Thesis

"Polymorphisms in the COMT and MAOA genes and their consequences for Clinical, Neuropsychological and Neuroimaging dimensions in a population at High Risk of Schizophrenia"

Thesis Abstract

Schizophrenia is a severe an enduring psychiatric condition occurring in around 1% of the general population. In addition to clinical symptoms, sufferers show neuropsychological deficits. Neuroimaging changes including deficits in frontal and temporal lobe structures can be seen in subjects with the condition. Of the many aetiological perspectives of Schizophrenia the heritability of the illness and the role of excess of the neurotransmitter dopamine are important. Dopamine is degraded by two enzymes COMT and MAOA. Thus mutations in the genes controlling the effectiveness of these enzymes may render subjects with a hyperdopaminergic state. This thesis will concentrate on two specific Single Nucleotide Polymorphisms in the MAOA and COMT genes and their consequences on the clinical, neuropsychological and neuroimaging phenotype. The study population for this thesis will be taken from the Edinburgh High Risk Study. This is a prospective cohort of individuals at high risk of schizophrenia due to having two or more relatives with the condition. It is in this population that the effects of the genes may be studied without the contaminating effects of psychotropic medication or other illness factors.

The results from this thesis show that COMT genotype can be related to structural and functional neuroimaging changes. Additionally MAOA genotype appears to have a significant effect on affective symptoms and neuropsychological traits. These findings suggest a mechanism for how a hyperdopaminergic state may impact on the Schizophrenia Phenotype.

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1. Introduction

Schizophrenia: Historical Perspectives and Aetiology

The historical origins of schizophrenia hinge critically on the phenomenological description of both symptoms and course of the illness. Emile Kraepelin (1) proposed the fundamental substrate of what is presently understood as Schizophrenia, which he termed "Dementia Praecox". While the clinical portrayal of the condition became more refined, its' genesis remained nebulous. By the time Kraepelin wrote the final (eighth) edition of his textbook in 1913, he had concluded that the causes of Dementia Praecox were wrapped in "impenetrable darkness". (2)

Over the past century however, the understanding of the illness has been accelerated due to important findings in genetics, neuroimaging and psychopharmacology. However, the symptomatic description of the illness remains critical to the diagnosis. Thus far genetic and neuroimaging findings have not been sufficient to overtake clinical diagnosis as the gold standard. Findings from genetic studies have demonstrated the importance of the heritability of the illness and current estimates suggest that approximately 80% of the aetiology of schizophrenia is considered to be genetic. (3) Neuroimaging findings have shown structural differences between the brains of schizophrenic patients and normal controls. Increased ventricular size, decreased hippocampi and temporal lobe structures and changes in frontal lobe function have all supported change in structural neuroanatomy as a basis for the illness. (4) The finding that the brains of schizophrenia

sufferers have decreased brain weight and size has lead towards the theory that schizophrenia may be a degenerative condition wherein these findings are a result of the illness process. (5)

Along with studies in genetics and neuroimaging, a further line of inquiry has followed the serendipitous finding that Dopamine receptor blockade has a therapeutic effect in Schizophrenia. Such findings have founded the idea that Schizophrenia (or at least Psychosis) is in some way related to excess of the neurotransmitter Dopamine. This theory founds the "Dopamine Hypothesis" first described by Carlsson and Lindquivst in 1965. (6) Further pharmacological work supports Dopamine receptor blockade as a treatment measure and critically, dopamine receptor "D2" blockade has been firmly established as being the main therapeutic measure. (7). Further studies have shown that post mortem brains of Schizophrenic patients have increased Dopamine receptor sensitivity (8). Furthermore Amphetamine, a compound which enhances Dopamine release, has been shown to cause an acute syndrome, clinically indistinguishable from Psychosis. (9)

Significant epidemiological findings in schizophrenia include a higher prevalence in those born in the winter (10), a higher rate of obstetric complications (11) and the apparent rise in cases experiencing a second trimester gestational insult such as maternal influenza (12) amongst those who develop Schizophrenia. As the early development of the central nervous system is characterised by neuronal migration, cell proliferation and cell death, intrauterine and prenatal complications could have major impact on

neurodevelopment. Preterm babies have been shown to develop a higher incidence of cognitive and behavioural disorders. (13) A number of early studies have noted mild neurological deficits in those who go on to develop Schizophrenia. These deficits have included gross neurological measures in infants (14) such as delayed motor milestones of development, lower educational test scores in adolescence and solitary play preferences in childhood. (15) Such work has supported the "Neurodevelopment Hypothesis of Schizophrenia" (16, 17). This suggests that the origins of Schizophrenia may be found long before the onset of symptoms which could be related to some early insult to the nervous system.

One strategy that has been employed more recently to investigate this is to look at individuals at high risk of Schizophrenia due to family history and study their phenotype prior to the onset of the illness. (18) This line of enquiry may demonstrate the occurrence of clinical, neuropsychological or neuroimaging changes as precursors to the illness.

In summary, the evidence described above is the foundation for five established perspectives of Schizophrenia. Firstly, that based on twin and adoption studies and a universally similar incidence, Schizophrenia has a strong genetic heritable determinant. Secondly, Schizophrenia is an illness which is associated with increased Dopaminergic metabolism. Thirdly, Schizophrenia is a brain disorder characterised by macroscopic neuroimaging and microscopic neuropathological changes. Fourthly, that neurological antecedents leading to cognitive and behavioural change predate the illness, lead to a widely held view that schizophrenia has the semblance of a neurodevelopmental disorder.

Lastly, these changes persist through the course of the illness and suggest that the illness may show the pattern of a neurobiological disorder.

However, a complete understanding of Schizophrenia remains elusive. Of the many questions that remain, some of the most important relate to which genes are involved, at what stage and how they play a part in the occurrence of symptoms, neurological and cognitive deficits and structural and functional brain changes.

Due to the known role of excess Dopamine in Schizophrenia, this thesis will concentrate on two genes associated with Dopamine degradation and how mutations in these genes may affect the symptoms, neuropsychological indices and neuroimaging findings. These will be studied in a population at high risk of Schizophrenia as it is in this population that a phenotype, uncontaminated by the chronic effects of the illness or medication, can be confidently related to the premorbid effects of these genes.

Schizophrenia: The Clinical Phenotype

The main clinical description of Schizophrenia is given in the International Classification of diseases volume 10 (ICD-10) (19) and Diagnostic and Statistical Manual of Mental Disorders (DSM IV) (20). These descriptions give an indication of best practice for diagnosis. The Schizophreniform disorders are characterized in general by fundamental and characteristic distortions of thinking and perception, and affects that are inappropriate or blunted. Clear consciousness and intellectual capacity are usually maintained although certain cognitive deficits may evolve in the course of time. The course of schizophrenic disorders can be either continuous, or episodic with progressive or stable deficit, or there can be one or more episodes with complete or incomplete remission. (19)

	ICD-10
Inclusion Criteria	**At least one of the following must be present** **1) Thought echo, thought insertion or withdrawal or thought broadcasting** **2) Delusions of control, influence or passivity, clearly referred to body or limb movements or specific thoughts actions or sensations** **3) Hallucinatory voices giving a running commentary on the patients behaviour or discussing the patient between themselves or other types of hallucinatory voices coming from some part of the body** **4) Persistent delusions of other kinds that are culturally inappropriate and completely impossible** **Or at least two of the following :** **1) Persistent hallucinations in any modality when occurring every day for at least 1 month, when accompanied by delusions (which may be fleeting or half formed) without clear affective content or when accompanied by persistent overvalued ideas** **2) Neologisms, breaks or interpolations in the train of thought, resulting in incoherence or irrelevant speech** **3) Catatonic behaviour such as excitement, posturing or waxy flexibility, negativism, mutism and stupor** **4) Negative symptoms such as marked apathy, paucity of speech and blunting or incongruity of affect**
Exclusion Criteria	**That the patient meets the criteria for manic episode or depressive episode the criteria must have been met before the mood disturbance developed** **The disorder is not attributable to organic brain disease or to alcohol or drug related dependence or withdrawal**

Table 1. ICD Criteria for Schizophrenia

As can be seen above, Schizophrenia is composed of positive symptoms (including hallucinations and delusions) and negative symptoms. The negative symptoms are characterised by social withdrawal and cognitive decline.

Neuroanatomical Findings in Schizophrenia

There existed an interest in the Neuropathology of schizophrenia as early as the time of Kraepelin. (21) Subsequent use of Pneumoencephalopathy (22) suggested differences between the brains of schizophrenics compared to controls though the methodology of such studies was limited. There have been many gross Neuroanatomical findings following the introduction of Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI) as research tools for psychiatric diseases. These include: a slight but significant reduction in brain mass, a significant reduction in the anteroposterior length of cerebral hemispheres and a decrease in cerebral mass including reduction of the cerebral hemispheres, cerebral cortex and central grey matter in patients with Schizophrenia compared to controls. (23)

One study (24) noted that the brain weight was reduced by 4.5%, brain length was reduced by 1cm (females) and 0.7cm (males) and ventricular size was increased. This study also noted that and excess of non-specific "focal pathology" (affecting the caudate and putamen) was detected. Lastly an excess of gliosis was noted, most significantly affecting the cerebral cortex, white matter and periventricular regions.

In a detailed review (25) of post mortem schizophrenic brains compared to controls, it was demonstrated that the volumes of the right and left hippocampal formation and internal pallidum were significantly smaller in the schizophrenia group. The hippocampus and pallidum in both hemispheres were equally affected. With the exception of the left

caudate, male patients were more affected than females. In addition to this, other research (26) found that the parahippocampal gyrus was significantly smaller in the schizophrenic group compared to controls. Other major studies have concluded that the Temporal lobe volume is reduced in schizophrenia. This finding specifically attributes the reduction to loss of grey matter at the level of the amygdala and anterior hippocampus.

There have also been findings regarding the histopathological changes in schizophrenic brains. Pyramidal cell disorientation in the hippocampus has been reported by two studies (27) (28). A further study found that schizophrenia patients had significantly lower pyramidal cell density than normal controls in the left CA4 hippocampal region. (29) Cytoarchitectural abnormalities in the entorhinal cortex have also been shown. (30) A meta-analysis (31) found that relative to the cerebral volume differences, the regional volumes of the subjects with Schizophrenia were 94% in the left and right amygdala, 94% in the left and 95% in the right hippocampus/amygdala, and 93% in the left and 95% in the right parahippocampus. Relative to the global ventricular system differences, the largest differences in ventricular subdivisions were in the right and left body of the lateral ventricle, where the volumes of schizophrenic subjects were 116% and 116%, respectively. (32)

The structural neuroimaging findings exemplify schizophrenia as a brain disease. While this might seems an obvious conclusion, an issue remains as to the direction of causality between the illness and the brain changes. On one hand, do the changes precede and thus lead to the clinical syndrome or, alternatively could some of the changes be attributed to a

compensatory reaction. A third possibility is that the brain changes are simply an epiphenomenon, unrelated to the central disease process, yet co-existent with cases.

Below is a table of established neuroimaging findings in Schizophrenia. This table also shows the findings from the subjects in the Edinburgh High Risk Study as genotype will be related to these findings in this thesis.

Replicated finding	**Evidence**	**EHRS population** (Lawrie et al. 2001) (41)
Ventricular enlargement	Johnstone 1976 (33), Pearlson 1993 (31)	Non significant reduction
Reduced Brain volume	Elkis 1995 (32)	
Hippocampal Reduction	Barta 1990 (34), Shenton 1992 (35)	See below
Prefrontal Cortex	Buchanan 1998 (36)	Non significant reductions
Superior Temporal Cortex	Barta 1990, (34) Shenton 1992 (35)	Left and right Amygdalohippocampal structures significantly reduced
Anterior Cingulate Cortex	Job 2002 (37)	Significant reduction
Reduced Thalamic Volume	Andreasen 1994 (38)	Both left and right thalamus are significantly smaller in High risk group

Table 2: Neuroimaging in Schizophrenia

Structural abnormality of anterior cingulate and medial prefrontal cortex is arguably one of the most consistently replicated findings in schizophrenia (39, 40, 41) and has previously been linked to increasing genetic liability, albeit to unidentified loci (42, 43).

Neuropsychological Findings in Schizophrenia

More recent evidence has accumulated showing that schizophrenia is associated with significant impairment in cognitive functioning. Specifically, deficits in attention, memory, and executive function have been consistently reported in patients with schizophrenia.

Many studies have shown that patients with schizophrenia have widespread neuropsychological impairments (44) compared to controls along with evidence of abnormal brain structure and function (45, 46).

Construct	Recorded test variables	Evidence from meta-analysis Mean effect size
Global Verbal Memory	CVLT trails 1-5 RAVLT trails 1-5 LNNB WMS	1.41
Selective Verbal Memory	CVLT, WMS RAVLT	0.90
Non-verbal Memory	ROCFT, WMS, RMT faces	0.74
Motor	FTT	1.30
Attention	WAIS-R digit span Trail marking test CPT Stroop	0.61 0.80 1.16 1.11
General Intelligence	WAIS-R full scale Verbal IQ Performance IQ	1.10 0.88 1.26
Spatial ability	Benton facial recognition WAIS- block design	 0.61
Executive function	WCST	0.88
Language function	Word fluency Token test Vocabulary	1.15 0.98 0.53

Table 3: Neuropsychological Findings in Schizophrenia

Above is a table of neuropsychological measures comparing normal controls to schizophrenia patients. The effect size is measured as an odds ratio. Taken from Heinrichs and Zakzanis 1998(44)

Construct	High risk subjects	Controls	Effect size	Significance
General intelligence				
Verbal IQ	**96.66**	**102.85**	**0.53**	**0.005**
Performance IQ	**99.85**	**107.68**	**0.54**	**0.005**
Full scale IQ	**97.82**	**105.47**	**0.59**	**0.002**
Premorbid IQ	**98.46**	**105.82**	**0.771**	**0.001**
Executive function				
Stroop incongruous condition	**23.58**	**21.36**	**-0.40**	**0.04**
Stroop difference between control and incongruous	**13.59**	**11.11**	**-0.46**	**0.02**
Verbal fluency (FAS)	**37.57**	**40.63**	**0.49**	**0.01**
Hayling sentence completion test (HSCT)	**17.72**	**15.61**	**0.47**	**0.02**
Verbal ability and language				
WAIS-R vocabulary	**8.38**	**9.38**	**-0.52**	**0.01**
Token test	**163**	**160**	**0.25**	**0.04**
Learning and memory				
RAVLT	**50.93**	**55.59**	**0.65**	**0.001**
RBMT	**22**	**23**	**0.50**	**0.01**
Perceptual motor speed	**10.93**	**12.37**	**0.44**	**0.025**

Table 4: Neuropsychological Findings in Schizophrenia in EHRS

Above is a table comparing subjects at high risk of schizophrenia due to family history with controls on a number of Neuropsychology measures. Taken from Byrne et al 2003(47)

In summary, schizophrenia is a condition with an established clinical phenotype. Neuroimaging and neuropsychological findings in schizophrenia are significant but not highly specific to the condition. For the purposes of the thesis psychological, Neuroanatomical and clinical features will be referred to as dimensions of the illness and it is these dimensions that will be related to genotype.

