Molecular genetic strategies to identify Obsessive-compulsive disorder (OCD) and schizophrenia candidate genes in a South African sub-population group

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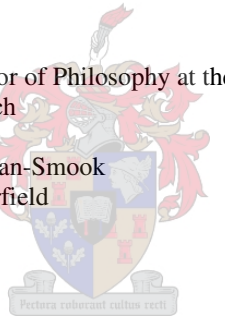

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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ABSTRACT

Obsessive-compulsive disorder is a severe, debilitating psychiatric disorder for which the underlying molecular aetiology still remains unclear. Evidence from family studies have suggested that OCD may be caused by a complex interplay of environmental and genetic factors.

In order to identify the genetic factors that mediate OCD susceptibility, several genetic association studies have been undertaken, which have yielded inconsistent findings. Moreover, the majority of these studies have focused on a small number of candidate genes that encode components of the serotonin and dopamine neurotransmitter pathways. However, based on the complexity of clinical manifestations observed in OCD, it is likely that its pathogenesis is mediated by a broader complex of interrelated neurotransmitter systems and signal transduction pathways; consequently there is a need to identify and assess novel candidate genes.

One method of identifying such novel OCD candidate genes is by utilising knowledge of diseases with phenomenological overlap with OCD, which lend themselves to better genetic dissection through linkage analysis and animal studies. Genetic loci for such disorders, identified though linkage analysis, could potentially harbour novel OCD candidate genes, while genes implicated through animal models may lead to the identification of additional susceptibility genes through delineation of pathways by, for instance, interactome analysis. One such disorder is schizophrenia, which manifests overlap in both symptoms and brain circuits with OCD. In schizophrenia, in addition to several case-control association studies having been performed, linkage data, studies of chromosomal aberrations and animal models have led to the identification of many chromosomal regions that may contain genes involved in its aetiology and thus may also contain OCD candidate genes.

In the present investigation, this approach was employed using previously reported schizophrenia susceptibility loci to identify novel OCD candidate genes. All genes residing in each of these loci were catalogued and individually analysed using a battery of bioinformatic techniques in order to assess their potential candidature for OCD susceptibility. These analyses yielded 13 credible OCD candidate genes.

Additional candidates were sought using information regarding a well-defined schizophrenia animal model, the heterozygous reeler mouse, that exhibits neurodevelopmental, neuroanatomical and behavioural abnormalities, similar to those displayed by patients with schizophrenia. The phenotype of these mice is caused by a mutation in *Reln,* which encodes reelin, a large extracellular matrix protein that plays a pivotal role in the ordered migration of neurons during the development of laminar brain structures. The fact that both reelin protein and mRNA levels have been shown to be reduced in post-mortem brain sections of schizophrenic patients, coupled with the observed behaviour and neurochemical similarities between the heterozygous reeler mouse and schizophrenic patients suggests that reelin may be involved in the pathogenesis of schizophrenia and hence also OCD. Furthermore, genes encoding proteins that interact with reelin may thus also be considered plausible candidate genes for both schizophrenia and OCD. For this reason, novel reelin-interacting proteins were sought using the N-terminal reeler-domain of reelin, a domain only found in proteins involved in neuronal migration, as "bait" in a yeast two-hybrid screen of a foetal brain cDNA library. Putative reelin ligands were subsequently re-evaluated using co-immunopreciptitation and mammalian two-hybrid analysis to corroborate the yeast two-hybrid findings. Results of these analyses showed that WDR47, a WD40-repeat domain protein, interacts with reelin via its reeler-domain; therefore, the gene encoding this ligand protein, as well as *RELN* itself, was also considered a credible OCD candidate gene.

Each of the candidate genes identified using the afore-mentioned strategies were assessed for their potential role in the aetiology of OCD by case-control association studies of a cohort of Afrikaner OCD patients and control individuals. Statistically significant associations were detected for two genes, *DLX6* and *SYN3,* with the disorder. These associations are exciting as they may point to novel mechanisms involved in OCD development.

The identification of WDR47 as a novel reelin-interacting protein has significant implications for our understanding of reelin-dependant signalling. Using this protein as the starting point, further novel components of the reelin signalling pathway may be unravelled, an investigation which may lead to the identification of novel roles for reelin in neurodevelopment. Such novel components may, of course, also be considered OCD and schizophrenia candidate genes, which may, in turn, augment the existing knowledge of the pathophysiologies of OCD, schizophrenia and other neurodevelopmental disorders.

Taken together, the current study yielded exciting results that warrants follow-up investigation in future. The identification of *DLX6* and *SYN3* as novel OCD susceptibility genes as well as the identification of WDR47 as a reelin-interacting protein may provide investigators with alternative avenues of research into potential pathological mechanisms involved both in OCD and schizophrenia, which may ultimately lead to alternative pharmacotherapy.

