corresponding deficit in spatial working memory in this study supported the theory that several neurotransmitter systems interact in the control of dopamine levels and associated cognitive functions. Similarly, Zahrt *et al.* (1997) found that 'supranormal' activation of D1 receptors in PFC, leading to an excess of cortical dopamine, was associated with poorer performance in rats on a number of cognitive measures including spatial working memory. The neurobiology of

this inverted U-shaped dose/response curve has been described in detail by Arnsten and colleagues (Amy F. Arnsten, 1998; Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007; Zahrt *et al.*, 1997). In brief, while dopaminergic stimulation of D_1 receptors appears to enhance spatial working memory, too much D_1 stimulation can disrupt calcium currents and prevent adequate signalling down the neuron. Impairments in spatial working memory tasks are then observed as new spatial information is not transferred from dendrite to soma, and response patterns are not updated (Amy F. Arnsten, 1998).

Noradrenaline also plays a role in spatial working memory via its activation of alpha-adrenergic receptors. Alpha-2 noradrenaline agonists improve performance of PFC-related tasks including spatial working memory (A. F. Arnsten, Cai, & Goldman-Rakic, 1988; Cai, Ma, Xu, & Hu, 1993; Rama, Linnankoski, Tanila, Pertovaara, & Carlson, 1996) but not PFC-unrelated tasks, including visuospatial attention. As with dopamine, there may be a critical range of noradrenaline availability for spatial working memory, as low levels of noradrenaline result in activation of the beneficial alpha-2 receptors, while increasing levels engage the alpha-1 adrenergic receptors (Amy F. Arnsten, 1998) which impair prefrontal cognition via activation of secondary messenger pathways (A. F. Arnsten, Mathew, Ubriani, Taylor, & Li, 1999).

Serotonin (5HT) is theorised to oppose and modulate the action of dopamine in limbic and cortical regions (M. Luciana, Collins, & Depue, 1998). Dopamine and serotonin both innervate cortical and subcortical areas including PFC, hypothalamus, basal

ganglia and limbic forebrain, and receptors for both neurotransmitters overlap in cortical and sub-cortical areas (P. S. Goldman-Rakic, Lidow, & Gallager, 1990). The role of dopamine is to enhance the signal-to-noise ratio of information flowing along these pathways (Sawaguchi, Matsumura, & Kubota, 1988), while serotonin acts to modulate and restrain this flow (Azmitia & Gannon, 1986). 5HT agonists induce impairment in cognitive performance, including spatial working memory performance, in rats (Winter & Petti, 1987) and in humans (Grasby *et al.*, 1992; M. Luciana, Collins, & Depue, 1998). Similar impairments have been observed following the administration of selective serotonin reuptake inhibitors to psychiatric patients (Bartfai, Asberg, Martensson, & Gustavsson, 1991; Sofuoglu & Debattista, 1996).

It may be seen from this section that the neurological basis of the executive functions is reasonably well understood, and research is ongoing in an effort to refine this understanding. In parallel with studies of the neurobiological correlates of executive functions described here, a large body of research has attempted to uncover their genetic foundations. A number of studies have identified genetic markers which are thought to be related to cortical neurotransmission and cognitive activity, however before any discussion of the genetic underpinnings of specific executive functions, it must be established to what extent these functions are heritable.

1.2 Heritability of executive functions

'Heritability' may be defined as the proportion of phenotypic variance accounted for by genetic variance. The 'broad-sense' definition of heritability includes all sources of

genetic variation, including dominance factors and epistasis. Narrow sense heritability, on the other hand, refers only to allelic variation, and it is this type of heritability which will be discussed in this thesis. The preferred method of establishing heritability of a cognitive function is the twin study. Twins (monozygotic or dizygotic) who have been raised together are assumed to have been exposed to similar environmental influences, both before and after birth. Monozygotic twins share 100% of their genetic material, while dizygotic twins share, on average, only 50%. Comparison of the average correlation between monozygotic and dizygotic twin pairs on a cognitive measure allows us to parse the variance due to genetic similarity, common (shared) environmental influences and non-shared environmental factors. This allows the calculation of h^2, an estimate of the heritability of performance on that measure. A number of studies have attempted to establish the heritability of executive functions, including conflict resolution, sustained attention and working memory. The studies described below were all conducted with samples of healthy monozygotic and dizygotic twins.

The literature on the heritability of executive control or conflict resolution is somewhat contradictory; while Fan, Wu, Fossella & Posner (2001) found conflict resolution in a flanker task known as the Attention Network Task (ANT) in which participants must respond to the direction of a central arrow while ignoring the direction of flanking arrows to be highly heritable ($h^2 = .72$), another larger study found no evidence for heritability of a similar task (Stins, van Baal, Polderman, Verhulst, & Boomsma, 2004). The authors did however find performance on a Stroop task to be heritable. The Stroop

task presents colour names (e.g. 'red', 'blue', 'green') printed in a variety of ink colours. Participants are instructed to respond to the colour of the ink only, and must therefore override the automatic tendency to react to the semantic meaning of the word. This task involves a high degree of conflict resolution as participants choose between competing responses. Stins *et al.* (2004) found both time to complete and the interference effect in the task to be highly heritable (h^2 = .7 and .5 respectively). The results of heritability studies employing the Wisconsin Card Sorting Test, a test of global executive functioning, are similarly inconclusive. While one study described moderate heritability of performance on the WCST (Anokhin, Heath, & Ralano, 2003), another found no evidence of heritability at all (Campana, Macciardi, Gambini, & Scarone, 1996). It should be noted, however that this study made use of a relatively small sample and may have been underpowered. Executive function deficits, as assessed by a questionnaire-style inventory, have been shown to be highly heritable, with a h^2 of .76 (Coolidge, Thede, & Jang, 2004). Discrepancies between the results described above may be due to several factors, including slight differences in the tasks used, the age of the participants and considerable variation in the sample sizes used.

Sustained attention appears to be moderately heritable, at least in children; Heiser, Heinzel-Gutenbrunner, Frey, Smidt, Grabarkiewicz, Friedel *et al.* (2006) found children's accuracy in a sustained attention task to have a h^2 of .28, while mean tempo on a sustained attention task was found to have a heritability of .46 for girls and .72 for boys (Groot, de Sonneville, Stins, & Boomsma, 2004). The conceptually similar process of alerting was also found to be somewhat heritable in a small sample of adult

twins (Fan *et al.*, 2001). Other facets of attention may also be heritable; for example, selective attention was shown in one study to have a heritability of .41 (Myles-Worsley & Coon, 1997). Another study was not able to demonstrate any heritability of selective attention, although familial resemblances were observed (Stins *et al.*, 2005). In contrast, orienting ability, as measured by the ANT, does not appear to be heritable at all (Fan *et al.*, 2001).

Working memory in its various forms appears to be moderately heritable, with h^2 values varying between .33 and .54. This includes analysis of the heritability of memory span, estimates of which vary from .33 (Wright *et al.*, 2001) to .52 (Jensen & Marisi, 1979), spatial and verbal storage, for which heritability has been calculated as .45 and .48 respectively (Ando, Ono, & Wright, 2001), and memory executive which displays heritability in the range .43-.48 (Ando, Ono, & Wright, 2001; Wright *et al.*, 2001).

Calculating the heritability of specific executive functions can be problematic for a number of reasons, not least of which is the fact that these functions frequently overlap in the tasks used to assess them. Tests of response inhibition, for example, require that the target be maintained in working memory, and also contain a sustained attention component. This overlapping does however allow the assessment of common factors influencing the various executive functions. One recent study employed a latent variable approach to establish that there is indeed a common factor underlying executive functions, and that it is almost entirely heritable (Friedman *et al.*, 2008). The

similarity between executive functions may be explained by this factor, while individual differences in performance of tasks tapping the various executive functions may be explained by other genetic influences specific to each task, and may themselves be heritable.

Given that the executive functions under discussion in this thesis are for the most part at least moderately heritable, analysis of the genetic architecture of these traits is appropriate.

1.3 fMRI as an endophenotype

The most common form of cognitive-genetic study strives to elucidate links between genetic polymorphisms and cognitive functions, as measured using neuropsychological tests. Many of these tests arose from the search for endophenotypes, measurable quantitative traits that lie intermediate between gene function and behaviour, and can be more clearly linked to genetic variation (Gottesman & Gould, 2003). While many valuable results have emerged from studies of this nature, they are subject to several criticisms. It was initially anticipated that these endophenotypes would result in larger effect sizes than diagnostic categories alone, however meta analyses have shown that this is not in fact the case for most endophenotypes that have been studied to date (Flint & Munafo, 2007). In addition, neuropsychological tasks are presumed to recruit specific brain networks, and interpretations of results based on this assumption across a variety of case and control populations. Neuroimaging technologies may therefore be a

more suitable assay of underlying genetic influences than purely behavioural measures, allowing us to determine more accurately the regions activated during specific task conditions within specific population groups.

A phenomenon frequently observed is the detection of genetic effects on fMRI measures, but not on the corresponding task performance (e.g. Bertolino, Blasi *et al.*, 2006; Fan, Fossella, Sommer, Wu, & Posner, 2003). Differences between groups on behavioural measures may not be observed for a number of reasons, not least of which is an insufficiency of statistical power in the analysis. The number of genes contributing to variation in an intermediate phenotype such as performance on a cognitive task may be smaller than that for a disease phenotype. Nevertheless, it is likely that any complex cognitive function will be under the influence of enough genetic factors that variation at each locus has only a small effect on performance (T. E. Goldberg & Weinberger, 2004). As a result, association studies generally require very large samples, in the order of hundreds, in order to achieve the necessary power to detect these small effects. As fMRI is in theory a more immediate phenotype than neuropsychological testing (Gottesman & Gould, 2003; Ahmad R. Hariri & Weinberger, 2003), under the influence of fewer intervening factors, genetic effect sizes may be larger, and therefore detectable with smaller samples. A number of studies have in fact demonstrated that fMRI can detect effects of specific genetic markers with much smaller samples than those required to find the same effects on behavioural measures (e.g. Egan *et al.*, 2001; Passamonti *et al.*, 2006).

While the disparity in neuropsychological task performance between clinical populations and controls is often very large and easy to detect, differences within healthy populations as a result of slight genetic variation can be much more subtle. Task performance in healthy populations is generally high to begin with, and ceiling effects in behavioural performance are not uncommon. As a result, many neuropsychological tests are simply not sensitive enough to discriminate between healthy participants with different genotypes, even with very large samples. Neuroimaging methods such as fMRI do not suffer from this problem and may therefore provide more sensitive endophenotypes for disorders of the executive system than behavioural testing alone.

1.4 Aims of the study

The first study described in this thesis (discussed in detail in Chapter 2) aims to investigate the genetic substrates of attention and spatial working memory. In examining the foundations of a cognitive function, it is often useful to rely on research into disorders in which those functions are compromised. Many of the hypotheses presented here regarding the attentional system are drawn at least in part from studies of attention-deficit hyperactivity disorder (ADHD), and occasionally from studies of schizophrenia. While research into the genetic underpinnings of these disorders is frequently very informative, care must be taken in extrapolating these results to healthy populations, as other mechanisms of disorder besides the system of interest may have played a role in determining the outcome for the patient group. The neurobiology of a

range of executive functions has been described in detail above. The critical role of the catecholamines in these functions points to the genes controlling the catecholamine system as likely targets for genetic studies of these functions. The role played by particular genes in controlling executive functions will be described in detail in later chapters.

The second study described here is an fMRI investigation of both the maintenance and manipulation aspects of spatial working memory. This study is discussed in Chapter 3. As discussed in section 1.3 above, fMRI may provide sensitive measure of genetic effects on executive functions such as spatial working memory. It is therefore predicted that this study will provide insights into the genetic architecture of spatial working memory greater than what can be achieved from behavioural testing alone.

Chapter 2

Behavioural Study

2.1 Introduction

In Chapter 1, the close relationship between executive functions and the neurotransmitters dopamine, noradrenaline and serotonin was described. The extent to which the genes controlling the action of these neurotransmitters influence individual cognitive abilities has been the subject of much study, and is the focus of this chapter. The COMT, DBH, DAT1, DRD4, MAOA and 5HTT genes are of particular interest here. This introduction provides a brief description of the function of each of these genes, and describes the literature relating them to the executive functions under discussion, *viz.* executive control of attention, sustained attention, spatial attention and spatial working memory. A wide variety of neuropsychological tests of executive function are described in this section. Some of these comprise detailed examinations of specific functions, while others are concerned with more general measures of executive function.

2.1.1 Genes of interest

2.1.1.1 COMT

The COMT gene codes for the catechol *O*-methyltransferase enzyme, which is involved in the degradation of extracellular dopamine. It is thought that COMT may play a role in prefrontal cognition as less dopamine transporter is found in the prefrontal cortex than in other areas, conferring greater responsibility on the COMT enzyme for the clearing of dopamine (Sesack, Hawrylack, Matus, Guido, & Levey, 1998). The principle functional polymorphism in the COMT gene is a Valine to

Methionine transition in Exon III. The enzyme produced by the Met allele of the gene is 4 times less active than the Val allele and breaks down dopamine more slowly (Lachman *et al.*, 1996). The Met allele is therefore thought to result in higher levels of prefrontal dopamine. The effect of this polymorphism on cognitive functions is still a matter for debate, and much of the research on this topic has been conducted on clinical populations, in particular patients with schizophrenia.

The Wisconsin Card Sorting Test (WCST`; Berg, 1948) is frequently considered to be a good general measure of frontal-executive functions, incorporating elements of attention, working memory and set-shifting. Studies of the effects of the COMT Val/Met polymorphism on performance in the WCST have repeatedly shown superior performance to be associated with the Met allele in individuals with schizophrenia (Bruder *et al.*, 2005; Egan *et al.*, 2001; Joober *et al.*, 2002; Malhotra *et al.*, 2002; Rosa *et al.*, 2004) their healthy siblings (Egan *et al.*, 2001; Rosa *et al.*, 2004) and healthy controls (Bruder *et al.*, 2005; Egan *et al.*, 2001; Joober *et al.*, 2002; Malhotra *et al.*, 2002), although other studies failed to replicate this finding in schizophrenic samples (Bilder *et al.*, 2002; Rosa *et al.*, 2004) and control samples (Aguilera *et al.*, 2008; Joober *et al.*, 2002; Tsai *et al.*, 2003). The effect of the Val/Met polymorphism on WCST performance has been shown to be unrelated to either clinical diagnosis (Egan *et al.*, 2001) or general attention and working memory ability (Bruder *et al.*, 2005), but a number of studies have indicated that it may be sensitive to population differences as the significance of some results was reduced when the analysis was restricted to Caucasian participants (Bruder *et al.*, 2005; Malhotra *et al.*, 2002).

The relationship between the COMT Val allele and less efficient executive functions was confirmed in a normal population where a single factor measure of executive function emerged from factor analysis of three tasks, including a working memory task, verbal fluency task and the Tower of Hanoi task (de Frias *et al.*, 2005). Possession of the Val allele was associated with poorer executive performance at both initial and follow-up testing, and greater decline in performance over a five-year period was observed in participants homozygous for the Val allele. Increasing Met allele dosage has also been associated with improved accuracy in a test of attentional control in a healthy population (Blasi *et al.*, 2005). One study in a schizophrenic population found that possession of the Met allele was associated with better cognitive stability, but poorer cognitive flexibility, as measured by a dual task (Nolan, Bilder, Lachman, & Volavka, 2004). COMT genotype accounted for 28-41% of variance in performance in that task. In contrast with these findings, Fossella, Sommer, Fan, Wu, Swanson, Pfaff *et al.* (2002) found homozygosity for the Met allele to be associated with a significantly *less* efficient executive control network, as measured by the Attention Network Task (ANT).

Sustained attention has been examined in relation to the COMT gene in healthy populations as well as in samples of individuals with schizophrenia and ADHD, however no clear trend can be discerned in the results of these studies. Possession of the Met allele was associated with poorer sustained attention in one sample of ADHD probands (M. A. Bellgrove, Domschke *et al.*, 2005) but not in another (Mills *et al.*, 2004) and had no effect in a sample of individuals with schizophrenia (T.E. Goldberg

et al., 2003). No effect of COMT was found on sustained attention in two studies with healthy populations (T.E. Goldberg *et al.*, 2003; Stefanis *et al.*, 2004). Similarly, the efficiency of the Alerting network (as measured by the ANT) was not affected by COMT genotype (Fossella *et al.*, 2002).

As discussed above, possession of the COMT Met allele has frequently been linked with superior performance on prefrontal-dependent cognitive tasks (Diamond *et al.*, 2004; Egan *et al.*, 2001; T.E. Goldberg *et al.*, 2003), however the relationship between COMT genotype and working memory is unclear, with many contradictory findings having been reported. Goldberg *et al.* (2003) found an effect of COMT on an n-back task, where participants respond to stimuli presented a specified number of trials previously. Requiring the manipulation and updating of information in working memory, this type of task is thought to rely heavily on the prefrontal cortex (Carlson *et al.*, 1998; Jansma *et al.*, 2000). Met/Met participants in this study performed better on both the 1-back and 2-back conditions than Val/Val individuals. There was no effect of genotype on the 0-back (control) condition or on a continuous performance task (CPT) tapping attention and vigilance, suggesting that the effects of COMT were important in the performance of the more difficult working memory conditions. Conversely, other studies have found no effect of COMT genotype on n-back task performance in both healthy populations (Bertolino, Blasi *et al.*, 2006; Bruder *et al.*, 2005; Stefanis *et al.*, 2004) and ADHD samples (Mills *et al.*, 2004). Bilder *et al.* (2002) similarly found no effect of COMT on memory measures, but did find an effect on measures of processing speed and attention.

2.1.1.2 DBH

DBH encodes the dopamine β-hydroxylase (DβH) enzyme which catalyses the synthesis of noradrenaline from dopamine (Kaufman & Friedman, 1965). Released into synapses at the same time as the catecholamines (A. D. Smith, de Potter, Moerman, & de Scaepdryver, 1970), DβH plays a critical role in controlling the balance of dopamine and noradrenaline available in the cortex. Lower levels of DβH in the plasma may result in elevated dopamine levels (Sternberg *et al.*, 1983) and have been associated with diagnoses of ADHD and conduct disorder (Rogeness *et al.*, 1984; Rogeness *et al.*, 1989).

A number of polymorphisms at and near the DBH gene have been associated with variation in DβH activity, most notably a C/T single nucleotide polymorphism (SNP) at position -1021 in the promoter region of the gene which accounts for 35-52% of variation in plasma DβH activity (Zabetian *et al.*, 2001). The T allele is considerably less frequent than the C allele, with a minor allele frequency of approximately .22 in European populations (Zabetian *et al.*, 2001). In all populations studied, individuals homozygous for the T allele, heterozygous, and homozygous for the C allele displayed very low, intermediate and high DβH plasma activity levels respectively. It has been suggested that the T allele of C-1021T diminishes gene transcription, resulting in lower levels of DβH than the C allele (Cubells & Zabetian, 2004). Although no direct research has been carried out into the effect of the DBH C-1021T marker on catecholamine levels in the human brain, a study on DBH knockout mice (Bourdelat-Parks *et al.*, 2005) has demonstrated that the gene directly affects the balance of

catecholamines in the prefrontal cortex. The T allele at the C-1021T locus is hypothesised to result in greater availability of dopamine, and comparatively lower availability of noradrenaline in the cortex.

No studies to date have described an association between the DBH gene and sustained attention in a healthy population, however the relationship of this gene to attention has been studied in ADHD populations. One of the tasks frequently used to assess sustained attention is the Sustained Attention to Response Task (SART`; I.H. Robertson *et al.*, 1997) where participants respond to presentation of the single digits 1-9, but withhold their response to presentation of the number 3. The SART involves elements of both sustained attention and response inhibition processes, as participants must remain vigilant throughout the task and continue to respond to all go trials, while withholding response to no-go trials. Two versions of the SART have been developed; in the 'Fixed' SART, the numbers 1-9 are presented sequentially, while in the 'Random' SART they are presented in no fixed order.

An association with the Fixed SART in an ADHD sample has been clearly demonstrated for a *TaqI* polymorphism in the fifth intron of the DBH gene (M. A. Bellgrove, Hawi, Gill, & Robertson, 2006), although studies examining the relationship of this polymorphism to ADHD diagnosis have produced contradictory results (Daly, Hawi, Fitzgerald, & Gill, 1999; Roman *et al.*, 2002; K. M. Smith *et al.*, 2003). The TaqI SNP is in linkage disequilibrium with the C-1021T marker (Tang *et al.*, 2006), which probably accounts for most of its influence on cognitive functions, however Tang *et al.* suggest that it may also have a small independent effect on plasma DβH

levels. Another study found that genotype at the functional C-1021T locus was associated with performance in a CPT in a sample of children with ADHD. In that study, participants in possession of two copies of the C allele made more errors of commission and omission than those with any copies of the T allele (Kieling, Genro, Hutz, & Rohde, 2008).

An effect of the G444A marker of the DBH gene on spatial working memory performance has been demonstrated (Parasuraman *et al.*, 2005). Increasing number of G alleles was linked with increasing accuracy on the higher loads of a spatial working memory storage task, but not on a visuospatial attention task. The G444A marker has previously been shown to be in strong LD with the putative functional marker in DBH, C-1021T, however no links with spatial working memory have been demonstrated for that polymorphism.

2.1.1.3 DAT1

The DAT1 gene codes for the dopamine transporter, responsible, in part, for the clearing of dopamine from the synapse. A number of polymorphisms have been identified in this gene. Although a variable number of tandem repeats (VNTR) polymorphism in Intron 8 of the gene has been examined by several groups, the majority of cognitive studies have examined a VNTR located in the 3′ untranslated region (UTR) of the gene. The fact that neither of these polymorphisms is located in a coding region of the gene has led to some debate over their functionality, particularly as several studies have reported an association of the 10-repeat allele of the 3′ UTR VNTR with a diagnosis of ADHD (Cook *et al.*, 1995; Daly *et al.*, 1999; Gill, Daly,

Heron, Hawi, & Fitzgerald, 1997). More recent research has indicated that, while the UTR of a gene does not alter amino acid sequence, it may regulate other functions of the gene including protein synthesis (Conne, Stutz, & Vassalli, 2000; Mignone, Gissi, Liuni, & Pesole, 2002; VanNess, Owens, & Kilts, 2005). The 10-repeat allele has been shown *in vitro* to result in 50% greater dopamine transporter density than the 9-repeat allele. This ought to result in a marked decrease in the level of dopamine at the synapse, however while some studies have described similar findings *in vivo* (e.g. Cheon, Ryu, Kim, & Cho, 2005; Heinz *et al.*, 2000), others have reported contradictory results (Martinez *et al.*, 2001; van Dyck *et al.*, 2005).

The 10-repeat allele of the DAT1 gene has been linked with poorer performance on sustained attention tasks within ADHD populations; participants homozygous for the 10-repeat allele committed more errors of commission in a CPT and displayed greater response variability than participants with one or no copies of the 10-repeat allele (Loo *et al.*, 2003). Similarly, 10-repeat homozygotes were also found to display greater response variability in the Fixed SART, although no effect of DAT1 genotype on performance accuracy was observed in that study (M. A. Bellgrove, Hawi, Kirley, Gill, & Robertson, 2005). There was no association of DAT1 genotype with a measure of sustained attention in a sample of healthy children (Cornish *et al.*, 2005), however children who were homozygous for the 10-repeat allele performed worse on a test of selective attention than those with only one copy of the allele. DAT1 3' VNTR also had no effect on performance on the alerting network of the ANT (Fossella *et al.*, 2002). Possession of the DAT1 10-repeat allele was however associated with slightly higher

(i.e. less efficient) executive control scores than those found in individuals with no copies of the allele (Fossella *et al.*, 2002).

Variation in the DAT1 gene has also been reported to affect spatial attention; Bellgrove and colleagues found that children with ADHD in possession of 2 copies of the 10-repeat allele showed an attenuated left spatial bias relative to those with one or no copies of the allele (M. A. Bellgrove, Hawi, Kirley, Gill *et al.*, 2005). This effect of homozygosity for the 10-repeat allele on left-sided inattention was most pronounced in those children who showed a very good response to treatment with methylphenidate medication, suggesting an interaction with an underlying hypodopaminergic state. A similar effect was found in a sample of healthy children who performed a spatial orienting task. Children homozygous for the 10-repeat allele displayed left inattention, while heterozygotes did not (M. A. Bellgrove, Chambers, Johnson, Dáibhis *et al.*, 2007).

Several studies have examined the relationship between the DAT1 gene and working memory, however DAT1 genotype was reported to have no effect on performance of a range of working memory tasks in healthy children (Cornish *et al.*, 2005) or on performance of an n-back task in healthy adults (Bertolino, Blasi *et al.*, 2006). Similarly, DAT1 had no effect on WCST performance in a sample of individuals with schizophrenia (Szekeres *et al.*, 2004)

2.1.1.4 DRD4

The DRD4 gene codes for the dopamine D4 receptor. Three polymorphisms in this gene are of interest in this study. A 48bp VNTR in Exon III has been identified, the 2- and 4-repeat alleles of which code for a D4 receptor that reacts twice as strongly to dopamine as the 7-repeat variant (Asghari, Sanyal, & Buchwaldt, 1995). This 7-repeat allele has been repeatedly associated with a diagnosis of ADHD, although some studies have failed to replicate this result (see Li, Sham, Owen, & He, 2006 for a meta analysis of this literature). Two SNPs in the 5′ promoter region of the gene have also been identified. One, a C to T change at position -521, has been shown to affect transcription, with the T allele resulting in 40% less transcription than the C allele (Okuyama, Ishiguro, Toru, & Arinami, 1999). The other SNP, a C to G change at position -616, has been associated with the diagnosis of ADHD (Lowe *et al.*, 2004). Although this SNP has not been shown to affect gene function, its location in the promoter region of the gene suggests a possible role in controlling transcription levels of the gene (Barr *et al.*, 2001).

Performance on the executive control network of the ANT has been found to be associated with possession of the 4-repeat allele of the DRD4 Exon III VNTR. Participants with one or two copies of the 4-repeat allele showed significantly higher executive control scores, indicating a less efficient network, than those without the 4-repeat allele (Fossella *et al.*, 2002). A trend was observed in the same study for higher executive control network scores (indicating poorer efficiency) with increasing T allele

dosage of the DRD4 -521 C/T SNP, however no effect of this marker on WCST performance was observed in a schizophrenic sample (Rybakowski *et al.*, 2005).

Variation in the DRD4 gene has been associated with differences in sustained attention capacity in an ADHD sample. Bellgrove, Hawi, Lowe, Kirley, Robertson and Gill (2005) found the T allele of the DRD4 -521 SNP, which results in lowered gene transcription levels, to be associated with more errors of commission on the Random SART than the C allele. There was no effect of the -616 C/G SNP in this study. The authors also found that ADHD probands in possession of the 7-repeat allele of the DRD4 Exon III VNTR performed better in terms of errors and response variability in the Random SART than the 7-absent group. The 7-present group did not differ from a control group without ADHD. This supports previous findings indicating that the 7-repeat allele may be protective against the reaction time variabilities and deficits generally seen in ADHD (Swanson *et al.*, 2000). In contrast, Langley, Marshall, van den Bree, Thomas, Owen, O'Donovan *et al.* (2004) found no effect of the Exon III VNTR on performance of a CPT in an ADHD population, and no association of any DRD4 marker with the efficiency of the ANT alerting network was observed in a sample of healthy participants (Fossella *et al.*, 2002).

While no main effect of DRD4 genotype on working memory performance has been reported, Alfimova, Golimbet, Gritsenko, Lezheiko, Abramova, Strel'tsova *et al.* (2007) have described an interaction between DRD4 and COMT genotype. In a sample of healthy controls and individuals with schizophrenia, participants homozygous for the

Val allele of the COMT gene and the G allele at position -809 on the DRD4 gene performed better on a working memory task than participants with other genotypes.

2.1.1.5 MAOA

The MAOA gene codes for the enzyme monoamine oxidase A which is responsible for the degradation of dopamine, noradrenaline and serotonin. The gene is located on the X chromosome; males therefore have only one copy of the MAOA locus while females have two copies, one of which is inactivated. A 30bp VNTR in the promoter region of the gene has been identified, the most common variants of which are the 3-repeat and 4-repeat alleles. The 4-repeat allele has been shown to result in five times more gene transcription than the 3-repeat allele (Sabol, Hu, & Hamer, 1998); individuals in possession of the 3-repeat allele would therefore be expected to have relatively higher levels of dopamine than carriers of the 4-repeat allele.

The MAOA 30bp promoter polymorphism has been shown to affect executive control, as measured by the ANT. Participants in possession of the 4-repeat allele (associated with lower levels of dopamine) performed better on the executive control component of the task than 3-repeat carriers (Fan *et al.*, 2003; Fossella *et al.*, 2002). Despite this evidence for the involvement of MAOA in executive functions, no association was found between MAOA genotype and WCST performance in a sample of Chinese females (Yu *et al.*, 2005).

The 4-repeat allele MAOA allele was associated with a more efficient ANT alerting network in a sample of healthy participants (Fossella *et al.*, 2002), however ADHD probands in possession of the long (4- or 5-repeat) alleles of this polymorphism made significantly more errors of commission on a test of sustained attention than those with the short (3-repeat) allele (Manor *et al.*, 2002). The fact that the long MAOA alleles are associated with increased MAOA activity may explain this, as the resulting decrease in cortical dopamine levels adds to the dopamine deficit already thought to be present in ADHD. In support of this, the attention deficit observed in this study was ameliorated by the introduction of methylphenidate (Manor *et al.*, 2002).