The Dopamine Hypothesis

The Dopamine hypothesis has been the pre-eminent hypothesis for the aetiology of schizophrenia and was first proposed by Carlsson and Lindqvist in 1963. (6)

Animal models have been extensively used to assess the dopamine hypothesis. Methods include sensitization of animal behavior by Amphetamine, Phencyclidine, or excess steroids; removing various genes (COMT, DBH, GPRK6, RGS9, RIIbeta); making brain lesions in newborn animals, or delivering animals abnormally by Caesarian section. All of these methods induce a marked behavioural super sensitivity to Dopamine and a marked rise in the number of dopamine D2 receptors in the high-affinity state for dopamine. (48) This work implies that there are multiple genes and neuronal pathways that can lead to Psychosis and that all these multiple psychosis pathways converge via the high-affinity state of the D2 receptor, the common target for all Antipsychotics.

In addition some of the best evidence for the theory is from the effect of drugs such as Amphetamine and Cocaine. Extensive reviews (49) highlight the fact that up to 75% of patients with schizophrenia have increased signs and symptoms of their Psychosis upon challenge with moderate doses of methylphenidate or amphetamine or other dopamine-like compounds, all given at doses at which controls do not have any psychologically disturbing effects.

This link between dopamine blockade and antipsychotic action was strengthened by evidence that the affinity of antipsychotic drugs for the D_2 dopamine receptor family seemed to be correlated with the reduction of psychotic symptoms. (49)This correlation between the therapeutic doses of antipsychotics and their affinities for the dopamine D2 receptor was reported by Delay and Deniker (50)

The original hypothesis was based on the understanding that Dopamine only innervated sub cortical regions and that these regions were over stimulated by excessive Dopamine transmission (51, 52).

It has been suggested that overactivity of dopamine systems in the mesolimbic pathway may contribute to the 'positive symptoms' of schizophrenia (such as delusions and hallucinations), whereas problems with dopamine function in the mesocortical pathway may be responsible for the 'negative symptoms', such as avolition, flat emotional response and alogia. (53)

However later work (52), demonstrated additional dopamine cortical projections and an opposite and reciprocal regulation of cortical and sub cortical dopamine projections. The revised dopamine hypothesis therefore proposed that hyperactive mesolimbic dopamine tracts contributed to the positive symptoms of schizophrenia and under active mesocortical dopamine played a part in negative symptoms and cognitive impairment (53)

Hypoactive cortical Dopaminergic pathways have been subsequently confirmed by functional imaging studies. Dopamine D1 receptor binding is reduced in the prefrontal cortex but not in the Striatum of drug naïve schizophrenic patients (56)

Hyperactive sub cortical dopaminergic activity has been confirmed by PET studies which have demonstrated an amphetamine-induced excessive release of Dopamine in the striatum in patients with schizophrenia (57). Over activity of the Striatum has been previously associated with the positive symptoms of schizophrenia. Studies of Raclopride (a selective dopamine D2 antagonist) suggested no evidence of altered D2 receptor levels in the basal ganglia in drug naïve schizophrenics. But studies using N-methyl Spiperone did indicate an elevation. An elevated endogenous level dopamine obscures an abnormally high concentration of D2 receptors.

It is postulated that this dopamine dysregulation arises from the development of "sensitisation", the process whereby repeated exposure to a drug induces not tolerance but rather reversed tolerance with progressively increased neurochemical and behavioural responses.

The Metabolic Pathway of Dopamine

Dopamine, like other catecholamine neurotransmitters, is synthesized from the amino acid precursor, tyrosine, which has to be taken up through the blood brain barrier by a transporter into the dopaminergic cells (58). The first step in the synthesis of catecholamines is the hydroxylation of tyrosine to DOPA, by tyrosine hydroxylase, which is also the rate limiting enzyme in the synthetic cascade.

In the cytoplasm of cells, DOPA decarboxylase transforms DOPA to dopamine, which is then carried by another active transporter to synaptic vesicles, where the molecules are protected from catabolizing enzymes. The synthesis rate of the dopamine is dependent on the activity of the tyrosine hydroxylase, an enzyme which is under the control of many mechanisms (59). The main short-term regulatory factors are end-product inhibition, firing rate of the neuron and autoreceptors located in the nerve-endings. The end-product of the dopaminergic neurons, dopamine, decreases the affinity of the enzyme's pteridine co-factor for tyrosine hydroxylase, which results in a decrease of enzyme activity. All of the above-mentioned mechanisms, as well as many other factors (59), regulate the phosphorylation state of tyrosine hydroxylase, which is the major factor in controlling its activity.

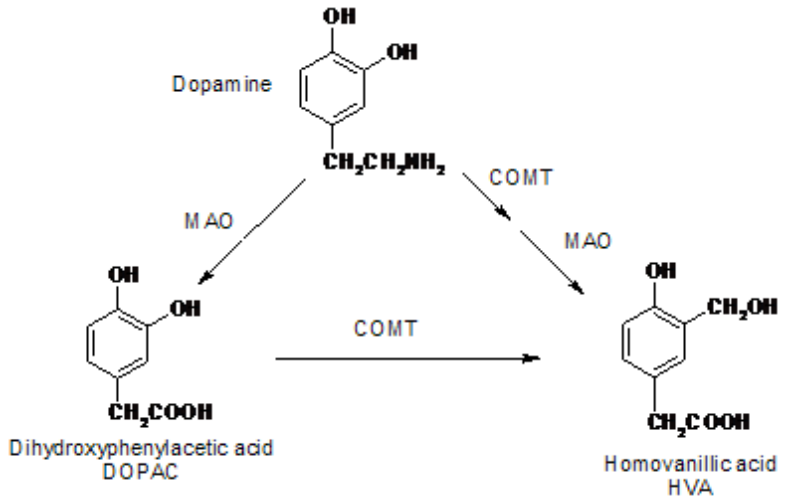


Figure 1: Dopamine Metabolism

Dopamine synthesis and its major metabolic routes. COMT = catechol-O-methyltransferase, MAO = monoamine oxidase, 3-MT = 3-methoxytyramine, HVA = homovanillic acid, DOPAC = 3,4-dihydroxyphenylacetic acid.

Inside the Dopaminergic cells the cytosolic dopamine is metabolized mainly by two successive reactions. First, monoamine oxidase (MAO) transforms dopamine to a corresponding aldehyde, which then can serve as a substrate for aldehyde dehydrogenase to produce 3,4-dihydroxyphenylacetic acid (DOPAC). DOPAC then diffuses out of the cells and can be either conjugated to glucuronides or transformed to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT) (60, 61, 62). A portion of MAO is located outside of the dopaminergic neurons, i.e. in the glial cells, while COMT is found only outside dopaminergic neurons. A fraction of the released dopamine, the size of which varies in different brain areas (63) is first O-methylated by COMT to 3-methoxytyramine (3-MT) and then oxidized by MAO to form HVA (64).

Since COMT does not exist inside the dopaminergic neurons (65, 66, 67), all 3-MT found in the brain should be derived from dopamine that is released from the nerve endings.

Based on this scheme, it has been suggested that the 3-MT concentration in the brain tissue (65, 66, 67) or interstitial fluid (68, 69) would be an indicator of dopamine release. Indeed, it has been shown that 3-MT levels in brain are elevated during electrical stimulation of the dopaminergic neurons and after drug treatments that increase dopamine release (69).

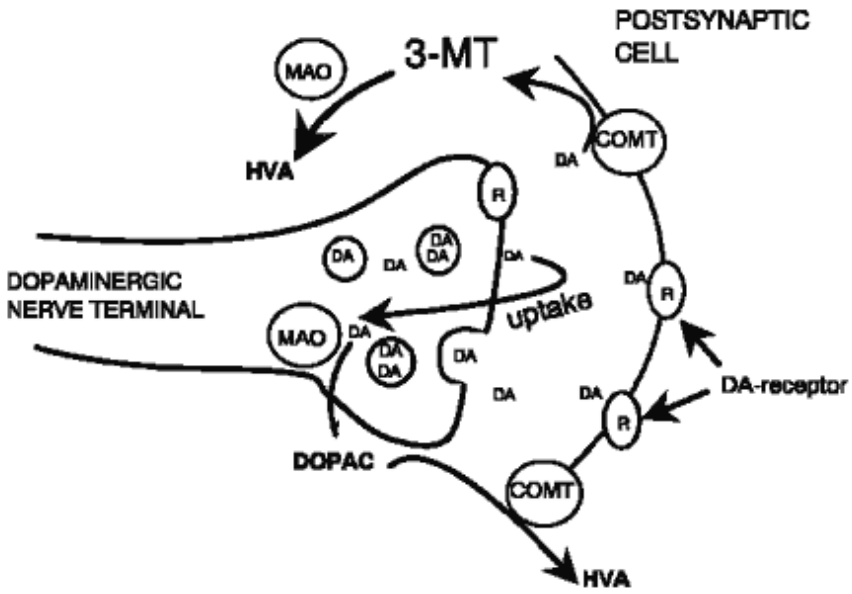


Figure 2: Dopamine in the Synapse

Metabolism of dopamine in the dopaminergic nerve terminal and synapse. DA = dopamine, COMT = catechol-O-methyltransferase, MAO = monoamine oxidase, 3-MT = 3-methoxytyramine, HVA = homovanillic acid, DOPAC = 3,4-dihydroxyphenylacetic acid.

In summary, the dopamine hypothesis provides an aetiological basis for psychotic symptoms and consequently schizophrenia but has notable limitations. Importantly for this thesis both COMT and MAO play a significant role in dopamine metabolism and merit study in the aetiopathology of schizophrenia

The Genetic Basis for Schizophrenia

As early as the 19th century, Sir Francis Galton, (70) among other medical innovations, is considered to be the first to use twins to study the "relative powers of nature and nurture". He described a pair of identical twin brothers concordant for some kind of psychosis, then termed "monomania", whose symptoms included paranoid delusions, auditory hallucinations and mood swings – a putative clinical description of schizophrenia. (70) In 1916, working with Kraepelin, Rudin performed the first systematic family studies, finding the increased prevalence of the illness amongst relatives. (71) Thus genetic factors appeared implicit in elucidating a biological basis for the disorder. The twin study method was subsequently developed by Siemens (72) in a more scientific format. In 1928 Luxenburger conducted the first systematic study of twins. While a genetic influence on the illness was established, Luxenburger sought to identify the degree of penetrance of an alleged gene given the pattern of inheritance did not conform to simple Mendelian theory. (72) Luxenburgers' results were largely inconclusive due to the small sample size and it was not until 1946 when a more robust demonstration of the method was executed by

Kallman. (72) In 1967 Eisenberg, noted that "these findings (adoption and twin studies) persuade me……not only that there is a genetic component in the transmission of schizophrenia but that it is a significant determinant of the occurrence of the disease". (72) Gottesman 1991 notes that "a renewed appreciation began for twin data" following this statement. (73)

Latterly, six major twin studies have been conducted from 1963 to 1987 in Finland, Norway, Denmark and the UK. These countries appear to show consistent, conservative diagnostic systems hence making their amalgamation valid. These studies combine to show that Monozygotic (MZ) twins appear 48% concordant for schizophrenia and Dizygotic (DZ) twins are only 17% concordant. The implication being that, when the environment is controlled, a higher degree of genetic similarity correlates to a higher clinical concordance.

A further arm in assessing the hereditable basis for schizophrenia is the use of adoption studies (74). Here, adoptees of schizophrenic parents are observed to have a higher likelihood of contracting the disorder, irrespective of the family they were brought up in.

The 1988 the World Health Organisation conducted a worldwide study of the epidemiology of Schizophrenia. (75). The findings showed that from as disparate cultures and climes as Denmark, India, Japan and Honolulu, the lifetime risk of schizophrenia was remarkably similar (0.8-1.4%). The natural conclusion is that, irrespective of environmental factors, there appears to be a biological, hereditable basis for

schizophrenia.

The studies above demonstrate the imperative implication of genes in schizophrenia. This necessitates the question of which genes are involved and how they affect both the cellular and clinical phenotype.

Since the advent of studies of candidate genes for schizophrenia, a question arises as to the function of these genes in the developmental neuropathology of illness. Some of the most promising candidates include COMT (76), NRG-1 (77) and BDNF (78). These genes have cellular products which, according to traditional hypotheses, could be involved in the pathological process of schizophrenia. However, a gap in knowledge exists in how the gene products bear any relation to as diverse manifestations of schizophrenia as working memory, schizoid personality traits and structural brain changes. The relatively new field of functional genomics in schizophrenia attempts to correlate candidate genes with neuroimaging, neuropsychological and clinical findings. With regards to schizophrenia there exist three main questions. Firstly, which characteristics of the illness are genetically mediated? These would be differentiated from prenatal or perinatal factors and environmental factors such as substance misuse and stress. Secondly, there arises the question about which genes correlate to which phenotypic characteristics and thirdly what the magnitude of the effect is. While we appear to be far away for understanding *how* genes cause the phenotypic characteristic directly, the focus in functional genomics has shifted to *whether* and *which*. Therefore the task lies in elucidating *which* genes are implicated in *which* changes in schizophrenia.

Different strategies have been employed to find whether genes have an effect on a particular trait. Twin and adoption studies have been the primary methods for this. High risk studies look at which characteristics precede illness and case control studies look at which traits appear disease specific. All of these methods can be used when choosing which characteristics should relate to the function of a candidate gene. One possibility is that the putative genes influence cortical migration (79). Thus if structural brain abnormalities in schizophrenia patients are genetically determined they could be a major biological marker for transmission of the schizophrenia genotype (80)

Although the genetic basis remains unclear, the evidence from family, twin and adoption studies indicates that the majority of risk is likely to be due to genetic factors. It is clear that unaffected family members share many of the neuropsychological (81, 82, 83) and imaging abnormalities (84, 85) found in affected individuals, albeit to a lesser degree, Further, some of these abnormalities are positively associated with the genetic proximity to an affected relative (86, 87, 88, 89). This suggests that the alleles which underlie the genetic risk to schizophrenia may primarily exert their effects on intermediate traits such as frontal lobe structure or function. Such 'intermediate phenotypes' may ultimately be more useful in understanding the biology of psychosis than diagnoses based on the subjective assessment of symptoms.

Twin Studies

Twin studies have provided the bedrock of assessing genetically mediated manifestations of illness and the magnitude of environmental effects. An important method to study genetic impact on schizophrenia phenotype is to look at monozygotic twins, discordant for schizophrenia. Follow-up study of the offspring of MZ twins with a diagnosis of schizophrenia and their MZ co-twins without schizophrenia demonstrated equal rates of schizophrenia; hence, each group of offspring carried equal genetic vulnerability for the illness. Indeed it is noted that "studies of differences between members of discordant MZ twins have great potential for shedding light on non genetic and epigenetic mechanisms that contribute to aetiology" (90). The main variables thus far studied include both structural and functional neuroimaging, neuropsychological tests and clinical variables.

Twin Studies, Neuroimaging and Neuropsychology

The inclusion of discussion about twin studies is important in this thesis as they, in conjunction with neuroimaging findings identify which traits in the disorder may be genetically mediated.