OPSOMMING

Obsessiewe kompulsiewe steuring (OKS) is `n ernstige, verswakkende psigiatriese steuring waarvan die onderliggende molekulêre etiologie steeds onbekend is. Bewyse verkry vanuit familiestudies het voorgestel dat OKS moontlik veroorsaak word deur `n komplekse interaksie van omgewings en genetiese faktore.

Om die genetiese faktore te identifiseer wat OKS vatbaarheid veroorsaak, is `n hele aantal genetiese assosiasie studies onderneem, wat teenstrydige resultate gelewer het. Wat meer is, die grootste hoeveelheid van hierdie studies het gefokus op `n klein aantal kandidaatgene wat vir komponente van die serotonien en dopamine neurotransmittor weë enkodeer. Dit is egter, gebaseer op die kompleksiteit van die kliniese manifestasies wat waargeneem word in OKS, heel moontlik dat die patogenisiteit van die siekte bemiddel word deur `n breër kompleks van interverwante neurotransmittor sisteme en seintransduksie weë. Daar is dus `n behoefte na die identifikasie en ondersoek van nuwe kandidaatgene.

Een metode om sulke nuwe OKS kandidaatgene te identifiseer, is deur die gebruik van bestaande kennis oor siektes wat fenomenologiese ooreenkomste het met OKS, siektes wat makliker geneties ontleed kan word deur koppelingsanalises en dierestudies. Genetiese lokusse vir sulke versteurings, geïdentifiseer deur koppelingsanalises, het die potensiaal om nuwe OKS kandidaatgene in te sluit, terwyl gene wat geïmpliseer word deur dierestudies mag lei tot die identifisering van bykomende vatbaarheidsgene deur die ondersoek van weë deur, byvoorbeeld, interaktoom analises. `n Voorbeeld van so `n versteuring is skisofrenie, wat in manifestasie oorvleuel in beide simptome en breinstroombane met OKS. In skisofrenie het, addisioneel tot verskeie geval-kontrole assosiasiestudies wat gedoen is, koppelingsdata, studies van chromosomale afwykings en dierestudies gelei tot die identifikasie van verskeie chromosomale gebiede wat gene mag bevat wat betrokke kan wees in die etiologie van die siekte, en dus ook OKS kandidaatgene mag bevat.

In die huidige ondersoek is hierdie benadering gevolg en is gebruik gemaak van voorheen gerapporteerde skisofrenie vatbaarheidslokusse om nuwe OKS kandidaatgene te identifiseer. Alle gene wat in hierdie lokusse voorkom is gekatalogiseer en individueel geanaliseer deur gebruik te maak van `n battery van bioinformatika tegnieke om hul potensiaal as kandidate vir OKS vatbaarheid te bepaal. Hierdie analise het 13 geloofwaardige OKS kandidate opgelewer.

Addisionele kandidate is gesoek deur inligting van `n goed gedefinieerde skisofrenie dieremodel te gebruik, naamlik die heterosigotiese "reeler" muismodel, wat neuro-ontwikkelings-, neuroanatomiese- en gedragsabnormaliteite vertoon, soortgelyk aan dié wat voorkom by pasiënte met skisofrenie.

Die feit dat daar aangetoon is dat beide reelin protein en bRNS vlakke verlaag is in post-mortem brein seksies van skisofrenie pasiënte, gekoppel aan die gedrags- en neurochemiese ooreenkomste wat gesien word tussen heterosigotiese "reeler" muise en skisofrenie pasiënte, stel voor dat reelin betrokke is by die patogenese van skisofrenie en dus ook OKS.

Vir hierdie rede is nuwe proteïene gesoek wat `n interaksie met reelin toon, deur gebruik te maak van die N-terminale reeler-domein van reelin, `n domein wat slegs gevind word in proteïene wat betrokke is by neuronale migrasie, as "aas" in `n gis-twee-hibried sifting van `n fetale brein cDNS biblioteek. Vermeende reelin ligande is vervolgens herevalueer deur gebruik te maak van ko-immunopresipitasie en soogdier twee-hibried analises om die gis-twee-hibried bevindings te bevestig. Resultate van hierdie analises het getoon dat daar interaksie is tussen WDR47, `n WD40-herhalingsdomein protein, met reelin via sy reeler-domein. Die geen wat hierdie ligand protein enkodeer, sowel as *RELN* self, is dus beskou as 'n geloofwaardige OKS kandidaatgeen.

Elkeen van die kandidaatgene wat geïdentifiseer is deur gebruik te maak van bogenoemde strategieë is ondersoek vir `n potensiële rol in die etiologie van OKS deur gebruik te maak van geval-kontrole assosiasie studies met `n groep Afrikaner OKVS pasiënte en kontrole individue. Statisties-betekenisvolle assosiasies met die versteuring is vasgestel vir twee gene, *DLX6* en *SYN3*. Hierdie assosiasies is opwindend aangesien hul nuwe meganismes betrokke by OKS ontwikkeling mag aantoon.