2.1.1.6 5HTT

The 5HTT (5-hydroxy-tryptamine transporter) gene controls the production and action of serotonin transporter molecules, which are primarily responsible for the reuptake of serotonin from the synaptic cleft. An insertion/deletion polymorphism has been identified in the 5HTT linked polymorphic region (5HTTLPR) in the promoter of the gene. The 'long' allele of the gene, which includes the 44bp insertion, results in roughly three times more gene transcription than the dominant 'short' allele (Heils *et al.*, 1996). Serotonin would therefore be expected to be cleared more slowly from the synapse in carriers of the short allele.

While 5HTT has been the subject of a number of studies of ADHD and emotional disorders, in particular depression, very little research has been conducted to examine its links to cognition and executive function. One study examined the effect of 5HTT

genotype on sustained attention in a healthy population, however no differences in performance on a CPT were observed (Fallgatter, Jatzke, Bartsch, Hamelbeck, & Lesch, 1999). Similarly, no effect of 5HTT was found on response inhibition in two studies (Clark *et al.*, 2005; Fallgatter *et al.*, 1999).

Summary

The findings described above are frequently confusing, and present difficulties for any attempt to draw conclusions about the role of the dopamine system, and the genes influencing it, in executive control. While studies of the COMT gene generally point towards improved performance of executive functions with the high-dopamine Met allele, the low-dopamine MAOA 4-repeat allele is also associated with better performance in the executive control domain. Findings are contradictory even within genes; Fossella *et al.* (2002) found decrements in executive attention with both the high-dopamine DRD4 4-repeat allele and the low-dopamine DRD4 -521T allele, and a number of studies (e.g. M. A. Bellgrove, Domschke *et al.*, 2005; Fossella *et al.*, 2002) have demonstrated impairments in performance to be associated with the generally beneficial COMT Met allele. Within the sustained attention domain, it is again difficult to discern clear trends, with later studies frequently failing to replicate apparently clear findings.

A number of possible explanations may account for the lack of consistent results from these studies. As discussed in Chapter 1, the extent to which specific facets of attention are heritable is by no means certain; for example, no study has clearly demonstrated the

heritability of spatial attention. Genetic results where heritability is uncertain should be treated with caution as the possibility exists that the results are entirely spurious. Another explanation for the lack of replication of many findings may simply reflect the inability of studies with very small samples to detect small genetic effects.

2.1.2 Aims and hypotheses

The aim of the present study was to examine the effect of a number of genetic polymorphisms in the dopaminergic and noradrenergic systems on performance in a battery of tasks designed to tap various aspects of executive function. The majority of these tasks are influenced by the same set of neurotransmitters, and the literature relating neurochemical factors to specific cognitive functions is frequently contradictory. This renders the formulation of discrete hypotheses regarding their genetic architecture rather problematic. Nevertheless, the following predictions are made, arising from previous findings and a knowledge of the neurological substrates of these functions.

1. The close relationship between sustained attention and noradrenaline was described in chapter 1. From this, it is hypothesised that DBH will affect sustained attention, as measured by the SART and the Alerting network of the ANT (SART and ANT alerting).
2. In keeping with previous research (particularly in ADHD populations) DAT1 genotype is predicted to affect sustained attention, as measured by the SART.

3. As DBH and DAT1 are both hypothesised to affect sustained attention, they may interact (additively or otherwise) to influence SART performance.
4. DAT1 will impact on spatial attentional bias as measured by the Landmark. This relationship has been observed in a number of previous studies.
5. Genes which directly alter the level of dopamine available in the cortex (e.g. DBH, COMT, and DAT1) will affect spatial working memory, independently and possibly in conjunction.
6. Reports from the literature vary, however an effect of COMT on executive control (as measured by the ANT) is tentatively predicted.
7. The MAOA 4-repeat allele has been linked with improved executive control and sustained attention in healthy participants, despite its 'low dopamine' nature. It is hypothesised that similar results will be observed here.
8. Although no associations have been reported between variation at the 5HTT locus and executive functions, administration of 5HT agonists has been linked with cognitive deficits. It is therefore tentatively suggested that the 'high serotonin' short allele may be associated with poorer performance in this sample.

2.2 Method

2.2.1 Participants

205 participants (84 male, 121 female) were recruited by means of an advertising campaign. 119 participants were paid a nominal sum (€10) for their participation, 80 received course credit and 6 were unpaid. All participants were right-handed, as determined by the Edinburgh Handedness Inventory, aged between 18 and 30 (mean age = 20.61 years, SD = 2.53) and had completed or were engaged in third level education at the time of testing. Exclusion criteria for participation included any history of ADHD, epilepsy or any other psychiatric or neurological disorder or serious head injury resulting in an open wound or loss of consciousness. Ethical approval for this project was obtained from the Ethics Committee of the School of Psychology, Trinity College Dublin.

A major consideration in the planning of genetic association studies is the problem of population stratification, whereby slight differences in performance across ethnic groups as a result of cultural and unaddressed genetic factors may confound the effect of the marker of interest. The best method of avoiding this problem is to test ethnically homogenous samples, and therefore every effort was made to restrict this sample to the Irish population. Ethnicity of participants was established by means of a self-report questionnaire detailing the national origins of the participants' 4 grandparents. 180 participants (89.5%) were classified as genetically Irish based on this measure. The

remaining 21 participants were classified variously as European (14), American (3), Australian (1), Indian (1) and Chinese (2).

2.2.2 Self-report measures

2.2.2.1 Cognitive Failures Questionnaire

The Cognitive Failures Questionnaire (CFQ`; Broadbent, Cooper, FitzGerald, & Parkes, 1982) measures everyday lapses in attention. The CFQ contains 25 items, and participants are asked to indicate on a 5 point Likert scale ranging from 'never' to 'very often' how frequently they have experienced particular slips of attention. The CFQ has been previously shown to be significantly correlated with lapses in sustained attention (I.H. Robertson *et al.*, 1997).

2.2.2.2 Hospital Anxiety and Depression Questionnaire

The Hospital Anxiety and Depression Questionnaire (HAD; Zigmond & Snaith, 1983) is a short measure of anxiety and depression, and was included as a screening measure to allow the exclusion of participants with levels of anxiety or depression that were likely to bias their cognitive performance. There are 7 items on each scale, with a possible maximum score of 21. A score of 16 on either scale is taken to indicate borderline anxiety or depression, while a score of 20 is considered severe.

2.2.3 Tasks

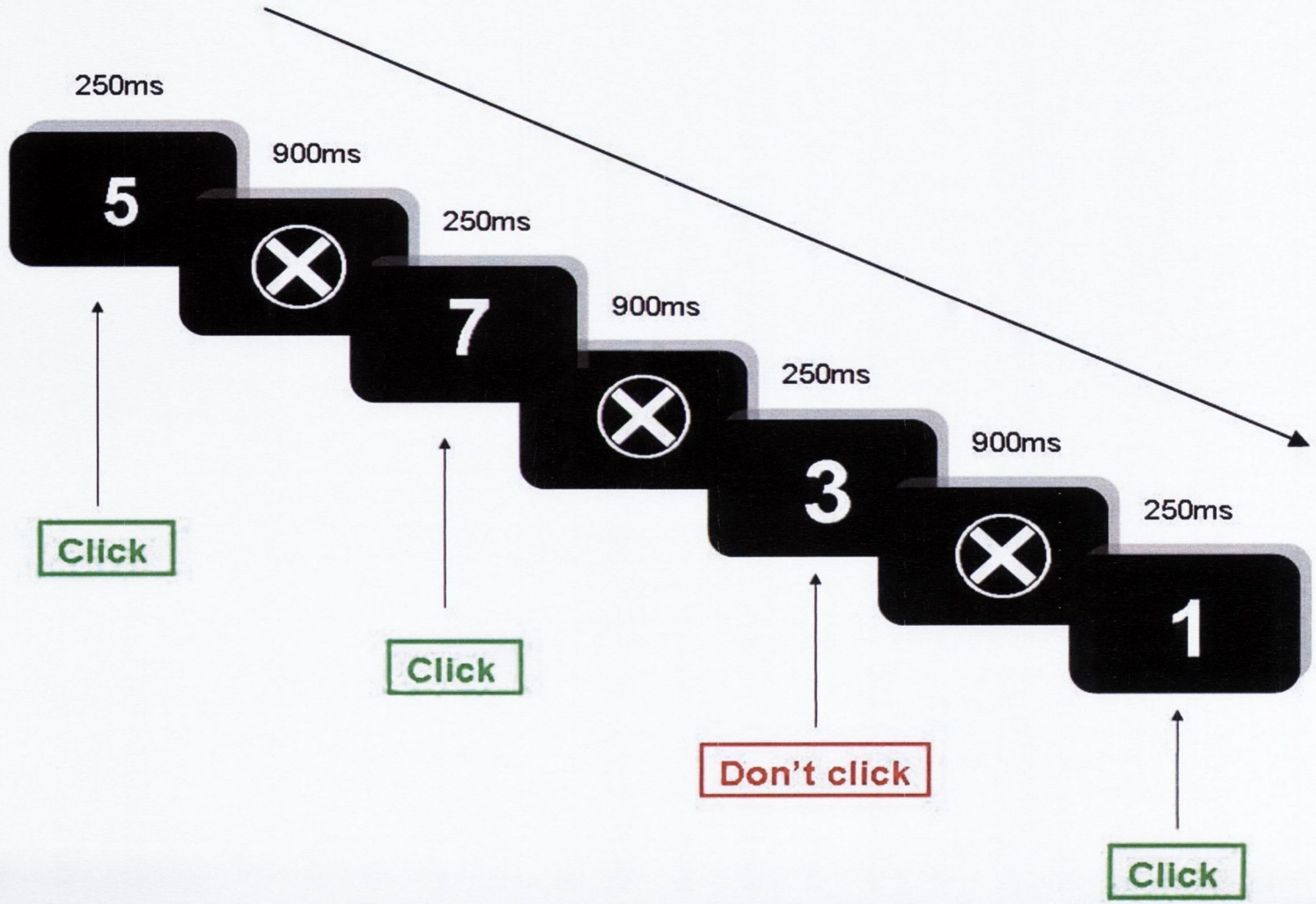
2.2.3.1 Sustained Attention to Response Task (SART)

Single digits between 1 and 9 were presented on screen and participants were required to respond with a button press to every number except 3. White digits in a variety of font sizes were presented for a period of 250ms on a black screen and were followed by a 900ms mask consisting of a white X in a circle that appeared to 'flash'. Participants were instructed to time their responses to coincide with this flash. Two versions of this task were performed; in the Fixed SART, the numbers 1 to 9 were presented in fixed numerical order such that the participant knew when to expect the target digit 3. In the Random SART, the digits were presented in pseudo-random fashion preventing the participant from anticipating the appearance of the target digit. Each block lasted approximately four minutes, and contained 225 trials. The number 3 was presented in 25 of these trials. Participants performed two blocks of each version of the SART. In each case, the number of errors of commission (button press when the number 3 was onscreen) and errors of omission (failure to press the button when any other number was onscreen) were calculated. Fig. 2.1 describes a sample sequence of the Random SART.

2.2.3.2 Attention Network Task (ANT)

The Attention Network Task (ANT), first described by Fan, McCandliss, Sommer, Raz and Posner (2002), allows the efficiency of the three attention networks (alerting, orienting and conflict) to be calculated. This test has been frequently used to assess

Fig. 2.1. Sample Random SART sequence

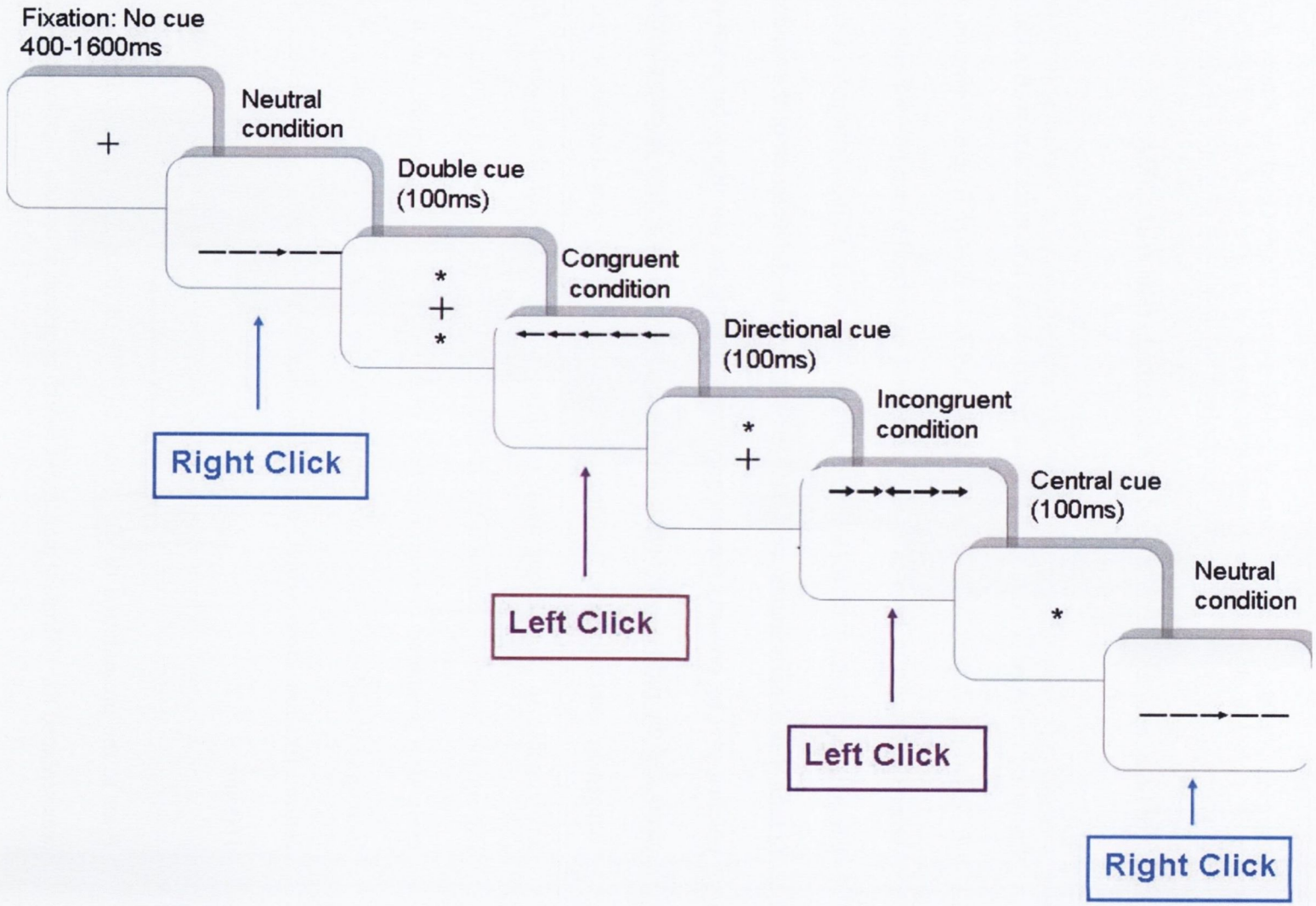


network efficiency in healthy controls (Fan *et al.*, 2005; Jennings, Dagenbach, Engle, & Funke, 2007), and people with ADHD (K. A. Johnson *et al.*, 2008; Oberlin, Alford, & Marrocco, 2005) and schizophrenia (Golimbet *et al.*, 2006; Gooding, Braun, & Studer, 2006). Test-retest reliabilities for the alerting, orienting and executive control networks have been reported as .52, .61 and .77 respectively (Fan *et al.*, 2002).

Each trial in the ANT consisted of a horizontal row of five black arrows (totalling 3.08° of visual angle) presented either above or below a central fixation point on a white screen. The target in each case was the central arrowhead which pointed either right or left and was 'flanked' on each side by two more arrows. Using a computer mouse held in both hands, participants were instructed to indicate the direction of the central arrow on each trial by pressing the left mouse button with the left thumb for a left-pointing arrow and the right mouse button with the right thumb for a right-pointing arrow. In the 'congruent' condition, the flanking arrows pointed in the same direction as the central arrow while in the 'incongruent' condition they pointed in the opposite direction. During the 'neutral' condition the flanking arrows were replaced by straight lines. 48 trials were presented in total; one third of these were congruent trials, one third were incongruent and one third were neutral. Before each trial, participants focused on a central fixation cross which remained onscreen for a period of between 400 and 1600ms.

Prior to the presentation of the arrows on 75% of the trials, the central fixation cross was replaced for 100ms by an asterisk which appeared at central fixation on some trials

Fig. 2.2. ANT task conditions

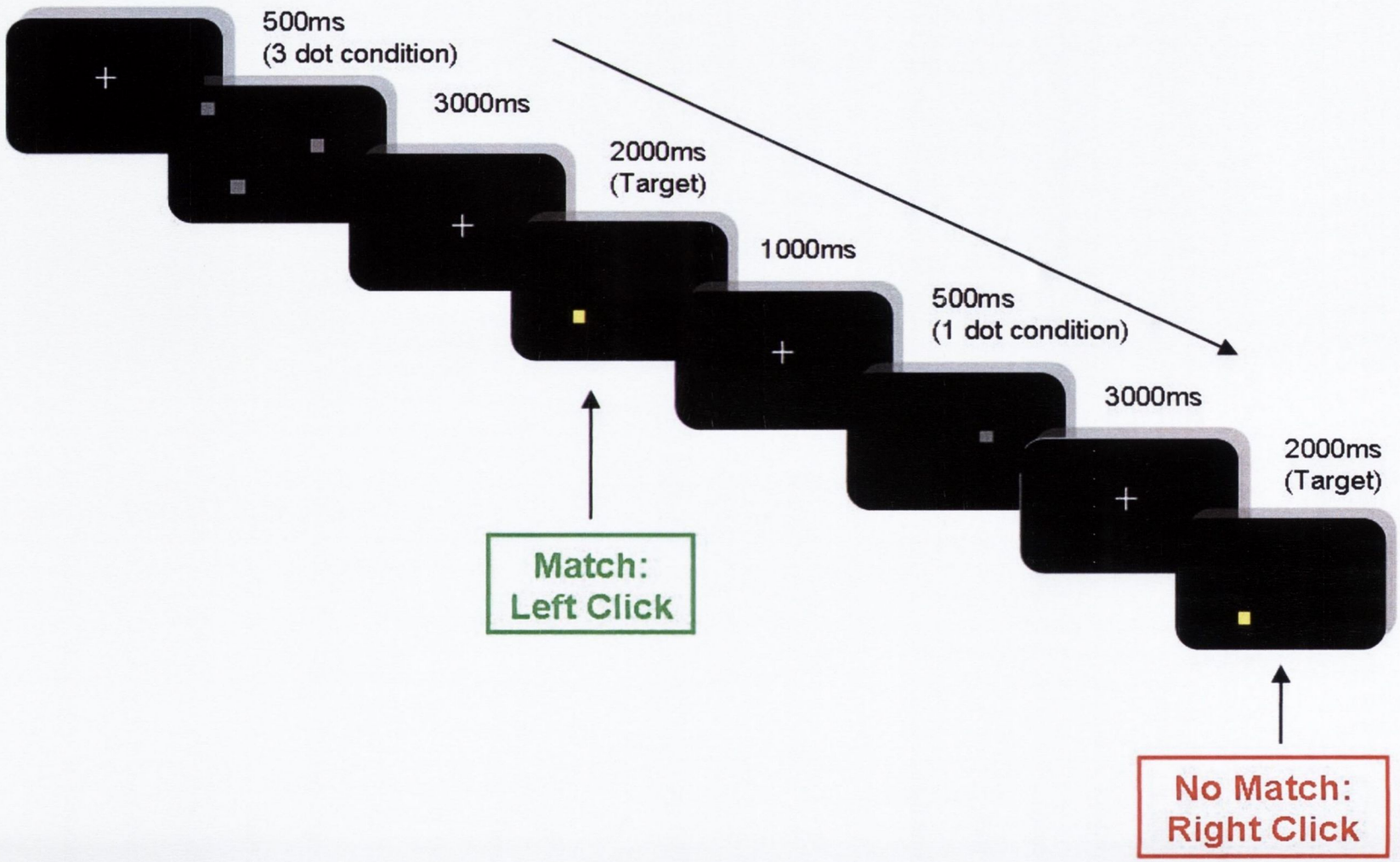


or above or below the fixation point on others. The presentation of this asterisk either centrally (centre cue, 12 trials) or simultaneously above *and* below the fixation point (double cue, 12 trials) acted as a temporal prompt and indicated that the arrows would shortly appear. Alternatively, its presentation *either above or below* fixation (up cue, 6 trials and down cue, 6 trials) provided a spatial cue to the onscreen location of the forthcoming arrows. The fixation cross then reappeared for 400ms before the presentation of the target and flanker arrows (see Fig. 2.2 for an illustration). The efficiency of the three attention networks was calculated from the resulting reaction time data.

2.2.3.3 Spatial Working Memory (SWM) Task

The spatial working memory task used here is based on that described in Parasuraman *et al.* (2005), a variant of one of the most frequently used tests of spatial working memory (J. Jonides *et al.*, 1993). This task, which requires participants to maintain in working memory the spatial locations of one, two or three stimuli over a short delay, has been shown to be a robust measure of spatial working memory. At the beginning of each trial, a white fixation cross (0.95° x 0.95° of visual angle) was presented centrally on a black screen for 1 second. Simultaneous with the disappearance of this cross, 1, 2 or 3 grey squares (0.67°) were presented in randomly distributed locations on the screen for a period of 500ms. After a 3 second delay, during which the fixation cross was presented, a yellow square (0.67°) appeared onscreen. Participants had 2 seconds to indicate by means of a mouse click whether the target (yellow) square was

Fig. 2.3. Spatial Working Memory Task



in the same location ('match'; 50% of trials) or a different location ('no match'; 50% of trials) as any of the grey squares. 252 trials were presented in total, 82 in each memory load category, and the order of presentation of the trial types was randomised. Participants identified 'match' trials with a left mouse click and 'no match' trials with a right mouse click. Mean accuracy (number of correct trials/total number of trials) and mean reaction time were calculated for each memory load level, defined by the number of grey squares presented. See Fig. 2.3 for an illustration of this task.

2.2.3.4 Landmark Task

Participants are presented with 20 sheets of paper containing a bisected line and are asked to indicate which side of the line is shorter. 50% of the lines are bisected exactly in the middle, while the remainder are bisected by a mark which is slightly offset to the left (25% of trials) or right (25% of trials). The degree of offset is varied; 6 of the 10 trials are offset by 1mm (3 to the right and 3 to the left), 2 are offset by 2.5mm (1 right, 1 left) and 2 by 5mm (1 right, 1 left).

2.2.4 Genotyping

2.2.4.1 DNA collection

Approximately 4ml of saliva was collected from each participant using Oragene DNA Self-Collection Kits, manufactured by DNA Genotek. Participants refrained from eating or drinking anything except water for at least one hour prior to collection of the sample, and were requested to rinse their mouths with water before depositing the saliva. In cases where participants experienced difficulty providing the sample, a small quantity of sugar was placed on the tongue to encourage salivation, in accordance with the manufacturer's advice.

2.2.4.2 DNA extraction

DNA was extracted from each saliva sample following the protocol provided by the manufacturers of the Oragene kits. Details can be found in Appendix I.

2.2.4.3 DNA quantification

The concentration of DNA in each sample (ng/µl) was determined by spectrophotometry using a NanoDrop^{TM} ND-1000 spectrophotometer. DNA concentration in the 205 samples tested ranged from 0.71ng/µl to 242.71ng/µl. A concentration of at least 10ng/µl was required for further analysis. 4 samples did not meet this requirement.

2.2.4.4 Genotyping process

Insertion / Deletions (Ins/Del) and Variable Tandem Nucleotide Repeats (VNTRs)

Four VNTRs (DAT1 3′ UTR and Intron 8, DRD4 Exon III and MAOA 30bp promoter region) and the 5HTT ins/del polymorphism were genotyped by polymerase chain reaction (PCR) and visualisation on agarose gels. The primers used are listed in Table 2.1 and details of the PCR conditions can be found in Appendix I.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Genotypes for the DBH TaqI, DBH G444A and MAOA 941 SNPS were ascertained using restriction fragment length polymorphism (RFLP) Analysis. This technique involves amplification of the region of the gene surrounding the marker of interest by PCR followed by digestion with a restriction enzyme. The enzyme cuts the fragment upon recognition of one allelic form of the marker but not the other, allowing the genotype of each sample to be ascertained. The PCR reaction mix for all three RFLP markers was composed similarly to that described for the VNTRs above. Restriction enzymes and buffers were obtained from New England Biolabs, Beverly, MA, USA. Details of the PCR and digestion conditions and restriction enzymes used for each marker can be found in Appendix I. The forward and reverse primers for each SNP are listed in Table 2.1.

Gel visualisation

PCR products were visualised on 2-3% (w/v) agarose gels stained with ethidium bromide. The gels were run in TAE buffer (x1) at -60 to -70V. The gels were then viewed on an ultra violet illuminator and photographed using either a Polaroid camera

or a Bio-Rad Molecular Imager Gel Doc XR System. PCR samples were mixed with 2µl loading dye prior to being loaded on to the gel along with appropriate size standards.

TaqMan Allelic Discrimination

The DBH C-1021T (rs1611115) and COMT Val/Met (rs4680) markers were genotyped using TaqMan assays for allelic discrimination (Applied Biosystems, Foster City, CA, USA). In each case, 3µl of DNA at 10ng/µl was analysed in a 6µl reaction with 0.125µl of the appropriate TaqMan probe and 2.5µl of TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were then run on the Applied Biosciences 7900HT Real Time PCR machine using the absolute quantification programme, and analysed using the SDS allele discrimination programme.

SNaPshot

Two SNPs in the promoter region of DRD4, -616 G/C and -521 C/T, were genotyped using the SNaPshot method (Applied Biosystems, Foster City, CA, USA). In this assay, a probe which is designed to lie adjacent to the SNP of interest is extended by one nucleotide using fluorescently labelled ddNTTPs. Each base is tagged with a different colour, which can then be read by an ABI Genetic Analyzer and used to identify the genotype of each sample. Details of the SNaPshot procedure for genotyping the DRD4 SNPs can be found in Appendix I, and the PCR and extension primers used are listed in Table 2.1.

Table 2.1: PCR primers for markers genotyped by PCR

Gene	Marker name	rs number	Primers
DAT1	3′ UTR VNTR	N/A	F – 5′ TGT GGT GTA GGG AAC GGC CTG AG 3′ R – 5′ CTT CCT GGA GCT CAC GGC TCA AGG 3′
	Intron 8 VNTR	N/A	F – 5′ GCT TGG GGA AGG AAG GG 3′ R – 5′ TGT GTG CGT GCA TGT GG 3′
DBH	G444A	rs1108580	F – 5′ GCA ATG AAT GCG GAG CTC TG 3′ R – 5′ GGC CCC AGA CTT ATC AGG GAC 3′
	TaqI	rs2519152	F – 5′ CTG TAT TTG GAA CTT GGC ATC 3′ R – 5′ AGG CAT TTT ACT ACC CAG AGG 3′
5HTT	Ins/del	N/A	F – 5′ GGC GTT GCC GCT CTG AAT GC 3′ R – 5′ GAG GGA CTG AGC TGG ACA ACC AC 3′
MAOA	941 G/T	rs6323	F – 5′ GAC CTT GAC TGC CAA GAT 3′ R – 5′ CTT CTC CTT CCA GAA GGC C 3′
	30bp promoter VNTR	N/A	F – 5′ CCC AGG CTG CTC CAG AAA C 3′ R – 5′ GGA CCT GGG CAG TTG TGC 3′
DRD4	Exon III VNTR	N/A	F – 5′ GCG ACT ACG TGG TCT ACT CG 3′ R – 5′ AGG ACC CTC ATG GCC TTG 3′
	-616 G/C	rs747302	PCR Primers: F – 5′ TCA ACT GTG CAA CGG GTG 3′ R – 5′ GAG AAA CCG ACA AGG ATG GA 3′ Extension Primer: 5′ TGG TCG CGG GGG CTG AG 3′
	-521 C/T	rs1800955	PCR Primers: F – 5′ TCA ACT GTG CAA CGG GTG 3′ R – 5′ GAG AAA CCG ACA AGG ATG GA 3′ Extension Primer: 5′ CTC GCC TCG ACC TCG TGC GC 3′

2.3 Results

2.3.1 Self-report measures

2.3.1.1 Cognitive Failures Questionnaire

Possible scores on the CFQ ranged from 0 to 100. Participants' scores ranged from 17 to 87 (mean = 42.49, SD = 11.96).

2.3.1.2 Hospital Anxiety and Depression Scale

The HAD contains 7 items in the depression scale and 7 in the anxiety scale. The maximum possible score on either scale is 21. All participants scored below 16 on both subscales (anxiety: mean = 7.8, SD = 3.63; depression: mean = 3.56, SD = 2.7). Levels of anxiety or depression were therefore unlikely to influence cognitive performance, and no participants were excluded from further analysis as a result.