With regard to Neuroimaging, results are varied. One study (91) found that hemispheric volumes were smaller in affected monozygotic (MZ) twins rather than unaffected twins. Schizophrenic twins, whether from concordant or discordant pairs, had smaller whole brain volumes than control twins. (92) The affected twins showed more abnormalities in

hippocampal, third and lateral ventricular volumes than concordant twins in the same study. Therefore whole brain volume appears under high genetic control and smaller whole brain volume is a reflection of the genetic liability to develop schizophrenia. The variation in hippocampal and ventricular volumes within discordant monozygotic pairs however, demonstrates the importance of environmental factors in causing these volume abnormalities in schizophrenia.

In one study (92) 15 sets of monozygotic twins who were discordant for schizophrenia were studied At the level of the pes hippocampi, the hippocampus was shown to be smaller on the left in 14 of the 15 affected twins, as compared with their normal twins, and smaller on the right in 13 affected twins. In the twins with schizophrenia, as compared with their normal twins, the lateral ventricles were larger on the left in 14 and on the right in 13. The third ventricle also was larger in 13 of the twins with schizophrenia. It was concluded that abnormalities of cerebral anatomy (namely, small anterior hippocampi and enlarged lateral and third ventricles) are consistent neuropathological features of schizophrenia and that their cause is at least in part not genetic.

In further studies (94) of hippocampal morphology, Hippocampal measures were used in statistical tests specifically designed to identify disease-associated genetic and non genetic influences on morphology. Smaller hippocampal volumes were confirmed in schizophrenia.. Disease-associated effects were not present between monozygotic discordant co-twins. Monozygotic, but not dizygotic, unaffected co-twins exhibited

smaller left hippocampi compared to control twins, supporting genetic influences. Results suggested that hippocampal volume reduction may be a trait marker for identifying individuals possessing a genetic predisposition for schizophrenia.

Other studies (95) of the hippocampus showed that Hippocampal volumes of affected twins were smaller than those of their unaffected MZ and DZ co-twins and healthy twins. Hippocampal volumes of affected' non-ill co-twins were smaller than those of healthy twins, but those of non-ill MZ and DZ co-twins of schizophrenic patients were similar.. The intra class correlation for healthy MZ pairs was larger than that for discordant MZ pairs, and the variance component estimate for additive genetic effects was lower in discordant twins than in healthy twins. Though hippocampal volume in healthy individuals is affected by genetic factors, it relates to greater modulation by environmental factors in schizophrenic patients and their relatives.

(96) Furthermore, like their affected twins, however, unaffected monozygotic co-twins of the schizophrenia twins exhibited significant callosal displacements. Lateral and third ventricle enlargements were related to callosal displacements. This demonstrates that genetic rather than disease-specific or shared environmental influences contribute to altered callosal morphology in schizophrenia. An upward bowing of the callosum may thus provide an easily identifiable neuroanatomic marker to screen individuals possessing a biological vulnerability for schizophrenia

A further study (97) found significantly increased caudate volumes in affected twins

compared to their unaffected co-twins, but no significant difference in thalamic volume. A study (98) looking at grey matter deficits in twins discordant for schizophrenia found disease-specific effects were present in a small fraction of the total surface area affected by genetic influences, corresponding primarily to middle frontal gyrus (dorsolateral prefrontal cortex) but contributed uniquely to gray-matter deficits in prefrontal regions involved in eye movements and motor planning and in temporal and parietal areas that support multimodal sensory and perceptual integration, auditory perception, and episodic memory.

With regards to functional neuroimaging, Prefrontal physiological deficit on WCST was studies for MZ twins discordant for schizophrenia. (98) It was noted that the entire distribution of rCBF is shifted towards lower values in schizophrenia population.
It was further noted that with MRI and rCBF measures, the more affected a twin differed from the unaffected twin in left hippocampal volume, the more they differed in prefrontal physiological activation during WCST. In affected twins, prefrontal activation was strongly related to both left and right hippocampal volume.

In summary, with regards to neuroimaging, findings that appear to be largely genetically modified include callosal displacements, thalamic volume and brain volume to a lesser degree. Factor which appear less genetically modified include right and left hippocampal and caudate volume. In functional imaging, prefrontal deficit appears less genetically related.

In a study of quantitative analysis of smooth pursuit eye tracking in discordant twins, measures for the affected twin were inferior to the unaffected twin and there was an increase in gain and amplitudes of saccadic intrusion. (99) This appeared to be associated with expression of illness.

When learning and memory was studied in a similar population, the affected group had deficits in story recall, pair association with learning, visual recall of designs and effortful and volitional retrieval. Mild impairments in episodic memory were also noted.(100) The same author also noted trend levels of significance with regards to the WCST between these groups. (101)

As an example of clinical data, significantly greater dysmorphological hand skin signs among schizophrenic MZ twins when compared with their non schizophrenic co-twins suggested an in utero second trimester fetal developmental abnormality for the schizophrenic subjects. (102)

Those traits which are only found in the schizophrenic twin (of discordant MZ twins), and appear illness related, must be largely non genetic or disease specific in origin. However, it would be impossible to state whether these changes predated or were subsequent to the onset of illness. Thus twin studies can tell us whether certain traits in schizophrenia are largely genetically modified but not whether these are state or trait markers. Twin studies show the chance of a trait being genetically mediated.

Candidate Genes for Schizophrenia

Schizophrenia research in the past decade has lead to fruitful findings with regards to Candidate genes for the illness. At this time no single gene has been shown to specifically associate with the condition but several candidates have shown promising associations. The basis for this line of enquiry has followed three major trajectories. Firstly, linkage studies (103, 104) have demonstrated genomic areas of interest including regions of chromosomes 5q, 3p, 11q, 6p, 1q, 22q, 8p, 20q, and 14p, and less significantly regions of chromosomes 16q, 18q, 10p, 15q, 6q, and 17q. (136) Further evidence (137) found the strongest evidence for susceptibility loci on 8p ($P < 2 \times 10(-4)$), 13q ($P < 7 \times 10(-5)$), and 22q ($P < 9 \times 10(-5)$) for schizophrenia. Thus the 8p and 22q regions were supported by both meta-analyses but eight other regions were supported by only one. There is greater consistency of linkage results across studies than has been previously recognized. The results here suggest that some or all of these regions contain loci that increase susceptibility to schizophrenia in diverse populations.

Secondly Association studies have found candidates with significant association to the schizophrenia phenotype although the results appear more equivocal when these candidates are studied in ethnically diverse populations. Below is a table containing the evidence and strength of association of the main candidate genes.

Candidate Gene	Evidence	Genomic Location
COMT	Shifman et al (105)	22q11
BDNF	Skibinska et al (106)	11p13
MAOA	Norton et al (107)	Xp11.23
NRG-1	Stefansson et al 2002 (108)	8p21-22
DISC-1	Zhang et al (109)	1q42
Dysbyndin	Straub et al (110)	6p22.3
DAOA	Ma et al 2005 (111)	13q22–34
DRD3	Jonsson et al 2003 (112)	11q21
GRM3	Chen et al 2003 (113)	7q21.1
RGS4	Chowdari et al 2002 (114)	1q22

Table 5: Candidate Genes

As can be seen above, only COMT and NRG-1 correspond to the areas of interest in the linkage studies, yet other candidate genes show association with schizophrenia albeit equivocal.

The third trajectory for finding appropriate candidates relates to the relevance of putative gene function in the condition. In this regard candidates such as the Dopamine receptor DRD3 would be an obvious choice to be investigated due to the known importance of dopamine in schizophrenia. COMT and MAO would, by the same token, necessitate investigation. Crucially, however, it must be stated that MAOA does not have high levels

of association with schizophrenia. Of all the above candidates the obvious choice for study in the present study's population would be COMT as it fulfils the requirements of being a good candidate in association studies, it lies within evidenced linkage regions and has a naturally relevant role in dopamine metabolism. Notably MAOA has been cited as a candidate for bipolar disorder and will be discussed later.

Catechol-O-Methyl Transferase (COMT) and Schizophrenia

The mechanism by which inheritance of the COMT val allele increases risk for schizophrenia may be related to its impact on prefrontal DA levels and prefrontal function. However, DA neurotransmission in PFC has also been shown in experimental animals to affect subcortical DA activity, which is implicated in both the psychotic symptoms of schizophrenia (115, 116, 117) and the therapeutic response to anti-dopaminergic drugs (118). DA flux in PFC modulates the activity of excitatory cortical neurons that project both directly and indirectly to mesencephalic DA neurons (119, 120, 121). Recent evidence in the rodent indicates that, whereas prefrontal neurons make direct contacts with DA neurons that project back to PFC, prefrontal projections do not contact directly DA neurons projecting to the striatum. This has led to speculation that prefrontal neurons tonically inhibit striatal DA projections perhaps via GABA intermediates in the striatum or in the brainstem (122, 123). Under experimental conditions in animals, reduced DA signaling in the PFC leads to increased responsivity of subcortically projecting midbrain DA neurons to stimuli such as stress (124, 125, 126). The notion that overactive populations of mesencephalic dopamine neurons might be a "downstream" effect of an abnormality in prefrontal function has been proposed as an explanation for the coexistence of both cortical and subcortical dopaminergic abnormalities in schizophrenia (160, 161, 157, 162). Therefore, to the extent that COMT genotype affects prefrontal function, it may contribute risk for schizophrenia not only because of its biological effects at the level of PFC but also because of indirect downstream effects on DA regulation.

The COMT Val allele has been shown to increase the risk of schizophrenia in some studies (131), particularly in samples of European origin. As discussed above, it has a pivotal role in the extracellular degradation of dopamine and there are many strands of evidence to link abnormal dopamine metabolism to the aetiology of schizophrenia. Firstly, there is evidence of increased dopamine transmission in people with schizophrenia, including some patients who were unmediated and not experiencing acute episodes (164). Secondly, there is evidence of state-dependent dysregulation during acute episodes (165). Thirdly, it is generally accepted that all currently available antipsychotic agents are antagonists at dopamine receptors and that the average daily dose is related to their affinity for the D2 receptor form (166).

Whilst alleles at several loci appear to raise the likelihood of developing schizophrenia, some members of affected families develop affective disorders or other illnesses which lie on the 'schizophrenia' spectrum (167, 168) and some of the children of these people may develop schizophrenia .

The extent to which each allele 'carrier' without schizophrenia shares the neuropsychological, imaging and other abnormalities of ill relatives is uncertain. Furthermore, whilst certain alleles are more common in people with schizophrenia, it is unclear whether the neuropsychological and imaging findings that they have are explained by their illness or risk states alone, or are better explained by the particular alleles that they carry. These issues are largely unaddressed in the literature although

there is evidence that the COMT Val^{158}Met polymorphism is related to executive function in healthy controls and in the siblings of affected subjects (169, 170). The neuropsychological and imaging correlates of specific loci are of considerable importance to the study of schizophrenia since they may define relatively homogeneous phenotypes that will permit a more pathophysiological approach to the investigation of affected subjects and ultimately inform disease management.

This thesis investigates the role of COMT in a sample of subjects from the Edinburgh High-Risk Study (EHRS) of individuals at high genetic risk of developing schizophrenia. The risk of schizophrenia associated with the Val allele was first estimated. This was followed by an investigation of the structural and functional MRI imaging associations of each COMT allele. The specific hypotheses were that the COMT Val allele would be related to structural and functional impairment of prefrontal cortex (PFC). This is based on a prior study examining the relationship of the COMT Val variant to perfusion of PFC (171) and on previous studies which found PFC volume to be related to genetic liability to schizophrenia (172, 173).

COMT and functional effects

Several lines of evidence suggest that working memory deficits and associated abnormalities in prefrontal cortical structure and function are reflective of an inherited diathesis to schizophrenia, and thus represent promising candidate phenotypic markers for the disorder. Spatial working memory deficits scale in severity with the number of

relatives affected with schizophrenia within families (174). This familial pattern has been confirmed to have a genetic basis, as performance on an experimental test of spatial working memory was observed to decrease with increasing genetic loading for schizophrenia among MZ and DZ twins discordant for schizophrenia and control twins (175). These results replicated findings from a previous study of the same sample using an extensive battery of clinical neuropsychological tests (176). Notably, in the latter study, multivariate methods were employed that accounted for the overlap in performance variation among the various tests; spatial working memory, divided attention, reaction time to visual targets, and intrusions during verbal memory retrieval were the only measures that were found to make unique contributions to the prediction of genetic loading for schizophrenia.

In parallel with these findings, structural abnormalities in polar and dorsolateral prefrontal regions on MRI varied in a dose-dependent fashion with a degree of genetic loading for schizophrenia in the same MZ and DZ twin sample (177). It has been suggested that a reduction of interneuronal neuropil in the prefrontal region in patients with schizophrenia results in impaired working memory functioning due to hypoactive dopaminergic (i.e., D1-receptor-mediated) modulation of pyramidal cell activity (178). This prediction has been supported by a recent PET investigation which found increased D1-receptor binding in the dorsolateral prefrontal cortex of schizophrenic patients compared to controls, which also correlated with reduced working memory performance in schizophrenia (179). This result was interpreted as suggesting D1 receptor up-regulation secondary to reduced prefrontal intracellular dopamine. Altered physiological

activity in the prefrontal cortex during performance of a working memory task has been observed in both patients with schizophrenia and their unaffected siblings (180, 181).

Given that abnormalities of working memory and prefrontal cortical structure and function are associated with genetic liability to schizophrenia, it should be possible to identify specific genes that underlie these disturbances. Weinberger and colleagues have reported evidence of one such genetic influence – the met/val polymorphism of the COMT gene (located in chromosome 22), with val alleles promoting more rapid breakdown of synaptic dopamine leading to prefrontal hypofunction in patients with schizophrenia (169, 170).

Together, these findings strongly implicate genetic factors as playing a role in the abnormalities of prefrontal cortex and working memory in schizophrenia. Nevertheless, patients with schizophrenia have been found to show even greater disturbances in DLPFC function and structure than their non-ill MZ co-twins (169). Thus, while genetic factors may cause patients and some of their first-degree relatives to share a certain degree of compromise in prefrontal cortical systems, non-genetic disease-related influences cause the DLPFC to be further deviant in the patients, and this added measure of deviance in prefrontal cortical function may be among the processes associated with overt symptom expression among those genetically predisposed.

The medial temporal lobe structures (i.e., hippocampus, amygdala) and adjacent temporal cortex are involved in learning and recall of episodic information, auditory perception,

emotion (especially the amygdala) and certain aspects of language processing (182). Reductions in temporal cortical and hippocampal volumes are present in both patients and their healthy biological relatives (183, 184, 185, 186, 187). From a neurocognitive perspective, impaired declarative memory processes that depend on the integrity of the hippocampus (188) have been reported in both high-risk adolescents (189) and non-psychotic relatives of schizophrenics (176, 184, 185, 190), suggesting that they derive in part from an inherited genotype. Importantly, two studies have shown a significant relationship between deficits in verbal declarative memory and smaller hippocampal volumes in relatives of schizophrenia patients (184, 185).

At the same time, however, hippocampal volume reductions and long-term memory deficits are specifically more pronounced in patients compared with their own healthy MZ co-twins (176, 186). Thus, non-genetic, disease-related factors must also be involved. The hippocampus in particular is acutely vulnerable to hypoxic-ischemic damage (191, 192).