Die identifikasie van WDR47 as 'n nuwe protein wat interaksie met reelin vertoon, het betekenisvolle implikasies vir die verstaan van reelin-afhanklike seining. Deur hierdie proteïn as die beginpunt te gebruik kan vêrdere nuwe komponente van die reelin seinweg ontdek word, `n ondersoek wat mag lei tot die identifisering van nuwe funksies vir reelin in neuro-ontwikkeling. Sulke nuwe komponente mag, natuurlik, ook in aanmerking kom as OKS en skisofrenie kandidaatgene, wat op sy beurt weer die bestaande kennis van die patofisiologie van OKS, skisofrenie en ander neuro-ontwikkelings versteurings mag verbreed.

In samevatting, hierdie studie het opwindende resultate gelewer wat opvolgondersoeke in die toekoms regverdig. Die identifikasie van *DLX6* en *Syn3* as nuwe OKS vatbaarheidsgene, sowel as die identifisering van WDR47 as ‘n protein wat interaksie vertoon met reelin, mag aan navorsers alternatiewe navorsingsweë voorsien om die moontlike patologiese meganismes wat betrokke is by beide OKS en skisofrenie te ondersoek, wat uiteindelik mag lei tot alternatiewe farmakoterapie.

INDEX PAGE

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LIST OF ABBREVIATIONS

3'-UTR	: 3 prime untranslated region
5'-UTR	: 5 prime untranslated region
5-HIAA	: 5 hydroxyindoleacetic acid
5-HT	: Serotonin
5-HT1A	: Serotonin receptor 1A
5-HT1Dβ	: Serotonin receptor 1Dβ
5-HT2A	: Serotonin receptor 2A
5-HT2C	: Serotonin receptor 2C
5HTR2C	: Serotonin receptor 2C gene
5-HTT	: Serotonin transporter protein
5HTTLPR	: Serotonin transporter promoter-liked polymorphism
(8-OH-DPAT	: 8-hydroxy-2(di-n-propylamino) tetralin
22qDS	: Chromosome 22q deletion syndrome
µl	: Microlitre
A	: Adenosine
AChR	: Acetylecholine receptor
ADAM33	: disintegrin and metalloprotease domain protein 33 encoding gene
AIMA	: Abnormal involuntary movement scale
ALD	: Acryl lick dermatitis
AMPA	: amino-hydroxy-5-methyl-4-isoxazole
ANOVA	: Analysis of Variance
APM	: Affected pedigree method
ApoER2	: Apolippoprotein E receptor 2
ASP	: Affected sib pair
ASREA	: Allele specific restriction enzyme analysis
BAS	: Barnes akathisia scale
BG	: Basal ganglia
BLAST	: Basic local alignment search tool
BLASTN	: Basic local alignment search tool (nucleotide)
BLASTP	: Basic local alignment search tool (protein)

BLASTX	: Basic local alignment search tool (translated)
bp	: Base pair
BZRP	: Peripheral benzodiazepine receptor
°C	: Degree Celsius
cAMP	: Cyclic adenosine monophosphate
cDNA	: Complementary DNA
CGI	: Clinical global impression scale
cm	: Centimetre
cM	: Centimorgan
CNR	: Cadherin-related neuronal proteins
CNS	: Central nervous system
CNTNAP2	: Contactin-associated protein
Co-IOP	: Co-immunoprecipitation
COMT	: Catechol-O-methyltransferase
CP	: Cortical plate
CSPD	: Chemiluninescent substrate
CT	: Computerised tomography
CTAFS	: Conotruncal anaomaly facial syndrome
CTD	: Chronic tic disorder
DAAO	: D-amino acid oxidase
Dab1	: Disabled 1
DALY	: Disability adjusted life year
DAT	: Dopamine transporter
DAT-KO	: Dopamine trnasporter knockout
dATP	: Deoxy-adenosine triphosphate
DBH	: Dopamine beta hydroxylase
dCTP	: Deoxy-cytosine triphosphate
DDC	: Dopa decarboxylase
dGTP	: Deoxy-guanosine triphosphate
DIS	: Diagnostic interview schedule
DISC	: Disrupted in schizophrenia
DLX6	: Distal-less like homeobox 6