2.3.2 Cognitive tests

An examination of the task results showed that many of the datasets were not normally distributed. Appropriate transformations were performed on the data, and subsequent analyses were conducted using the transformed datasets. A list of mean scores for all task measures before and after transformation may be found in Table 2.2. One-way analyses of variance indicated no significant differences in performance of any measure between male and female participants, or between participants classified as Irish and those from other ethnic backgrounds. As participants were all in the 18-30 age range,

with the majority of participants aged between 18 and 22, the effects of age on performance were not assessed.

Table 2.2: Means and standard deviations of performance scores before and after transformation

Measure	Raw mean	Raw SD	Transformation performed	Transformed mean	Transformed SD	N[1]
Landmark:						
Accuracy	82.3	11.23	None	-	-	204
Spatial bias	.071	.433	None	-	-	204
Random SART:						
Errors of commission	16.97	10.14	Square root	3.94	1.29	202
Errors of omission	3.29	6.08	Square root	1.25	1.3	205
Reaction time (ms)	386.7	87.28	Square root	19.55	2.16	205
Fixed SART:						
Errors of commission	4.06	3.34	Square root	1.86	.85	203
Errors of omission	5.08	6.7	Square root	1.74	1.43	198
Reaction time (ms)	358.38	95.34	Square root	18.78	2.43	203
ANT:[2]						
Conflict network	.197	.06	None	-	-	204
Alerting network	.079	.041	None	-	-	200
Orienting network	.093	.045	None	-	-	203
Accuracy - congruent	.988	.036	None	-	-	204
Accuracy – incongruent	.936	.065	None	-	-	204
Accuracy - neutral	.985	.031	None	-	-	204
SWM:						
Load 1 Accuracy	.89	.08	Square root	.31	.12	203
Load 1RT (ms)	581.9	132.87	Log_{10}	2.75	0.97	203
Load 2 Accuracy	.82	.09	Square root	.41	.1	203
Load 2 RT (ms)	665.11	141.46	Log_{10}	2.81	0.92	203
Load 3 Accuracy	.78	.09	Square root	.46	.1	203
Load 3RT (ms)	701.25	149.66	Log_{10}	2.84	0.93	203

[1]Sample size for each measure following exclusion of outliers.
[2]All ANT network scores are in the form of ratio scores (raw network score/overall mean RT).

2.3.2.1 Fixed and Random SART

205 participants performed the SART. Errors of commission (EoC; button presses when the target digit 3 was presented), errors of omission (failures to respond when any other digit was presented) and mean reaction time were calculated for both fixed and Random SART. Despite the instruction to participants that responses should be time-locked with the flashing X, mean reaction times varied between 239 and 696 ms in the Random SART and between 216 and 852 ms in the Fixed SART. None of the measures were normally distributed, displaying positive skew and kurtosis and significant Kolmogorov-Smirnov tests.

Table 2.3: Correlations between Fixed and Random SART and CFQ score

	Fixed SART			Random SART			*CFQ*
	EoCs	*EoOs*	*Mean RT*	*EoCs*	*EoOs*	*Mean RT*	*Score*
Fixed SART							
EoCs	1						
EoOs	.32**	1					
Mean RT	-.004	-.190**	1				
Random SART							
EoCs	.39**	.355**	-.49**	1			
EoOs	.25**	.329**	.121	.152*	1		
Mean RT	-.026	-.132	.671**	-.745**	.194**	1	
CFQ score	.067	.131	.049	-.011	.170*	.155*	1

EoCs = errors of commission
EoOs = errors of omission
Mean RT = mean reaction time

*Significant at .05 level
**Significant at .001 level

Large outliers, which were felt to reflect a failure to perform the task correctly rather than lapses in attention, were removed from the dataset. Square root transformations were performed on each of the measures, following which they more closely approximated a normal distribution. Performance on the different measures of the SART was quite strongly intercorrelated. Within both fixed and Random SART, errors of commission and omission were significantly correlated, while mean reaction time and errors of commission were very highly correlated in the Random SART, indicating that participants who made more errors also reacted more quickly. Standard deviation of reaction time, a measure of performance variability, was also calculated.

Errors of commission and omission between Fixed and Random SART also showed significant correlation at the .001 and .05 levels. Table 2.3 details these correlations. Correlations between SART performance and score on the CFQ, purported to measure lapses in everyday attention, are also listed. No significant correlations were observed between CFQ score and performance on the Fixed SART. While there was no correlation between Random SART errors of commission, significant correlations at the .05 level were observed between CFQ score and errors of omission and mean reaction time.

2.3.2.2 ANT

204 participants performed the ANT. The alerting network score was calculated by subtracting the mean reaction time (RT) of the double cue trials from the mean RT of the no cue trials. Neither of these conditions included any spatial element. A higher

score for this network indicates more efficient alerting. The orienting effect was calculated by subtracting the mean RT of the spatial cue trials from the mean RT of the centre cue trials to isolate the effect of a spatial cue. A higher score for this network indicates more efficient orienting. The score for the executive control network, responsible for resolving conflict, was calculated by subtracting the mean RT of the congruent trials from the mean RT of the incongruent trials. As participants tend to respond more slowly during uncued trials, some conflict resolution may take place during the resulting extended reaction time (Fossella *et al.*, 2002). In order to reduce the effect of this, the raw attention network scores were divided by the overall mean reaction time to give a ratio score. A higher score for this network indicates less efficient executive control.

Four outliers were removed from the alerting network, and one from the orienting network datasets, after which the data from all three networks were normally distributed and no transformations were required. The mean ratio scores and standard deviations calculated for each network are listed in Table 2.2. No significant correlations among the three attention networks were observed (see Table 2.4).

Table 2.4: Correlations between attentional networks in the ANT task

	Alerting	Orienting	Conflict
Alerting	1		
Orienting	.062	1	
Conflict	-.012	.052	1

Accuracy measures during the different trial conditions were also calculated. Accuracy was very high overall (mean = .975, SD = .023). Mean accuracy in the congruent, incongruent and neutral conditions is listed in Table 2.2. Accuracy data was not normally distributed, and so non-parametric tests were performed. A Friedman's ANOVA indicated that the three conditions differed significantly from one another [χ^2 (2) = 246.52, p<.001]. Wilcoxon's Signed-Rank tests were conducted post-hoc. A Bonferroni correction was applied to the post-hoc tests, resulting in an alpha level of .05/3 = .0167. Accuracy in the congruent condition found to be significantly higher than that in the neutral condition (T=2311, p<.0167) or the incongruent condition (T=214, p<.0167). The neutral condition also resulted in significantly higher accuracy than the incongruent condition (T=15166.5, p<.0167).

2.3.2.3 Spatial Working Memory

203 participants performed the spatial working memory (SWM) task. Accuracy measures at all 3 loads of the SWM task displayed very high levels of negative skew, and so were subjected to a square root transformation of the reversed data (1 – raw data value). The mean scores for each memory load are listed in Table 2.2. A repeated measures ANOVA indicated that accuracy decreased significantly with increasing memory load [F (2, 404) = 385.345, p<.001]. The reaction time data on all loads (see Table 2.2) displayed some positive skew and kurtosis. A $\log_{10}$ transformation on these data corrected the abnormality. A repeated measures ANOVA indicated that reaction time increased significantly with increasing memory load [F (2, 404) = 721.25, p<.001].

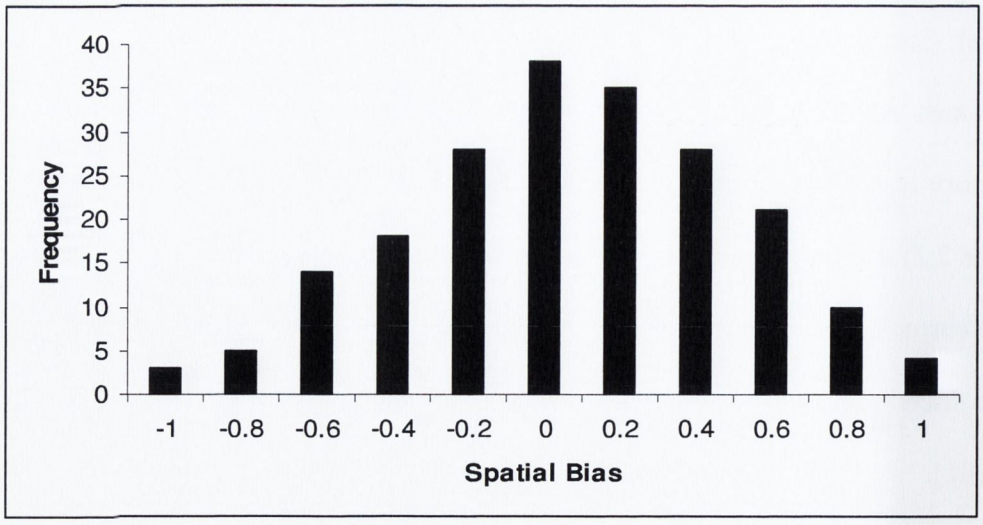
2.3.2.4 Landmark Task

204 participants performed the Landmark task. Mean accuracy (percentage of offset trials answered correctly) and spatial bias were calculated. Spatial bias was computed using the formula

$$\frac{L - R}{10}$$

where L is the number of evenly bisected lines which participants perceived to be shorter on the left and R is the number of evenly bisected lines perceived to be shorter on the right. This resulted in a score between -1 and 1, where positive numbers indicated right spatial bias, negative numbers indicated left spatial bias and 0 indicated no bias. Means and standard deviations for accuracy and spatial bias are listed in Table 2.2. All Landmark data were found to be normally distributed. The mean score of .071 indicated that participants on average displayed a slight rightwards bias. The distribution of spatial biases across the sample is depicted in Fig. 2.4.

Fig. 2.4. Frequency of Landmark bias scores



2.3.2 Genetic results

2.3.2.1 Hardy-Weinberg Equilibrium

Allele frequencies at each marker were tested for Hardy-Weinberg equilibrium. The Hardy-Weinberg principle assumes that, in a randomly mating population, allele frequencies are in equilibrium and are distributed in accordance with the equation

$$p^2 + 2pq + q^2 = 1$$

where p^2 = the probability of being homozygous for allele 1

$2pq$ = the probability of being heterozygous

q^2 = the probability of being homozygous for allele 2.

Deviation from Hardy-Weinberg equilibrium can be assessed using a Chi-square test according to the equation

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

where O = observed genotype frequency

E = expected genotype frequency.

A significant χ^2 value indicates deviation from Hardy-Weinberg equilibrium. This may result from genotyping errors, or may imply a lack of conformation with the Hardy-Weinberg assumptions, which are as follows: 1) an infinite population size, 2) discrete

generations, 3) random mating, 4) no selection, 5) no migration, 6) no mutation, 7) equal initial genotype frequencies in the two sexes.

Genotype frequencies and the results of Hardy-Weinberg analysis for all SNPs are displayed in Table 2.5. The allele frequencies of all markers except the MAOA 941 G/T SNP were found to be in Hardy-Weinberg equilibrium. The disequilibrium in the MAOA 941 marker appeared to have arisen as a result of problems in the genotyping process. As the results of any analysis on this polymorphism would therefore be unreliable, the marker was omitted from further study.

Table 2.5: Genotype frequencies and Hardy-Weinberg equilibrium for all SNPs

Gene	Marker	Genotype	Frequency	Hardy-Weinberg equilibrium
DBH	C-1021T	CC	113 (56.2%)	Frequencies in equilibrium
		CT	75 (37.3%)	
		TT	13 (6.5%)	
	G444A	GG	52 (26.4%)	Frequencies in equilibrium
		GA	103 (52.3%)	
		AA	42 (21.3%)	
	TaqI	CC	42 (21.3%)	Frequencies in equilibrium
		CT	109 (55.3%)	
		TT	46 (23.4%)	
DRD4	-521 C/T	CC	32 (16.7%)	Frequencies in equilibrium
		CT	85 (44.5%)	
		TT	74 (38.8%)	
	-616 C/G	CC	84 (47.5%)	Frequencies in equilibrium
		CG	75 (42.4%)	
		GG	18 (10.1%)	
COMT	Val/Met	Val/Val	53 (26.5%)	Frequencies in equilibrium
		Val/Met	94 (47%)	
		Met/Met	53 (26.5%)	
MAOA	941	GG	13 (6.9%)	Frequencies ***not*** in equilibrium
		GT	46 (24.3%)	
		TT	130 (68.8%)	
5HTT	Ins/Del	Ins/Ins	60 (31.9%)	Frequencies in equilibrium
		Ins/Del	97 (51.6%)	
		Del/Del	31 (16.5%)	

Calculation of Hardy-Weinberg equilibrium can be problematic for polymorphisms with rare alleles as all possible genotypes are likely to be represented only in very large samples, and Chi-square analysis is inappropriate where there are empty cells in the matrix. Genotypes in multi-allelic systems have therefore been collapsed into groups based on the presence of the allele of interest. This allows the polymorphism to be treated as a biallelic system for the purposes of Hardy-Weinberg analysis. Allele frequencies and Hardy-Weinberg analysis for the four VNTRs analysed are described below.

DAT1 3′ UTR VNTR

Four alleles of the DAT1 3′ UTR were observed. The 9- and 10-repeats were by far the most common, with allele frequencies of .32 and .67 respectively. The 8-repeat and 11-repeat variants were very rare, with frequencies of .0025 and .01 respectively. Genotypes for all participants were collapsed into categories based on the presence or absence of the 10-repeat allele. Allele frequencies were then found to be in Hardy-Weinberg equilibrium.

DAT1 Intron 8 VNTR

Only two alleles of the Intron 8 VNTR were observed; the 2-repeat allele was present at a frequency of .29 while the 3-repeat allele was present at a frequency of .71. Allele frequencies were in Hardy-Weinberg equilibrium.

DRD4 Exon III VNTR

Five alleles were present in the following frequencies: 2-repeat: 0.13; 3-repeat: 0.07; 4-repeat: 0.7; 5-repeat: 0.005; 7-repeat: 0.09. Allele frequencies for this marker were not in Hardy-Weinberg equilibrium when collapsed into categories based on the presence of the 2-repeat, 4-repeat or 7-repeat allele. This is most likely due to the existence of a number of low-frequency alleles which are inadequately represented in a sample of this size. As the failure to comply with the assumption of Hardy-Weinberg equilibrium was not thought to have arisen from inaccuracies in the genotyping process, further analysis was conducted on this marker. The results of this analysis should however be treated with caution.

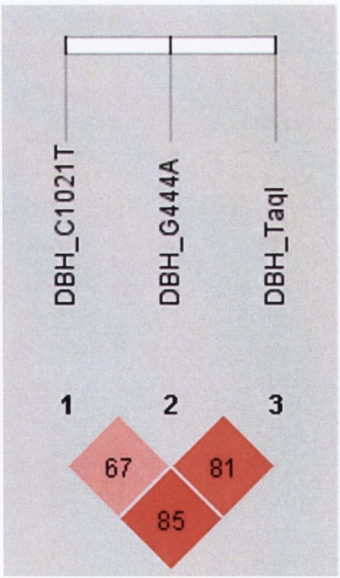
MAOA 30bp VNTR

Across the sample as a whole, the two most common alleles of the MAOA 30bp promoter were the 3-repeat (0.28) and the 4-repeat (0.67). Two less frequent alleles, the 3a (frequency = 0.015) and 5-repeat (frequency = 0.036) were also observed. As this polymorphism is located on the X chromosome, further analysis was conducted separately for males and females. In the male subgroup, the 3-repeat allele was present at a frequency of 0.3 while the 4-repeat allele was present at a frequency of 0.66. Among the female participants, the 3-repeat and 4-repeat alleles were present at frequencies of 0.295 and 0.7 respectively. These frequencies were in Hardy-Weinberg equilibrium.

2.3.2.2 Linkage Disequilibrium

Linkage disequilibrium (LD) may be defined as the non-random association of alleles at two or more loci within a population when particular alleles occur together more frequently than would be dictated by chance (Ardlie, Kruglyak, & Seielstad, 2002). Markers that are located close together on a chromosome are more likely to occur together as the impact of recombination will be less extreme than on markers separated by large distances. This is not always the case, however; some markers located very close together will shown no LD, while LD has been observed between markers located at some distance from one another (Abecasis *et al.*, 2001). An analysis of linkages disequilibrium is important as markers in strong LD with one another would be expected to show similar relationships with quantitative traits. The extent of LD is measured using the statistic D′, where D′=1 indicates complete LD.

Fig. 2.5. Linkage disequilibrium results (D′) from Haploview for DBH markers.



Linkage disequilibrium for SNPs in the DBH gene was calculated using the program Haploview (http://www.broad.mit.edu/haploview/haploview), and LD measures are displayed graphically in Fig. 2.5. It can be seen from this figure that the 3 DBH markers appear to be in reasonably tight LD with one another.

LD between multiallelic VNTRs was calculated using the PowerMarker program (http://statgen.ncsu.edu/powermarker). Moderate LD (D′ = 0.62, r2 = 0.31) was observed between the DAT1 3′ UTR and Intron 8 markers. Table 2.6 summaries the LD results (D′ and r^2) for the DRD4 gene, including the two promoter region SNPs and the VNTR in Exon III. It can be seen that the three DRD4 markers are not in strong LD with one another.

Table 2.6. Linkage Disequilibrium in the DRD4 gene

Marker	*DRD4 -521 C/T*	*DRD4 -616 C/G*	*DRD4 Exon III*
DRD4 -521 C/T		0.217	0.174
DRD4 -616 C/G	0.035		0.098
DRD4 Exon III	0.016	0.003	

Yellow: D′ measures, where 0 = complete equilibrium and 1 = complete disequilibrium
Green: r^2 measures

2.3.2.3 Multiple comparisons

A serious issue with all research of this nature is the number of comparisons made, and the resulting inflation of the probability of Type I error. A common, and rather conservative, method of resolving this issue is to apply a Bonferroni correction, whereby the critical alpha for the experiment is divided by the number of independent tests performed. While simple in theory, this method presents a number of problems. Not the least of these is the difficulty of determining the number of independent comparisons, given the high level of correlation among the behavioural measures and genetic markers examined. Correlations within genes may be measured using linkage disequilibrium tests (see Section 2.3.2.2), however measuring the level of interdependency of measures within and between behavioural tasks is rather more complicated.

The battery of tasks performed here are all measures of frontal-executive function, and by definition have much in common. The Alerting network of the ANT is, for example, considered to have many theoretical similarities to the sustained attention network assessed by the SART. Treating these tests as entirely independent would therefore be inappropriate, and the application of a Bonferroni correction incorporating all measures within all of these tasks would be overly stringent, resulting in a very conservative alpha level that no test could hope to surpass. The results described below have consequently not been corrected for the overall number of comparisons made, although Bonferroni corrections have been applied within each family of comparisons and to post-hoc tests. As the research conducted here is somewhat exploratory in nature, and

to provide the clearest possible pattern of results, p values both before and after correction are reported.

2.3.2.4 Statistical Analysis

Where the data met with assumptions for parametric analysis, analysis of variance (ANOVA) and linear regression analysis was performed to determine any effects of genetic markers on the behavioural measures. Where appropriate, non-parametric statistical equivalents were used. Multiple regression (backwards stepwise method) was used in cases where the combined impact of a number of genes on a variable was of interest. This analysis was only conducted in the investigation of specific hypotheses to avoid contributing to the problem of multiple comparisons. In a backwards stepwise regression, all predictor variables of interest are entered into the initial regression. Those which do not make a significant contribution to the ability of the model to predict of the dependent variable are iteratively removed. By the final step, only variables which add to the prediction of the dependent variable are retained. This method, while powerful, is limited in that it identifies only linear relationships between the variables.

2.3.2.5 Genetic associations with behavioural tasks

SART

As described in section 2.3.2.2, participants appeared to find the Fixed SART very easy to perform, and only made an average of 4.06 errors of commission across two blocks of the task. The Fixed SART is therefore unlikely to be sufficiently sensitive to detect slight differences in sustained attention performance due to genetic factors. Genetic analyses were consequently restricted to the Random SART.

Simple linear regressions were performed to determine the effect of DBH C-1021T genotype on errors of commission, errors of omission, mean reaction time and standard deviation of reaction time in the Random SART. C-1021T genotype was found to significantly predict errors of commission ($F(1,197) = 6.905$, $p<.01$; $r^2 = .034$) and standard deviation of reaction time ($F(1,197) = 4.507$, $p<.05$, $r^2 = .022$). Following Bonferroni correction for this family of comparisons, only the effect on errors of commission remained significant at the corrected alpha level of .0125.

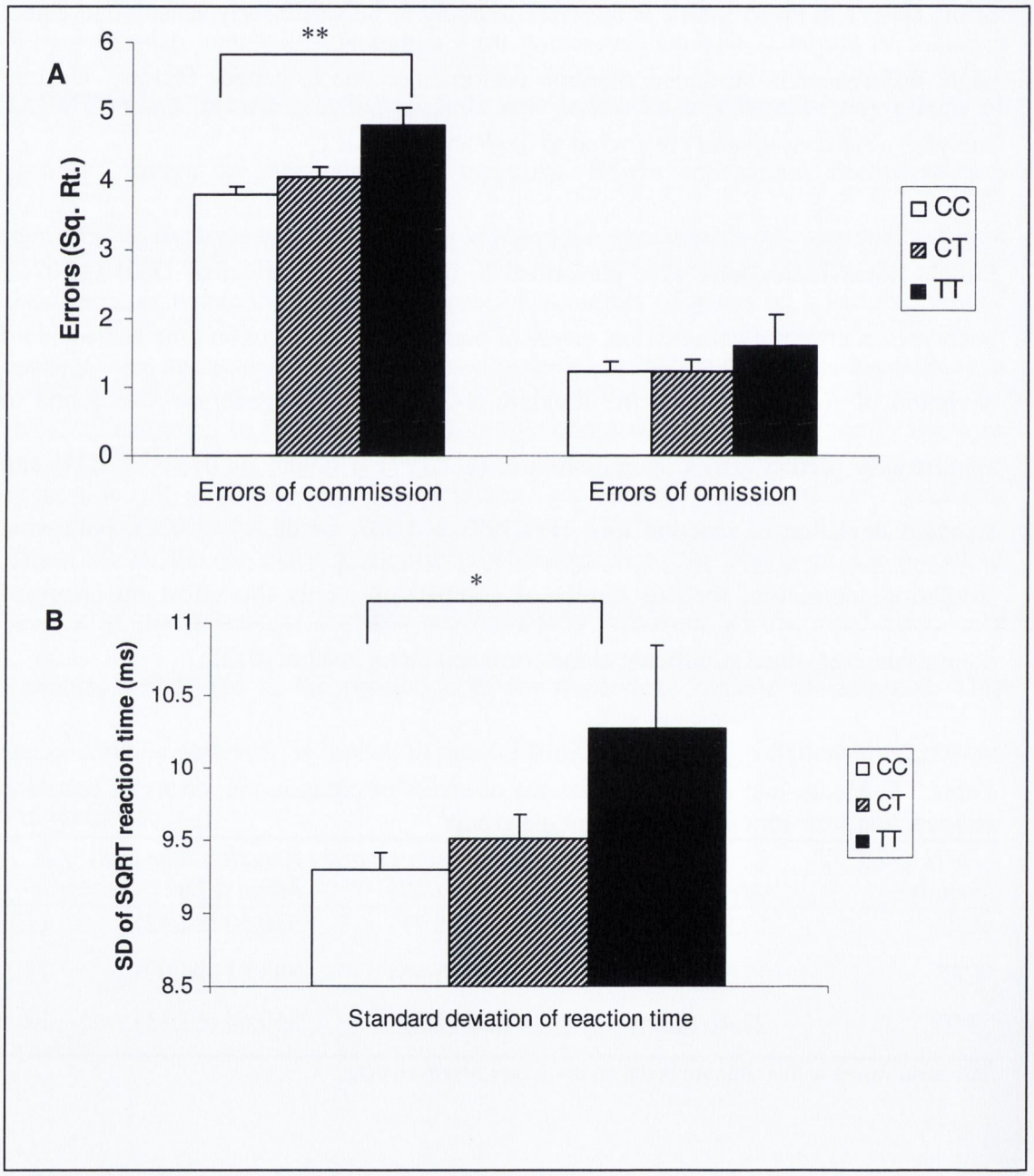
Table 2.7: Means and standard deviations of errors of commission, errors of omission and reaction time for each DBH genotype group[1]

DBH C-1021T genotype	*Commission errors Mean (SD)*	*Omission errors Mean (SD)*	*Reaction time (ms) Mean (SD)*	*N*
C/C	15.92 (9.5)	3.05 (6.7)	386.50 (84.42)	113
C/T	17.69 (10.75)	3.40 (5.45)	388.81 (94.09)	73
T/T	22.46 (9.36)	4.54 (7.5)	359.64 (62.11)	12

[1]All mean values in this table are based on raw (untransformed) data

Fig. 2.6 displays a linear increase in the square root of errors of commission with increasing T allele dosage.

Fig. 2.6. Effect of DBH C-1021T genotype on (A) errors of commission and omission and (B) standard deviation of reaction time in the Random SART



* Significant at p<.05 level (uncorrected)
**Significant at p<.0125 level (corrected)

It can be seen from this figure that participants homozygous for the T allele made more errors of omission than participants with 1 or 2 copies of the C allele, however this difference was not significant.

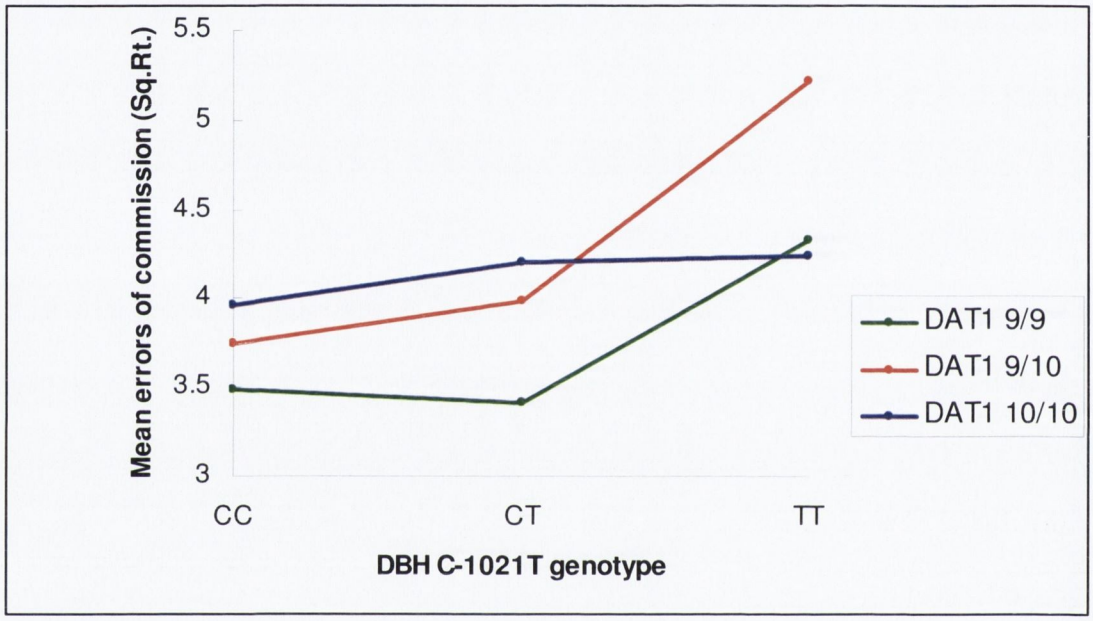
As indicated in Table 2.7, T homozygotes also tended to use a faster and more variable reaction time than participants possessing any copies of the C allele. The difference in reaction time between genotype groups did not reach statistical significance. No effect of the DBH *TaqI* or G444A markers on SART performance was observed.

Two VNTRs in the DAT1 gene, one in the 3′ untranslated region and one in Intron 8, were examined to establish whether they had any effect on performance in the Random SART. No significant differences were observed on any measure. Although no main effect of DAT1 on sustained attention performance was observed, it was hypothesised that the combined effect of these genes on SART performance might be greater than the effects of the genes when considered separately.

DBH C-1021T and DAT1 3′ UTR genotypes were entered as regressors into a backward stepwise regression where the dependent variable was the square root of mean errors of commission. Both variables were retained in the model, and a significant result ($F(2,188) = 3.982$, $p<.05$, $r^2=.041$) was obtained. The r^2 value of .041, signifying the proportion of variance explained by the regressors, was larger than that obtained from the regression of DBH alone on SART errors of commission, indicating that the addition of the DAT1 variable increased the predictive ability of the model.

Fig. 2.7 provides a graphic representation of this interaction. Errors of commission are seen to increase with increasing number of DBH T alleles, as described above. Of particular interest to this analysis is the fact that errors also increase with number of DAT1 10-repeats *except* in participants with two copies of the DBH T allele.

Fig. 2.7. Mean errors of commission in the random SART as a function of DBH and DAT1 genotype.