Mono Amine Oxidase (MAO) and Schizophrenia

Monoamine oxidase (MAO) is a mitochondrial enzyme involved in the degradation of biological amines including serotonin, dopamine and noradrenaline. In the 1970's platelet MAO activity was thought to be a biological marker for vulnerability to schizophrenia (193, 194). Chronic schizophrenic patients, but not those in the acute phase were found to have lower activity of platelet MAO when compared to controls (195, 196). Subsequently antipsychotic medication was shown to be the likely source of this activity. (197)

In humans two isoenzymes exist: Monoamine oxidase A (MAOA) which degrades serotonin and noradrenalin preferentially and Monoamine oxidase B (MAOB) which is solely responsible for phenylethylamines and benzyl amine (198, 199). In humans both enzymes participate in the degradation of dopamine. Both the MAO genes are located on chromosome Xp11.23-Xp11.4 (200). This genomic area has been put forward as a region of interest in schizophrenia based on some linkage results in genome screeens of sib-pairs and multiplex families (201, 202, 203, 204, 205)

In the brain MAOA is expressed at highest levels in catecholaminergic neurons whereas MAOB is most expressed in astrocytes and serotinergic neurons (206, 207, 208). The regional variations in the relative contributions of the two forms to the oxidation of dopamine result in differences in the proportions of MAOA and MAOB in the different regions of the brain (209). Low level of MAOA expression in blood cells has limited the study of MAOA in neurological and psychiatric disorders. Both twin (210) and

segregation analyses (211) have established that platelet and fibroblast MAO activities are controlled by genetic factors. Segregation analysis studies have indicated that this genetic control involves a single major locus and molecular studies have indicated that this locus is the MAO locus itself (212). A mutation associated with a complete deficiency of MAOA enzymatic activity and abnormal behaviour with impulsive control disorder has been described within the coding sequence of the MAO gene in one family (213). Several DNA polymorphisms have been characterised both for MAOA (214, 215) and MAOB (216, 217,218, 219, 220). While these polymorphisms did not affect the amino acid sequence of the MAOA protein they did influence the enzyme activity level.

Previous studies of allele frequency of MAOA in the population showed the following results (221)

Subjects	MAOA	MAOA	MAOB	MAOB
	Allele 1	Allele 2	Allele 1	Allele 2
Controls (87)	0.70	0.30	0.47	0.53
Patients (110)	0.65	0.35	0.50	0.50

Table 6: MAOA alleles

Here no statistically significant difference was observed between patients and controls (MAOA, X=0.61, MAOB, X=0.19 p>0.05 for both)

However trend level of significance did emerge when the clinical subtypes were elaborated. This association came closer to significance when the relationship was

studied in males alone (due, in part, to the location of the gene being on the X chromosome). Since it had been shown previously that the level of MAO activity is proportional to the amount of enzyme protein rather than to different catalytic properties of molecules (222), it can be hypothesized that the sequence variation is located within the non-coding regulatory region of the gene, which controls the amount of enzyme

The MAOA promoter harbours a Variable Number Tandem Repeat (VNTR) which has been shown to influence transcription (223, 224). Alleles with the 3.5 repeats and 4 repeats were found to transcribe the protein more efficiently than the three repeat allele. The 4 repeat allele was shown to induce higher MAOA enzyme activity in human male skin fibroblasts than the 3 repeat allele (225). The 3.5 and 4 repeat alleles were also associated with higher levels of the serotonergic and dopaminergic degradation products 5-Hyroxyindoleatic Acid (5-HIAA) and homovallinic acid (HVA) in healthy women. (226).

With regard to Monoamine metabolism in MAOA and MAOB deficient mice, gene knockout techniques have been a useful tool in understanding the functions of these enzymes in vivo. In brains of MAOA knockout mice 5HT levels were only increased two fold partly due to the development of MAOB. Noradrenaline levels were increased up to two fold and importantly Dopamine levels were found to have increased (230). Dopamine levels were not found to have increased in MAOB knockout mice.

MAOA genotype has been associated with other psychiatric conditions**.** One of the most

interesting findings relating to the function of MAOA relates to conduct disorder in a study of eight males from a Dutch family (213). Here a single nucleotide polymorphism associated with conduct disorder.

MAOA mutations may underlie the susceptibility to alcoholism because MAOA alleles have been associated with alcoholism among European populations (233)

One study (234) showed increased salivary MAOA and MAOB is correlated with stress. MAOA knockout mice showed increase response to stress in the force swim test (230) which would be consistent with the increased levels of Dopamine and Noradrenaline in this same population.

Several lines of enquiry have linked 5HT systems with aggressive behaviour (235, 236). MAOA knockout mice have elevated brain levels of 5HT and a distinct behavioural syndrome including enhanced aggression is manifested by adult males. Similarly elevated levels of 5HT may be important in the enhanced emotional learning that the adult knockout mice exhibit (237). It is possible that aggression related to MAOA deficiency may related to structural changes in the somatosensory cortex (238). MAO A deficiency may render mice more susceptible to the effects of environmental substances, drugs or biogenic amines (such as dopamine).

More recently MAOA genotype has been found to associate with bipolar disorder (239). It must be noted that this action could be mediated through MAOA action upon 5HT or

noradrenalin levels.

Monoamine Oxidase A Functional Effects

Some studies have demonstrated the phenotype effects of variants in MAOA genotype. One study looked at the effects of MAOA genotype on brain structure and function. (240) A common variable number of tandem repeats (VNTR) polymorphism of the MAOA gene has been described that strongly impacts transcriptional efficiency: enzyme expression is relatively high for carriers of 3.5 or 4 repeats (MAOA-H) and lower for carriers of 2, 3, or 5 repeats (MAOA-L) (202).

Analysis of brain structure revealed that allelic variation in MAOA was associated with noticeable regionally specific changes in grey matter volume. When compared with MAOA-H subjects, MAOA-L individuals showed a significant reduction in volume that encompassed virtually the entire cingulate gyrus and bilateral amygdalae, and a maximum in anterior cingulate cortex. Additionally, significant reductions in insula and hypothalamus were found. Relative to the volume in MAOA-H subjects, reductions averaged around 8% in MAOA-L subjects. Furthermore, a sex-specific (males only) increase of approximately 14% in bilateral lateral Orbito Frontal Cortex (OFC) volume in MAOA-L men, relative to MAOA-H men was seen. The OFC was the only brain region where an interaction effect was found.

In this study fMRI analysis of perceptual matching of angry and fearful faces showed

significant activation of amygdala and task-related deactivation of limbic and paralimbic regions implicated in emotion processing, a pattern seen previously (203, 204). MAOA-L individuals showed significantly increased activity in left amygdala and decreased response of subgenual (BA 25) and supragenual (BA 32) ventral cingulate cortex, left lateral OFC, and left insular cortex, relative to MAOA-H subjects. It was postulated that it is unlikely that the fMRI effects were related to smaller volume in these small structures because both increased and decreased reactivity was seen in these various limbic regions that showed reduced structural volume in MAOA-L individuals.

MAOA genotype was also studied in relation to emotional memory, i.e., the encoding and retrieval of aversively, compared with neutrally, valenced information. A pronounced effect was found of genotype and sex in left amygdala and hippocampal formation; men, but not women, carrying the low-expression MAOA genotype showed increased reactivity during retrieval of negatively valenced emotional material. This effect was present for aversive, but not neutral, material and only during retrieval.

This study also probed the neural correlates of cognitive inhibitory control using a no-go "flanker task." In men only, MAOA genotype had a pronounced effect on activation during response inhibition in dorsal anterior cingulate: MAOA-L hemizygotes showed deficient activation. This finding is compatible with a previous observation in a small group of male subjects (205). Women had no significant effect of genotype, resulting in a significant sex-by-genotype interaction at this locus. Again, this functional change was located within the cingulate region of maximal structural change related to genotype.

Monoamine oxidase A has several areas of interest with regard to variable expression. Gene fusion and transfection experiments have suggested that a VNTR located in the MAOA promoter affects the transcriptional activity in the transfected cells (223, 224). The two main areas of interest include a VNTR at MAO at intron 1 and a VNTR in the MAO promoter. Furthermore a SNP8 is located in a region of four exons containing five polymorphisms that had been identified at the MAOA locus. Since the polymorphisms were in complete linkage disequilibrium only one was selected for the present studies. The choice of polymorphism ultimately was that at exon 8 (rs6265) as there is an easy dichotomous variable that could relate to a relatively small population as in this study. (140)

The Edinburgh High Risk Study

High risk studies offer a useful adjunct to understanding functional genomics. High risk studies are prospective studies of individuals in whom the risk for schizophrenia is enhanced. These studies concentrate on neuropathological and clinical variables that pre date the onset of illness. Five main studies have been conducted to date: the Copenhagen Project (202), the Helsinki High Risk Study (203), the Israeli High Risk Study (204), New York High Risk Project (205) and the Edinburgh High Risk Study (EHRS) (206). The difficulty with such studies has been the long interval between identification of cases and the clinical manifestations of the illness. The Edinburgh high risk study recruited subjects at a much late age thus shortening the follow up period.

The main findings of these studies are in the domains of structural and functional neuroimaging, neuropsychological and clinical data.

The data for the EHRS has been collected serially starting even years prior to the onset of illness thus enabling a time axis to be considered in relation to the illness. This allows study of which traits are predictive of illness and whether the neurological deficits can be seen long before onset or appear in close proximity to illness. This allows investigation of the “early lesion” verses “late lesion” debate with regards to neurodevelopmental hypothesis of schizophrenia. Additionally, the populations of these studies include individuals who, while at high risk of the disorder, remain clinically well. Should this population reveal a spectrum of deficits, these individuals could be deemed as belonging

to both an intermediate phenotype and genotype. These populations also strengthen the assessment of State verses Trait characteristics.

In the Israeli high risk study, current and lifetime psychopathology was assessed in 50 Israeli children of parents with schizophrenia and re-evaluated at the age of 31. The findings support the concept that both familial and environmental factors operate in the expression of psychopathology.

The results suggest that attention skills of the adult children of a parent with schizophrenia fall between those of schizophrenia patients and controls, and that measures of sustained attention and the ability to focus and execute provide the best discrimination among groups. Low scores on a digit cancellation test at age 11, but not at age 17, predicted which of the children at genetic risk became unwell (24)

In the New York high risk project offspring of schizophrenia mothers were tested at 7-12 years and further assessed in mid adulthood. Findings include childhood deficits in verbal memory, gross motor skills and attention in those who went on to develop schizophrenia. It was also noted that substance abuse had a significant interaction with the clinical outcome groups. In subjects without substance abuse, those with schizophrenia-related psychoses had exhibited significantly more behavioural problems as children than had adult offspring with affective or anxiety disorder or with substance abuse only or no disorder. The confounding effects of substance abuse should be statistically controlled in studies of longitudinal associations between childhood behavioural disturbance and axis I

outcomes.

The Edinburgh high risk study has remarked on several premorbid findings including higher levels of psychopathology and aberrant neuropsychological performance. Neuroimaging findings include smaller right and left hippocampal volumes, larger right and left lenticular nuclei, smaller left and right thalami and smaller third ventricles. Differences were also noted in the amygdala and gyrification index. (208)

Hypothesis

Two family-based association studies have provided evidence for a role of COMT in schizophrenia (209, 210, 211), but it has been unclear how either protein variation would increase risk for schizophrenia (212, 213).

One approach used by Egan and colleagues (214) that may improve power to find genes for complex disorders is to target biological traits found in ill subjects and their unaffected relatives, so-called intermediate phenotypes, rather than clinical diagnosis (215, 216). Such traits may be more directly related to the biological effects of susceptibility genes. As described, abnormal function of the prefrontal cortex, an important aspect of schizophrenia, also may represent an intermediate phenotype related to genetic risk for schizophrenia (217, 218). Stable deficits in cognitive functions referable to the dorsolateral prefrontal cortex and cortical physiological abnormalities during performance of such tasks have been consistently reported in studies of schizophrenia (219, 220). Other evidence indicates that healthy siblings of patients, including monozygotic co-twins, show similar cognitive and physiological abnormalities (211, 221, 222). The subjects from the EHRS would be suitable candidates to test this hypothesis.

Electrophysiological studies in primates (223, 224) and rodents (225), and neuroimaging studies in humans (226, 227), have shown that dopamine plays an important role in

modulating the activity of prefrontal circuitry during performance of working memory tasks. Although there are many proteins involved in the biological actions of dopamine, COMT, because it metabolizes released dopamine, may be an important factor during such prefrontally mediated tasks. Despite COMT's widespread distribution in nondopaminergic neurons and glia, pharmacological studies have shown that catabolic flux of synaptic dopamine through the COMT pathway is characteristic of the prefrontal cortex in contrast to the striatum (227). Studies of COMT knockout mice, similarly, have demonstrated that dopamine levels are increased only in prefrontal cortex (228) and, remarkably, that memory performance is enhanced. These findings strongly support the notion that variation in COMT activity may have neurobiological effects specific to the prefrontal cortex.

As discussed, genetically determined variations in COMT activity might affect prefrontal cortical activity, especially during executive and working memory tasks. The primary hypothesis for this thesis that the high-activity Val allele, because it leads to increased dopamine catabolism, would be associated with relatively compromised prefrontal function, and, by virtue of this effect, would increase risk for symptoms of schizophrenia and neuropsychological deficits.

It has been previously shown using the Wisconsin Card Sorting Test (WCST) that deficits in WCST performance are enduring and core features of schizophrenia and predict long term-disability, independent of other cognitive deficits (225); healthy siblings of patients with schizophrenia also perform abnormally on it (226, 227).

Functional neuroimaging studies have found that the WCST activates the dorsolateral prefrontal cortex (228) and that dopamimetic drugs improve performance on this task in patients with schizophrenia and enhance the signal to noise of the prefrontal physiological response (229, 230). It has been shown that COMT genotype would affect WCST performance and that Val/Val individuals would have the poorest performance. In this thesis this will be measured using the Hayling Sentence Completion Test.

In one study, to assay prefrontal physiology, functional MRI (fMRI) was used while subjects performed the N-back task. This task has been shown to activate dorsolateral prefrontal cortex as well as a distributed cortical working memory network (231). FMRI results have been described in unaffected siblings, suggesting that inefficient prefrontal information processing is related to genetic risk for schizophrenia.

In contrast, the efficiency of the N-back fMRI response in dorsolateral prefrontal cortex is enhanced by the dopamimetic drug, amphetamine, in healthy individuals whose performance remains stable (232). Thus, deviations of prefrontal physiology can be appreciated with this in vivo fMRI assessment even if there is compensation at the level of performance accuracy, and changes in cortical dopaminergic function impact on physiological efficiency during this task.

It was hypothesized, therefore, that COMT genotype would affect the efficiency of the prefrontal fMRI response during this neuropsychological task and would predict an allele dosage relationship with activation, with Val/Val individuals being least efficient.

With regards to MAOA genotype, when compared with low/high enzymatic activity genotype subjects, low activity genotype subjects individuals showed a significant reduction in volume that encompassed virtually the entire cingulate gyrus and bilateral amygdalae with a maximum reduction in anterior cingulate cortex. (179) It is hypothesized that this finding could be replicated in the high risk population. Low activity genotype subjects are likely to show increased levels of dopamine thus increasing the efficiency of neuropsychological performance in these brain areas. Just as with COMT Val Val genotype it is hypothesized that MAOA "TT" subjects will show different neuropsychological performance on Hayling Sentence Completion in areas such as the cingulate cortex.