DLPFC : Dosolateral prefrontal cortex
DMSO : Dimethyl sulphoxide
DNA : Deoxyribonucleic acid
DNTBP1 : Dysbindin
dNTP : Deoxy- nucleotide triphosphate
DOPAC : 3,4-dihydroxyphenylacetic acid
DOPEG : Dihydroxyphenylglycol
DRD2 : Dopamine receptor 2
DRD3 : Dopamine receptor 3
DRD4 : Dopamine receptor 4
DSM-IV : Diagnostic and Statistical Manual of Mental Disorders
dTTP : Deoxy-thymidine triphosphate
DZ : Dizygotic
ECA : Epidemiological catchment area
ECM : Extracellular matirx
EDTA : Ethylene-diamine-tetra-acetic acid
EEG : Electroencephalogram
EMD : Eye movement dysfunction
ERE : Oestrogen response elements
ERP : Event related potential
ESRS : Extrapyramidal symptom rating scale
FACS : Fluorescence activated cell sorting
FAK : Focal adhesion kinase
Fig : Figure
GABA : Gamma-aminobuteric acid
GABHS : Group A B-haemolytic streptococcus
GAD_{67} : Glutamate decarboxylase
GAF : General assessment of functioning
GBR : Gamma-aminobuteric acid receptor 1
GBRβ2 : Gamma-aminobuteric acid receptor β 2
GPCR : G-protein-coupled receptors
GRIA4 : amino-hydroxy-5-methyl-4-isoxazole receptor subunit GluR1

GRIN1 : N-methyl-D-aspartate NR1 subunit
GRM3 : Metabotropic glutamate receptor 3
GTP : Guanine triphosphate
H_2O : Water
HLA : Human leukocyte antigen
HLOD : heterogeneity logarithm of odds
HoxB8 : homeobox protein B8
HRM : Heterozygous reeler mouse
HRR : Haplotype relative risk
HVA : Homovanillic acid
IBD : Identity by descent
IHC : Idiopathic haemachromatosis
IMMP2L : Inner membrane peptidase subunit 2
IQ : Intelligence quotient
ISHDF : Icelandic schizophrenia high density families
IZ : Intermediate zone
K : Potassium
kb : Kilobase
kDA : Kilo Dalton
LB : Luria-Bertani broth
LD : Linkage disequilibrium
LOD : Logarithm of odds
LSD : lysergic acid diethylamide
LTD : Limited
M : Molar
M2H : Mammalian two-hybrid
MAO : Monoamine oxidase
MAO-A : Monoamine oxidase A
MAO-B : Monoamine oxidase B
MAP2B : Microtubule associated protein 2
Mb : Megabases
MB-COMT : Membrane-bound COMT

m-CCP : Meta-chlorophenyl piperazine
MCS : Multiple cloning site
MD : mediodorsal
mg : Magnesium
$MgCl_2$: Magnesium chloride
ml : Millilitre
MLS : Multipoint logarithm of odds score
mm : Millilitre
mM : Millimolar
MOPEG : 3-methoxy-4-hydroxyphenylethyleneglycol
MPA : Minor physical anomalies
MRC : Medical Research Council
MRI : Magnetic resonance imaging
mRNA : Messenger ribonucleic acid
ms : Milliseconds
MZ : Monozygotic
NAT : Negative automatic thought
ng : Nanograms
NIMH : National institute of mental health
NMDA : n-methyl-D-aspartate
NMDA-R : n-methyl-D-aspartate receptor
NOD2 : nucleotide-binding domain
NPL : Non-parametric logarithm of odds
NR1-KO : n-methyl-D-aspartate receptor 1 knock out
NR2A-KO: : n-methyl-D-aspartate receptor 2A knock out
NRG1 : Neuregulin 1
OC : Obsessive-compulsive
OCD : Obsessive-compulsive disorder
OCS : Obsessive-compulsive symptoms
OCT7 : Octamer binding transcription factor 7
OD : Optical density

ORF	: Open reading frame
PAGE	: Polyacrylamide gel electrophoresis
PANDAS	: Paediatric autoimmune neuropsychiatric disorder associated with streptococcal infection
PANNS	: Positive and negative syndrome scale for schizophrenia
PCI	: Phenon chloroform isoamyl
PCP	: phencyclidine
PCR	: Polymerase chain reaction
PCR-SSCP	: Polymerase chain reaction single strand conformational polymorphism
PET	: Positron emission tomography
PFC	: Prefrontal cortex
PITANDS	: Paediatric infection-triggered autoimmune neuropsychiatric disorder
POU3F2	: POU domain, class 3, transcription factor 2;
PPI	: Pre-pulse inhibition
PRODH2	: Proline dehydrogenase
PSE	: Present state examination
QNP	: Quinpirole
RARE	: Retinioic acid response elements
RELN	: Reelin
REM	: Rapid eye movement
RET	: Rational emotive therapy
RF	: Rheumatic fever
RGS4	: Regulator of G-Protein signalling 4
RNA	: Ribonucleic acid
RT-PCR	: Reverse transcriptase polymerase chain reaction
RXRβ	: Retinoid X receptor beta
SANS	: Scale for the Assessment of Negative Symptoms
SAPS	: Scales for the Assessment of Positive symptoms
SAS	: Simpson-Angus Scale
SC	: Sydenham's chorea
SCID-I	: Structured clinical interview for axis I disorders
SCID-I/P	: Structured clinical interview for axis I disorders patient version
SCID-II/P	: Structured clinical interview for axis II disorders patient version