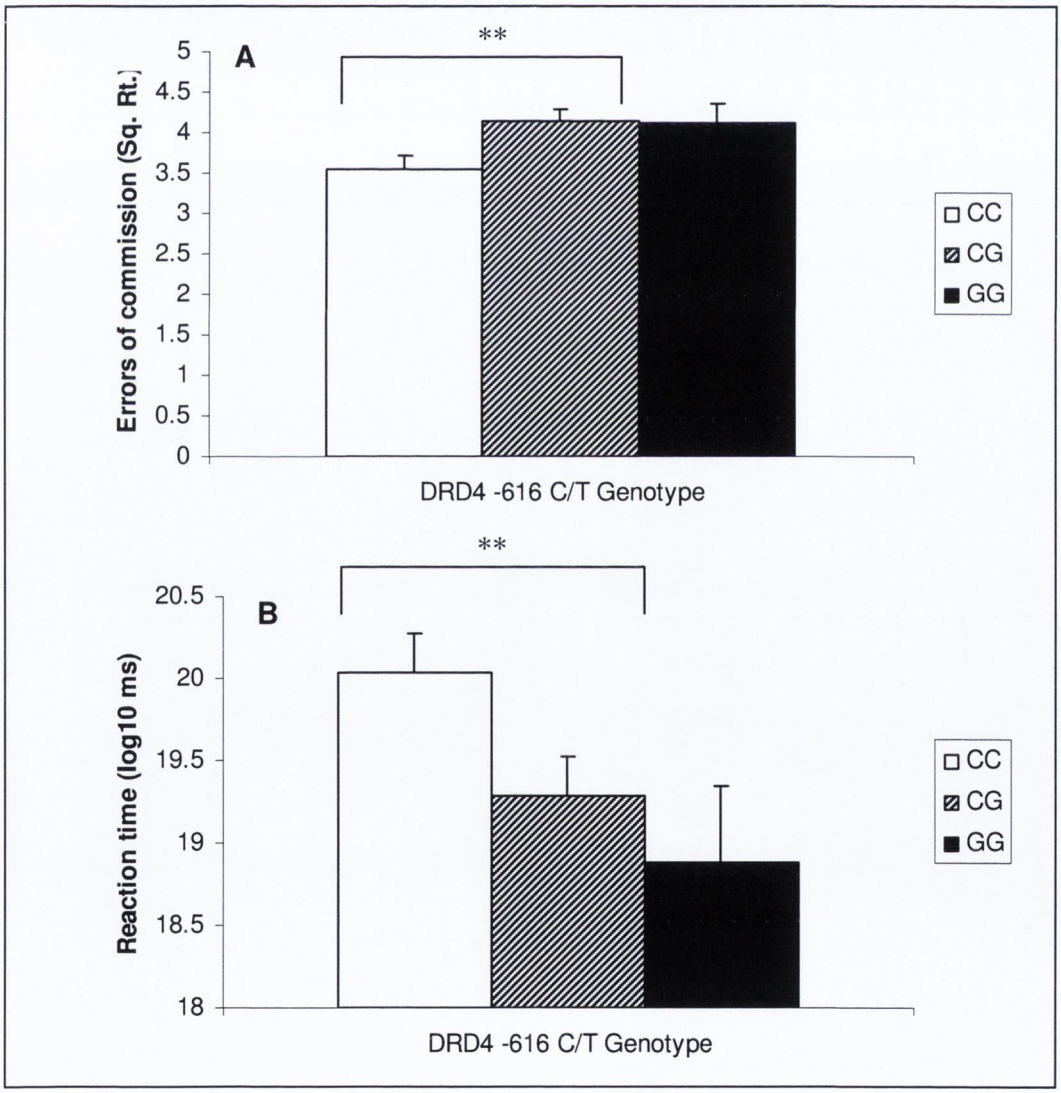
Linear regression analysis of the DRD4 gene indicated that genotype at the -616 locus significantly predicts errors of commission ($F(2,188) = 6.016$, $p<.05$, $r^2=.033$) and mean reaction time ($F(2,188) = 6.537$, $p<.05$, $r^2=.036$) in the Random SART.

Following Bonferroni correction, both results survived the new alpha level of .0125. As shown in Fig. 2.8, increasing number of G alleles was associated with greater number

of errors and a faster reaction time. There was no significant effect on SART measures of variation at the DRD4 -521 locus.

No effect of variation at the COMT Val/Met, MAOA promoter or 5HTT ins/del loci were observed on any SART measure.

Fig. 2.8. DRD4 -616 genotype and Random SART performance. (A) Effect of DRD4 -616 SNP on SART errors of commission. (B) Effect of DRD4 -616 SNP on SART reaction time.

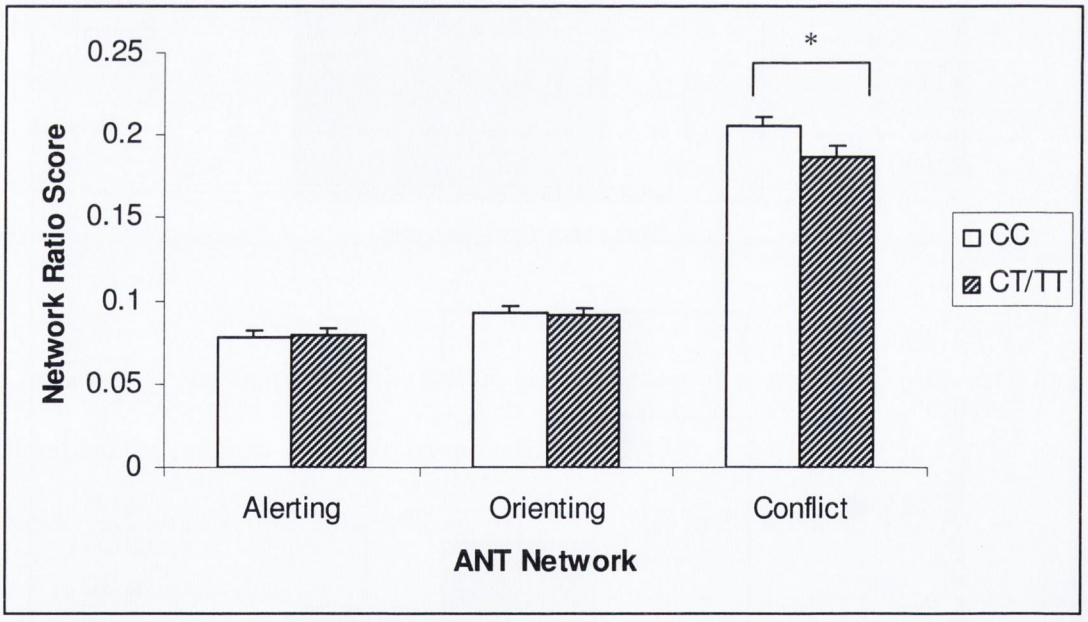


**Significant at p<.0125 level (corrected)

ANT

The effect of the three DBH markers on the alerting, orienting and executive control (conflict) networks of the ANT was examined. No significant difference in alerting or orienting scores among DBH genotype groups was observed. The DBH C-1021T marker had a significant effect on ANT conflict; when analysed under a codominant (additive) model, this effect was borderline (p=.052), however a clear effect was observed under a dominant model, whereby participants with one or two T alleles performed significantly better than those carrying no copies of the allele ($F(1,199) = 5.527$, $p<.05$; see Fig. 2.9).

Fig. 2.9. Effect of DBH C-1021T genotype on performance on the 3 attention networks of the ANT.

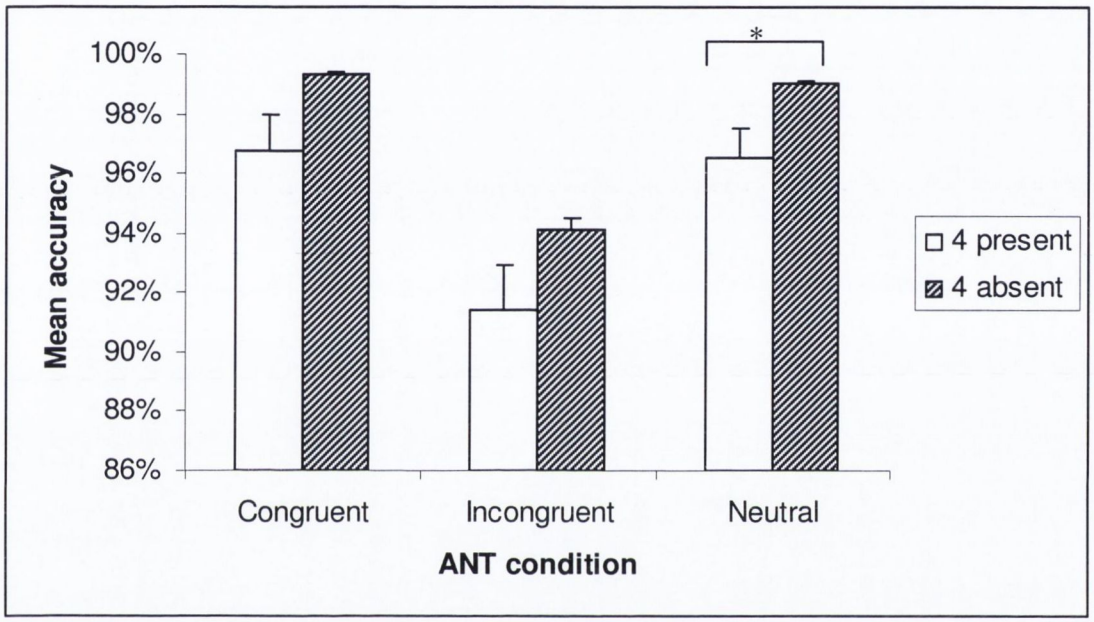


*Significant at p<.05 level (uncorrected)

Bonferroni correction for this family of comparisons resulted in a critical alpha level of .0167; this result did not survive the correction. The non-parametric Kruskal-Wallis test

was used to examine the effect of DBH on accuracy in the ANT. No significant effect of any DBH variant on accuracy in any of the trial types (congruent, incongruent or neutral) was observed.

Fig. 2.10. Accuracy in congruent, incongruent and neutral ANT conditions as a function of presence or absence of the DRD4 4-repeat allele.



*Significant at p<.05 level (uncorrected)

No significant effects of DAT1 3′ UTR or Intron 8 genotype were observed on any ANT network or accuracy measure.

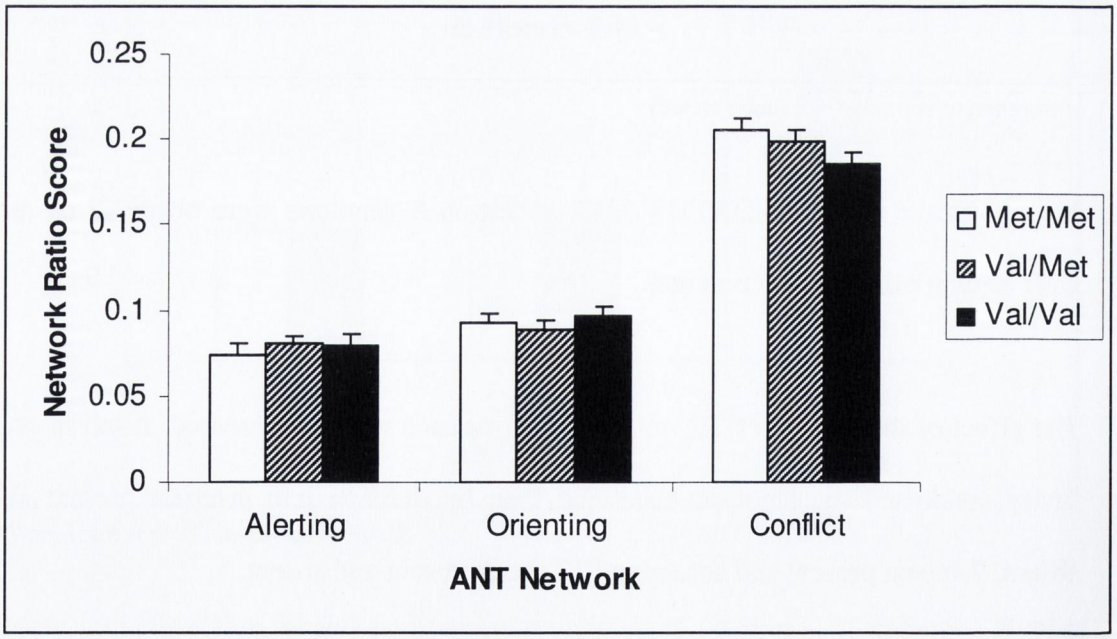
The effect of the DRD4 VNTR on ANT performance was also assessed. Analysis was firstly conducted by genotype class and then by division into 4-repeat present and absent, 7-repeat present and absent and 2-repeat present and absent.

There was no significant effect of any classification on the efficiency of any ANT network. An analysis of the effect of this marker on accuracy in the congruent,

incongruent and neutral conditions of the ANT was conducted. Accuracy was very high overall, with a mean of above 90% for all conditions. The accuracy data was not normally distributed, and so non-parametric Mann-Whitney U tests were performed. As indicated in Fig. 2.10, participants in possession of the 4-repeat allele tended to perform more accurately than those without that allele. Accuracy was found to differ significantly between participants with and without the 4-repeat allele only in the neutral condition ($U=2482.5$, $p<.05$), although this result did not survive correction for multiple comparisons (critical $\alpha = .0167$)

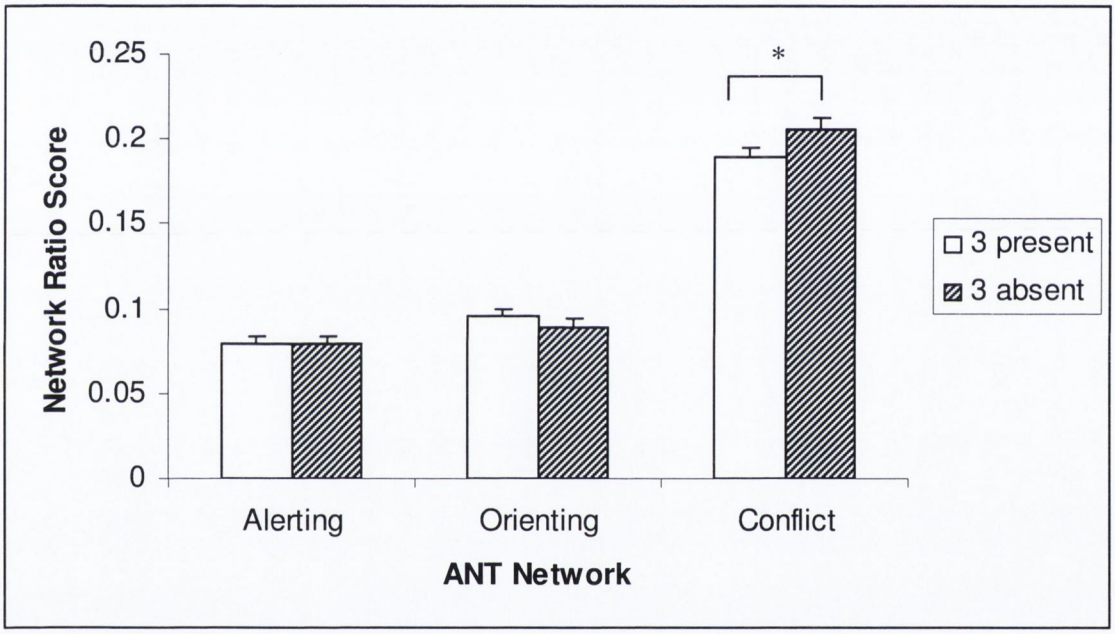
There was no significant effect of the DRD4 -616 or -521 markers on any ANT network or accuracy index.

Fig. 2.11. ANT network efficiencies as a function of COMT Val/Met genotype.



No significant effects of the COMT Val/Met polymorphism on the efficiency of any of the ANT networks were observed. A clear trend can however be discerned in the conflict network, where increasing number of COMT Val alleles is linked with lower executive network ratio score (see Fig. 2.11). There was no effect of COMT genotype on accuracy in any condition of the ANT.

Fig. 2.12. Attention Network efficiencies as a function of genotype at the MAOA promoter VNTR.

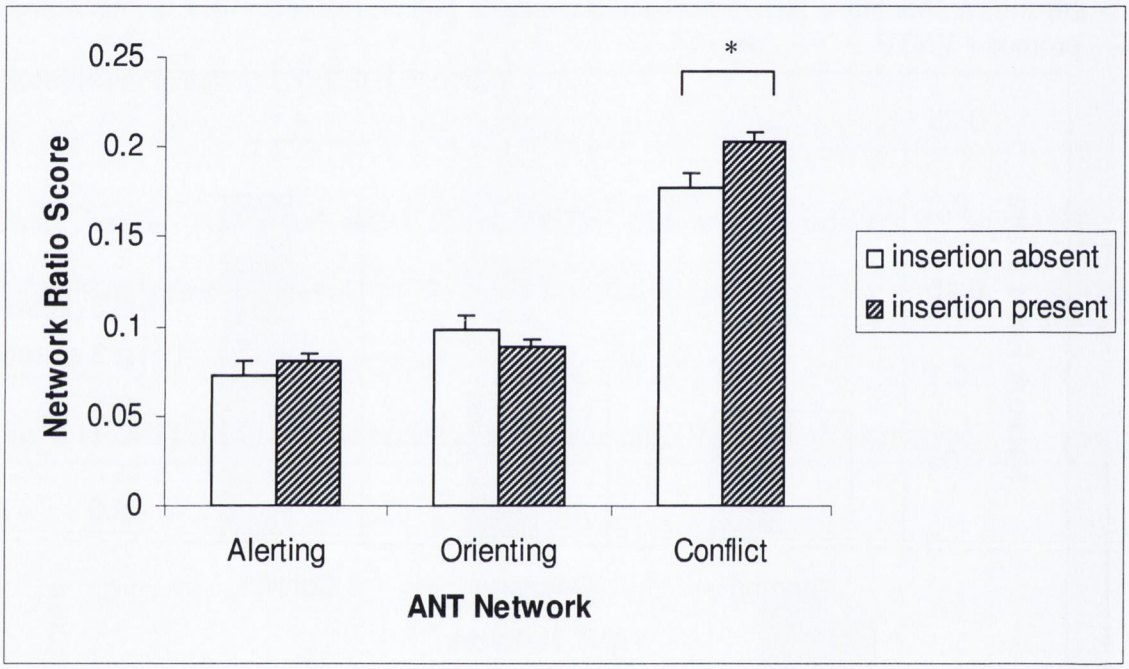


*Significant at p<.05 level (uncorrected)

Presence or absence of the 3-repeat allele of the MAOA 30bp promoter region polymorphism was found to have a significant effect on the conflict network of the ANT ($F(1,194) = 3.95$, $p<.05$) whereby participants in possession of the 3-repeat allele achieved a higher conflict network score than those without any copies of the allele (see Fig. 2.12). This result did not survive Bonferroni correction.

Similarly, participants possessing 1 or 2 copies of the insertion allele of the 5HTT ins/del polymorphism showed significantly higher conflict network scores than participants with no copies of the insertion allele ($F(1,186) = 4.174$, $p<0.5$; see Fig. 2.13). Once more, the significance of the result did not survive Bonferroni correction.

Fig. 2.13. Attention Network efficiencies as a function of 5HTT insertion/deletion genotype.

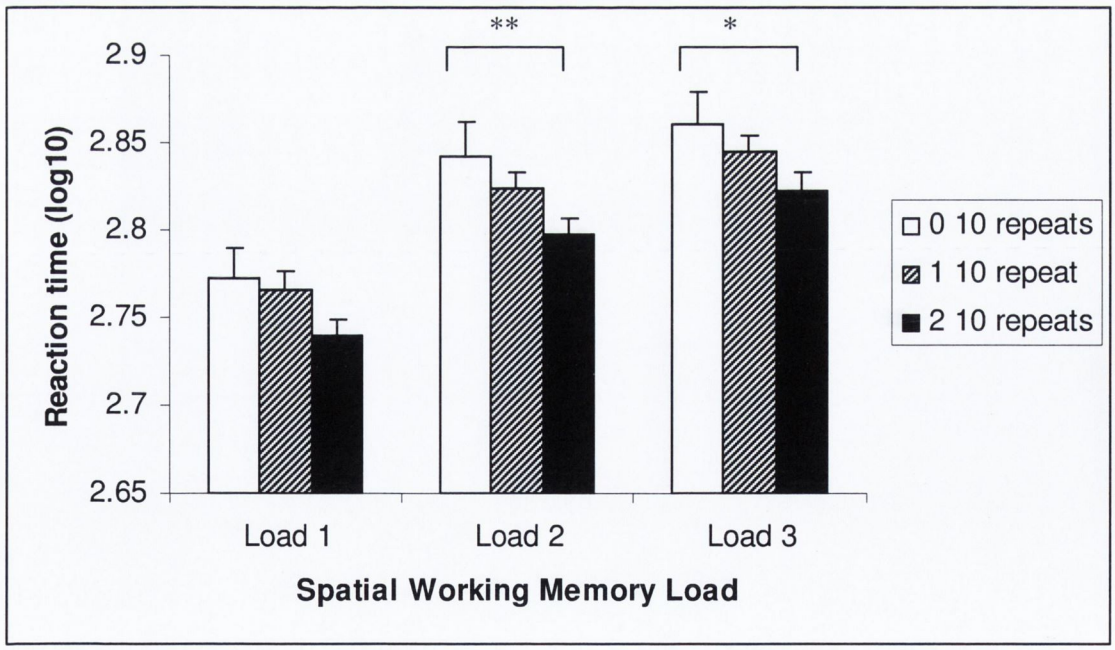


*Significant at p<.05 level (uncorrected)

Spatial Working Memory (SWM) task

The DBH gene was hypothesised to have an effect on spatial working memory, however linear regression and analyses of variance indicated no effect of any DBH marker on either accuracy or reaction time in the SWM task. A trend towards decreasing reaction time with increasing number of DAT1 10-repeats was observed on all memory loads of the spatial working memory task (see Fig. 2.14 for an illustration). Linear regression analysis indicated that this trend was nominally significant at loads 2 and 3 ($F(1,194) = 5.674$, $p<.05$, $r^2=.028$; $F(1,194) =4.055$, $p<.05$, $r^2=.02$) but did not quite reach statistical significance at load 1 ($F(1,194) =3.72$, $p=.055$, $r^2=.019$). Only the effect at load 2 survived correction for multiple comparisons ($p<.0167$). No effect of DAT1 3′ UTR genotype on accuracy in the SWM task was observed.

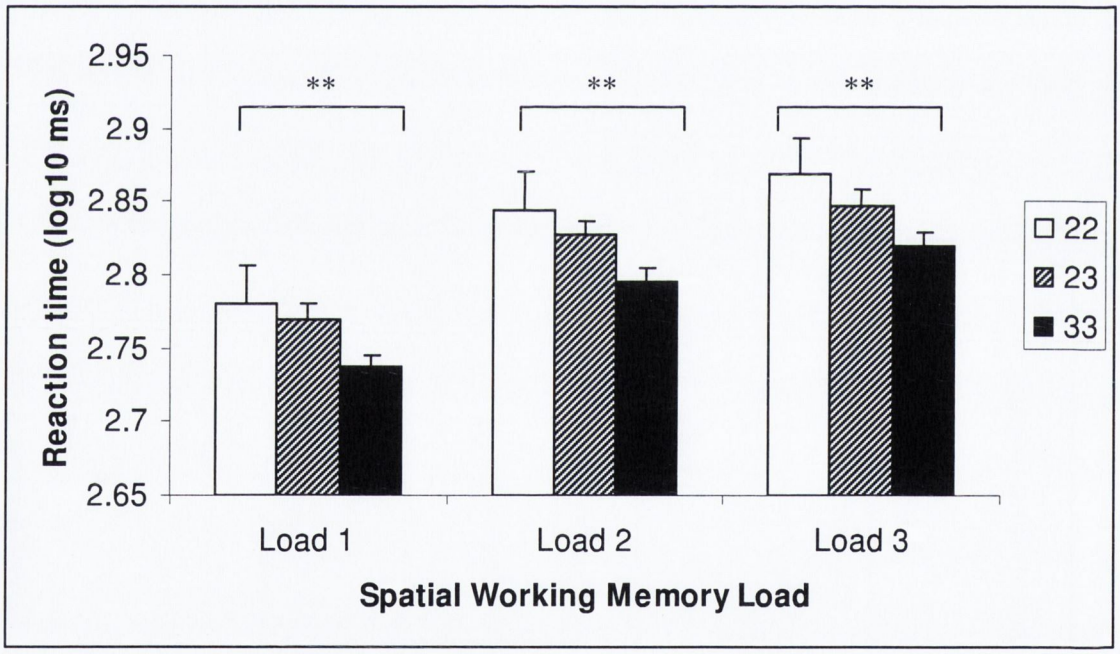
Fig. 2.14. Effect of number of DAT1 10-repeat alleles on SWM reaction time.



*Significant at the $p<.05$ level (uncorrected)
**Significant at the $p<.0167$ level (corrected)

Linear regression analysis also indicated a significant effect of the DAT1 Intron 8 polymorphism on reaction time at all 3 spatial working memory loads whereby reaction time decreased with increasing number of 3-repeat alleles (Load 1: $F(1,196) = 6.348$, $p<.05$, $r^2=.031$; Load 2: $F(1,196) = 7.072$, $p<.05$, $r^2=.03$; Load 3: $F(1,196) = 5.472$, $p<.05$, $r^2=.027$). The effect at all three loads remained significant at $p<.0167$ following Bonferroni correction (see Fig. 2.15). Again, no effect of this marker was observed on accuracy in the SWM task.

Fig. 2.15. Spatial working memory reaction time at 3 memory loads as a function of DAT1 Intron 8 genotype



**Significant at the $p<.0167$ level (corrected)

Following this finding of variation in SWM reaction times as a function of genotypes at both DAT1 loci, the effect of a haplotype of the 3′ UTR 10-repeat allele and Intron 8 3-repeat allele was examined. 108 participants were homozygous for the 10/3 haplotype,

70 carried one copy of it and 25 did not possess any copies of the haplotype. There was no significant effect of this haplotype on accuracy or reaction time at any load of the spatial working memory task.

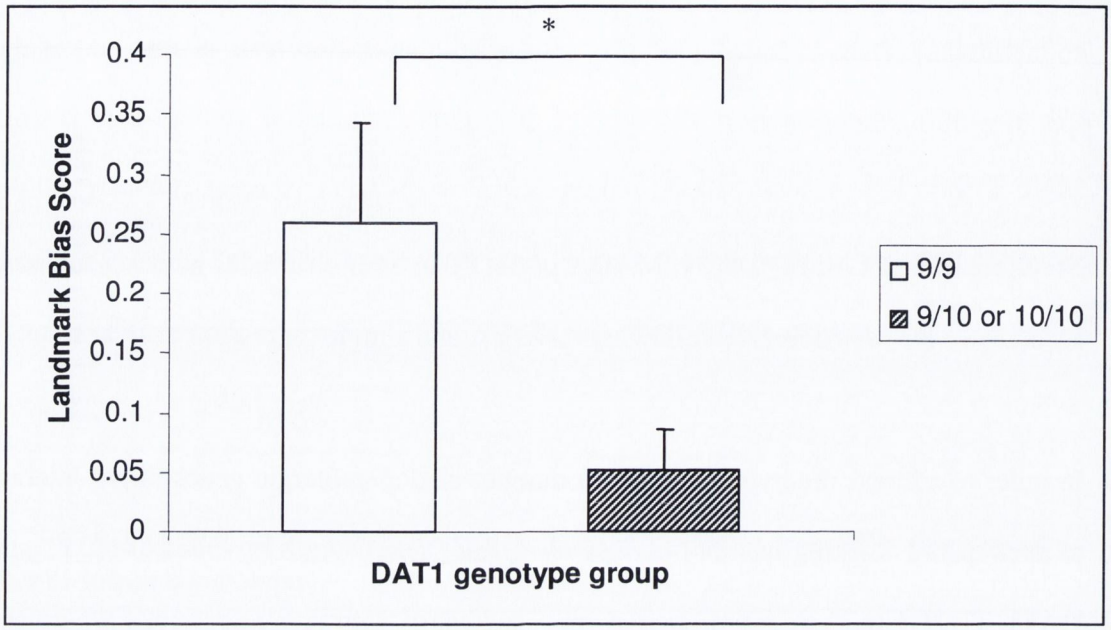
No effect of any COMT, DRD4, MAOA or 5HTT marker on spatial working memory accuracy or RT was observed following ANOVA and simple regression analyses.

In order to address the hypothesis that a number of dopaminergic genes might interact to alter spatial working memory performance, the COMT Val/Met, DBH C-1021T and DAT1 3′ UTR markers were entered into backwards stepwise regressions where the dependent variables were accuracy and reaction time at the three spatial working memory loads. The addition of the extra genotype variables did not significantly improve the predictive ability of the models.

Landmark task

The effect of the DAT1 3′ UTR marker on spatial attention (as measured by the Landmark task) was assessed. Under an additive model (0 vs. 1 vs. 2 10-repeat alleles), analysis of variance indicated a borderline effect of DAT1 on Landmark bias ($F(2,194) = 2.828$, $p=.062$), however a significant difference was observed under a dominant model ($F(1,195) = 4.618$, $p<.05$), where the sample was divided into groups with and without any copies of the 10-repeat allele.

Fig. 2.16. Landmark Bias Score for participants with and without the 10-repeat allele.