Just as COMT genotype was hypothesized to possible relate to development of schizophrenia and psychotic symptoms, it is hypothesized that MAOA genotype may relate to affective symptoms amongst the subject population.

2. Methodology

The main advantages of high risk studies are that various abnormalities can be studied that do not reflect the consequences of illness nor medication. The critical brain and clinical changes that occur prior to the onset of illness can be studied. Given the evidence that schizophrenia may be neurodevelopment in origin it is essential to uncover what happens in the brain prior to symptom development.

The disadvantage with high risk studies is the resources required to find a large enough population who will go on to develop schizophrenia. This population will require to be followed for the longest period of time to study all precursors to illness. Ideally this population would be studied from birth but the chances of not losing a large number of individuals by the time they reach the risk period for illness is very high.

In the Edinburgh high risk study participants were studied from the age of 15. These individuals were selected on the basis of their family history. Each participant had atleast 2 close relatives with schizophrenia. From previous work it could be estimated that these individuals would carry a 15% chance of developing the illness.

Study population

Details of the recruitment process have been described in previous papers (251, 252). Briefly, individuals with schizophrenia, with a family history of schizophrenia and with adolescent relatives, were identified from psychiatric hospital case records in several regions of Scotland. Case-note diagnoses of schizophrenia were verified with the Operational Criteria Checklist (210). We then approached their unaffected relatives, and from them, recruited high-risk subjects aged 16-25 who agreed to participate. A control group of healthy individuals without any family history of schizophrenia was recruited from the same regions and communities as the high-risk participants. These people were of similar age to the high-risk group and their numbers were comparable to the number of people expected to develop psychosis.

Members of the high risk and control groups were given a detailed clinical, neuropsychological and brain imaging assessment at baseline. The clinical assessment included the Present State Examination (PSE, 9th edition), a structured psychiatric interview schedule which has been widely used in studies of schizophrenia (211). All these assessments were repeated after approximately 2 years in consenting participants who had enrolled in the first 2 years of the study. Further clinical, imaging and neuropsychological assessments were conducted at further 2-yearly intervals up until 2004. These included many of the assessments performed earlier, including structural MRI, and a functional MRI scan of the brain using the Hayling sentence completion test (HSCT). High risk subjects who did not develop schizophrenia were further subdivided

into those with or without psychotic symptoms at any assessment (referred to as HR with symptoms and HR without symptoms respectively).

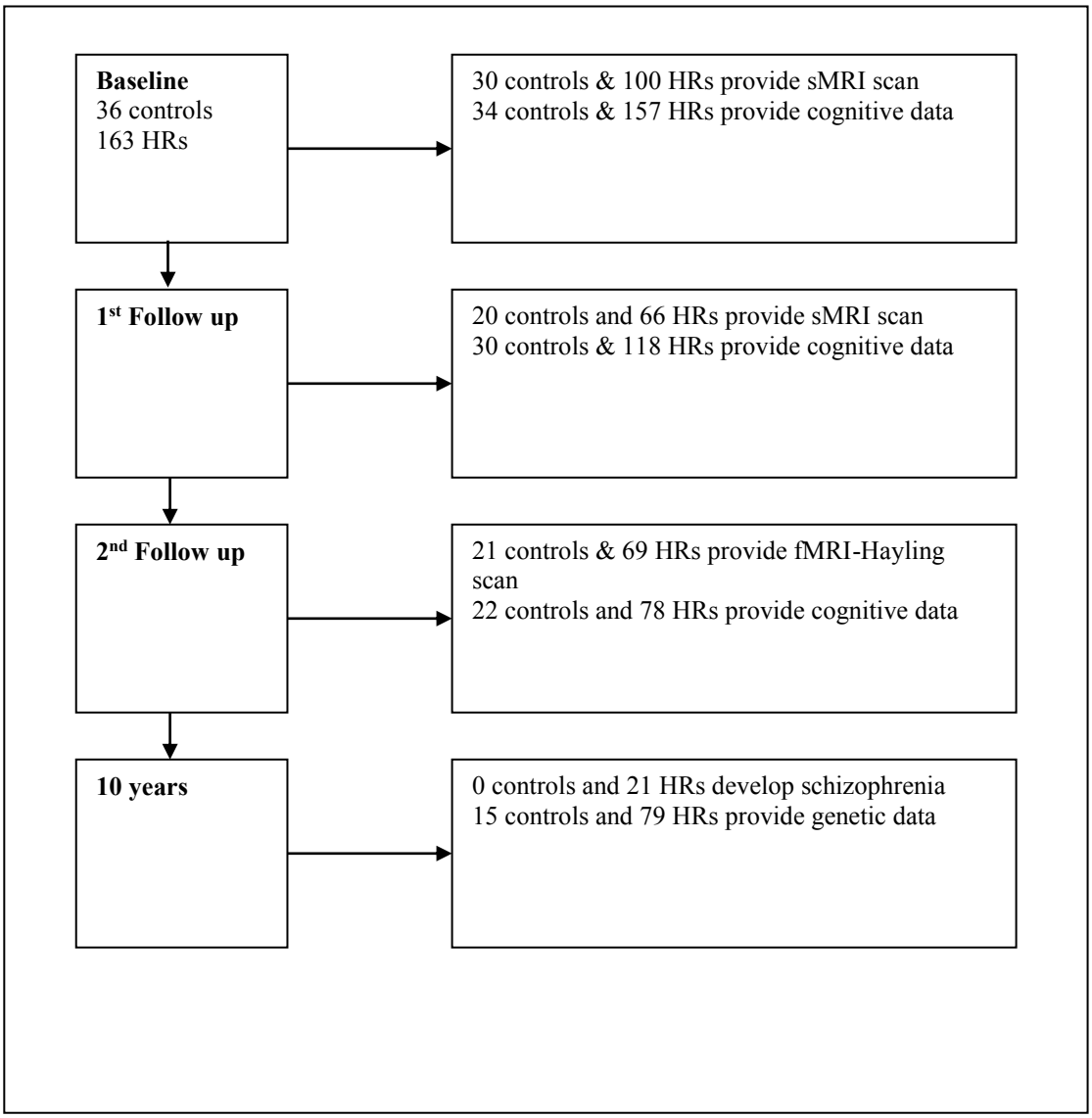


Figure 3: Study Population Analysis

Clinical and Neuropsychological Data

All subjects, as documented, underwent standardized battery of neuropsychological testing including measures of Premorbid intellectual function (National Adult Reading Test NART), Full scale IQ (FSIQ), Current intellectual function, Total Verbal Recall, Verbal Fluency, Executive Function as measured by the Stroop Test, Word processing, Rey Auditory Verbal Learning Test, Rivermead Behavioural Learning Test
Verbal Recall and the Hayling Sentence Completion Test. MAOA genotype was related to mean scores on each of the neuropsychological variables using Independent T-tests. Only subjects with Val Val homozygote and Met Met homozygote were compared from COMT genotype due to constraints of numbers.

Genotype analysis

Genomic DNA was isolated from whole blood and the COMT and MAOA genotype of each subject then determined. The COMT gene has a common functional polymorphism resulting in a Met to Val substitution in the translated protein (212). The COMT Val allele has been shown to raise the likelihood of developing schizophrenia compared to the Met form. The target sequence for the Val158Met SNP was submitted to the Assay By Design Service for ABI SNP Genotyping Assays. Assays from the service consist of a mix of unlabelled primers and TaqMan MGB probes.

Genotyping was performed at the Genetics Core of the Wellcome Trust Clinical Research Facility, Edinburgh (www.wtcrf.ed.ac.uk) in 384 well-plates, using the TaqMan polymerase chain reaction-based method. Allelic discrimination using this chemistry is based on the design of two TaqMan probes, specific for the wild type allele and the

mutant allele. Each of the two probes is labelled with a different fluorescent tag (FAM and VIC), and each is designed with the gene mutation affecting the middle part of the probe sequence. The binding efficiency of the wild type TaqMan probe to the mutant allele and vice versa is low due to the mismatch within the TaqMan probe and the target sequence; therefore, mismatched binding is highly reduced. The final volume PCR reaction was 5µl using 20ng of genomic DNA, 2.5µl of TaqMan Master Mix and 0.125µl of 40x Assay by design Genotyping Assay Mix. The cycling parameters are as follows: 95° for 10 minutes, followed by forty cycles of denaturation at 92° for 15 seconds and annealing/extension at 60° for 1 minute. PCR plates were then read on ABI PRISM 7900HT instrument with SDS v2.1 software.

The relationship of each allele to the risk of schizophrenia was determined by comparing the genotype frequencies across controls, and high risk subjects who had or had not developed schizophrenia at the study's 10-year conclusion (213). The significance of any difference was determined using a $\chi 2$ test. The relative risk of developing schizophrenia in high risk subjects with a Val-Val or Val-Met genotype was also calculated.

MAOA 941T > G PCR and RFLP Assay

Twenty-four ng genomic DNA was amplified with 1.4 pmol each primer: forward, 5′ GAC CTT GAC TGC CAA GAT 3′ and reverse, 5′ CTT CTT CTT CCA GAA GGC C 3′, 1X Qiagen buffer, 1.5 mM magnesium chloride, 100 µM each dNTP, 1 unit Qiagen taq polymerase, in a final volume of 12 µL, for 1 cycle of 94°C 3 min, 35 cycles of 94°C 30 sec, 55°C 30 sec, and 1 cycle 72°C 5 min. To the PCR product, 1 unit of the restriction

enzyme Fnu 4HI, 1.5 µL 10X NEB2 buffer and 1.3 µL water were added to give a final volume of 15 µL. The PCR product was digested for 4 hr at 37°C to give fragment sizes of 135 bp (uncut allele 1) and 65 bp (cut allele 2). Digested PCR product was electrophoresed on 2% metaphor agarose: 1% agarose.

Neuroimaging: Structural

The methods applied to the acquisition, pre-processing and analysis of images are detailed in previous papers from this study (214, 215). Each participant underwent MRI scanning on a 1T Siemens (Erlangen, Germany) Magnetom scanner. The scan, for the volumetric analysis, was a three-dimensional magnetisation prepared rapid-acquisition gradient echo sequence consisting of an 180 degrees inversion pulse followed by a fast low-angle shot collection (flip angle 12O, TR=10 ms, TE=4 ms, TI=200 ms, relaxation delay time 500 ms, field of view 250 mm) giving 128 contiguous 1·88 mm thick slices in the coronal plane orthogonal to the Talairach plane. We corrected for any inhomogeneity in the head coil.

Voxel-Based Morphometry was performed using the SPM99 toolbox (Wellcome Department of Imaging Neuroscience, London). A study-specific template and a priori probability maps for grey matter, white matter and cerebral spinal fluid were constructed. Using these study specific templates all of the images in the control and high-risk group were spatially normalised and segmented, eliminating large-scale differences between the subjects, using a 12 parameter affine transformation. All of the resulting GMD segments were then smoothed at 12mm full width at half maximum (FWHM) before being entered

into our statistical analysis (214).

Average structural images were compared between groups (defined by genotype) on a voxel by voxel basis using SPM99. Cluster results are reported for contrast images thresholded at T=3, after adjustment for the underling smoothness as described in Moorhead et al (2005) (216). The effect of COMT Val allele dose was estimated using a 2-tailed linear contrast (1 0 -1;-1 0 1) where the alleles are given in ascending order of risk (MM MV VV, where M=Met allele and V=Val allele) with correction for multiple comparisons as implemented in SPM99 (225, 226). Individuals homozygous for the Val or Met allele were also compared to the other groups combined. Subject status (HR with no symptoms, HR with symptoms, HR who had progressed to schizophrenia), age, sex and number of grey matter voxels (nvox) were included as covariates. Where a positive association was found, the parameter estimates were inspected graphically to check that their estimates were ranked in order of increasing or decreasing magnitude. The effect of MAOA genotype was measured with a 2-tailed linear contrast (1 0 -1;-1 0 1) in which the GG genotype was compared to GT with correction for multiple comparisons. The analysis was corrected for multiple hypotheses testing in the imaging data.

Neuroimaging: Functional MRI

Functional imaging was carried out approximately 4 years later than the structural scans at the Brain Imaging Research Centre for Scotland (Edinburgh, UK) on a GE 1.5 T Signa scanner (GE Medical, Milwaukee, WI, USA) equipped with 23mT/m Echospeed gradients having a rise time of 200μs. Subjects were imaged with axial gradient-echo

planar images (TR/TE=4000/40ms; matrix 64x128; FOV 220x440mm) acquired continually during the experimental paradigm. Thirty-eight contiguous 5mm slices were acquired within each TR period. Each EPI acquisition was run for 204 volumes, of which the first four volumes were discarded. Visual stimuli were presented using a screen (IFIS; MRI Devices, Waukesha, WI, USA) placed in the bore of the magnet; corrective lenses were used where necessary.

The participants in the study performed the verbal initiation section of the Hayling sentence completion test (HSCT, 218) in the scanner. Subjects were shown sentences with the last word missing and were asked to silently think of an appropriate word to complete the sentence (i.e. without speaking the word), and press a button when they had done so, giving a reaction time measure. The task was adapted for fMRI to have four levels of difficulty, according to the range of suitable completion words suggested by the sentence context (see Whalley et al. 1999 (220) for further details). Sentences were presented in blocks of fixed difficulty; each block lasted 40 s and included eight sentences. Sentences were presented for a period of 3s followed by a fixation cross for 2s. Subjects were instructed to respond at any time by button press until the next sentence appeared. The rest condition consisted of viewing a screen of white circles on a black background for 40s. The order of the blocks was pseudo-random, and each block was repeated four times (different sentences were used for each sentence block). This design allowed both a standard subtraction (sentence completion versus rest) and parametric analysis (examining areas of increasing activation with reducing constraint/increasing task difficulty). Immediately after scanning, subjects were given the same sequence of

sentences on paper and requested to complete each sentence with the word they first thought of in the scanner. Scores for word appropriateness and reaction time were determined for each constraint level.

Scan analysis was performed using SPM99 (Wellcome Department of Cognitive Neurology and collaborators, Institute of Neurology, London, UK), and was performed blind to the group status of the individual. For each subject, EPI volumes were realigned to the first volume in the series using rigid body transformations. The images were then normalized to the SPM99 EPI template using a linear affine transformation followed by non-linear deformations, and resampled using sinc interpolation to cubic voxels of size 8mm3. Normalized images were spatially smoothed with a 6mm FWHM kernel to minimize residual inter-subject differences, and in order to meet assumptions for statistical analysis.

At the individual subject level the data were modelled with five conditions (the four difficulty levels and the rest condition), each modelled by a boxcar convolved with a synthetic hamodynamic response function. The estimates of the subject's movement during the scan were also entered as 'covariates of no interest'. Before fitting the model, the subject's data were filtered in the time domain using both a low-pass (4s) and a high-pass (400s) filter. Contrasts were constructed to examine all four sentence completion conditions versus rest, and areas of increasing activation with increasing task difficulty (the parametric contrast).