S-COMT	: Soluble COMT
SD	: Synthetic dropout
SDS	: Sodium dodycyl sulphate
SDS-PAGE	: Sodium dodycyl sulphate polyacrylamide gel electrophroresis
SEAP	: Secreted alkaline phosphatase
sec	: Seconds
SEP	: Smooth eye persuit
SIT	: Self-instructional training
SLC6A4	: Solute carrier protein family 6 member 4
SNAP25	: Synaptosomal associated protein of 25kDa
SNAP29	: Synaptosomal associated protein of 29kDa
SNP	: Single nucleotide polymorphism
SSCP	: Single strand conformational polymorphism
SSLP	: Simple sequence length polymorphism
SSRI	: Selective serotonin reuptake inhibitors
SWM	: Spatial working memory
SynIII	: Synapsin three
Ta	: Annealing temperature
TAE	: Tris acetic acid and EDTA buffer
TBE	: Tris, boric acid and EDTA buffer
TD	: Denaturing temperature
TDT	: Transmission disequilibrium test
Te	: Extension temperature
TE	: Extension temperature
TH	: Tyrosine hydroxylase
TPH	: Tryptophan hydroxylase
TS	: Tourette's syndrome
TTM	: Trichotillomania
UK	: United Kingdom
US	: United States
UV	: Ultraviolet
V	: Volts

VFCS : Velocardiofacial syndrome
VLDLR : Very low density lipoprotein receptor
VMA : Vanillylamandelic acid
VNTR : Variable number of tandem repeats
VZ : Ventricular zone
W : Watts
WCST : Wisconsin card sorting test
www : World wide web
Y2H : Yeast two-hybrid
YAC : Yeast artificial chromosome
Y-BOCS : Yale-Brown obsessive-compulsive scale
YGTSS : Yale global tic severity scale
YWHAH : Chaperone protein 14-3-3η

LIST OF FIGURES

FIGURE **PAGE**

CHAPTER 3

FIGURE **PAGE**

CHAPTER 4

LIST OF TABLES

TABLE **PAGE**

CHAPTER1: INTRODUCTION

INDEX **PAGE**

CHAPTER 1: INTRODUCTION

1. PYCHIATRIC DISORDERS

Psychiatric disorders are among the most widespread and disabling of all illnesses in developed societies. However, since they are not listed among major causes of death, they rarely receive the attention given to diseases such as cancer or AIDS which have high mortality rates.

In terms of their overall prevalence, economic burden and the long-sustained suffering they cause, these disorders exceed most forms of ill health. In the United States of America (U.S.A) alone, the estimated collective cost per year is around $400 billion (Cowan *et al.,* 2002). Furthermore, on the basis of a large epidemiological study conducted in the U.S. in 1991, the life-time prevalence of mental illness was estimated at 32% and in the year preceding the study, as many as 20% of the population was affected. (Robins and Reiger, 1991). More recent surveys estimate the number of affected individuals in the U.S at 43 million adults (over the age of 18 years) (US Department of Health and Human Services, 1999). In addition, four of the ten leading causes of disability in the U.S. and other developed countries are mental disorders such as major depression, bipolar disorder, schizophrenia, and obsessive-compulsive disorder (Murray and Lopez, 1996).

Comment [MB1]: Robins, L.N. and Regier, D.A. Psychiatric Disorders in America. New York: The Free Press, 1991

Moreover, the "Disability Adjusted Life Year" or "DALY" scale (a scale that measures the years of healthy life lost due to mortality and disability) shows that psychiatric disorders are responsible for a significant number of years lost due to disability (with mortality excluded) (Murray and Lope., 1996, Michaud *et al.,* 2001). When mortality is included, psychiatric illnesses rank second only to cardiovascular disease on the DALY scale (Murray and Lopez, 1996).

These statistics emphasise the severity and prevalence of mental illness and stress the importance of getting a clear handle on the pathophysiology of these disorders, in order to develop better diagnostic tools and treatment regiments. In the last 20 years, much progress has been made in improving diagnosis and treatment of many psychiatric disorders (Cowan *et al.,* 2002). However, in the majority of psychiatric disorders, little knowledge exists about the cellular and molecular abnormalities and their relationship to the nervous system's structure and function.

Recent years have seen many major advances in biomedical research and, like the rest of medicine, psychiatry has entered the "molecular medicine revolution" with all its exponentially improving technologies (Gould and Manji, 2004). However, the field of psychiatry still lags behind other medical conditions, with respect to delineating pathophysiology, for a number of reasons. These include the lack of a clearly defined pathogenesis, the sheer complexity of human behaviour and of the central nervous system (CNS), and the multifactorial molecular pathophysiology of psychiatric illnesses (Gould and Manji, 2004). Compared to organs such as the liver where the cells are nearly all identical, have similar phenotypes, transcriptomes and proteomes, and have homogeneous interactions, cell types in the brain are quite different from each other, have different transcriptomes and proteomes and display heterogeneous interactions (Gottesman and Gould, 2003).