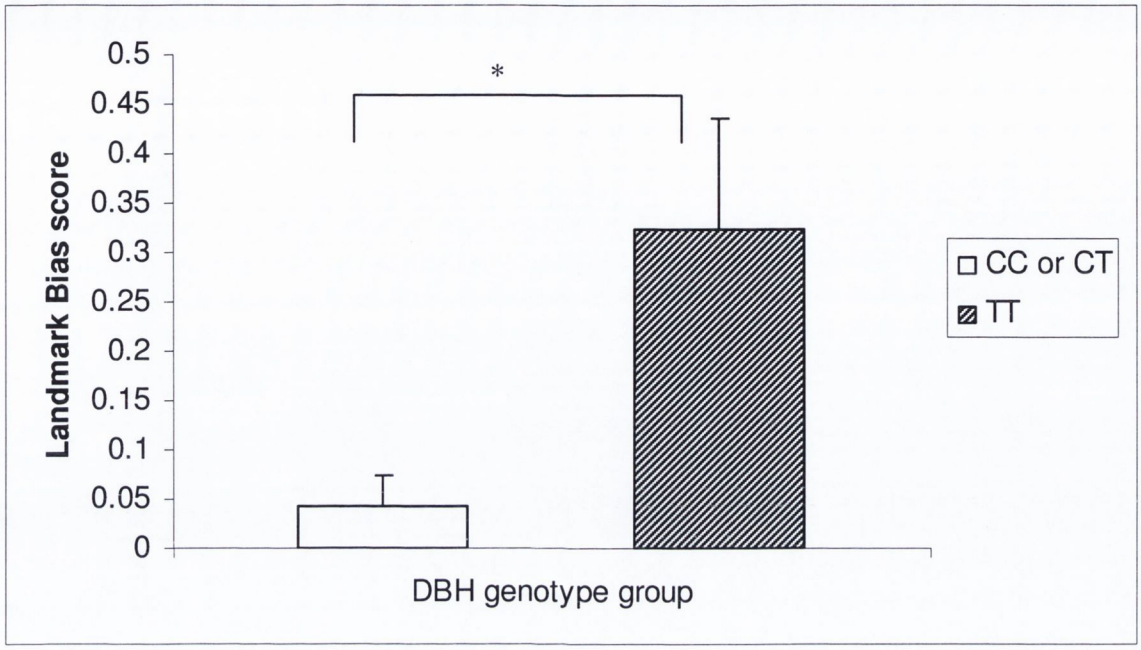


*Significant at p<.05 level

While a mild degree of right bias (.071) was observed in the sample as a whole, the 10-repeat absent group were found to be significantly more right-biased (i.e. displayed greater left inattention) than the 10-repeat present group (see Fig. 2.16 for an illustration).

Spatial bias was not significantly affected by DAT1 Intron 8 genotype. The only other marker to show any effect on spatial bias was DBH C-1021T; participants in possession of 2 copies of the T allele were significantly more right-biased than those with 0 or 1 copy ($F(1,199) = 5.301$, $p<.05$; see Fig. 2.17).

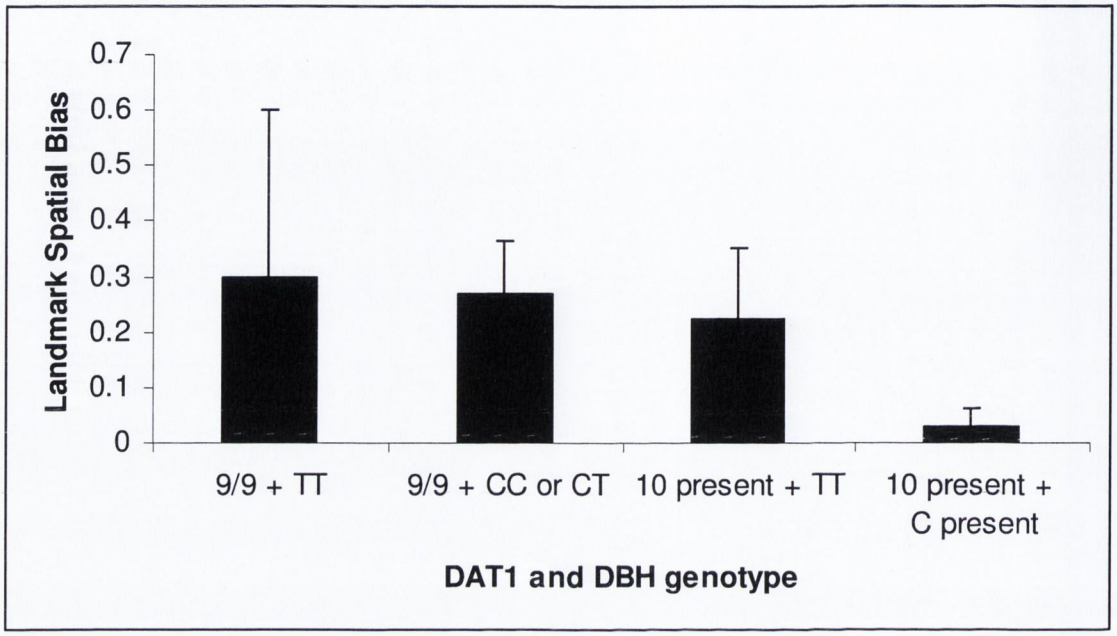
Fig. 2.17. Landmark Bias Score as a function of DBH C-1021T genotype



*Significant at p<.05 level

To determine whether there was an additive effect of the DAT1 and DBH genes on spatial bias, the combined genotype groups were assigned weights based on their proposed effect on extrasynaptic dopamine levels. Group 1 contained participants homozygous for both 'high dopamine' alleles (DBH -1021T and DAT1 9-repeat), while group 4 contained participants in possession of the 'low dopamine' DBH -1021C and DAT1 10-repeat alleles. Groups 2 and 3 were theorised to result in intermediate dopamine levels. These values were entered into a linear regression where the dependent variable was Landmark spatial bias. A significant effect of combined DBH and DAT1 genotype was observed ($F(3,198) = 6.757$, $r^2=.035$, $p<.01$). The mean spatial bias in each genotype group is depicted in Fig. 2.18.

Fig. 2.18. Spatial attentional bias in the Landmark task as a function of combined DBH and DAT1 genotype



2.4 Discussion

A number of interesting findings emerged from this study, and they will be dealt with here in turn.

Among the most interesting results to emerge from this study was the finding that DBH genotype predicts lapses in sustained attention, as measured by the Random SART. Participants with more copies of the T allele of the DBH C-1021T polymorphism committed more errors of commission in the SART than those with fewer copies. These participants also tended to make more errors of omission and to react more quickly and more variably, but these differences were not statistically significant following correction for multiple comparisons. This is the first study to demonstrate an association between a functional DBH gene polymorphism and sustained attention in healthy participants. Despite the reasonably strong linkage disequilibrium between the C-1021T marker and the other two SNPs genotyped in the DBH gene, neither the TaqI nor G444A markers had significant effects on any measure in this study.

The T allele leads to a slower rate of dopamine-noradrenaline conversion than the C allele (Zabetian *et al.*, 2001), and is therefore presumed to result in higher levels of extrasynaptic dopamine and relatively lower levels of noradrenaline. Noradrenaline is thought to improve alerting during sustained attention tasks (J. T. Coull, Middleton *et al.*, 1995), and so decreased levels of noradrenaline may reduce participants' capacity to remain alert throughout the task. This, in combination with previous findings that noradrenaline plays a role in target detection (Nieuwenhuis, Aston-Jones, & Cohen, 2005) and reduces susceptibility to distractors (Robbins, 1984), may at least in part

explain the failure of participants with greater T allele dosage to withhold their response to the target digit.

The Landmark task, which measures spatial bias, usually reveals the phenomenon known as pseudoneglect, whereby normal healthy participants display a slight degree of inattention to the right side of space (i.e. an attentional bias towards the left) as a result of the dominance of the right hemisphere in spatial attention (Bowers & Heilman, 1980). In the present study, however, participants tended on average to show some right bias, or left inattention. This bias, while unusual, is very slight, and the distribution of results indicates a roughly even proportion of participants showing left and right spatial biases. This is not unexpected, as even in studies where an overall tendency towards left spatial bias is seen in healthy participants, a significant proportion of individuals display consistent right bias (e.g. Jason B. Mattingley *et al.*, 2004).

An effect of the DBH C-1021T polymorphism on Landmark bias was observed; participants in possession of two copies of the high-dopamine T allele were significantly more right-biased than individuals with one or no copies, whose average bias score was very close to zero. When combined with the finding described above that participants homozygous for the T allele also display lapses in sustained attention, this result is particularly interesting. As described in Chapter 1, sustained and spatial attention are closely related, and sustained attention efficiency modulates spatial attention performance (M.A. Bellgrove *et al.*, 2004). This finding that a single genetic

polymorphism in a gene controlling the balance of dopamine and noradrenaline in the cortex affects both sustained and spatial attention provides important confirmation of this relationship. It is suggested that the lapses in sustained attention occasioned by the low-noradrenaline, high-dopamine T allele are the driving force behind the increase in left inattention found in participants homozygous for the T allele.

Previous research, in particular in ADHD populations, led to the hypothesis that the DAT1 gene might affect sustained attention. No main effect of DAT1 on SART performance was observed; an interaction with DBH C-1021T genotype was however observed whereby errors of commission increased with increasing number of DAT1 10-repeats, except in the case of participants with two copies of the DBH T allele. Participants with the DBH TT genotype who were also homozygous for the DAT1 10-repeat allele committed fewer errors than participants with either the 9/9 or 9/10 genotype. As discussed previously, participants homozygous for the DBH T allele have very high dopamine and relatively low noradrenaline levels. The fact that the presence of two copies of the low-dopamine DAT1 10-repeat allele dramatically reduces the number of errors made by these participants suggests that it is not only an insufficiency of noradrenaline, but also an overabundance of dopamine which drives the increase in errors accompanying the DBH TT genotype. The T allele is considerably less frequent than the C allele, and so very few participants were homozygous for both the DBH T allele and DAT1 10-repeat allele. As a consequence, this result may not be reliable, and requires replication in a sample containing a higher proportion of participants with two copies of the T allele.

Participants without any copies of the DAT1 10-repeat allele displayed a significantly higher degree of right bias on the Landmark task than those with one or two copies of the allele, whose spatial bias score was very close to zero. Furthermore, the DAT1 and DBH genes appeared to have an additive effect on spatial attentional asymmetry, with the most right-biased scores found in participants homozygous for the high-dopamine 9-repeat and T alleles. The finding with regard to DAT1 is in direct contrast to previous research (M. A. Bellgrove, Chambers, Johnson, Dáibhis *et al.*, 2007; M. A. Bellgrove, Hawi, Kirley, Fitzgerald *et al.*, 2005) showing an association between the 10-repeat allele and left-sided inattention. In the present study, the 10-repeat allele, rather than resulting in left neglect, appears to normalise spatial attention.

The DAT1 10-repeat allele is associated with increased DAT density *in vitro*, and possibly *in vivo*, although other factors may intervene to upregulate DAT function (VanNess, Owens, & Kilts, 2005). The 10-repeat allele may therefore be associated with decreased levels of dopamine being made available at the synapse. Much of the previous research into the effect of DAT1 on spatial attention was conducted on children with ADHD, a population frequently associated with hypodopaminergia, and with increased dopamine transporter binding (Dougherty *et al.*, 1999). Where the underlying level of extrasynaptic dopamine is low, the increase in dopamine transporter density thought to be occasioned by the DAT1 10-repeat allele could result in the net dopamine level dropping below the threshold required for the maintenance of the right hemisphere's dominance over spatial attention, resulting in the symptoms of left neglect described in ADHD (see M. A. Bellgrove, Hawi, Kirley, Fitzgerald *et al.*,

2005). In a healthy population, who presumably do not suffer from any dopaminergic abnormalities, an increase in dopamine transporter activity would not result in similar symptoms.

An association of the 10-repeat allele with increased left inattention has however been demonstrated in a sample of healthy children (M. A. Bellgrove, Chambers, Johnson, Dáibhis *et al.*, 2007). The discrepancy in results between this study and the present work may lie in the fact that dopamine transporter density is known to decrease with age (Lavalaye, Booij, Reneman, Habraken, & van Royen, 2000; Mozley *et al.*, 1999), such that the young adults included in the present study will have less dopamine transporter, and therefore higher levels of dopamine than the children in the Bellgrove study. Variation in the DAT1 gene will consequently have different effects on dopamine levels and spatial attention in these two populations.

Interestingly, large increases in rightward bias relative to other groups have been observed at both extremes of the dopamine transporter binding spectrum. Children with ADHD who were homozygous for the 10-repeat allele are likely to have increased DAT binding and decreased dopamine levels as a result of their age, diagnosis and genotype. In contrast, healthy adults homozygous for the 9-repeat allele show low levels of DAT binding (and therefore increased dopamine) due to the same three factors. The fact that left inattention is observed in both of these groups suggests a non-linear pattern of association between dopamine availability and spatial attention. As dopamine is known to have an inverted-U shaped relationship with some prefrontal

functions, whereby an excess or insufficiency of dopamine may lead to deficits in task performance, it is possible that this same dose/response curve may influence spatial attentional asymmetry.

The alerting network as measured by the ANT is thought to be analogous to the sustained attention network (Sturm *et al.*, 1999), and so similar genetic mechanisms might be expected to operate on both processes. Nevertheless, no effect of the DBH C-1021T SNP was found on ANT alerting despite the clear association with SART performance described above.

A similar discrepancy was observed in the analysis of the DRD4 gene. Genotype at the -616 locus significantly predicted SART errors of commission and mean reaction time, but not ANT alerting. The mechanism by which this DRD4 marker might have affected sustained attention performance is unclear, as the -616 SNP has never been shown to alter gene function. Its position in the promoter region of the gene suggests a possible role in transcription, and the effect of the DBH C-1021T SNP on the same indices indicates that an advantage in sustained attention may be conferred by low-dopamine alleles. In the absence of functional studies of this marker, however, this interpretation is purely speculative.

The question remains as to why the theoretically similar alerting and sustained attention networks (measured by the ANT and SART respectively) do not appear to be affected by the same genetic markers. While the possibility exists that the two tasks actually tap

entirely separate processes and are therefore not controlled by the same genetic mechanisms, it is more likely that there was simply insufficient power for detecting weak genetic effects on the ANT. Fossella *et al.* (2002) suggest that a sample of up to 600 participants may be required to detect genetic links to ANT networks. The SART therefore appears to be a more sensitive measure of genetic effects on alerting and sustained attention in smaller samples.

Contrary to previous findings (Parasuraman *et al.*, 2005), no effect of any DBH polymorphism was observed on spatial working memory accuracy or mean reaction time. While it is possible that this analysis was simply underpowered, the task administered here was identical to that used by Parasuraman *et al*, and was performed by more than double the number of participants. Rather than a lack of power in the present study, this discrepancy may indicate that the result reported by Parasuraman *et al.* was unreliable. An effect of the DAT1 gene on reaction time in the spatial working memory task was however observed. Possession of an increasing number of the putative low-dopamine 10-repeat alleles predicted a linear decrease in reaction time on loads 2 and 3 of the task. A similar pattern was observed on all loads with increasing number of 3-repeat alleles at the DAT1 Intron 8 locus. As this marker is located in a non-coding region of the gene, this effect may be due to its moderate linkage disequilibrium with the 3′ UTR marker, or to LD with another functional polymorphism in the gene.

The purported decrease in dopamine at the synapse as a result of the greater dopamine transporter densities associated with the 10-repeat allele appears to speed reaction times to spatial working memory stimuli without any corresponding alteration in accuracy. In accordance with research indicating that the relationship between dopamine levels and working memory performance is best described by an inverted-U shaped dose/response curve (Amy F. Arnsten, 1998; Vijayraghavan *et al.*, 2007; Zahrt *et al.*, 1997), it was hypothesised that other markers directly affecting dopamine levels might interact with DAT1 on this measure. The addition of COMT Val/Met and DBH C-1021T markers, both of which have functional effects on relative dopamine levels, did not however improve the ability of the model to predict performance.

Despite the observation of Fossella *et al.* (2002) that samples of 600 or more may be required to produce significant genetic effects on ANT networks, a number of polymorphisms were found to significantly affect the efficiency of the conflict/executive control network. Participants in possession of the MAOA 3-repeat allele, associated with reduced gene transcription and consequently increased levels of dopamine, were found to have a less efficient network than those without any copies of that allele. A similar nominally significant effect of the DBH C-1021T allele on ANT conflict was also observed, whereby possession of the high-dopamine, low-noradrenaline T allele reduced the efficiency of the conflict network. This result did not survive correction for multiple comparisons. Taken together, these results indicate that an overabundance of dopamine is detrimental to executive control.

Participants in possession of one or two copies of the long allele of the 5HTT gene were found to have less efficient executive control networks than participants who were homozygous for the short allele. The long allele, which results in increased gene transcription, leads to serotonin being cleared more quickly from the synapse than the short allele (Heils *et al.*, 1996), and so this result indicates that greater serotonin availability at the synapse is associated with improved executive control. This supports previous research linking the long allele with poor performance on a battery of executive function tasks (Roiser, Rogers, Cook, & Sahakian, 2006). The mechanism by which serotonin affects cognitive function remains unclear, although it has been suggested that the effects of serotonin on emotional factors such as depression and anxiety may play a role in cognition (Strobel *et al.*, 2007). An alternative explanation relates to the mechanism by which the serotonin system (specifically 5HT receptors) modulates the degree of dopamine release in the striatum (Porras *et al.*, 2002).

No genetic effects on the ANT alerting or orienting networks were observed, and only one marker (the DRD4 Exon III VNTR) was associated with accuracy in the ANT. Allele frequencies at this locus were not in Hardy-Weinberg equilibrium, and so this result should be treated with caution. It should also be noted that as the ANT accuracy data were not normally distributed, non-parametric tests, which lack the power of their parametric equivalents, were conducted. Under these conditions, it is perhaps not surprising that small genetic effects were not detected.

One of the more surprising results to emerge from this study is the lack of association between the COMT gene and performance on any task. An effect of COMT might have been predicted particularly on executive control (as measured by the ANT) and on spatial working memory, as similar results have been observed in previous studies (e.g. Fossella *et al.*, 2002; T.E. Goldberg *et al.*, 2003). A non-significant trend was observed in the ANT executive control network whereby the highest scores (indicative of a less efficient network) were observed in participants with the Met/Met genotype and the lowest in participants with the Val/Val genotype. Val/Met participants' scores were intermediate between these two. This trend is in the opposite direction to that usually seen, where the Met allele results in better cognitive performance. Given that the functional nature of the COMT Val/Met polymorphism is known and that executive control is known to be under the influence of dopamine (J. T. Coull, Sahakian *et al.*, 1995), the most likely explanation for this absence of significant results is that the analysis was simply underpowered. A study with a larger sample might be in a better position to detect the effect of COMT genotype.

The results described here merely hint at the complexity of the genetic architecture of executive functions. While many of the findings mentioned above are interesting in themselves, it should be noted that the genetic effects described explain comparatively little of the variance in performance on each task. The examination of interactions between genes within a particular neurotransmitter system goes some way towards addressing this problem, however it is virtually impossible to control for all of the genetic, environmental and individual differences that contribute to variation in task

performance. It is possible that some of the noise resulting from these factors may be eliminated by directly examining genetic links to the brain activation associated with task performance, rather than to behavioural measures alone. The higher signal-to-noise ratio provided by endophenotypes such as fMRI may increase the chance of detecting genetic effects on cognitive networks, and it is this hypothesis that the next chapter will investigate.

Chapter 3

fMRI Study: Spatial Working Memory

3.1 Introduction

The neurological bases of spatial working memory were discussed in detail in Chapter 1, while genetic associations with performance of spatial working memory tasks were described in Chapter 2. The aim of the present chapter is to examine potential genetic links to cortical activation associated with the 'maintenance' and 'manipulation' aspects of spatial working memory. These elements of memory will be assessed by two tasks, one requiring participants to remember spatial information during a delay and another requiring them to manipulate that information in working memory.

A number of studies have attempted to distinguish between cortical regions required for spatial and non-spatial working memory. Early fMRI studies, following research with non-human primates, hypothesised that spatial working memory would recruit dorsolateral prefrontal regions, while non-spatial tasks would involve more ventrolateral regions (Wilson, Ó Scalaidhe, & Goldman-Rakic, 1993). This has since been proved inaccurate, and both spatial and non-spatial working memory tasks have been shown to recruit regions of middle frontal gyrus (Callicott *et al.*, 1999; Carlson *et al.*, 1998; D'Esposito *et al.*, 1998). It has also been suggested that spatial working memory may disproportionately recruit regions of the right hemisphere, while non-spatial memory is associated with more left hemispheric involvement (D'Esposito *et al.*, 1998). Certainly, the right hemisphere has repeatedly been linked with spatial attention and awareness (Corbetta *et al.*, 2000; Fan *et al.*, 2005; Heilman *et al.*, 1986; Mesulam, 1981), and so a strong involvement in spatial working memory would not be unexpected. Nevertheless, some studies (e.g. B. R. Postle, Stern, Rosen, & Corkin,

2000) have failed to detect a difference in right-hemisphere activations between spatial and non-spatial working memory, although left-hemisphere regions related to language processing do show increased activation in non-spatial working memory tasks, probably as a result of sub-vocalisation while committing letter or number-based stimuli to memory (Paulesu, Frith, & Frackowiak, 1993).

The dorsolateral prefrontal cortex (DLPFC) is located primarily in the middle frontal gyrus, covering Brodmann's areas (BA) 9 and 46, although regions of BA 8 and 10 are included in some definitions. The DLPFC is known to be recruited when manipulation of information in working memory, such as that which takes place during performance of an N-Back task, is required (Bradley R. Postle *et al.*, 2006; E. E. Smith, Jonides, & Koeppe, 1996). This is the case during both spatial and non-spatial tasks. A growing number of studies has however indicated that intensifying demands on working memory maintenance, whether by increasing memory load (Todd S. Braver *et al.*, 1997; J. Jonides *et al.*, 1997; Manoach *et al.*, 1997; Rypma *et al.*, 1999) or lengthening the delay between stimulus presentation and recall (Barch *et al.*, 1997; Kruggel, Zysset, & von Cramon, 2000) also leads to recruitment of the DLPFC. A parametric increase of working memory load has been shown to result in increased activation of a number of regions besides the DLPFC, including right ventrolateral PFC and bilateral regions of posterior parietal, premotor and supplementary motor cortex (Altamura *et al.*, 2007).

Cortical activation will be measured in this study using functional magnetic resonance imaging (fMRI). The activation of a particular neural network results in the increased

flow of oxygenated blood to the appropriate region. As oxygenated and deoxygenated blood have different magnetic properties this increase in oxygenated blood flow, known as the blood-oxygen-level dependent (BOLD) effect, can be detected by fMRI. fMRI studies suffer even more than behavioural studies from problems associated with multiple testing and increased Type I error as analyses are conducted simultaneously on thousands of voxels. The thresholding techniques used to address this issue will be described later in the chapter. In an effort to reduce the number of comparisons made, only the effects of the DAT1, DBH and COMT genes will be assessed here as strong theoretical reasons exist why these genes might impact on cortical activity associated with spatial working memory.

As discussed in Chapter 1, working memory is highly dopamine-dependent, and an inverted-U shaped curve has been suggested to best describe the relationship between dopamine and working memory efficiency. Although brain regions differentially activated by spatial and non-spatial working memory are difficult to identify, dissociations in the neurochemistry of different forms have been suggested; administration of D_2 receptor agonists modulated spatial working memory (Monica Luciana & Collins, 1997; Monica Luciana, Depue, Arbisi, & Leon, 1992), but not object working memory (Monica Luciana & Collins, 1997), which appears to be primarily under the control of D_1 receptors. Links between dopaminergic genes and spatial working memory task performance are described in Chapter 2, however very few studies have been published which examine genetic effects on cortical activation associated with spatial working memory. Several of these studies (e.g. Bertolino, Blasi

et al., 2006; A. R. Hariri *et al.*, 2002; Tan *et al.*, 2007) did however find genetic effects on brain activation when no significant differences in behavioural performance could be detected.

Results with regard to the direction of the effect of COMT genotype on spatial working memory task performance are somewhat inconsistent (see section 2.1.1), and no effect whatsoever of the COMT Val/Met polymorphism was found on cognitive performance measures in the behavioural study described in this thesis. Despite this, studies examining the effect of the gene on brain activation have almost uniformly reported an association of the COMT Met allele with reduced BOLD signal intensity during performance of a working memory n-back task. This trend has been observed across a range of brain regions including the DLPFC in individuals with schizophrenia (Bertolino, Caforio *et al.*, 2006; Egan *et al.*, 2001), their unaffected siblings (Egan *et al.*, 2001) and healthy controls (Bertolino, Blasi *et al.*, 2006; Caldú *et al.*, 2007; V. S. Mattay *et al.*, 2003; Meyer-Lindenberg *et al.*, 2006). The reduction of BOLD signal, when coupled with a lack of group differences in performance, is generally interpreted as indexing a more efficient, or focused, cortical network. Mattay *et al.* (2003) also reported that administration of the dopamine agonist amphetamine led to improved efficiency of the Val/Val group's working memory network, presumably by removing the disadvantage occasioned by possession of the Val allele, which results in reduced levels of dopamine at the synapse (see section 2.1.1).

Two studies (Bertolino, Blasi *et al.*, 2006; Caldú *et al.*, 2007) have investigated the effects of DAT1 gene variants on cortical activation during working memory. In both studies, participants who were homozygous for the 10-repeat allele of the DAT1 3′ UTR displayed a more focused prefrontal response during performance of an N-Back task than participants with one or two copies of the 9-repeat allele. Furthermore, the most focused response of all in both studies was found in those participants who were homozygous for both the DAT1 10-repeat and the COMT Met allele. The 10-repeat allele is thought to result in faster clearing of dopamine from the synapse (Cheon *et al.*, 2005; Heinz *et al.*, 2000), and may therefore be considered a 'low dopamine' allele. DAT1 is expressed primarily in the striatum, where working memory is facilitated by the filtering of irrelevant sensory inputs (Newman & Grace, 1999). Higher dopamine concentrations in the striatum have the effect of increasing activity in cortical-subcortical pathways (Tisch, Silberstein, Limousin-Dowsey, & Jahanshahi, 2004). DAT1 genotype may therefore affect the nature of inputs along a cortico-striatal-thalamic pathway to the prefrontal cortex where the COMT gene is heavily expressed (Bertolino, Blasi *et al.*, 2006). These two genes may therefore interact in their effects on working memory.

No studies to date have explicitly examined the effect of DBH genotype on brain activation during working memory task performance. One study did examine the effect of several SNPs in DBH, among other genes, on activation associated with performance of an auditory oddball task in a sample of individuals with schizophrenia, their relatives and healthy controls (Windemuth *et al.*, 2008). While no effect of the

DBH C-1021T marker was observed, a significant effect of another marker, a coding SNP in Exon 5 of the gene, was found to significantly affect BOLD signal in regions of temporal cortex. Although DBH is primarily a noradrenergic gene, its role in the management of dopamine levels makes it a likely gene target for working memory. Indeed, one study (Parasuraman *et al.*, 2005) did find an effect of the DBH G444A marker on spatial working memory performance, although this finding was not replicated in the behavioural study described in this thesis (Chapter 2).

In accordance with the literature in the area, it was expected that BOLD signal intensity would increase with increasing memory load during both the spatial working memory maintenance task and the N-Back task. It was also expected that the COMT Val allele would result in increased BOLD signal intensity relative to the Met allele in both tasks. The hypothesised direction of effect of the DAT1 and DBH genes was less certain as little research has been conducted on the effect of these polymorphisms on cortical activations. Nevertheless, it was expected that, as both genes directly affect dopamine levels, genetic variation at the 3′ UTR and C-1021T loci would modulate BOLD signal intensity during spatial working memory performance.

3.2 Methods

3.2.1 Participants

40 right-handed participants (15 male, 25 female; mean age = 23.28, SD = 3.3) took part in the fMRI study. These participants had previously participated in the behavioural phase of the study (see Chapter 2) and had provided DNA samples for genotyping (see section 2.2.4). Participants' ethnicity was determined by means of a questionnaire detailing their grandparents' nationality. All participants in the fMRI study had four Irish grandparents, and so were deemed genetically Irish. These participants were selected from the sample described in Chapter 2 with a view to maximising the number of participants homozygous for the rare DBH T allele and were paid €20 for their participation. Ethical approval for this project was obtained from the Ethics Committee of the School of Psychology, Trinity College Dublin.