For each contrast of interest (sentence completion versus rest, and parametric effects) one contrast image per subject was entered into a second level random effects analysis to examine areas of activation within each of the three groups, and differences in activation between the groups. As the groups were matched on demographic variables, and there were no significant differences in movement parameters, we did not include these factors as potential confounds in the model.

Statistical maps were thresholded at a level of $p=0.01$ uncorrected and regions were considered significant at $P<0.05$ cluster level corrected for multiple comparisons, unless otherwise stated. All P-values quoted in the text are at the corrected cluster level. Co-ordinates are given using the Montreal Neurological Institute (MNI) convention. The effect of COMT Val allele dose was estimated using a 2-tailed linear contrast (1 0 -1;-1 0 1) where the alleles are given in ascending order of risk (MM MV VV). Subject status (HR with symptoms, HR without psychotic symptoms, control) was included as the only covariate since age, sex and handedness had been shown in a previous analysis to exert no statistically significant effect on the results (220). The small numbers of genotyped HR subjects progressing to schizophrenia with fMRI data (n=3) unfortunately precluded them from any meaningful statistical analysis. Where a positive association was found, the parameter estimates were inspected graphically to check that their estimates were ranked in order of increasing or decreasing magnitude.

Finally, for each significant result reported in respect of the fMRI paradigm, we tested for subject group (HR with symptoms, HR without symptoms, control) by COMT status

interactions to check whether any effect of COMT was generalisable to all HR subjects, or whether it was specific those with psychotic symptoms alone

3. Results

Groups at Baseline

Below is data relating to COMT and MAOA genotype groups at baseline.

1. COMT analysis Groups at baseline

	HR (no symptoms)			HR (with symptoms)			HR (schizophrenia)		
	MM	MV	VV	MM	MV	VV	MM	MV	VV
Subjects (n)	12	17	6	13	14	5	1	3	7
Families (n)	11	15	6	13	11	4	1	3	6
Age $\bar{x}$ (SD)	22.7 (3.1)	21.6 (2.9)	20.9 (2.8)	21.6 (2.8)	21.5 (2.9)	18.9 (2.9)	16.3 (IC)	18.9 (3.7)	19.4 (2.1)
Male n/N	3/12	10/17	3/6	5/13	5/14	0/5	1/1	1/3	3/3
SES† n,n	2,10	8,9	3,3	3,10	3,11	1,4	0,1	1,2	2,5
Height†† $\bar{x}$ (SD)	168 (8.8)	173.9 (12.5)	174.4 (10.9)	168.8 (9.6)	167.7 (7.0)	164.2 (7.2)	175 (IC)	171 (3.8)	170 (5.9)
NART* $\bar{x}$ (SD)	99.2 (6.5)	101.9 (9.9)	100.3 (8.5)	98.3 (9.1)	99.2 (7.5)	101.0 (6.2)	95.0 (IC)	107.3 (24.5)	96.4 (8.0)
sMRI	12	17	6	13	12	5	1	2	7
fMRI	7	15	4	12	14	5	-	-	-

MM: Met-Met, MV: Met-Val, VV: Val-Val, HR: high risk subjects. SES†: paternal socio-economic status (non-manual, manual), Height††: height in cm, NART*: predicted full-scale IQ from National Adult Reading Test. s/fMRI refers to the number of subjects providing s or fMRI data in each group. The number of genotyped subjects with schizophrenia providing a useable fMRI scan was insufficient to allow meaningful statistical analysis. The numbers for these groups are omitted from the table.

2. MAOA analysis groups at baseline

	HR (no symptoms)		HR (with symptoms)		HR (schizophrenia)	
	GT	GG	GT	GG	GT	GG
Subjects (n)	50	7	7	2	10	0
Families (n)	45	6	6	2	9	0
Age $\bar{x}$ (SD)	22.7 (3.1)	21.6 (2.9)		21.5 (2.9)	16.3 (IC)	18.9 (3.7)
Male n/N	27/50	6/7	2/7	0/2	4/10	0/0
SES† n,n	2,10	8,9	3,10	3,11	0,1	1,2
Height†† $\bar{x}$ (SD)	168 (8.8)	173.9 (12.5)	168.8 (9.6)	167.7 (7.0)	175 (IC)	171 (3.8)
NART* $\bar{x}$ (SD)	99.2 (6.5)	101.9 (9.9)	98.3 (9.1)	99.2 (7.5)	95.0 (IC)	107.3 (24.5)
sMRI	45	7	5	0	4	
fMRI	43	6	5	2	-	-

GG: Met-Met, GT: Met-Val, , HR: high risk subjects. SES†: paternal socio-economic status (non-manual, manual), Height††: height in cm, NART*: predicted full-scale IQ from National Adult Reading Test. s/fMRI refers to the number of subjects providing s or fMRI data in each group. The number of genotyped subjects with schizophrenia providing a useable fMRI scan was insufficient to allow meaningful statistical analysis. The numbers for these groups are omitted from the table.

Results Genetics

Seventy nine high risk subjects and 15 control subjects provided blood for genetic analysis collected over a 6 month period in 2004. Whilst there was no evidence that the COMT nor MAOA genotype were associated with being at high-risk of schizophrenia ($\chi^2_{2\,df}$=0.42, p=0.78) or with the presence of psychotic symptoms ($\chi^2_{2\,df}$=0.07, p=0.87), there was evidence that the COMT Val allele was associated with an increased risk of schizophrenia within high risk subjects (Fisher's exact test, p=0.01). Within the high risk group alone, the VV compared to the MM genotype had an estimated crude relative risk (RR) of 8.2 (95%CI 1.2 to 57.3). The estimated crude RR of the VM compared to MM genotype was somewhat smaller (RR=2.5; 95%CI 0.3 to 21.2) suggestive of a dose-response relationship, albeit based on low expected cell counts.

Clinical Variables Results

It can be seen here that there are no significant differences with regard to clinical dimensions of the schizophrenia between COMT Val Val and Met Met genotype. The variations in Clinical symptom scores between genotypes are calculated using Independent T-tests.

1. COMT Val Val genotype (n=24) verses COMT Met Met genotype (n=20)

Clinical Variable	*Genotype*		*Significance p(2 tailed)*
	Val Val Mean Symptom score	**Met Met Mean Symptom score**	
Situational anxiety	.67	1.25	.146
Tension	1.83	1.70	.842
Depression	.58	1.05	.242
Manic	.25	.10	.323
Perceptual Change	.17	.15	.899 .
Hallucinations	.33	.15	.359
Thought Disorder	.00	.05	.278

Table 9: Clinical Results COMT

2. MAOA genotype GT Heterozygote (n=65) verses GG homozygote (n=8)

It can be noted that there are several significant differences with regards to MAOA genotype between GT heterozygote and GG homozygote. Most notably affective symptoms such as Mania, over activity, Situational Anxiety and Incoherence are significantly different with regards to genotype. Here Independent T-tests were used to calculate differences between groups.

Clinical Variable	*Genotype*		*Significance (2 tailed)*
	GT Mean Symptom score	**GG Mean Symptom score**	
Situational anxiety	.73	.78	**.046**
tension	1.91	.78	.181
depression	.99	1.00	.104
manic	.13	.67	**.001**
overactivity	.04	.56	**.01**
incoherence	.00	.22	**.02**
Perceptual change	.18	.11	.366
Hallucinations	.16	.00	**.055**
Thought disorder	.01	.00	.462

Table 10: MAOA Clinical result

Neuropsychology Results

1. MAOA genotype GT Heterozygote verses GG homozygote

Here, the only significant finding with regards to neuropsychological data is the deficit in executive function as shown by the Stroop Test and Speed of comprehension. This pattern is consistent with frontal lobe deficit seen in schizophrenia and noted to be associated with dopaminergic levels.

Table 11 Neuropsychological Variables by MAOA genotype. Each variable is has specific scoring system. GG (n=8) compared with GT (n=65). Significant findings are in bold**.**

Neuropsychological Variable	*Genotype*		*Significance p value (2 tailed)*
	GT (mean Score on variable)	**GG (mean Score on variable)**	
National Adult Reading Test	99.75	99.88	.921
Full scale IQ	99.24	94.63	.612
Total Verbal Recall	39.29	32.00	.218
List animals	16.33	15.75	.430
Stroop Test 3	24.212	24.988	.775
Stroop Test 31	13.374	15.088	**.045**
Stroop Test 32	10.441	12.513	.069
Speed of comprehension	64.15	64.63	**.008**
Ravli Rey Auditory Verbal Learning Test	6.63	5.88	.800

Ravlvi Rey Auditory Verbal Learning Test	11.52	9.88	.859
Rey Rey Auditory Verbal Learning Test	53.16	48.38	.875
Rbmtstan Rivermead Behavioural Learning Test Learning and memory	21.71	20.38	**.053**
Storyim Rivermead Behavioural Learning Test Learning and memory	9.188	8.250	.575
Storydel Rivermead Behavioural Learning Test Learning and memory	8.223	7.875	.823
Verbal recall total 1	35.42	33.75	.603
Verbal recall total 2	32.95	28.63	**.052**
Ravlrec Rey Auditory Verbal Learning Test	6.06	5.88	.913
Hayling Sentence Completion errors A	1.33	1.86	.911
Hayling Sentence Completion errors B	2.71	2.57	.511

2. Neuropsychology Results for COMT genotype.

Table 12: Neuropsychological Variables by COMT genotype. Each variable is has specific scoring system. Met Met (n=23) compared with Val Val (n=19). Significant findings are in bold**.**

Neuropsychological Variable	*Genotype*		*Significance p value (2 tailed)*
	Met Met (mean Score on variable)	**Val Val (mean Score on variable)**	
National Adult Reading Test	99.13	98.47	.784
Full scale IQ	96.75	95.37	.685
Total Verbal Recall	38.48	37.42	.787
List animals	14.43	15.05	.637
Stroop Test 3	24.223	28.126	.339
Stroop Test 31	13.836	14.922	.512
Stroop Test 32	11.177	12.278	.476
Speed of comprehension	66.22	60.21	.348
Ravli Rey Auditory Verbal Learning Test	6.91	6.26	.285
Ravlvi Rey Auditory Verbal Learning Test	11.00	10.63	.691
Rey Rey Auditory Verbal Learning Test	53.39	50.42	.299
Rbmtstan Rivermead Behavioural Learning Test Learning and memory	21.96	21.11	.216
Storyim Rivermead Behavioural Learning Test Learning and memory	8.217	8.458	.804
Storydel Rivermead	7.435	7.605	.853

Behavioural Learning Test Learning and memory			
Verbal recall total 1	35.86	33.16	.**031**
Verbal recall total 2	33.14	30.68	.115
Ravlrec Rey Auditory Verbal Learning Test	6.00	5.95	.915
Hayling Sentence Completion errors A	1.29	2.17	.205
Hayling Sentence Completion errors B	2.88	3.00	.864

Table 12: COMT Neuropsychology Results

Key to Neuropsychology Results

National Adult Reading Test NART Premorbid intellectual function
(**FSIQ) Full scale IQ** Current intellectual function
Total Verbal Recall (verbal IQ) Temporal Lobe Function
Animals (Verbal Fluency) Executive Function
stroop3, stroop31, stroop32 (Executive Function) Prefrontal Cortex/Cingulate
Speedcr (word processing) Temporal Lobe Function
Rey, Ravli, Ravlvi ravldelr ravlrec Rey Auditory Verbal Learning Test Learning and memory, Fronto-Temporal Function
Rbmtstan, storydel, storyim Rivermead Behavioural Learning Test Learning and memory (hippocampal function)
Vrtotal1, vrtotal2 Verbal Recall Total Fronto-parietal Function
Errsan/errsbn Hayling Sentence Completion Test Errors (Executive function) Prefrontal cortex/cingulate

Neuroimaging Results

Structural Differences at baseline

Analysis was performed on 74 HR subjects with both genetic and baseline sMRI data. Reductions in grey matter density contingent upon the Val genotype were found in right (peak coordinates=8, 36, 24, T=4.10, p=0.04, cluster size=2969 voxels) and left (peak coordinates=-13, 16, 28, T=4.30, p=0.04, cluster size=5537 voxels) anterior cingulate cortex using a linear contrast for trend. The plot of parameter estimates at the voxel of peak difference suggests that the relationship is dose-dependent with increasing levels of the Val allele being associated with decreasing density of anterior cingulate cortex. Comparing the Val homozygotes to subjects with at least one Met allele revealed a similar region of anterior cingulate reduction (BA 32) in those homozygous for the Val allele (Peak Coordinates 21, 40, 4, T=4.49, $p<0.01$, cluster size=4183). Plotting the parameter estimates at the voxel of peak difference confirmed that the difference was not dose-related. No significant differences were found between Met homozygotes and those with at least one Val allele. No significant differences were found with regards to MAOA genotype.

Functional imaging

Analysis was performed on the 57 HR subjects with both genetic and baseline fMRI data. This excluded 3 subjects with genetic data who subsequently became unwell. No significant differences in reaction time or word appropriateness scores were found between the COMT and MAOA groups for any of the 4 levels of sentence difficulty. In addition, there were no significant differences in average or maximum head motion along or around the x, y or z axes (6 measurements) during the fMRI scanning sequence.

On subtraction analysis (sentence completion versus rest), the COMT Val allele was not associated with any significant differences in activation using the linear contrast. Similarly, by comparing individuals homozygous for Val or Met with the other two groups combined, no differences in activation were found. Contrasting the parametric images between COMT genotypes revealed differing patterns of prefrontal activation with increasing sentence completion difficulty. Using the linear test for trend (VV>MM) demonstrated a significantly greater activation in anterior cingulate cortex (figure 2: peak coordinates -10, 52, -2, T=5.62, $p<0.001$, cluster size=1414 voxels) and posterior cingulate cortex (figure Y: peak coordinates -6, -68, 18, T=4.05, $p=0.003$, cluster size=946 voxels). The plot of parameter estimates for each peak within the cluster revealed a trend for greater activation in the rank order VV>VM>MM. Comparing individuals homozygous for Val with the other two groups combined revealed a region of increased activation in anterior cingulate cortex with the same voxel maxima as stated above (peak coordinates -10, 52, -2, T=5.07, $p<0.001$, cluster size=1468 voxels) and additional region of increased activation in BA 45 ventrolateral prefrontal cortex (peak

voxels 60, 18, 4, T=3.96, p=0.015, cluster size=721 voxels). Comparing Met-Met homozygotes to those with at least 1 Val allele demonstrated no additional regions of difference, although increases in both anterior (peak coordinates -10, 52, -2, T=5.62, $p<0.001$, cluster size=1441 voxels) and posterior (peak coordinates 4, -48, 28, T=4.81, $p<0.001$, cluster size=1536 voxels) cingulate activation in those with at least 1 Val allele compared to the MM group were found.