The complex interactions of the brain are not only limited to genes, proteins and cell types, but varying individual experiences all contribute to phenotype.

Despite these obstacles, many researchers have sought to elucidate the multifaceted pathophysiology of psychiatric disorders using a variety of approaches, including identifying genetic loci involved in the development of these disorders. The focus of this thesis is the identification of novel susceptibility genes for obsessive-compulsive disorder (OCD). One method of identifying such novel OCD candidate genes is by utilising knowledge of diseases with phenomenological overlap with OCD, which lend themselves to better genetic dissection through the approach of linkage analysis and animal studies. Genetic loci for such disorders, identified though linkage analysis, could potentially harbour novel OCD candidate genes, while genes implicated through animal models of the "overlapping disorder" may lead to the identification of additional susceptibility genes through delineation of pathways by, for instance, interactome analysis. One such overlapping disorder is schizophrenia, which manifests some similarities both in symptoms and the brain circuits with OCD. For this reason, the sections that follow will describe each of these disorders, focusing on symptoms and theories regarding their pathogeneses, the evidence for a genetic component to their etiology, as well as some of the approaches used in the identification of susceptibility genes to date will be reviewed.

1.1 SCHIZOPHRENIA

Schizophrenia is a devastating mental illness that impairs some of the most advanced functions of the human brain(reviewed in Picchioni and Murray, 2007). Its lifetime prevalence has been estimated at 1% worldwide and an annual incidence of 0.16-0.42 per 1000 population has been predicted (Jablensky, 2000). Symptoms usually appear during the second decade of life, but cases of late-onset schizophrenia have also been reported.

Comment [MB2]: Picchioni MM, Murray RM.

Related Articles, Links Schizophrenia. BMJ. 2007 Jul 14;335(7610):91-5.

The symptoms of shcizophrenia can be divided into three main categories (Kelly *et al.,* 2000; Hales *et al.,* 1994) namely, psychotic (or positive symptoms) symptoms, deficit (or negative symptoms) symptoms and cognitive impairment. The negative and cognitive symptoms are more persistent and chronic, while the psychotic symptoms have an episodic pattern that, when active, is usually the reason for hospitalization of patients (Andreasen *et al.,* 1995). A complete summary for Diagnostic and Statistical Manual on Mental Disorders (DSM-IV) diagnostic criteria for schizophrenia is shown in Table 1.1. Genetic studies of schizophrenia often differ with respect to definition of phenotype, eg. some studies include individuals with schizophrenia spectrum disorders, while others include only individuals with narrowly defined schizophrenia, and yet others make use of intermediate phenotypes, it is necessary to discuss these phenotypic concepts below.

Comment [MB3]: Hales R.E, Yudofsky S.C, Talbott J.A Eds. The American psychiatric press textbook of psychiatry. Washington, D.C: American Psychiatric Press, Inc; 1994

1.1.1. Psychotic symptoms

Psychotic symptoms, a feature of a number of brain disorders, fall into three main groups (Hales *et al.,* 1994), namely hallucinations, delusions and thought disorder. In schizophrenia, the hallucinations experienced are usually auditory, in the form of human speech, i.e. "hearing voices" (Andreasen and Black., 1991). The typical schizophrenic delusions are usually paranoid and include delusions of persecution, grandiosity, external

control, having thoughts inserted or withdrawn from one's head, ideas of reference and mind reading (Stompe *et al.,* 1999). Thought disorder refers to the abnormalities in the form of thought. Its cardinal features are improper use of semantic and rational aspects of language, which the listener experiences as disorganised speech (Goldberg *et al.,* 1998).

1.1.2. Negative symptoms and cognitive impairments

Negative symptoms consist of severe disturbances in social interaction, motivation, expression of affection, ability to experience pleasure and spontaneous speech (Hale *et al.,* 1994). Thus the negative symptoms can be described as a loss of normal functions (Andreasen and Olsen, 1982; Andreasen, 1990). Cognitive impairment in schizophrenia affects executive functions, memory, attention and general intellectual functioning (Wiekert *et al.,* 2000).

Table 1.1 **The DSM-IV diagnostic criteria for Schizophrenia.**

A. *Characteristic symptoms:* Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
(1) delusions
(2) hallucinations
(3) disorganized speech (e.g., frequent derailment or incoherence)
(4) grossly disorganized or catatonic behavior
(5) negative symptoms, i.e., affective flattening, alogia, or avolition

Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other.

B. *Social/occupational dysfunction:* For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. *Duration:* Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. *Schizoaffective and Mood Disorder exclusion:* Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive, Manic, or Mixed Episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. *Substance/general medical condition exclusion:* The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. *Relationship to a Pervasive Developmental Disorder:* If there is a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

1.1.3. Schizophrenia spectrum disorders

The concept of schizophrenia spectrum disorders dates back to the observations of Kraepelin, who noted some less severe schizophrenia-like characteristics in families of patients with schizophrenia (described in Lichtermann *et al.,* 2000). These characteristics were termed schizophrenia spectrum disorders and are thought to share a familial-genetic aetiology with schizophrenia (Lichtermann *et al.,* 2000).