3.2.2 Tasks

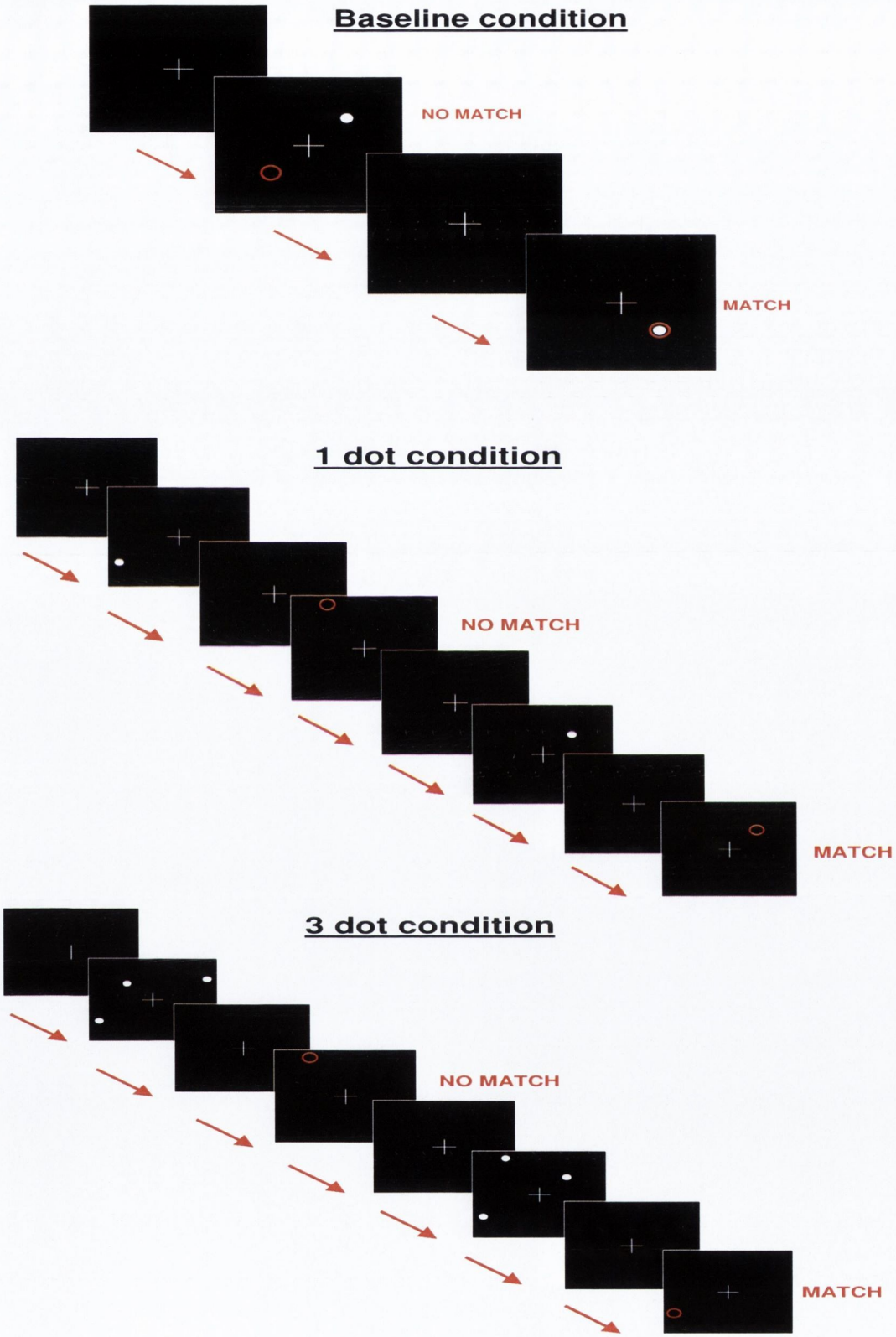
Participants performed two spatial working memory tasks while undergoing magnetic resonance imaging. Both tasks employed similar stimuli and response requirements in order that the results be as comparable as possible. Participants performed practice versions of each task prior to entering the scanner to ensure they were familiar with the task requirements.

3.2.2.1 Spatial Working Memory (SWM) Maintenance Task

Participants were required to maintain the spatial locations of a specified number of visual stimuli in working memory over a short delay. A block design was employed in this study comprising one baseline and two experimental conditions (see Fig. 3.1). At the beginning of each trial, a white fixation cross was presented centre-screen 1 second prior to the presentation of the task stimuli. This cross remained onscreen for the duration of the task. Each trial of the two experimental conditions consisted of a 500ms presentation of the target stimulus (one or three white dots, each of which was displayed in one of 12 possible onscreen positions), followed by a 3 second delay and a 500ms presentation of the probe stimulus (red circle). During the baseline condition, the red circle and one white dot were presented simultaneously. The interstimulus duration was 2 seconds, and 6 trials were performed per block.

Participants performed 4 blocks of each condition, presented in counterbalanced order. Regardless of condition, participants were instructed to respond (using their right hand only) by pressing the left button on an MRI compatible response pad if the probe location matched one of the target locations and with a right button press if the probe was in a different location. 50% of trials were defined as 'match' trials, where the target and probe stimuli were presented in the same location. A further 20% of trials (confined to the 1dot and 3dot conditions) were defined as 'difficult' trials, where the probe was in a different location to the stimulus, but near the target location (i.e. in the same quadrant).

Fig. 3.1. Spatial Working Memory Task – fMRI version

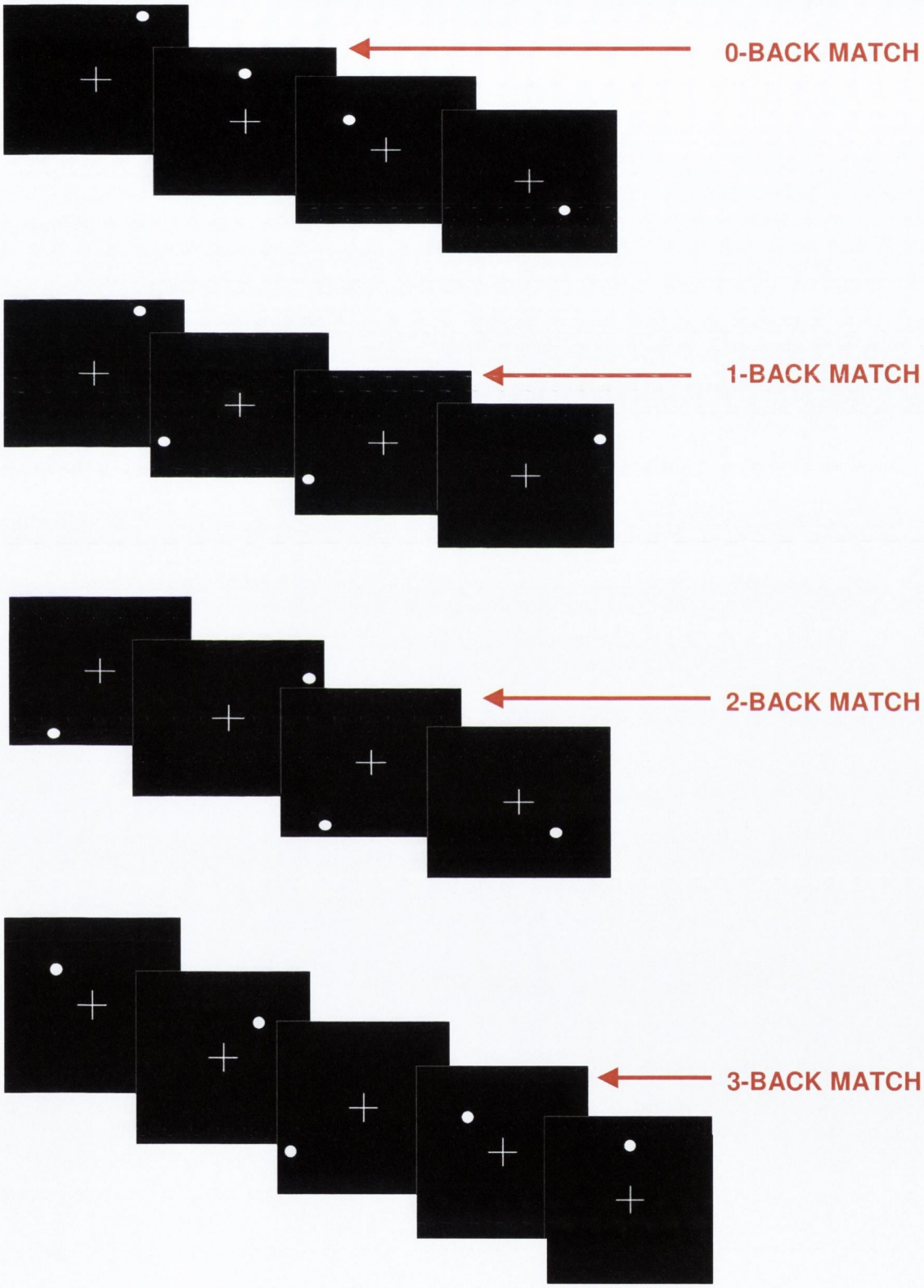


3.2.2.2 N-Back Task

In the N-Back task, participants were requested to respond to the spatial location of a stimulus presented a specified number of trials previously (see Fig. 3.2). Participants performed three experimental conditions (designated 1-back, 2-back and 3-back) and a baseline condition, referred to as '0-back'. In each condition, single white dots were presented onscreen for 500ms, with a 2500ms interstimulus interval. In the 1-back, 2-back and 3-back conditions, participants were instructed to respond with a left button press if the white dot was in the same spatial location as the dot 1, 2 or 3 trials previously, and with a right button press if the dot was in a different location. Participants were instructed to respond to every trial using their right hand only. In the 0-back condition, participants were asked to respond with a left button press if the white dot was in a predefined location (directly above the fixation cross) and otherwise to press the left button.

Participants performed 60 trials of each condition, divided into 4 sub-blocks. The order of presentation of the four task conditions was counterbalanced across the sample. 20% of trials (3 per sub-block, 12 per condition) were defined as n-back 'match' trials, where the target stimulus location matched the location of the stimulus presented n trials previously. A further 20% of trials were defined as 'foil' trials, where the stimulus was presented a different number of trials previously than that required by the task condition. The 2-back condition for example included four trials where the stimulus matched the trial 1 back, and four where it matched the trial 3 back.

Fig. 3.2. N-back Task for fMRI



3.2.3 fMRI Methods

3.2.3.1 fMRI parameters

Scanning was conducted using a Philips Intera Achieva 3T MR system. A mirror mounted on the MR scanner reflected the visual display which was projected onto a screen behind the scanner. Foam padding was placed around the participant's head to reduce movement during scanning. 180 T1 weighted anatomical axial images were obtained over 6 minutes with a field of view of 230 x 230mm and a slice thickness of 0.9mm. This data was used for spatial normalisation and accurate localisation of BOLD signal changes. SENSE (SENSitivity Encoding) is a parallel imaging technique employing multiple RF receiver coils to reduce MRI scanning time. A reference scan (to test for sensitivity of the SENSE coil) and SENSE scan were conducted prior to the T1 scan. During the functional runs for the experiment, a T2* weighted echo planar imaging sequence was used to obtain 32 non-contiguous slices covering the whole brain (3.5 mm slices with 0.3mm interslice gap; TR = 2000ms; TE = 35ms; FOV = 224 x 224mm; matrix size in Fourier space = 64 × 64 mm). One run of the spatial working memory task (220 dynamic scans; 440s) and one run (141 dynamic scans; 282s) for each of the four N-Back task conditions were conducted. Due to a technical failure, the data from 2 participants was lost during scanning.

3.2.3.2 fMRI data processing

All MRI analyses were conducted using the AFNI analysis package (Cox, 1996). Before processing, the four N-Back runs were concatenated into one dataset. Only one

run of the SWM maintenance task was conducted, and so the concatenation step was not required. All data sets were then subjected to the same data processing steps. 3D volume registration was conducted to correct for small amounts of participant head motion around 3 axes (anterior-posterior, superior-inferior, left-right). A volume towards the beginning of the scan was chosen as the reference volume, and subsequent volumes were aligned with it. Edge detection was then performed to remove activation outside the brain. Regressors detailing the time series for each condition were convolved with the haemodynamic response function, and percentage signal change for each experimental condition was calculated relative to baseline. Voxels with greater than ±3% signal change relative to baseline were defined as outliers and removed from the dataset. Greater than 99% of signal change values lay within the ±3% range. The T1 anatomical datasets were warped into a common stereotaxic (Talairach) space using the ICBM452 brain atlas (Rex, Ma, & Toga, 2003). The functional data was then aligned with the anatomical data and warped into Talairach space. Following a second edge detection using the Talairached anatomical data as a mask, the functional data was blurred using a Gaussian kernel with a root mean square (rms) of 3mm. This step was performed to allow for slight discrepancies in anatomy and to increase the chance of activated regions overlapping between participants.

3.3 Results

3.3.1 Task Results

Mean accuracy and reaction time at the three SWM maintenance conditions are listed in Table 3.1. Repeated measures ANOVAs indicated that accuracy decreased significantly with increasing memory load ($F(2,76) = 37.897$, $p<.001$). Memory load also significantly affected reaction time ($F(2,76) = 20.956$, $p<.001$), although planned contrasts indicated that the trend that best fit the data was quadratic, as the reaction time observed during the 1dot condition was faster than that during either the baseline or 3dot conditions. 50% of errors in the 1dot condition, and 33% in the 3dot condition, were found to have been committed on 'difficult' trials.

Table 3.1. Descriptive statistics for Spatial Working Memory maintenance task

	Accuracy	*Total errors*	*% of errors on difficult trials*	*Reaction time*
Baseline	0.94 (.18)	1.41 (4.2)	-	741.48 (167.8)
1dot	0.91 (.18)	2.05 (4.33)	50%	680.23 (184.4)
3dot	0.82 (.18)	4.31 (4.29)	33%	786.32 (208.7)

Descriptive statistics for the four N-Back task conditions are listed in Table 3.2. Mean accuracy was found to decrease with increasing N-Back load ($F(2,76) = 54.023$, $p<.001$) as mean reaction time increased ($F(2,76) = 115.966$, $p<.001$). The percentage of errors made on 'foil' trials increased steadily from 0-back to 2-back, but declined in the 3-back condition. No significant differences as a result of participant gender were observed in either task.

Table 3.2. Mean and standard deviation of performance measures on all N-Back conditions

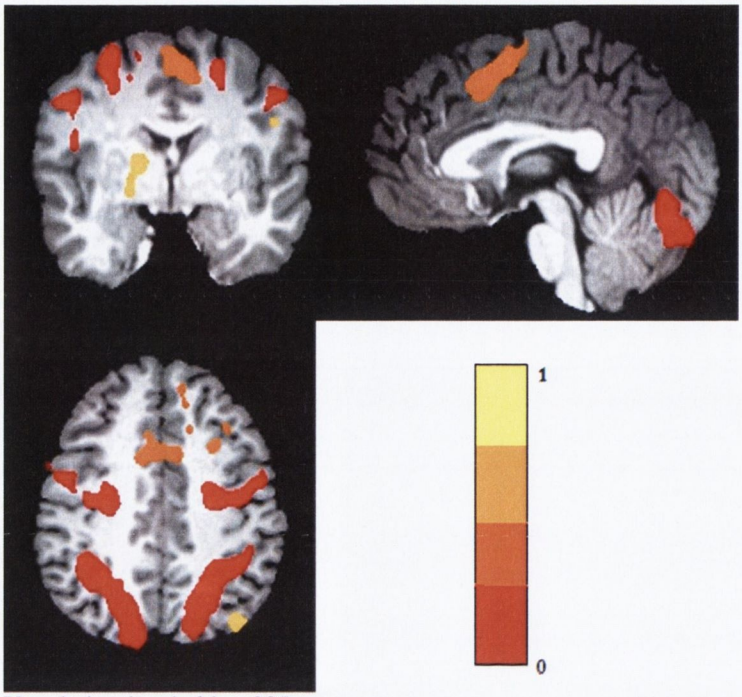
	Accuracy	*Hits*	*Misses*	*Incorrect hits*	*% of errors on foil trials*	*Reaction time (ms)*
0Back	0.98 (.04)	11.49 (.65)	0.51 (0.6)	0.65 (2.1)	11%	527.86 (115.5)
1Back	0.96 (.04)	10.78 (1.2)	1.22 (1.2)	1.16 (1.6)	16%	690.53 (157.8)
2Back	0.90 (.07)	10.24 (2.2)	1.76 (2.2)	3.35 (2.7)	32%	861.19 (231.9)
3Back	0.84 (.08)	9.27 (2.2)	2.59 (2.1)	4.78 (3.2)	18%	962.20 (280.3)

3.3.2 fMRI Results

3.3.2.1 SWM Maintenance Task

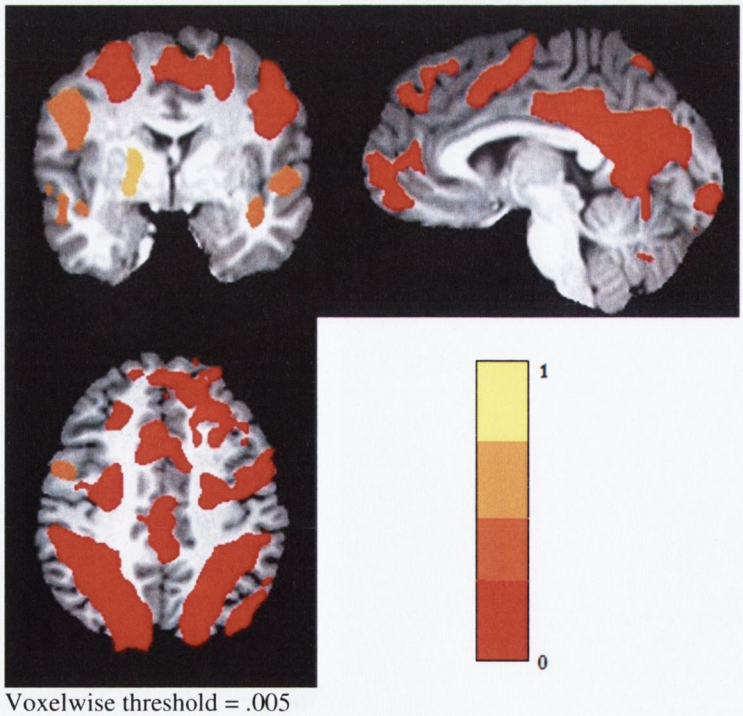
In order to compare BOLD signal intensity in the two active conditions of the spatial working memory task, activation maps were first created separately for the 1dot and 3dot conditions. One-sample t-tests were conducted against zero for each voxel within each condition. No clear guidelines for the selection of voxelwise thresholds in fMRI analysis exist, however voxelwise alphas of .001, .005 and .0001 are the most frequently reported. A reasonably conservative alpha level of .005 was chosen as the significance threshold for each voxel in the analysis of the SWM maintenance task. With 38 degrees of freedom, the associated critical *t* value was 2.98029. To increase power to differentiate signal from noise, clusters of contiguous activated voxels were identified. Monte Carlo simulations were conducted to establish the minimum cluster size not due to chance. With a voxelwise threshold of .005, clusters of 289 voxels or greater would have a less than 5% probability of occurring by chance alone. Distinct activation maps displaying supra-threshold clusters were then created for the 1dot and 3dot conditions. These maps are displayed in Figs. 3.3a and 3.3b.

Fig. 3.3a. Clusters activated in the 1dot condition relative to baseline



Voxelwise threshold = .005

Fig. 3.3b. Clusters activated in the 3dot condition relative to baseline



Voxelwise threshold = .005

A single activation map containing regions activated in either the 1dot or 3dot condition was created using AFNI's 'OR map' command. 28 clusters with a volume exceeding 289 voxels survived the .005 threshold and were included in this map. Using the combined map as a mask, BOLD signal intensities during both conditions were produced for each participant within each cluster.

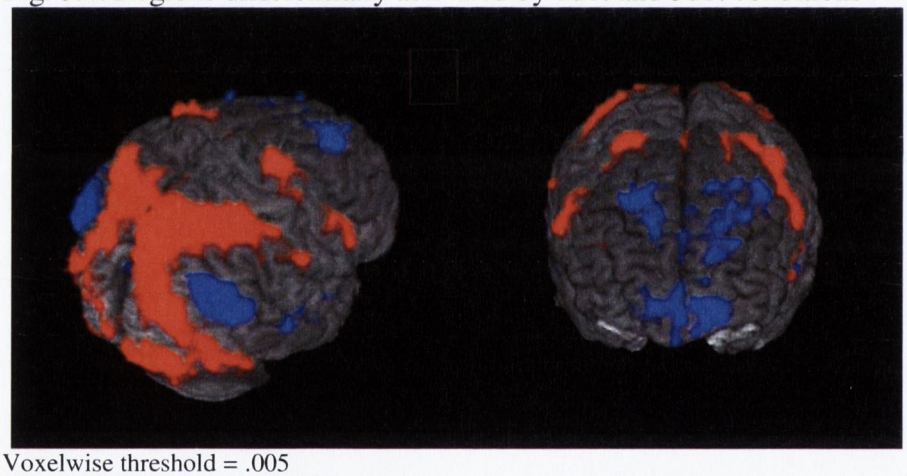
Table 3.3. Regions differentially activated by 1dot and 3dot conditions

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
3dot > 1dot					
Superior and inferior parietal lobule (BA 7/40); middle occipital gyrus (BA18/19); precuneus (BA31)	170612	Right	2	-68	20
Superior and inferior parietal lobule (BA 7/40); middle occipital gyrus (BA18/19); precuneus (BA31)	50291	Left	-2	-1	46
Middle frontal gyrus (BA 9/46)	1716	Right	41	35	23
Inferior frontal gyrus (BA47)	1107	Left	-30	24	0
Inferior frontal gyrus (BA44/45)	512	Left	-54	20	12
Parahippocampal gyrus	579	Left	-26	-26	-4
Inferior occipital gyrus; fusiform gyrus (BA10)	3815	Left	-27	-88	-21
Substantia nigra	326	Right	11	-14	-13
Substantia nigra	417	Left	-8	-24	-12
Thalamus	1232	Right	24	-26	0
1dot > 3dot					
Posterior cingulate (BA23); cingulate gyrus (BA31); cuneus (BA18)	37913	Bilateral	-2	-53	21
Medial and superior frontal gyrus (BA8)	22606	Bilateral	-10	33	46
Medial frontal gyrus (BA10)	17056	Bilateral	-2	58	4
Middle temporal gyrus (BA39)	15383	Left	-47	-67	27
Middle temporal gyrus (BA39)	8464	Right	50	-66	23
Superior temporal gyrus (BA22/41)	12495	Left	-49	-16	4
Superior temporal gyrus (BA22/41)	10037	Right	50	-20	1
Inferior temporal gyrus (BA21)	303	Right	50	-66	23

Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
Voxelwise α= .005; differences between conditions significant where $p < .05$ (uncorrected)

Repeated measures t-tests were conducted in SPSS 14 to compare average BOLD signal in each cluster between conditions. Table 3.3 contains a list of all regions where activation differed significantly between the 1dot and 3dot conditions ($p<.05$, uncorrected). These differences are displayed graphically in Fig. 3.4. Red voxels denote regions where BOLD signal intensity during the 3dot condition exceeded that during the 1dot condition. Blue voxels indicate regions where average BOLD signal was greater during the 1dot than 3dot condition.

Fig. 3.4. Regions differentially activated by 1dot and 3dot conditions



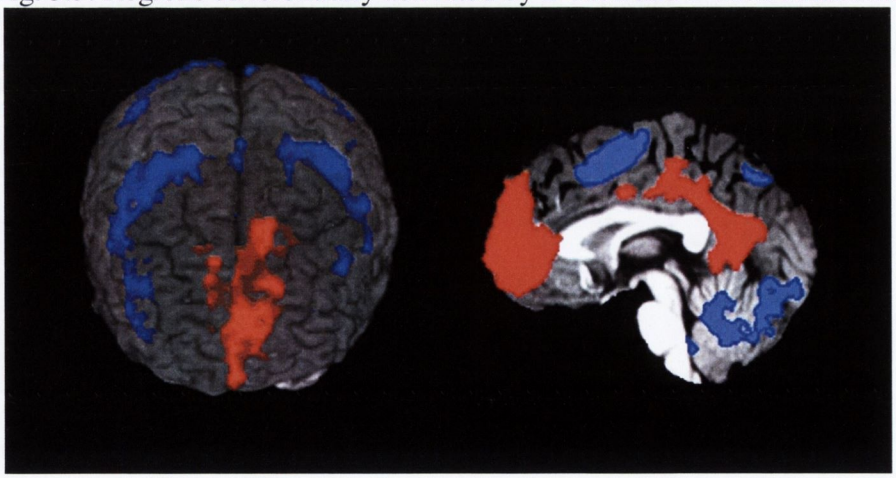
Voxelwise threshold = .005
Differences between conditions are deemed significant where $p<.05$
Red regions: 3dot BOLD signal > 1dot
Blue regions: 1dot BOLD signal > 3dot

3.3.2.2 N-Back Task

Activation maps for the 1, 2 and 3-back conditions were created relative to the 0-back (baseline) condition in the same manner as for the SWM maintenance task. A voxelwise threshold of .005 was found to be overly liberal in this case as very large clusters covering the majority of the brain were produced. A threshold of .0001 was therefore decided upon. With 37 degrees of freedom, the critical *t* value was 4.359 and Monte Carlo simulations indicated that clusters of 66 voxels or greater had a less than 5% probability of occurring by chance. An activation map composed of regions activated during the 1-back, 2-back or 3-back conditions was then generated.

An initial inspection of the BOLD signal associated with each task condition indicated that while activation tended to increase from 1-back to 2-back, there was a sharp decrease in many clusters from 2-back to 3-back.

Fig. 3.5. Regions differentially activated by 2-back and 3-back conditions



Voxelwise threshold = .0001
Differences between conditions are deemed significant where $p<.05$
Red regions: 3-back BOLD signal > 2-back
Blue regions: 2-back BOLD signal > 3-back

Paired sample t-tests were conducted to compare BOLD signal relative to baseline in the 2-back and 3-back conditions. The regions differentially activated by the two conditions are listed in Table 3.4 and displayed in Fig. 3.5.

Table 3.4. regions differentially activated by 2-back and 3-back conditions.

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
3-back > 2-back					
Medial frontal gyrus (BA10/32); superior frontal gyrus (BA 9); anterior cingulate (BA24)	40707	Bilateral	-3	48	21
Posterior cingulate (BA 23/29/30); cingulate gyrus (BA31)	26346	Bilateral	-2	49	21
Superior temporal gyrus (BA38)	451	Left	-30	13	-25
Superior temporal gyrus (BA41); insula (BA13)	4106	Right	44	-19	5
Insula (BA13)	753	Left	-37	-22	11
Fusiform gyrus (BA18); inferior occipital gyrus (BA17)	4515	Right	25	-89	-20
Fusiform gyrus (BA18); inferior occipital gyrus (BA17)	3839	Left	-25	-88	-22
Fusiform gyrus (BA37)	228	Left	-33	-51	-5
2-back > 3-back					
Inferior and superior parietal lobule (BA 7/40); middle occipital gyrus (BA 17/18/19)	115303	Bilateral	3	-66	18
Middle, superior and inferior frontal gyrus (BA 8/9/10/46); medial frontal gyrus (BA6)	63383	Bilateral	9	2	47
Middle frontal gyrus (BA9)	1194	Left	-47	26	34
Middle frontal gyrus (BA10)	370	Left	-34	46	21
Inferior frontal gyrus (BA47); insula (BA13)	5149	Right	39	18	0
Insula (BA13)	2248	Left	-32	21	3
Thalamus	3201	Left	-15	-20	9
Thalamus	2474	Right	10	-20	4
Thalamus; caudate	830	Right	14	0	10
Putamen; lentiform nucleus	2221	Left	-17	5	3

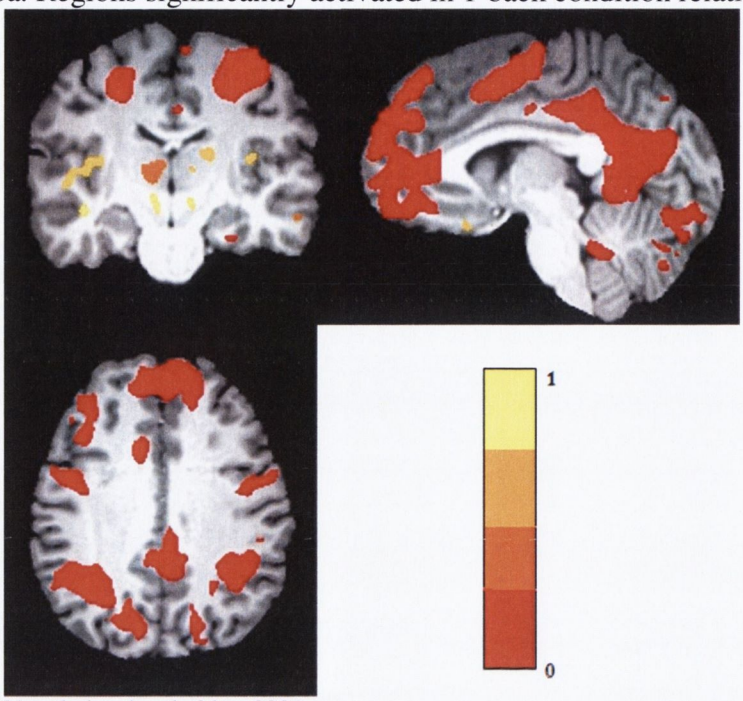
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
Voxelwise $\alpha = .0001$; differences between conditions significant where $p < .05$ (uncorrected)

BOLD activation in regions associated with spatial working memory, including middle frontal gyrus, inferior frontal gyrus and inferior and superior parietal lobule was found to be reduced in the 3-back condition relative to the 2-back condition. In contrast, activation in medial frontal and posterior cingulate regions was greater during the 3-back than the 2-back. Examination of the statistics for each cluster indicates that this increase in activation from 2-back to 3-back is actually a decrease in deactivation in these regions during the more difficult task condition.

These medial regions form part of the default network, generally found to be active when participants are engaged in self-directed, non-goal-oriented thinking (Buckner, Andrews-Hanna, & Schacter, 2008). This, coupled with the decrease in activation in spatial working memory regions, indicates that participants were not actively engaging with the task during the 3-back condition, and had effectively given up. Consequently, all further analysis was restricted to the 1-back and 2-back conditions. Regions significantly activated relative to baseline (with a voxelwise threshold of .0001) in the 1-back and 2-back conditions are displayed in Figs. 3.6a and 3.6b respectively.