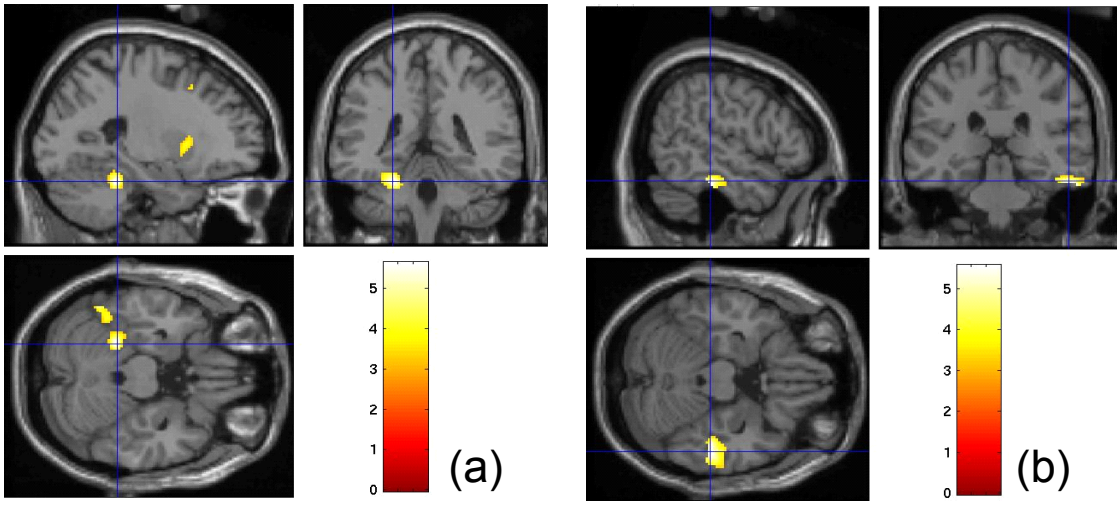
The parameter values were extracted at the voxel of maximum difference for each significant result (for both the structural and functional imaging results). These values were then imported into SAS (version 9.1, SAS Institute, Cary, NC) and the effects of group status (HR with symptoms, HR with without symptoms) by COMT group interactions investigated by analysis of variance. No significant group*COMT interaction was found for the anterior cingulate, posterior cingulate or ventrolateral prefrontal cortex results. However, the main effect of COMT remained significant after the addition of the symptom and symptom by COMT interaction terms to the model.

MAOA Results

Table 13: Brain areas showing increased activation on fMRI during Hayling Sentence Completion Test with GT and GG genotype from MAOA G941T SNP

Comparison	*Genotype comparison*	*Region*	*Z score*	*Tal co-ords*	*P value*
Active versus rest:	GT > G	N/A			n/s
Active versus rest:	GT < G	**Left fusiform/parahippocampal gyrus, BA36/35**	5	-24 -40 -18	0.013
Active versus rest:	GT < G	**Left superior frontal gyrus, BA8**	4.48	-10 28 58	0.016
Active versus rest:	GT < G	**Left insula**	4.22	-28 12 5	0.017
Active versus rest:	GT< G	**Left fusiform/inferior temporal gyrus, BA37**	5	-24 -40 -18	0.064
Parametric:	GT > G	**Right inferior temporal gyrus, BA20**	4.95	53 -30 -17	0.002
Parametric:	GT < G	N/A			n/s

Figure 3: Brain Map of functional Magnetic Resonance Imaging during Hayling Sentence Completion Task comparing GT genotype with GG genotype for the MAOA G941T SNP. (a) Active v rest, (b) Parametric (p=0.001 uncorrected, K_E=100, K_E=200 respectively).Areas highlighted of increased activation in GG genotype compared to GT genotype.



COMT Results

Figure 4: Structural Magnetic Resonance Imaging in Met Met genotype compared with Val Val genotype of the COMT Val158Met SNP: Figure shows regions of significant grey matter density reduction associated with Val allele compared to Met using linear contrast (-1 0 1).

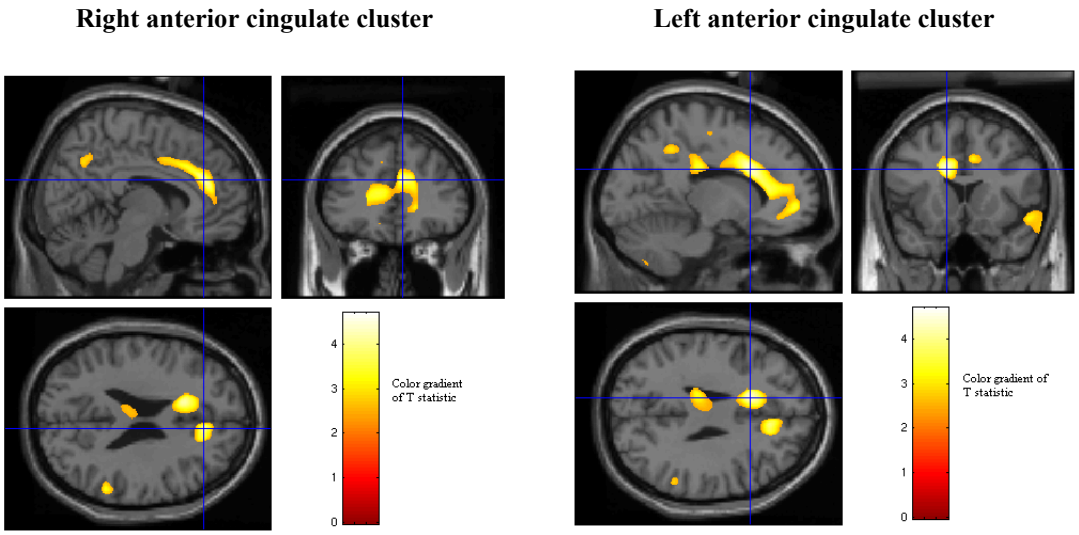
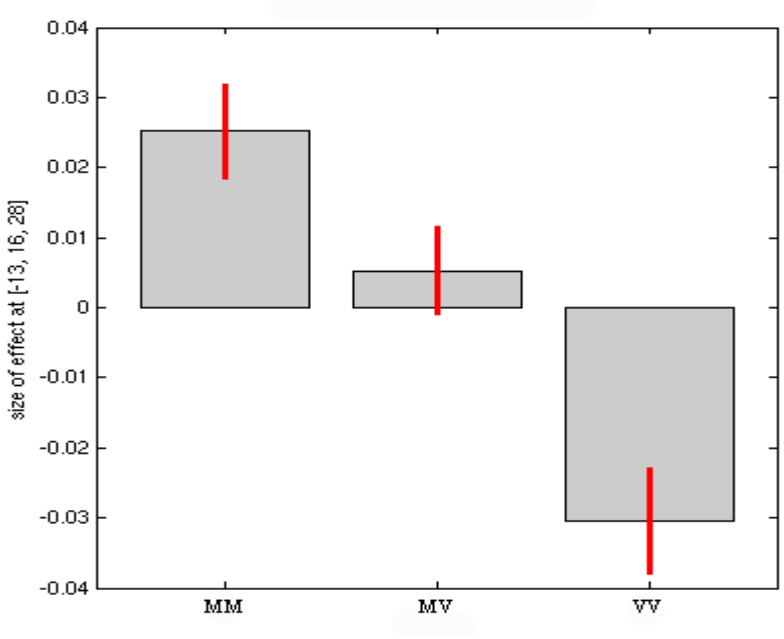


Figure 5: Parameter estimates are plotted beneath to show intermediate status of heterozygous group for Met Met (MM), Met Val (MV) and Val Val (VV)

Anterior Cingulate Cluster



Posterior Cingulate Cluster

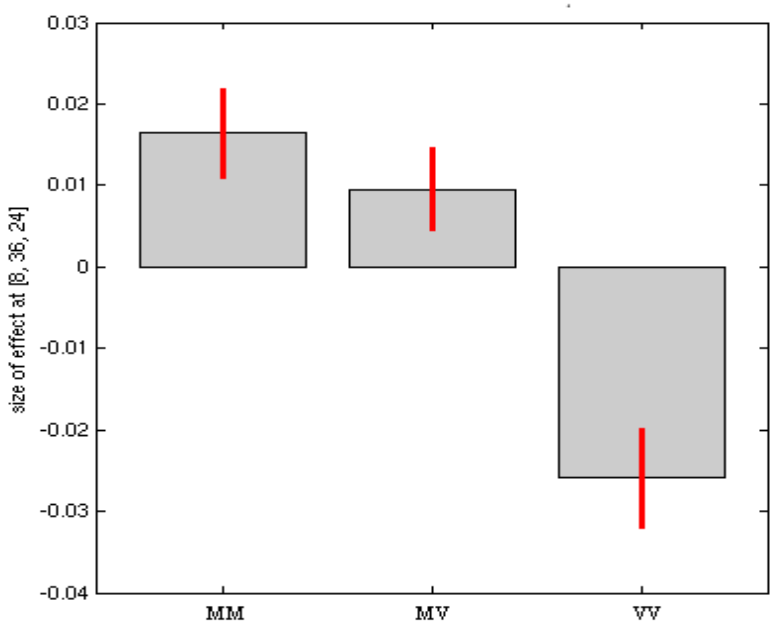


Figure 6: Functional Magnetic Resonance Imaging during Hayling Sentence Completion Test showing increased activation in the Anterior and Posterior Cingulate cortex in Val Val genotype compared with Met Met genotype of the COMT Val158Met SNP:

Anterior cingulate cortex **Posterior cingulate cortex**

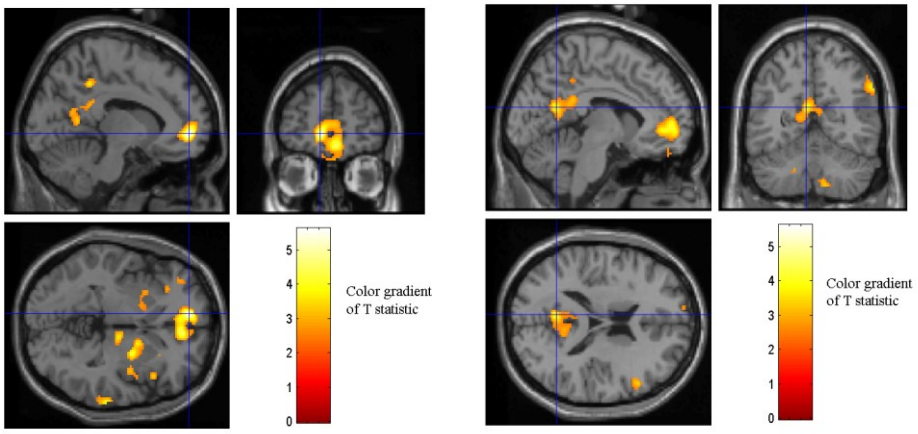
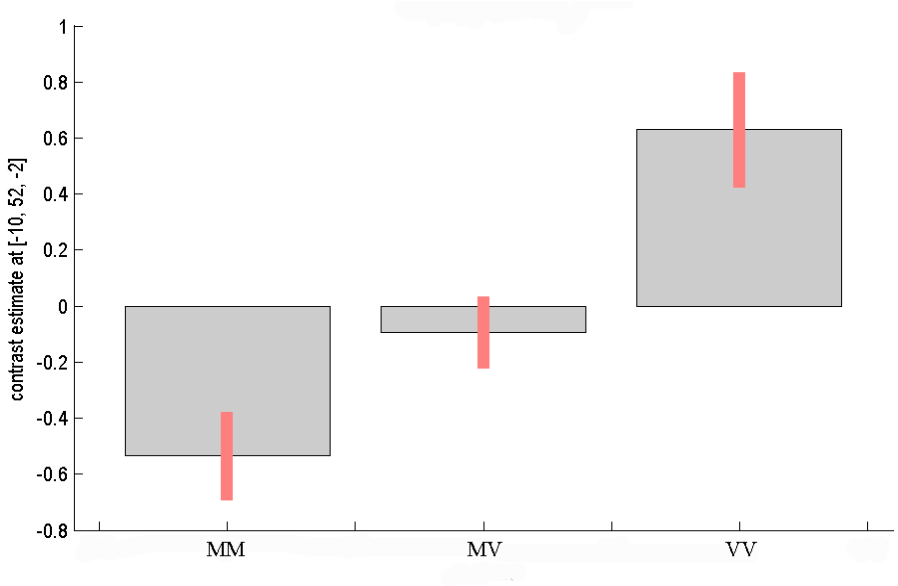


Figure 7: Parameter estimates to show intermediate status of heterozygous group of activation on fMRI; Met Met (MM), Met Val (MV), Val Val (VV)

Anterior Cingulate Cortex



Posterior Cingulate Cortex

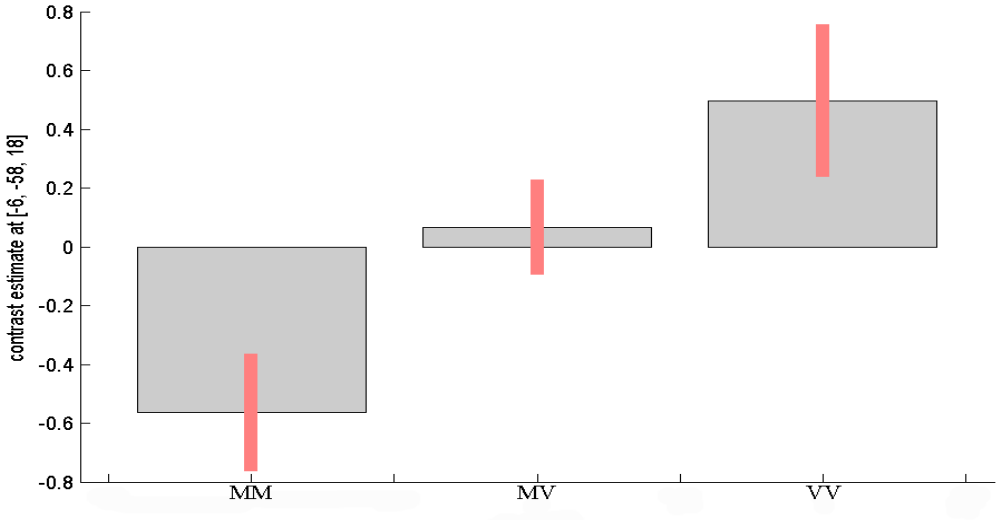


Figure 8: Brain areas showing increased activation of Val Val genotype compared to Met

Met genotype on fMRI for an increasing level of sentence completion difficulty during the Hayling Sentance completion test ($p<0.01$). Blue crosshairs are placed in voxel of maximum difference within significant clusters.