Valid members of the spectrum included schizoaffective disorder and schizotypal personality disorder (Kendler *et al* .,1993; Maier *et al.,* 1994). Biometric analysis of available family data has confirmed that schizophrenia spectrum disorders do indeed share common familial factors with schizophrenia (Baron and Risch, 1987; Kendler *et al.,* 1995). Moreover, biometric analysis of the Copenhagen adoption study cohort confirmed a genetic link between schizophrenia and schizotypal personality disorder (Tyrka *et al.,* 1995).

1.1.4. Schizophrenia endophenotypes

In the study of Mendelian disorders, genotypes are usually found to be to a greater or lesser extent indicative of phenotypes (Gottesman and Gould, 2003). Even this degree of genetic certainty is, however, not applicable to complex disorders where a complex interplay between genetic factors, epigenetic factors and the environment give rise to the phenotype.

Many investigations have been undertaken to investigate the genetic aetiology of various psychiatric disorders, with little success. Undoubtedly, this is partly due to the fact that current diagnostic criteria describe a group of heterogeneous disorders rather that a single phenotypic entity (Andreasen, 1999, 2000; Lewis, 2002). In psychiatry, the phenotype, ie., behaviour, is complex and therefore classification of psychiatric disorders on the basis of overt phenotypes may not be optimal for genetic elucidation. Thus, the concept of endophenotypes or intermediate phenotypes was introduced to bridge the gap between genotype and phenotype. Gotessman and Shields describe endophenotypes as "internal phenotypes discoverable by a biological test or microscopic examination" (Gottesman and Shields, 1973).

Endophenotypes are traits that are associated with the expression of a disorder and are believed to represent a genetic liability among non-affected individuals. They can be biochemical, neurophysiological, neuroanatomical, cognitive or neuropsychological in nature. (Leboyer *et al.,* 1998; Glahn *et al*., 2007). The rationale for dissecting a condition into endophenotypes is that, if phenotypes associated with a disorder are very specialized and represent biologically measurable phenomena, the number of genes involved in the manifestation of variation of these traits may be fewer than those producing the particular psychiatric diagnostic entity (Fig. 1.1) (Leboyer *et al.,* 1998).

Comment [MB4]: Glahn DC, Thompson PM, Blangero J.

Related Articles, Links Neuroimaging endophenotypes: strategies for finding genes influencing brain structure and function. Hum Brain Mapp. 2007 Jun;28(6):488-501

It should, however, be noted that putative endophenotypes do not always reflect a genetic vulnerability and may in fact have epigenetic or environmental origins (Gottesman and Gould, 2003). Therefore, Gottesman and Gould, in their review of endophenotypes in psychiatry, adapted criteria useful in identification of markers in psychiatric genetics (Gershon and Goldin, 1986) to apply to endophenotypes (Gottesman and Gould, 2003). These criteria state that endophenotypes should be associated with illness in populations, be heritable, primarily state-dependent, ie. manifest within an individual in a family whether or not the illness is active, and be found in non-affected family members at a higher rate than in the general population.

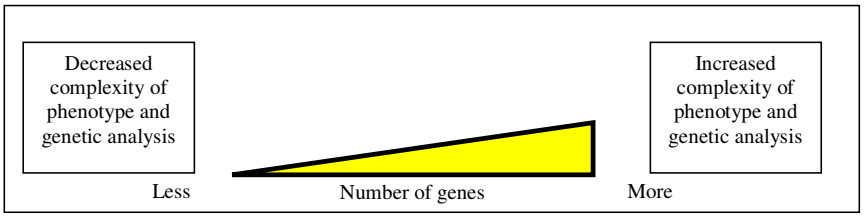


Fig 1.1. The rationale for using an endophenotype approach for genetic analysis of complex disorders (Adapted from Gottesman and Gould 2003).

An example of how endophenotypes help to dissect complex genetic problems comes from studies of epilepsy, a complex disorder that shows similar difficulties to those experienced in psychiatric disorder in terms of dissecting complex genetic aetiologies: Greenberg and colleagues by using electro-encephalographic (EEG) signature of seizures identified a genetic susceptibility factor for juvenile myoclonic epilepsy (Greenberg *et al.,* 1988). Schizophrenia lends itself to sub-stratification into a number of endophenotypes. As these are pertinent to the discussion of schizophrenia genetic locus identification, some of these endophenotypes will be briefly discussed.

1.1.4.1. Sensory motor gating

In schizophrenia, deficiency in sensory motor gating is a consistent neurobiological finding (Braff and Freedman, 2002; Braff *et al.,* 2001). Sensory motor gating refers to the regulation of sensitivity to sensory stimuli and is a crucial psychophysiological mechanism in brain function. It is the mechanism underlying one's ability to process information selectively in order to screen or "gate out" trivial stimuli, so that one is able to focus on the most salient aspects of the environment (Broadbent, 1971).