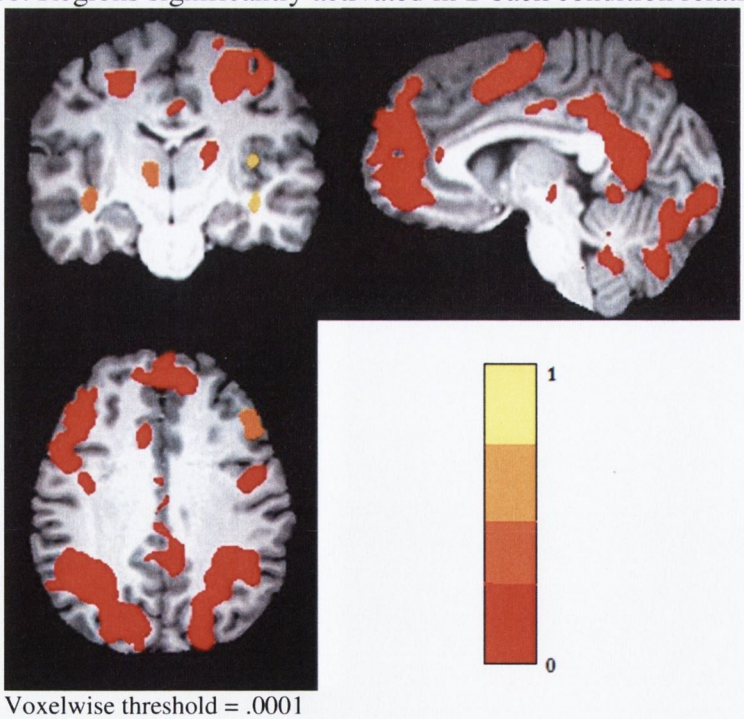
A new OR map containing regions activated during the 1-back or 2-back conditions was created and used for comparisons between the two conditions. With a voxelwise alpha level of .0001, only 3 clusters displayed significant differences in BOLD signal between the 1-back and 2-back conditions. In all three clusters, BOLD signal intensity was higher in the 2-back than in the 1-back condition.

Fig. 3.6a. Regions significantly activated in 1-back condition relative to baseline.



Voxelwise threshold = .0001

Fig. 3.6b. Regions significantly activated in 2-back condition relative to baseline.



Voxelwise threshold = .0001

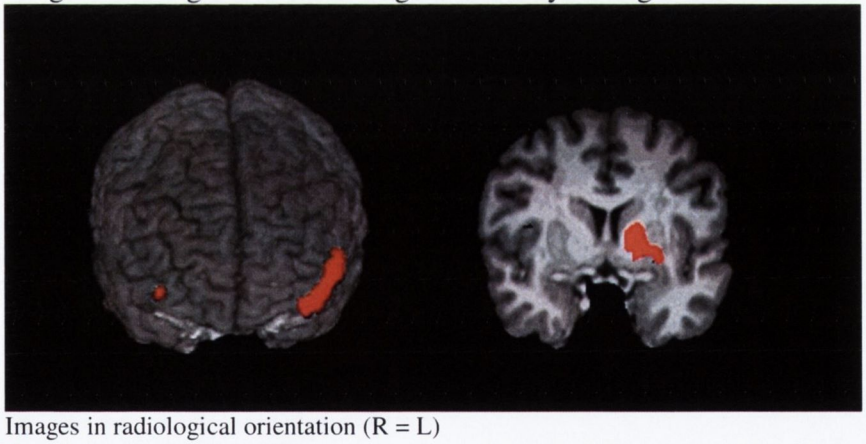
Table 3.5. Regions differentially activated by 1-back and 2-back conditions

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
Inferior frontal gyrus (BA46/47)	2180	Left	-46	31	-4
Middle frontal gyrus (BA10)	112	Right	40	49	4
Putamen; lentiform nucleus	2072	Left	-17	5	3

Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
Voxelwise α= .0001; differences between conditions significant where $p < .05$ (uncorrected)

These regions are listed in Table 3.5 and displayed graphically in Fig. 3.7. This contrast was also examined at the more liberal .001 and .005 thresholds, however no other significantly different clusters emerged.

Fig. 3.7 Regions with greater BOLD signal intensity during 2-back than 1-back condition



Images in radiological orientation (R = L)
Voxelwise threshold = .0001
Differences between conditions are deemed significant where $p<.05$.

3.3.3 Genetic results

3.3.3.1 Behavioural-genetic comparisons

DBH, DAT1 and COMT genotype frequencies within the sample are listed in Table 3.6. No significant effects of variants in these genes were observed on any SWM or N-Back task performance measure, including accuracy, reaction time, or proportion of errors made on foil or difficult trials. As described in Chapter 1 (section 1.3), a failure to observe genetic effects on behavioural measures is not unusual in neuroimaging studies as the samples used are frequently small. Significant effects are however frequently seen on brain activation associated with the task. The absence of significant effects of genetic variants on task performance does not therefore preclude analysis of the BOLD signal intensity observed during the task.

Table 3.6. DBH, DAT1 and COMT genotype frequencies for variants in the DBH, DAT1 and COMT genes within the fMRI study sample

COMT Val/Met		*DBH C-1021T*		*DAT1 3' UTR*	
Val/Val	12 (31.6%)	CC	16 (42%)	9/9	7 (19%)
Val/Met	17 (44.7%)	CT	16 (42%)	9/10	15 (40.5%)
Met/Met	9 (23.7%)	TT	6 (16%)	10/10	15 (40.5%)
Total	38	*Total*	38	*Total*	37

3.3.3.2 fMRI-genetic comparisons

Regions of interest in the assessment of specific genetic effects on BOLD signal intensities during SWM were calculated using OR maps containing regions activated by any genotype group in any condition within the task. An OR map of activation in both conditions was first generated for each of the 3 genotype groups (homozygous for

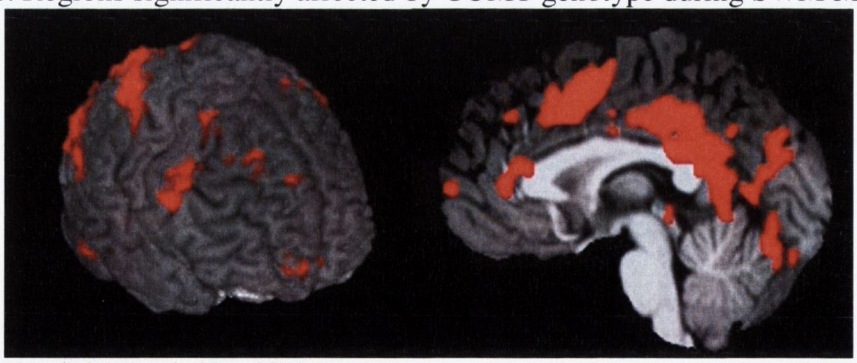
allele 1, heterozygous, or homozygous for allele 2). These were then merged to form a single map which was used as a mask for further analysis. This method is more powerful than voxelwise statistics as the analysis is restricted to objectively selected regions of interest, reducing the number of comparisons performed. The method also has the advantage that regions activated by only one genotype group are included in the merged activation map where they might otherwise be obscured by the averaging of signal intensities across all groups. The main disadvantage to the method lies in the fact that the critical *t* value associated with the chosen voxelwise threshold is dependent on sample size. Small groups will require a higher critical *t*, reducing the chance of activated voxels exceeding the threshold. Small groups will therefore contribute less to the final activation map than larger groups. The BOLD signal change statistics from each cluster in this map were then entered into a mixed model ANOVA where the between subjects variable was genotype and the within subjects variable was SWM load. This allowed the analysis not only of genetic effects on each task condition but also of interactions between task condition and genotype.

3.3.3.3 fMRI-genetic analysis: SWM Maintenance Task

An activation map containing regions activated by any COMT Val/Met genotype group during the 1dot or 3dot SWM conditions was created. Following voxelwise thresholding at .005 with a critical *t* value of 3.83252, 3.25199 and 3.49661 for the Met/Met, Val/Met and Val/Val groups respectively, 38 significantly activated clusters were retained. Only 9 participants were homozygous for the Met allele, as a result of which the Met/Met group contributed less to the OR map than the other two groups.

Significant differences were observed between the Met/Met and Val/Met groups in very few clusters; these two groups were therefore collapsed into one group (Met carriers) which was then compared to the Val/Val group. 27 clusters showed a significant effect of COMT genotype; in all clusters, the Met carrier group displayed significantly higher BOLD signal intensity than participants homozygous for the Val allele (see Table 3.7 and Fig. 3.8).

Fig. 3.8. Regions significantly affected by COMT genotype during SWM Maintenance



Voxelwise threshold = .005
Red voxels = regions where BOLD signal was greater in Met carriers group than Val/Val group

Table 3.7. Clusters displaying significant effects of COMT genotype on BOLD activation during SWM maintenance.

Region	*Volume (mm^3)*	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
Met carriers > Val/Val					
Superior and inferior parietal lobule (BA 7/40); middle occipital gyrus (BA18)	83285	Right	22	-61	28
Superior and inferior parietal lobule (BA 7/40); middle occipital gyrus (BA18)[1]	66984	Left	-29	-68	20
Middle frontal gyrus and precentral gyrus (BA6)[1]	14797	Left	-39	-4	44
Middle frontal gyrus and precentral gyrus (BA6)[1]	6980	Right	26	-6	50
Middle frontal gyrus; inferior frontal gyrus; precentral gyrus (BA6/9)	4938	Right	49	3	35
Middle frontal gyrus (BA10/46)	353	Left	-42	44	12
Superior frontal gyrus (BA8)	1899	Right	22	27	50
Superior frontal gyrus (BA10)[1]	794	Right	17	63	11
Inferior frontal gyrus (BA47)[1]	1165	Right	33	25	-7
Inferior frontal gyrus (BA47)[1]	322	Right	44	14	-9
Inferior frontal gyrus (BA47)[1]	312	Left	-32	27	-8
Medial frontal gyrus (BA10)[2]	1288	Right	6	59	0
Medial frontal gyrus (BA6/32); cingulate gyrus (BA24)	10439	Bilateral	0	14	44
Anterior cingulate (BA24/32)	1260	Bilateral	2	38	12
Cingulate gyrus (BA24)[2]	324	Left	-4	-6	36
Superior temporal gyrus (BA22)[2]	5147	Left	-46	-15	-2
Superior temporal gyrus (BA22)[2]	381	Right	58	-14	2
Middle temporal gyrus; inferior temporal gyrus (BA20/21)[1]	2866	Left	-62	-40	-11
Postcentral gyrus (BA5)	313	Left	-26	-39	60
Insula (BA13)[2]	416	Right	44	-15	-5
Parahippocampal gyrus (BA35)[2]	351	Right	24	-17	-26
Lentiform nucleus; putamen[1]	1712	Left	17	-2	4
Thalamus[1]	1277	Right	23	-28	0
Culmen[1]	934	Left	-10	-26	-11
Culmen[1]	449	Right	3	-32	-2
Culmen	301	Left	-17	-46	-20
Declive[1]	752	Left	-29	-55	-15

Voxelwise $\alpha = .005$; group differences significant where $p < .05$ (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis.
[1]Significant at 1dot condition only
[2]Significant at 3dot condition only

An OR map composed of regions activated by any DBH C-1021T group during any SWM maintenance task condition was generated. With a voxelwise threshold of .005, critical *t* for the CC, CT and TT groups was 3.28604, 3.28604 and 4.77334 respectively. Following thresholding, 30 significantly activated clusters were retained. As very few participants were homozygous for the T allele the CT and TT groups were collapsed into a single (T carriers) group.

Table 3.8. Clusters displaying significant effect of presence or absence of DBH T allele on BOLD activation during SWM maintenance.

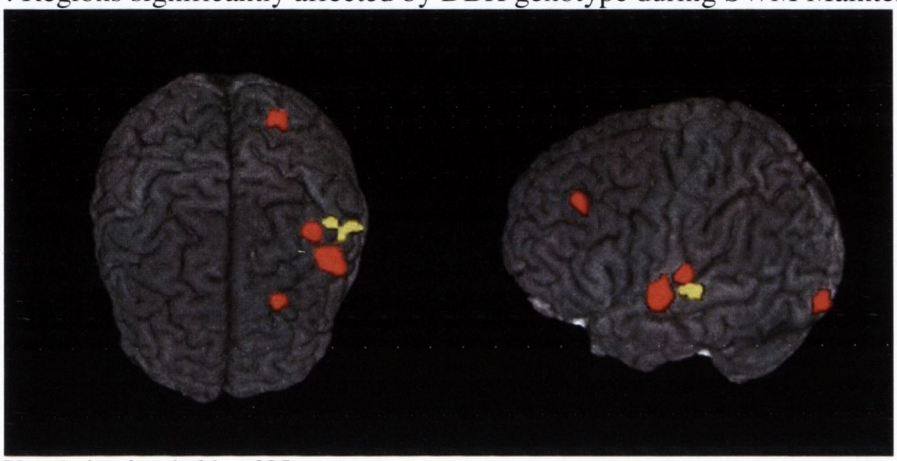
Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
T carriers > CC					
Middle frontal gyrus (BA8)[1]	470	Left	-27	33	44
Superior temporal gyrus (BA13)[1]	643	Left	-44	-18	8
Superior temporal gyrus (BA22)[2]	1704	Left	-54	-5	2
Uvula	626	Left	-26	-90	-21
CC > T carriers					
Superior temporal gyrus (BA42)[2]	572	Left	-60	-21	6

Voxelwise α= .005; group differences significant where p <.05 (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis.
[1]significant only at 1dot condition
[2]significant only at 3dot condition

Mixed model analysis of variance was conducted to compare this combined group with the CC group, and the results are summarised in Table 3.8. Five clusters, all in the left hemisphere, displayed significant effects of DBH genotype. Carriers of the T allele displayed increased BOLD signal relative to participants with 2 copies of the C allele in the middle frontal and superior temporal gyrus and uvula, while the opposite trend was

observed in another superior temporal gyrus cluster (see Fig. 3.9). The majority of these effects were only significant at one level of the spatial working memory task.

Fig. 3.9. Regions significantly affected by DBH genotype during SWM Maintenance



Voxelwise threshold = .005
Red voxels = regions where BOLD signal was greater in T carriers group than CC group
Yellow voxels = regions where BOLD signal was greater in CC group than T carriers group

At a voxelwise threshold of .005, 42 clusters were retained in an activation map containing regions activated by any DAT1 group during the 1dot or 3dot SWM conditions. The critical *t* values for the 9/9, 9/10 and 10/10 groups were 4.31683, 3.3257 and 3.3257 respectively. 13 clusters showed a significant effect of DAT1 genotype. Participants with two copies of the 10-repeat allele displayed increased BOLD signal relative to other genotype groups in clusters in the frontal cortex, while clusters in temporal regions displayed greater activation in those participants with one or two copies of the 9-repeat allele (see Fig. 3.10).

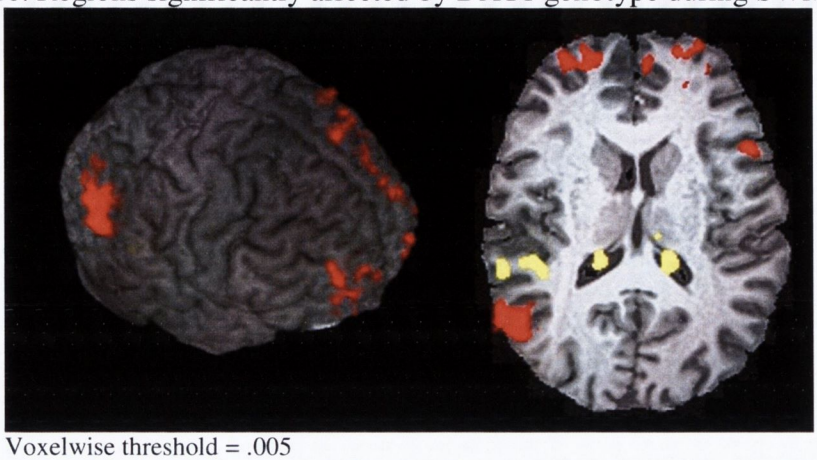
Table 3.9. Clusters displaying significant effects of DAT1 genotype on BOLD activation during SWM maintenance.

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
10/10 > 9-repeat carriers					
Middle and superior frontal gyrus (BA 8/9/10)	8151	Left	-22	51	26
Superior frontal gyrus (BA8)	4534	Left	-31	17	49
Middle and inferior frontal gyrus (BA46)[1]	2429	Left	-48	39	0
Middle frontal gyrus (BA9); inferior frontal gyrus (BA 44/45)[1]	573	Left	-51	18	-18
Middle frontal gyrus (BA10)[2]	6211	Right	16	57	9
Middle temporal gyrus (BA39)[1]	8093	Right	51	-62	26
Caudate[1]	322	Left	-11	9	1
Cerebellum[2]	788	Right	1	-50	-31
9-repeat carriers > 10/10					
Superior temporal gyrus (BA22); insula (BA13)	1063	Right	49	-36	17
Insula (BA13)[2]	417	Right	42	-16	-7
Thalamus[2]	684	Left	-15	-31	17
Caudate[2]	309	Right	17	-34	16
Culmen[1]	460	Left	-33	-47	-15

Voxelwise $\alpha = .005$; group differences significant where $p < .05$ (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
[1]Significant at 1dot condition only
[2]Significant at 3dot condition only

Many of these effects were observed at only one level of the SWM task (see Table 3.9 for details). Significant effects of DAT1 were also observed in clusters in the cerebellum and striatum, although these effects did not follow a consistent pattern.

Fig. 3.10. Regions significantly affected by DAT1 genotype during SWM Maintenance



Voxelwise threshold = .005
Red voxels = regions where BOLD signal was greater in 10/10 group than 9-repeat carriers group
Yellow voxels = regions where BOLD signal was greater in 9-repeat carriers group than 10/10 group

3.3.3.4 fMRI-genetic analysis: N-Back Task

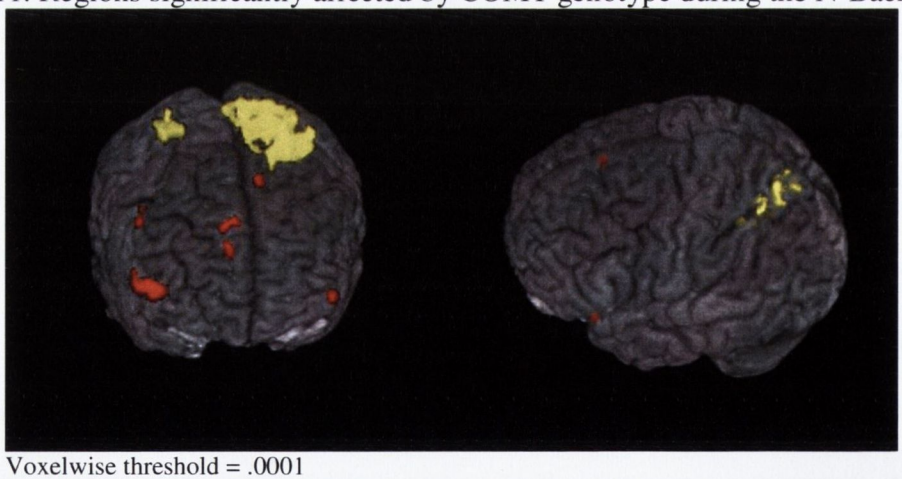
An activation map composed of regions activated by any COMT Val/Met genotype groups during the 1-back or 2-back conditions of the N-Back task was produced. 59 significantly activated clusters were observed with a voxelwise threshold of .0001. With a voxelwise threshold of .0001, critical *t* for the Val/Val, Val/Met and Met/Met groups was 5.92119, 5.23909 and 7.12 respectively. Met carriers were found to display increased BOLD signal relative to Val/Val participants in several frontal clusters and one cluster in the cingulate gyrus during the 2-back condition. The opposite trend was observed during the 1-back condition in clusters in the parietal lobule and precuneus. These findings are summarised in Table 3.10 and depicted graphically in Fig. 3.11.

Table 3.10. Clusters displaying significant effects of COMT genotype on BOLD activation during N-Back

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
Met carriers > Val/Val					
Inferior frontal gyrus (BA47)[2]	602	Right	46	12	-3
Inferior frontal gyrus (BA47)[2]	86	Left	-46	29	-8
Superior frontal gyrus (BA8)[2]	109	Right	11	49	37
Superior frontal gyrus (BA6)[2]	92	Right	18	23	51
Superior frontal gyrus (BA6)[2]	80	Left	-9	23	61
Cingulate gyrus (BA32)[2]	205	Right	6	23	37
Val/Val > Met carriers					
Superior and inferior parietal lobule (BA7/40)[1]	6381	Left	-31	-59	42
Precuneus (BA7)[1]	502	Right	22	-67	45

Voxelwise $\alpha = .0001$; group differences significant where $p < .05$ (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
[1]Significant during 1-back condition only
[2]Significant during 2-back condition only

Fig. 3.11. Regions significantly affected by COMT genotype during the N-Back task



Voxelwise threshold = .0001
Red voxels = regions where BOLD signal was greater in Met carriers group than Val/Val group
Yellow voxels = regions where BOLD signal was greater in Val/Val group than Met carriers group

An OR map composed of regions activated by any DBH C-1021T group during either the 1-back or 2-back condition contained 53 significantly activated clusters at a voxelwise threshold of .0001. The critical *t* value was 5.23909 for the CC group, 5.36341 for the CT group and 11.1777 for the TT group.

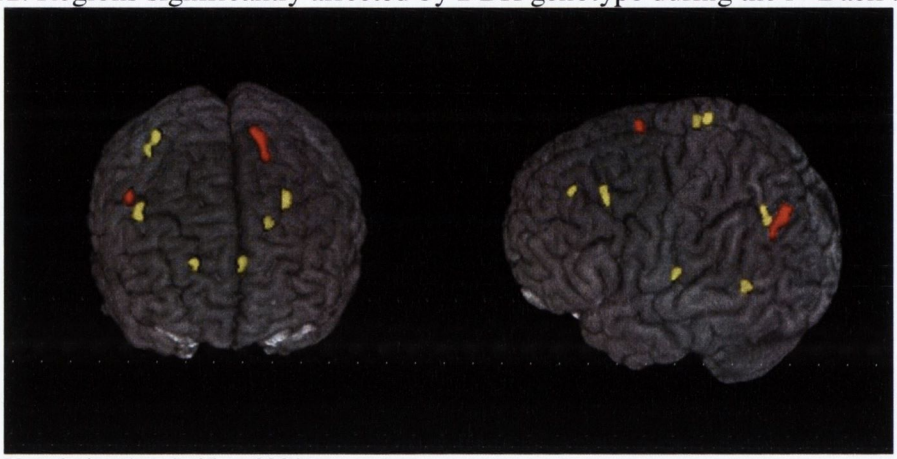
Table 3.11. Clusters displaying significant effect of DBH genotype on BOLD activation during N-Back.

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
T carriers > CC					
Cuneus[1]	378	Left	-22	-72	30
Superior temporal gyrus (BA22)[1]	125	Right	53	-8	-3
CC > T carriers					
Superior frontal gyrus (BA8)[2]	165	Left	-28	19	48
Postcentral gyrus (BA 3/40)[2]	209	Right	36	-32	56
Superior parietal lobule (BA7)[2]	75	Right	28	-46	58
Inferior temporal gyrus (BA37)[2]	170	Right	41	-64	4
Superior temporal gyrus (BA22)[2]	125	Left	-23	23	52
Substantia nigra[1]	106	Left	-8	-18	-10
Cerebellum[1]	110	Right	13	-54	-30

Voxelwise α= .0001; group differences significant where $p < .05$ (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
[1]Significant at 1-back only
[2]Significant at 2-back only

The CC group displayed increased BOLD signal relative to the T carriers group in several frontal and parietal regions during the 2-back condition. The opposite trend was observed in the superior temporal gyrus and cuneus during the 1-back condition (see Table 3.11 and Fig. 3.12).

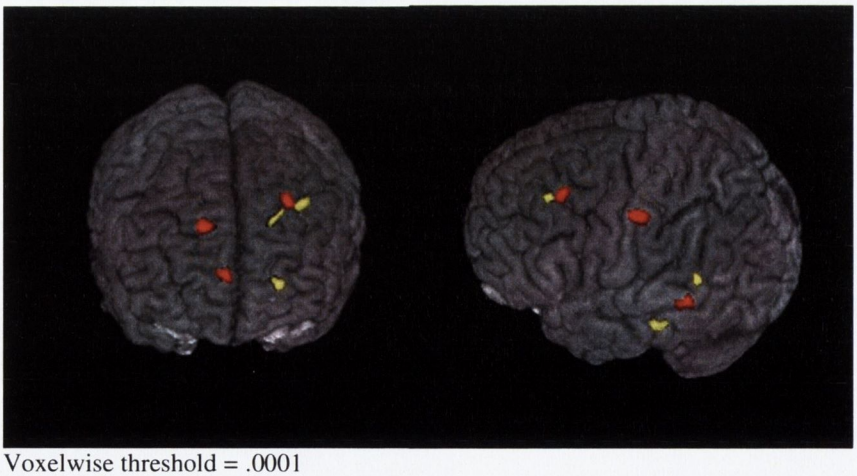
Fig. 3.12. Regions significantly affected by DBH genotype during the N-Back task



Voxelwise threshold = .0001
Red voxels = regions where BOLD signal was greater in T carriers group than CC group
Yellow voxels = regions where BOLD signal was greater in CC group than T carriers group

An OR map of all clusters activated by all DAT1 groups during the 1- and 2-back conditions was generated with a voxelwise threshold of .0001. Critical *t* for the 9/9, 9/10 and 10/10 groups was 9.08235, 5.51252 and 5.36341 respectively. 55 significantly activated clusters were retained.

Fig. 3.13. Regions significantly affected by DAT1 genotype during the N-Back task



Voxelwise threshold = .0001
Red voxels = regions where BOLD signal was greater in 10/10 group than 9-repeat carriers group
Yellow voxels = regions where BOLD signal was greater in 9-repeat carriers group than 10/10 group

BOLD activation in six clusters was found to be significantly affected by DAT1 genotype (see Fig. 3.13). The direction of the effect of DAT1 on activation in each cluster is listed in Table 3.12. Participants with the 10/10 genotype displayed greater activation in middle frontal gyrus during the 1-back condition, and reduced activation relative to the 9-repeat carriers group during the 2-back condition.

Table 3.12. Clusters displaying significant effects of DAT1 genotype during N-Back.

Region	*Volume* (mm^3)	*Hemisphere*	*Cluster coordinates*		
			x	*y*	*z*
10/10 > 9-repeat carriers					
Middle frontal gyrus (BA8)[1]	176	Left	-29	20	47
Thalamus[1]	309	Right	10	-19	11
Cerebellum[1]	166	Left	-2	-43	-33
9-repeat carriers > 10/10					
Middle frontal gyrus (BA8)[2]	87	Left	-22	27	41
Middle temporal gyrus (BA39)[2]	127	Left	-43	-49	4
Culmen[1]	130	Left	-28	-29	-31

Voxelwise $\alpha = .0001$; group differences significant where $p < .05$ (uncorrected).
Cluster coordinates denote centre of mass of each cluster in LPI format where x = left-right axis, y = posterior-anterior axis and z = inferior to superior axis
[1]Significant during 1-back condition only
[2]Significant during 2-back condition only

3.4 Discussion

3.4.1 Task-related activations

Two spatial working memory tasks were performed by participants in this study as they underwent functional magnetic resonance imaging. In the introduction (section 3.1) it was hypothesised that BOLD signal intensity would increase with increasing memory load in both the SWM maintenance and N-Back tasks. In accordance with the hypotheses, performance of the spatial working memory maintenance task produced load-dependent activations in regions of parietal and frontal cortex, including DLPFC. This provides further support for the involvement of the dorsolateral prefrontal cortex in maintenance memory, as suggested by a number of previous studies (Todd S. Braver *et al.*, 1997; J. Jonides *et al.*, 1997; Manoach *et al.*, 1997; Rypma *et al.*, 1999). Performance measures indicated that accuracy decreased and response time increased with increasing memory load, indicating that DLPFC was recruited as the task became more difficult.

Performance of the SWM maintenance task at the higher memory load also led to deactivations in regions of the medial frontal gyrus, lateral temporal cortex and posterior cingulate. These regions have been shown to form part of the 'default network' (Raichle *et al.*, 2001), a group of brain regions that are found to be active when individuals are engaged in non goal-oriented, internally directed thinking (Buckner, Andrews-Hanna, & Schacter, 2008; Schneider *et al.*, 2008). These regions are usually found to be deactivated during demanding task conditions where participants must direct their attention towards achieving a goal. The deactivation of

these regions during the 3dot condition of the task, combined with the increase in activation in prefrontal and parietal regions and the decrements in performance accuracy, indicates that participants found this condition more difficult to perform and devoted more cognitive resources to completing it successfully.

The results of the N-Back task were not in line with expectations, and were more difficult to interpret. Accuracy and response time measures indicated a steady decline in performance with increasing N-Back memory load. BOLD signal intensities were expected to increase in frontal and parietal regions with increasing task difficulty, however this was not found to be the case. Increases in activation between the 1-back and 2-back conditions were observed in only three clusters, two of those in the DLPFC. Activations declined sharply in parietal and prefrontal clusters (including DLPFC) between the 2-back and 3-back conditions, while default network regions showed an increase in BOLD signal intensity.