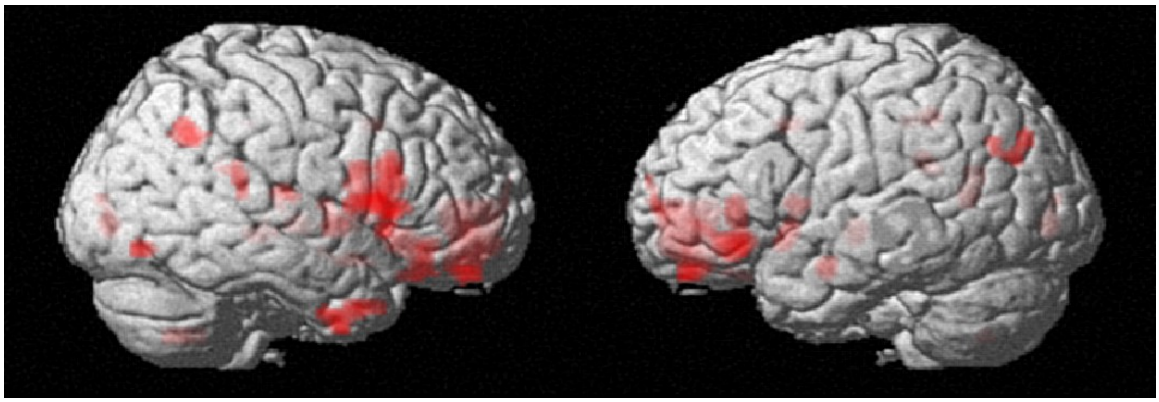
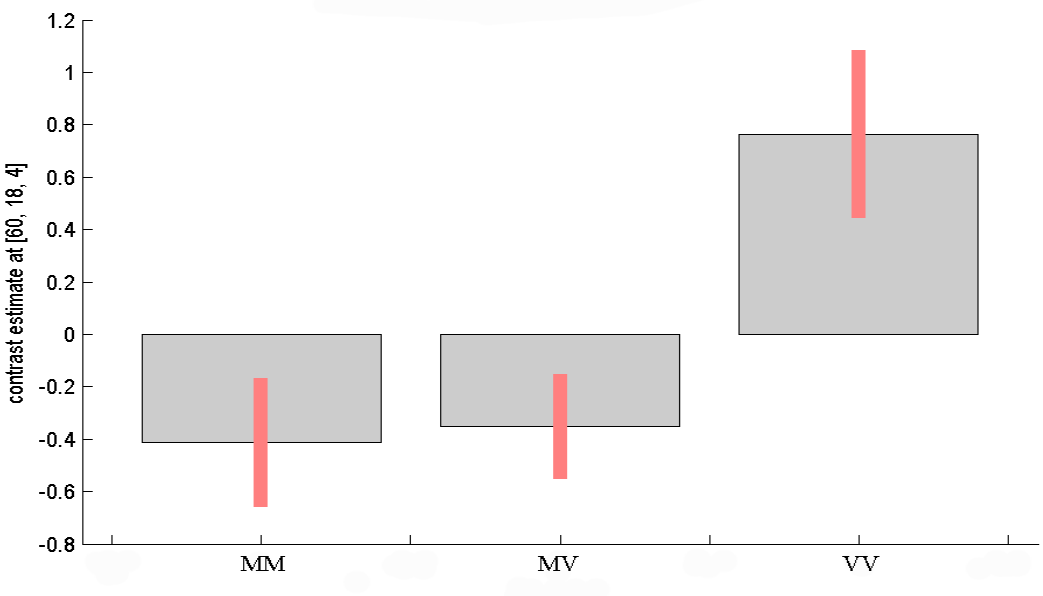


Figure 9: Areas of Increased Activation on fMRI of brain regions by COMT genotype in Met Met (MM), Met Val (MV) or Val Val (VV) Parameter estimates are plotted beneath figures to show intermediate status of heterozygous group



4. Discussion

This study demonstrates effects of COMT and MAOA genotype on clinical, neuropsychological and neuroimaging variables in a population at high risk of Schizophrenia

Methodology

It has been generally acknowledged that to study the effects of candidate genes, large investigations have been necessary to produce significant clinical findings. Candidate genes in schizophrenia have, as a rule, small effect sizes and thus large populations have been used. However, this study population, although small by genetic studies standards, has yielded statistically significant results. The extensive characterisation of this sample demonstrates many dimensions of the illness including the neuropsychological and neuroimaging components of the illness. Furthermore, most genetic studies only discriminate between a gross dichotomous phenotype of disordered verses non-disordered individuals. Here, the in depth study of different symptom variables demonstrates how genes may affect some symptoms but not others. The Edinburgh High Risk Study has the advantage of data from individuals before the onset of illness, thus being able to measure traits uncontaminated by the effect of medication or disease process. In addition to this, it is possible that subjects with familial schizophrenia may be more heavily loaded with candidate alleles.

One of the limitations of the present study is the small sample size. It is indeed possible that with more subjects some of the results may have become significant. Of the original 157 individuals who entered into the study only 79 were successfully genotyped. Those who were genotyped may have been clinically well relative to those who were not as it is likely that subjects who were lost to follow up showed a greater clinical deterioration. However, 11 of the sample did meet criteria for schizophreniform disorder and only 21 of the high risk sample did. It must be noted that this was not a study of gene effect on diagnosis so the sample are still a valid cohort in which to study the effect of these genes. Of the 79 individuals who were genotyped only 59 had available scans performed. While this would be an acceptable number for a neuroimaging study one can question how valid this number would be for a study relevant to genetics.

Ultimately, in spite of the sample size, this was a well characterised cohort which was assessed blindly and prospectively. Standardised, well validated neuropsychological and neuroimaging tools were used to assess this population.

It can be argued that confounding factors such as haplotype from other candidate genes may have played a role in the findings but due to the size of the sample this could not be adequately controlled for. We cannot address this argument within this investigation.

With regards to the choice of candidates, there can be little question that COMT was an obvious gene to study given its role in Velo-Cardio-Facial Syndrome, its linkage findings at 22q11 and its well evidenced association. It is perhaps not surprising that this gene

yielded the most significant results. Many other studies have failed to find an association between COMT and schizophrenia. However, it must be stated that all candidates appear to show heterogeneity with respect to ethnic group. Furthermore, this study looked at familial schizophrenia and it has been suggested that association may be stronger in such samples.

The choice of MAOA as a candidate can certainly be questioned given the fact that it has shown very little association with schizophrenia. Additionally it has not been shown to be situated in the linkage areas found in the meta-analyses (129, 130). The MAOA knockout mice do not show a phenotype that relates to traditional animals models of psychosis. MAOA does relate to other disorders as has been described above such as bipolar illness and conduct disorder. Its lack of association with schizophrenia cannot in itself be a reason for it not be relevant for study in this population however as the well characterised sample could (and did) yield significant results. Its known association with affective disorder and impulsive traits make it worth studying this gene in a population where these dimensions of phenotype are studied. Following the lack of association with schizophrenia MAOA has not been used in functional genomic imaging studies in the way that COMT and NRG-1 have. Thus this is the first study to relate MAOA genotype with Neuroimaging and Neuropsychology in a population at high risk for schizophrenia.

The fact remains however that, as discussed above, the dopamine hypothesis remains as a principal theory in the aetiology of schizophrenia. As COMT has been shown to affect dopamine levels it would seem only logical to study MAO which does metabolise

dopamine as well. (It can be noted that Dopamine receptor and Transporter gene polymorphisms would, by the same token, be equally justified lines of enquiry.) The initial premise in this study was not that MAO genotype would be shown to be an independent risk factor in aetiology but that in combination with COMT genotype it could yield measurable effects through the extra burden of excessive dopamine.
A further valid question regarding the study of MAO was both the choice of enzyme and the choice of mutation. Both MAOA and MAOB are known to metabolise dopamine but MAOA was chosen. The main reason for this was due to its brain distribution and the fact that mouse MAOA knockout mice showed more phenotypic variation than MAOB knockouts. Furthermore, MAOA has been continually cited as relevant to other psychiatric conditions compared to MAOB which has not.

There have been 4 noted gene mutations in the MAOA. These include two VNTRs in the MAOA promoter region and two SNPs in exon 8 (rs6362) and exon 14. Again none of these have specifically been shown to associate with schizophrenia. The choice of the SNP at exon 8 was for one simple reason. The VNTR polymorphisms exhibit up to 5 different haplotypes. Given the size of this sample group, to divide a sample of 79 into 5 would be extremely unlikely to produce group sizes that would be likely to show significant results. Therefore rs6362 was chosen as it has a simple dichotomous genotype that could easily divide a cohort into two reasonable size groups. It must be noted that it would indeed be worthwhile to study the other SNP and VNTRs of MAOA and also the SNPs in MAOB. Certainly this is a topic for future work.

Clinical Findings

With regards to allele frequency COMT yielded a higher proportion of Val Val genotype amongst the high risk sample. This study found evidence that the COMT Val allele raises the risk of developing schizophrenia in subjects from families where there is more than one affected member. This supports an increasing body of literature implicating this locus and is plausibly linked to the aetiology of schizophrenia through the action of COMT on extra-cellular dopamine (223) Studies have generally failed to find an association between COMT and risk of schizophrenia (224). However, in a population at increased risk of schizophrenia for presumed genetic reasons, we have shown that the COMT Val allele is associated with an increased risk of developing schizophrenia.

Studies have generally found little association between MAOA and schizophrenia and this study adds to this literature that independently MAOA is not an obvious susceptibility gene. However, MAOA does produce significant effects with regards to other measured symptoms in this cohort such as depressive symptoms, manic symptoms incoherence and over activity. This evidence is consistent with the association between MAOA genotype and Bipolar Disorder. Notably there was no association with hallucinations or delusions. Situational anxiety was found to be relevant at trend level of significance. This finding would be consistent with the evidence that MAOA is primarily linked to affective symptoms. It is likely that these symptoms are mediated by the failure of MAOA to metabolize serotonin or noradrenalin. When COMT and MAOA genotype are combined there is an interesting finding that situational anxiety becomes a significant

result. (Here carriers of low functioning COMT and MAOA enzymes have significantly higher situational anxiety than carriers of the high functioning enzymes).

Neuropsychology Findings

With regards to the Neuropsychology results there are also interesting findings that relate to genotype. Working Memory (WM) has been consistently demonstrated as being deficient in schizophrenic patients compared to controls and WM has also been related to COMT genotype suggesting that WM is an endophenotype for COMT. This has not been demonstrated in this study population. With regards to MAOA there are several significant findings. The finding that MAOA GG genotype scores significantly less on Stroop Tests may relate to the finding that some aspect of frontal lobe dysfunction. Verbal recall was found to be different at a trend level of significance.

These findings are the first evidence of the MAOA endophenotype and may give some understanding of the underlying neuropsychological traits behind conditions such as conduct disorder

Neuroimaging Findings

The COMT Val allele was also associated with reduced grey matter density in anterior cingulate and with increasing perfusion of the same region during tasks of increasing difficulty. The finding of increasing prefrontal activation for an equivalent level of task performance is consistent with impairment of anterior cingulate cortex and with the

consequent need for greater perfusion given an equivalent level of task difficulty and behavioural response. The COMT Val allele was also associated with an increased perfusion of posterior cingulate cortex and ventrolateral prefrontal cortex (BA45) during sentences of increasing difficulty, although the latter result was only found in individuals homozygous for the Val allele when compared to the remaining groups combined.

Structural abnormality of anterior cingulate and medial prefrontal cortex is arguably one of the most consistently replicated findings in schizophrenia (225, 226, 227) and has previously been linked to increasing genetic liability, albeit to unidentified loci (228, 173). The functional correlates of this region mirror the structural abnormality, although are in opposite directions and are suggestive of a compensatory mechanism. Medial prefrontal cortex is innervated by a diversity of neurones from different brain regions, including dopaminergic neurones from the striatum to which it also sends efferent connections. These neurones are part of the fronto-striatal pathway and are the structural substrate of the dopamine hypothesis of schizophrenia. Increased dopamine receptor availability in the striatum has been shown to be related to genetic liability to schizophrenia in unaffected subjects (227). It is therefore possible that the finding of reduced medial prefrontal grey matter and impaired function are related to striatal dysfunction. These Val associated structural and functional changes in medial prefrontal cortex have not been found in larger studies of COMT in people with schizophrenia (229). This suggest that the structural and functional differences are likely to relate to the effects of COMT val allele load on prefrontal dopamine levels and physiology (228)

The COMT Val allele has been linked to impaired perfusion of DLPFC in previous studies (228). Although the results of the present study did not replicate this finding exactly, subjects with the Val allele activated more ventrolateral prefrontal cortex for an equivalent level of task performance and difficulty. This area is adjacent, to that reported by Egan and colleagues, and given the spatial resolution of fMRI, may be an overlapping region of cortex. In contrast, the association between the COMT-Val allele and increasing posterior cingulate activation for increasing level of sentence difficulty is novel, so far as any of the authors are aware. This region, in combination with the adjacent precuneus, is associated with episodic memory retrieval in healthy human subjects (229, 230), a function impaired in the relatives of schizophrenic subjects (258). Abnormalities of dopamine metabolism in posterior cingulate have previously been reported in medication-free schizophrenic subjects (229). Our finding suggests that dopamine-related dysfunction in this region may be related to COMT status and be applicable to the wider group of subjects at increased familial risk.

With regard to MAOA findings, the significant finding concerning increased left superior frontal gyrus is consistent with the neuro-cognitive deficits and clinical data found from the genotype. The left fusiform area is connected with verbal recognition which again relates to the neuropsychological deficits. Thus far no other studies have validated this finding. As the frontal and temporal lobes are highly implicated in schizophrenia this finding is both relevant and important.

Future Work

The EHRS is one of the largest prospective imaging investigations in psychiatry to date. However, there are practical limitations in any study from a single geographical region. In order to increase sample size, whilst maintaining the quality of recorded information, multicentre investigation will need to become routine. Secondly, since the imaging associations of the COMT or MAOA genotype may not be specific to schizophrenia, it would be helpful to further refine the behavioural associations of this allele; either by examining the associations quantitatively, or by examining the allelic associations in other psychiatric disorders.

The main limitations of this study relate to the cohort size. An automatic area of future work would be to relate COMT and MAOA genotype in a large population to neuroimaging and neuropsychological findings. Other COMT variants should be identified, genotyped and studied and importantly the other MAOA SNPs and VNTRs would need to be studied. MAOB would also be of great interest. A further line of enquiry as mentioned above would be the studying of other genetic mutations of proteins in the dopamine pathway including DRD3, Dopamine Transporter and Tyrosine Hydroxlase.

Conclusion

In conclusion, this study demonstrates the effect of COMT and MAOA genotype in a population at high risk of schizophrenia. Because the sample has been extensively characterized the genotype effect can be seen in clinical, neuropsychological and

neuroimaging dimensions of the illness. The strength of this study lies in the prospective cohort design and the amount of data relating to each subject. COMT can be seen to be more obviously involved in the pathogenesis of Schizophrenia but MAOA can only be seen to relate to certain dimensions of the illness. Both genes importantly can be studied as to their functional effects, irrespective of their place in the pathology of schizophrenia. The MAOA data is the first of its kind and may, hopefully lead to a more extensive study of this gene in psychiatric illness. Furthermore this study emphasizes the need to extensively characterize phenotypes to see a genotype related effect.

The combination of high risk studies with genetics is relatively new in the study of schizophrenia. Much work has already been done in the area of functional genomics but in these studies it is important to realise that measured brain or psychological changes may relate to secondary effects of the illness or indeed medication. High risk studies control for this potential confounder by taking subjects prior to the onset of symptoms. MAOA has been implicated in a wide variety of disorders but high risk studies only exist in schizophrenia. Hence work looking at conduct disorder/antisocial personality disorder and anxiety conditions (and indeed Parkinsons disease) will be confounded by many factors.

Both genes have been implicated in affective disorders and the results from these studies both enhance what has already been known and concentrate on the endophenotypic underpinnings of these disorders. It is interesting to find that MAO and COMT in combination have effects on anxiety. This finding has already been noted relating MAOA

and COMT to neuroticism in the population. (232) Although we report on a schizophrenic population these results are still valid due to the extensive measures used to characterise the sample.

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