This pattern of activations implies that participants were not performing the task during the 3-back condition and had reverted to an internally focused cognitive state. Confusingly, however, performance measures do not appear to support this interpretation. While accuracy during the 3-back was reduced relative to the 2-back, participants still responded correctly on 84% of trials. One possible explanation for this is that participants may have employed an alternative, but still largely successful, strategy in selecting their responses to the 3-back trials. While more incorrect hits were made during the 3-back condition than the 2-back, 32% of all errors in the 2-back

occurred on 'foil' trials, where the trial stimulus matched the location of the stimulus presented 1 or 3 trials previously. It was expected that participants would make a high proportion of errors on these foil trials, particularly during the more difficult conditions as they juggled an increasing number of stimuli in working memory. The fact that the average percentage of errors made on foil trials dropped to 18% in the 3-back suggests that participants may have resigned the usual method of mentally counting back three trials, and resorted to a more intuitive strategy. Research has shown that even when participants do not attend to visual stimuli, or are unaware of having seen them at all, unconscious perception of these stimuli influences subsequent responding (see Merikle, Smilek, & Eastwood, 2001 for a review). A mechanism similar to this may explain the incompatibility of the performance measures and BOLD signal measures observed during the 3-back.

3.4.2 Genetic associations with task-related activations

The observed effects of the COMT Val/Met polymorphism on BOLD signal intensity during spatial working memory are in direct contrast to the hypotheses (see section 3.1) and to much of the published research in this area. Increased activation with the high-dopamine Met allele across the whole brain, including DLPFC and parietal areas, was observed here, while previous studies reported a decrease in BOLD signal to be associated with this allele (Bertolino, Blasi *et al.*, 2006; Caldú *et al.*, 2007; Heinz & Smolka, 2006; V. S. Mattay *et al.*, 2003; Meyer-Lindenberg *et al.*, 2006). The fact that the trend reported here was observed in a large number of clusters during both the SWM maintenance task and the N-Back task indicates that this result is unlikely to be

the result of Type I error but is in fact a real finding. Several possible explanations may account for the discrepancy between these results and those found in the COMT literature.

The first of these relates to the differences in task design between previous studies and the present study. The majority of research into COMT effects on brain activation has been conducted by one research group using one particular form of the n-back task (e.g. Bertolino, Blasi *et al.*, 2006; Egan *et al.*, 2001; V. S. Mattay *et al.*, 2003; Meyer-Lindenberg *et al.*, 2006). The stimuli in that task consist of the numbers 1-4 presented in the corners of a diamond-shaped box. Each number is always presented in the same place (e.g. 1 in the top corner, 2 in the left corner). This task is sometimes interpreted as a test of *spatial* working memory, but the fact that the numbers are presented in predefined locations means that participants may simply hold the number in working memory rather than the location. Both the SWM maintenance and N-Back tasks used in the present study make use of 12 distributed spatial locations that can not be easily verbalised. These tasks are therefore both more difficult and truer tests of spatial working memory than that described in the literature.

While a simplistic interpretation would have it that the increased net dopamine occasioned by the Met allele generally leads to an improvement in working memory, the reality is of course more complex. The Met allele results in increased tonic and reduced phasic dopamine subcortically, and increased overall cortical dopamine levels relative to the Val allele (Bilder, Volavka, Lachman, & Grace, 2004). As discussed

previously (section 1.1.3), working memory has an inverted-U shaped relationship with dopamine, requiring that dopamine levels fall within a certain range for optimal performance (Amy F. Arnsten, 1998). D_2 receptors are thought to influence spatial working memory but not non-spatial working memory (Monica Luciana & Collins, 1997). As the present task is unarguably an example of a spatial working memory task, it is possible that the effects of COMT genotype on phasic and tonic dopamine interacted differently with D_2 receptors than during the n-back task used in previous studies, thereby producing an altered pattern of COMT effects on brain activation.

Increased dopamine levels brought on by administration of bromocriptine, a D_2 receptor agonist, reduced activity during the encoding phase of a working memory task and increased activity during the response phase (Gibbs & D'Esposito, 2005). The present task required a continuous response, such that encoding of new trials and responding to old ones occurs simultaneously. Among participants with the high-dopamine Met allele, the net reduction of BOLD signal during the task block may therefore have been the result of a decrease in activation while recalling the location of the stimulus. Unfortunately the block design of this task prevents any more detailed examination of this possibility. An event-related study would be required to examine the effects of COMT genotype on the different task phases.

In addition to variations in task design, individual differences may account for some of the discrepancies between the results reported here and those in the literature. It is worth noting that no effect of COMT was observed on spatial working memory

behavioural performance, or indeed on any other cognitive measure, in a sample of over 200 participants in the behavioural phase of this study (see Chapter 2). A variety of factors including education, intelligence, ethnicity or underlying dopamine levels within the population sampled may therefore have reduced the effect of variation at the COMT locus on working memory. Dopamine agonists (such as dextroamphetamine and bromocriptine) have been shown to improve performance and reduce cortical activation in participants with poor baseline working memory capacity, while worsening the performance and increasing the BOLD signal of those with high baseline working memory capacity (Kimberg, Aguirre, Lease, & D'Esposito, 2001; V.S. Mattay *et al.*, 2000). Participants in the present study demonstrated very high accuracy, indicative of good working memory capacity, even on the more difficult task conditions. As a result of this, participants with the high-dopamine Met allele may have been at a disadvantage relative to the participants with the low-dopamine Val/Val genotype.

As this was the first study to examine the effects of DBH genotype on cortical activation during spatial working memory, no hypotheses regarding the direction of any effect were formed, and the results reported here will require replication. Participants with the high-dopamine T allele displayed increased BOLD signal intensity in left hemisphere prefrontal and temporal regions during the SWM maintenance task, but decreased activation in prefrontal and parietal regions during the 2-back condition of the N-Back. The implication of this is that the high levels of dopamine resulting from possession of the T allele were detrimental to network efficiency during the easier

maintenance task, but improved efficiency during the more demanding 2-back task condition. Levels of dopamine receptor stimulation that may be ideal for one task may be detrimental to performance of another (Robbins, 2000). The optimal level of dopamine availability would therefore appear to be higher for performance of the N-Back task than for performance of the maintenance task.

An additional factor here may be the role of noradrenaline in performance of the SWM maintenance and N-Back tasks. As described previously, noradrenaline improves performance on tasks where exogenous alerting is low, and participants must maintain an internal state of alertness throughout the task (J. T. Coull, Middleton *et al.*, 1995; A. Smith & Nutt, 1996). The SWM maintenance task is subjectively easier to perform than the N-Back, requiring no manipulation of information in working memory. Those participants who possessed the T allele (linked with lowered noradrenaline availability) may therefore have required more effort, indexed by increased BOLD signal in prefrontal regions, to remain alert during the maintenance task. The fact that no significant effect of the DBH marker was observed in the right hemisphere, traditionally associated with sustained attention and alerting (Pardo, Fox, & Raichle, 1991; Tomas Paus & Zatorre, 1997), does not support this interpretation. It is however possible that this effect was present at a level below the .005 threshold, and a more liberal voxelwise alpha level might be in a position to detect it.

No specific hypotheses regarding the direction of any effect of DAT1 on task-related activation were presented. Regions of interest for a comparison of brain activation

between DAT1 genotype groups include both cortical and subcortical regions as a result of the role played by the dopamine transporter in the striatum. Increased activation in frontal regions, including DLPFC, was observed with the low-dopamine 10-repeat allele during the SWM maintenance task and in the 1-back condition of the N-Back task. The opposite trend was observed during the 2-back condition. Within the striatum, the 10/10 group displayed increased activation during the SWM maintenance 1dot and N-Back 1-back condition, and reduced activation during the SWM maintenance 3dot condition. No effect of DAT1 was observed in the striatum during the 2-back condition, although this may have been as a result of the stringent voxelwise threshold applied. Although no effect of DAT1 genotype was observed on task performance in this study, increasing number of 10-repeat alleles was associated with faster SWM reaction time at the higher memory loads in the larger behavioural study described in Chapter 2 (section 2.3.2.5), and homozygosity for the 10-repeat allele has previously been associated with reduced prefrontal activation during working memory performance (Bertolino, Blasi *et al.*, 2006; Caldú *et al.*, 2007). Taken together, these results suggest that reduced dopamine levels resulting from increased dopamine transporter binding may result in a reduction of working memory efficiency at low memory loads but an improvement of network efficiency at higher memory loads.

Several interesting effects of the COMT, DBH and DAT1 genes have been reported here which go some way towards explaining the role of dopaminergic genes in the control of spatial working memory. It should not be forgotten however that these genes account for only a small proportion of the variance in working memory performance

and the associated cortical activity. It is likely not only that these genes interact with one another, but also that they interact, in both linear and non-linear fashion, with other neurotransmitter systems involved in the modulation of executive function. The methods used here unfortunately do not allow for the comprehensive analysis of these interactions in the absence of very large samples. The results described here form part of a body of research attempting to elucidate the genetic architecture of executive function. The implications of these findings for the field as a whole, and suggestions for future research directions are discussed in Chapter 4.

Chapter 4

General Discussion

4.1 Summary of main findings

This thesis described a study in two parts aimed at uncovering associations between executive functions and variants within genes involved in the synthesis and management of catecholamines. The first phase of the study, conducted with a sample of 205 participants, examined the effects of a range of genes on behavioural performance of a battery of cognitive tasks. The second phase consisted of an fMRI investigation of the effects of three of these genes on spatial working memory-related brain activation in a subset of the previous sample. The main results from both of these studies, and their relationship to one another, are summarised below.

Among the most interesting findings to emerge from the behavioural phase of the study was the relationship between DBH and sustained attention. DBH genotype was found to predict errors on the SART, with the TT genotype (associated with relatively higher levels of dopamine and lower levels of noradrenaline) resulting in the worst performance. Participants were found to show a slight right spatial bias on average, and this bias (as measured by the Landmark task) was significantly affected by variants in both the DBH and DAT1 genes. The fact that both SART and Landmark measures were affected by DBH genotype is an important finding, as strong links have been reported between sustained and spatial attention. The fact that both forms of attention are influenced by the same genes provides confirmation of this relationship. The only gene found to affect spatial working memory performance was DAT1, with both the 3′ UTR and Intron 8 variants exerting significant effects on reaction time during the task.

Two spatial working memory tasks were performed during the fMRI phase of the study. One was concerned only with the maintenance or storage element of working memory, while the other examined the effects on brain activation of manipulating information in working memory. The two tasks used in this study employed very similar stimuli and response requirements, ensuring that their results are comparable. The results of this study indicate that, contrary to one popular theory (e.g. McCarthy *et al.*, 1994; Owen, Evans, & Petrides, 1996), the dorsolateral prefrontal cortex (DLPFC) is not activated only during tasks where manipulation is required, but also in a load-dependent manner during maintenance tasks. COMT genotype was found to have a very strong effect on cortical activation associated with both forms of spatial working memory, in the absence of any discernable differences in task performance. This effect was in the opposite direction to that generally reported in the literature (see section 3.1), possibly as a result of differences in task design. Significant effects in left hemisphere regions, including prefrontal cortex, were observed as a function of DBH genotype; carriers of the T allele displayed increased activation in these regions during easy task conditions, but decreased activation during more difficult conditions. On balance, it appears likely that this result is due to the role of DBH in sustained attention and not in spatial working memory *per se*. Variation in the DAT1 gene was also found to result in differences in task-related activation in both cortical and subcortical regions. Increased dopamine transporter binding, resulting in reduced dopamine availability at the synapse, led to reduced working memory efficiency at low memory loads but improved efficiency at higher memory loads.

The fMRI phase of this study provided support for the idea (discussed in full in section 1.3) that neuroimaging studies may be more powerful assays of cognitive-genetic associations than behavioural studies alone. Significant effects of variants in the DBH, DAT1 and COMT genes were found on fMRI measures in this study where no effect on behavioural performance was observed. While some genes (e.g. DBH, DAT1 and 5HTT) were found to significantly affect behavioural performance in the larger sample examined in the behavioural study, no genetic effects were observed in the sub-group of 40 participants who took part in the fMRI study. Most strikingly, a very clear effect of COMT genotype was observed on BOLD signal across the whole brain during performance of both MRI tasks yet, surprisingly, no effects whatsoever of that gene were observed on behavioural measures.

4.2 Methodological issues

Before any analysis of the implications of the findings described above, a number of methodological issues must be addressed. The most surprising finding in this study was the increase of BOLD activation during SWM task performance with the COMT Met allele. The discrepancies between the direction of this effect in the results presented here and in those described previously (see Chapter 3, section 3.1) bring the validity of these findings into question. The possibility exists that these results were false positives, as a result of either methodological or Type I error, however a closer examination of the data indicates that this is unlikely. The SWM maintenance and N-back tasks were conducted and analysed independently; this acts as an internal control, reducing the likelihood that the unusual pattern of results observed emerged as a result

of methodological error. In addition, this pattern of results was observed in a great many regions across the brain, and the probability of this trend occurring with such frequency as a result of chance alone is extremely small. The results presented here are therefore unlikely to be false positives, and may be considered to reflect a real association between genotype and task-related activation. With this reassurance, the impact of other methodological issues may be considered.

The study described here examined the effects of deliberately chosen 'candidate' genes. In recent years, whole genome studies of psychiatric disorders have become increasingly common, and it has occasionally been suggested that these should replace the traditional candidate gene format. Genome-wide studies are entirely data-driven and have the power to detect novel associations not previously anticipated. They are however very expensive, often difficult to conduct, and require very large samples. Even real associations from one of the multiple genes of small effect that are frequently hypothesised to affect individual differences in executive function may not survive correction for the hundreds of thousands of comparisons conducted. Candidate gene studies, on the other hand, are generally designed on the basis of a priori hypotheses relating to the function of the gene. With fewer genes under investigation, less power is needed to uncover significant effects. Studies of these genes can therefore be reasonably conducted with considerably fewer participants than would be required in a whole genome study.

Sample size is directly related to the power of a statistical test to detect significant effects, and so larger samples are of course preferable, particularly when looking for small effects. As complex cognitive functions are generally thought to be under the influence of multiple genes of small effect, this issue is of prime importance here. In order to have adequate power to detect genetic effects within healthy populations, samples in the order of several thousand participants may be required. In Chapter 2, however, small effects of specific genes on cognitive performance were detected with a sample of just over 200. While this is cause for satisfaction, the main issue with regard to underpowered studies is that no conclusion can be drawn about the relationship between variables where no significant effect is detected. While it is possible that no effect exists, it is also possible that a real effect can not be detected with the sample size used. The negative results presented in this study must therefore be considered as exploratory as no definitive conclusion can be reached.

The rise in popularity of genomewide studies also gives rise to questions regarding appropriate significance thresholds in candidate gene studies. If convincing results in genomewide studies require very high p values, it is reasonable to ask whether the same stringency ought to be applied to studies examining just a few markers. The argument has been made that the statistical analyses performed in a candidate gene study are effectively a subset of all the analyses which could potentially be performed, and ought to be treated as such. This is not a trivial argument; nevertheless, the presence of a strong biological hypothesis adds weight to the nominally significant findings in

candidate gene studies, even in the absence of genomewide significance levels, and the knowledge obtained from studies of this nature may outweigh their disadvantages.

One difficulty that arose in the course of this study was the question of examining interactions between genes in their effects on brain activation. Genes within the same neurotransmitter system may well operate in an interdependent fashion, but any assessment of patterns in these interactions is problematic. Very few papers have been published to date which have attempted to do this. Those that have done so have generally employed some form of multiple regression (e.g. Bertolino, Blasi *et al.*, 2006; Caldú *et al.*, 2007), using genotypes as regressors. This method is limited in its usefulness in that it imposes a linear trend on the interaction which is not necessarily the best way of explaining the relationship between the variables. In small samples such as those frequently used in MRI studies, it is really only practical to examine interactions between two or three genes using this method; this therefore assumes an a priori assumption as to which genes will interact, placing limits on the scope of the investigation.

Ideally, interactions would be examined using the OR maps method described in Chapter 3. Using this method, an interaction between two genetic variants, A and B, would result in nine genotype groups (i.e. three at locus A x three at locus B). Average BOLD signal in each of these groups during the task would be calculated separately, and then merged to form a single activation map. While it has the clear advantage of including regions activated by only one subgroup, this method is very dependent on

sample size. Further, the use of this method to assess interactions between genes is not practical with small samples as sub-groups with very few or no participants will inevitably arise, and t-tests can not be conducted using these groups. New methods which may alleviate this problem are discussed in Future Directions (section 4.5) below.

4.3 Implications of findings in this thesis

A vast quantity of data was presented over the course of this thesis, some of which has very real implications for the continued study of genetic links to cognitive functions. Crucially, this study provided important information that could inform research into the different neurochemical requirements of the executive functions. The 'high dopamine' DBH T allele led to poor sustained attention and reduced spatial working memory network efficiency during the SWM maintenance task, but improved efficiency during the more demanding 2-back task condition. In contrast, the 'low dopamine' DAT1 10-repeat allele had similar effects, decreasing memory efficiency during easy task conditions, but improving it at higher memory loads. These results may appear contradictory at first glance, however it should be recalled that these genes operate primarily on different brain regions, with DBH altering cortical catecholamine levels, while the effects of DAT1 take place mainly in the striatum. These genes therefore appear to exert their effects somewhat independently of one another. Where they do interact, there is no reason to assume that they would do so in an additive fashion. Indeed, an examination of the DBH and DAT1 genes, described in Chapter 2, section

2.3.2.5, showed that changes in dopamine levels resulting from variants in the two genes interacted in a non-additive manner in their effects on sustained attention.

Of course, DBH is not primarily a dopaminergic gene, and its role in noradrenaline synthesis probably drives much of its effects. During easy task conditions which are not intrinsically arousing, such as the lower-load SWM maintenance conditions, increased levels of arousal and alertness are required to maintain focus on the task (I.H. Robertson *et al.*, 1997). Noradrenaline levels interact with underlying states of arousal (J. T. Coull, Middleton *et al.*, 1995; A. Smith & Nutt, 1996), and greater prefrontal activation may have been required among participants with the low-noradrenaline T allele to maintain task-appropriate levels of attention during these task conditions. Combined with the fact that no effect of DBH genotype on SWM performance was observed even in a sample of over 200 participants, this would imply that variation in DBH does not affect spatial working memory *per se*, but rather operates on the alertness and attention systems that allow performance of working memory tasks. In other words, despite the very close links between attention and memory described in Chapter 1 (section 1.1.1), a trade-off may exist between the neurochemical requirements of the various executive functions. While increased levels of noradrenaline appear to be beneficial for tasks requiring sustained attention, dopamine levels appear to be the crucial factor in spatial working memory tasks.

Results from the neuroimaging phase of the study also have direct implications for the imaging genetics field. The fact that the direction of the effect of COMT genotype on

BOLD signal was opposite to that generally described in the literature indicates that the neurological substrates of spatial working memory may not be as well understood as has been assumed. This study demonstrated clearly that differences in task design, and possibly in individual and population characteristics, can result in dramatic alterations in the pattern of activation associated with specific genotype groups. This inevitably begs the question as to what extent the results of studies from different groups can be usefully compared.

The outcome of any study is inevitably dependent on the methods used, and the results produced at the end of an analysis reflect the decisions taken at each step of the analysis as much as they reflect the patterns within the data. Within fMRI studies, the choice of voxelwise thresholds will impact significantly on the pattern of activations observed. The choice of threshold is somewhat arbitrary, and no strict conventions exist. The choice of threshold involves a trade-off between the degree of activation of individual voxels and the size of clusters detected; a liberal threshold will reveal large clusters of moderately activated voxels but may miss small clusters of highly activated voxels, while a more conservative threshold will retain those small, highly activated clusters but will fail to detect large clusters of voxels which do not quite reach the threshold.

Within the present study, a more stringent threshold was applied to the N-Back data than to the SWM maintenance data; the rationale behind this was explained in section 3.3.2.2. Although the thresholds chosen for each analysis were appropriate to the data,

this decision has ramifications for the interpretation of results. As a higher threshold was chosen for the N-Back data, fewer significant differences between conditions and between genotype groups were observed in this task than in the SWM Maintenance task. A careless reading of these findings could lead to the interpretation that COMT genotype has less impact on the manipulation aspect of working memory than on tasks involving maintenance only. This example illustrates the point that great caution must be used in comparing the findings of disparate studies without careful consideration of the design and analysis choices made by the researchers at every step of the investigation.

4.4 Cognitive-genetic-imaging studies and developing understanding

Studies like the one described in this thesis may be of particular benefit in building models of executive functioning, drawing as they do on the fields of neuropsychology, neuroimaging and cognitive genetics. This interdisciplinary approach provides a clearer understanding of the relationship between the executive functions than any one line of research; a neuropsychological theory of cognition which is informed by both genetic factors and an understanding of brain anatomy and function will inevitably be more comprehensive than one based on behavioural measures alone. It was this goal which first led to the development of the concept of endophenotypes, discussed in Chapter 1. Although the endophenotype experiment has in one sense been unsuccessful, having failed to deliver the promised increases in effect sizes (Flint & Munafo, 2007), it has resulted in a clearer understanding of the connections between brain, behaviour and genome in healthy individuals. This leaves us in a better position to form hypotheses

about the causes of neuropsychological disorders, and aids in the development of treatments. In the past, theories of disorder frequently arose from the almost coincidental neurological effects of drugs with known effects on symptomology. An example of this is the hypodopaminergic theory of ADHD, which emerged from efforts to explain the efficacy of methylphenidate in treating symptoms of hyperactivity and inattention. An increasing level of knowledge of the biological substrates of disorder introduces the possibility of developing treatments from theories, rather than theories from treatments.

An interdisciplinary approach may also reveal or confirm hypothesised relationships between cognitive functions. One clear example of this is reported in this thesis. As described in Chapter 1, close links, both functional and anatomical, are thought to exist between attention and memory. The DBH gene, shown in the Behavioural phase of the study (Chapter 2, section 2.3.2.2) to influence sustained attention, and through it, spatial attention, was shown in Chapter 3 to have an impact on the degree of cortical activation in prefrontal regions during spatial working memory. This provides important confirmation, via three converging lines of inquiry, that the sustained attention system, which is largely under noradrenergic control, exerts an influence on a range of executive functions, including spatial working memory.

4.5 Future directions

Several possibilities for future research are suggested by the outcomes of this study. One of the most striking results to emerge from this study was the clear effect of DBH

genotype on tasks requiring sustained attention. An fMRI study of this effect is therefore a promising next step. The T allele, which results in decreased noradrenaline availability at the synapse, would be expected to lead to reduced efficiency of the sustained attention network. In addition, it would be interesting to establish whether the direction of any DBH effect would interact with task difficulty, as was the case during the spatial working memory task. The findings of such a study could contribute significantly to our understanding of the neurological substrates of sustained attention.

Several possible explanations for the unexpected direction of the COMT effect on cortical activation during spatial working memory were discussed in Chapter 3. Verification of one or more of these explanations could be achieved through a study designed to gauge the effects of variations in task and individual characteristics on brain activation. Such a study would require a number of conditions in which number and type of stimuli were varied in parametric fashion. The use of an event-related design would allow assessment of the effects of genotype on the various phases of memory, including encoding, delay and retrieval. Individual characteristics such as baseline working memory span could also be included as covariates.

One possibility mentioned in explanation of the unusual pattern of results emerging from analysis of the N-Back task (see section 3.3.2.2) was that participants had effectively given up on performing the 3-back condition of the task because they believed it to be too difficult. Future studies using this task could include a longer period of training prior to scanning. This might increase participants' confidence in

their ability to perform all conditions of the task, and ultimately improve adherence to task goals during 3-back.

As described above (section 4.2), one of the main limitations of the present study was the difficulty of examining the effects of gene-gene interactions on BOLD signal in small samples. Machine learning methods such as support vector learning or kernel canonical correlation analysis (Hardoon *et al.*, in press) are currently being developed to examine the effects of large numbers of SNPs on neuroimaging data. To date, these methods have been applied only to genetic analysis of structural MRI data, but investigations into their application of functional imaging data are ongoing. This promising line of research may confirm hypotheses relating to the interactions of neurotransmitters systems, and may also reveal unexpected relationships between genes.

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Appendix I

DNA extraction and genotyping details

I.1 Extraction of DNA from Oragene DNA Self-Collection Kits

Samples were incubated overnight at 50°C before being divided into 1ml portions in autoclaved microcentrifuge tubes. 40µl of Oragene buffer, provided by the manufacturers, was added to each 1ml sample and mixed gently by inversion, resulting in the precipitation of impurities. The samples were incubated on ice for 10 minutes and then centrifuged at 13,400 x g for 3 minutes at room temperature. The supernatant from the entire sample was placed in a 15ml tube, and equal volume (approx 4ml) of 95% ethanol was added. The contents of the tubes were mixed gently by inversion, and left to stand at room temperature for 10 minutes, allowing the DNA to precipitate. The samples were then centrifuged at 3,500 x g for 10 minutes at room temperature. The supernatant from each tube was discarded, leaving a DNA pellet. Any remaining ethanol was allowed to evaporate off overnight. Each pellet was dissolved in 250µl of TE buffer, and incubated at 4°C for 2-3 days, with occasional tapping to encourage dissolution of DNA. Samples were subsequently stored at 4°C when not in use.

I.2 Genotyping of Insertion / Deletion (Ins/Del) and Variable Tandem Nucleotide Repeat (VNTR) polymorphisms

The polymorphic region in each gene was amplified in a 25µl volume containing 5µl DNA at 10ng/µl, 2.5µl standard PCR buffer, 2µl $MgCl_2$, 4µl dNTPs, 1 unit of Taq polymerase and 1µl of each of the appropriate forward and reverse primers listed in Appendix I. The DRD4 Exon III VNTR is located in a region containing a high number of G-C bonds; dGTP in the master mix was therefore replaced with the same quantity

of 7-deaza-dGTP. The thermocycler conditions for these markers can be found in Table I.1.

Table I.1: PCR conditions for VNTRs and Insertion/Deletions

Marker	*PCR thermocycler conditions*
DAT1 3′ UTR	95°C for 9.5 minutes; 40 cycles of 95°C (60 sec), 65°C (60 sec), 72°C (60 sec); 72°C for 5 minutes.
DAT1 Intron 8	95°C for 9.5 minutes; 40 cycles of 95°C (60 sec), 65°C (60 sec), 72°C (60 sec); 72°C for 5 minutes.
MAOA 30bp promoter	94°C for 4.5 minutes; 35 cycles of 94°C (40 sec), 59°C (40 sec), 72°C (60 sec); 72°C for 10 minutes.
DRD4 Exon III	95°C for 9.5 minutes; 40 cycles of 95°C (15 sec), 58°C (15 sec), 72°C (20 sec); 72°C for 10 minutes.
5HTT ins/del	Touch-down cycle. 95°C for 9.5 minutes; 9 cycles of 95°C (30 sec), 63°C → 55°C (30 sec, -1°C per cycle), 72°C (30 sec); 28 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec); 72°C for 10 minutes.

Appendix I

Table I.2: PCR conditions and restriction enzymes for RFLPs

Marker	*PCR thermocycler conditions*	*Restriction enzyme*	*Digest thermocycler conditions*
DBH TaqI	95°C for 9 minutes; 30 cycles of 95°C (30 sec), 59°C (30 sec), 72°C (30 sec); 72°C for 10 minutes.	TaqαI	65°C for 2 hours
DBH G444A	Touchdown cycle. 94°C for 10 minutes; 6 cycles of 94°C (30 sec), 72°C → 62°C (30 sec, -2°C per cycle), 72°C (30 sec); 29 cycles of 94°C (30 sec), 62°C (30 sec), 72°C (30 sec); 72°C for 5 minutes.	EcoNI	37°C for 4 hours
MAOA G941T	94°C for 9 minutes; 28 cycles of 94°C (30 sec), 54°C (30 sec), 72°C; (30 sec); 72°C for 10 minutes.	Fnu4HI	37°C for 4 hours

I.3 SNaPshot genotyping procedure for DRD4 -521 and -616 SNPs

The region containing both SNPs was amplified by PCR in a 20µl reaction containing 10µl of Failsafe™ Buffer G, 1 unit of Taq polymerase and 1µl each of the PCR primers listed in Table x at 20pmol/µl. The PCR conditions were as follows: an initial denaturing step of 9.5 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 56°C, and 30 seconds at 72°C, and a final extension step of 72°C for 10 minutes. Unused primers and dNTPs were removed from 5µl of the PCR product in a reaction with shrimp alkaline phosphotase (SAP) and the Exonuclease I enzyme, which was incubated at 37°C for 60 minutes followed by 80°C for 20 minutes. 1.5µl of this ExoSAP product was then added to the SNaPshot reaction, containing 1µl SNaPshot Reaction Mix, 1.5µl SNaPshot Reaction Buffer and 1µl of the SNP-specific extension primer at 5pmol/µl. The mixture was heated to 95°C for 2 minutes followed by 49 cycles of 95°C for 5 seconds, 43°C for 5 seconds and 60°C for 5 seconds. 5µl of the resulting product was added to a reaction containing SAP which was incubated at 37°C for 60 minutes and 80°C for 20 minutes to prevent further primer extension. The resulting fluorescently labelled probes were then added to a mixture containing formamide and Liz Ladder 120 size standard and run by electrophoresis on an ABI 3130 XL Genetic Analyser. The resulting data was analysed using the GeneMapper software (Applied Biosystems, Foster City, CA, USA).