One strategy to evaluate sensory gating is to measure the decrement in the brain's evoked response to repeated auditory stimuli (Callaway, 1973). This measure is known as pre-pulse inhibition (PPI) of the startle response. When auditory stimuli are repeated at close intervals, the evoked response is normally diminished or "gated" (Davis *et al.,* 1966). The first sound (or the pre-pulse) activates inhibitory neuronal pathways, so that the response to the second sound (pulse) is diminished. A positive event-related potential is then measured using an EEG.

1.1.4.2. Eye movement dysfunction

Another neurobiological dysfunction that has received much attention in the study of schizophrenia pathophysiology is eye-tracking (or ocular motor) dysfunctions. At the turn of the last century, Diefendorf and Dodge observed that patients with dementia praecox, later to be known as schizophrenia, had difficulty following a swinging pendulum with their eyes (Diefendorf and Dodge., 1908), later termed "eye movement dysfunction" (EMD) (Holzman *et al.,* 1973). It is consistently observed that non-psychotic first-degree relatives of schizophrenic patients also exhibit EMD; this suggests that EMD may shed some light on the genetic mechanisms involved in schizophrenia pathogenesis (Calkins and Iacono, 2000).

Comment [MB5]: Braff DL, Freedman R: (2002) Endophenotypes in studies of the genetics of schizophrenia, in Davis KL, Charney DS, Coyle JT, Nemeroff C (eds) N*europsychopharmacology: The Fifth Generation of Progress*.. Philadelphia, Lippincott Williams & Wilkins, 2002, pp 703–716

Comment [MB6]: Broadbent D.E (1971): Decision and Stress. New York: Academic Press

Comment [MB7]: Callaway E (1973): Habituation of average of average evoked potentials in man. In Peeke H.V.S and Herz M.J (eds), *Habituation, II.* New York: Academic Press

Comment [MB8]: Diefendorf AR, Dodge R: An experimental study of the ocular reactions of the insane from photographic records. Brain 1908; 31:451–489

The investigations of EMDs in schizophrenia have focused on the smooth eye pursuit (SEP) and saccade eye movement systems, all indicating that patients with schizophrenia have significant impairments in SEP (Reviewed by Calkins and Iacono, 2000; Lee and Williams, 2000). In general, these deficiencies are manifest as corrective saccade, which follow SEP movements that are slightly slower than the target object (Calkins and Iacono, 2000). The SEP dysfunction was found to be stable over time and is present before onset of schizophrenia symptoms and during symptom remission (Gooding *et al.,* 1994; Iacono *et al.,* 1982, 1992). The heritability of this trait has been investigated extensively and the generated data have suggested that relatives of schizophrenic patients have increased rates of SEP dysfunction. Furthermore, 40%-80% of schizophrenic patients, and 25%-45% of their first degree relatives show this trait, compared to approximately 10% of the general population (Calkins and Iacono, 2000; Lee and Williams, 2000). These results indicate that SEP dysfunction can be considered a schizophrenia endophenotype.

Investigations have also focused on the saccadic system in schizophrenia. Saccadic eye movements are composed of several subtypes that include voluntary (intentional) and reflexive. Voluntary saccades, including the antisaccade and the memory guided saccade, are eye movements intentionally triggered by an individual to achieve a goal (eg. examine details in a photograph), while reflexive saccades are triggered externally in response to a suddenly approaching object (Calkins and Iacono, 2000). Schizophrenic patients and their biological relatives have shown a replicated deficiency in their capacity to inhibit reflexive saccades to the target object (Clementz *et al.,* 1994; Katsanis *et al.,* 1997; McDowell and Clementz, 1997; Ross *et al.,* 1998; Curtis *et al.,* 1999; McDowell *et al.,* 1999; Curtis *et al.*, 2001). The few studies on memory guided saccade EMD have shown that schizophrenic patients and their biological relatives are slow to move their eyes toward a remembered target once the cue for the saccade has been issued. Furthermore, schizophrenic patients often generate inappropriate reflexive saccades to the initial target (McDowel and Clementz, 1996).

These data suggests a familial component to both types of voluntary saccade eye movement; this coupled with the fact that it is comorbid with schizophrenia, makes voluntary saccade EMD a pertinent schizophrenia endophenotype.

1.1.4.3. Spatial working memory

Spatial working memory (SWM) has also been used as a schizophrenia endophenotype in a number of investigations (Pisculic *et al*., 2007). Spatial working memory is the temporary storage and manifestation of spatial information in the service of 'higher" cognitive processing (Glahn *et al.,* 2003). Impairments of SWM in schizophrenia sufferers, as well as their biological relatives, have been well documented (Park and Holzman, 1992; Park *et al*., 1995).

Comment [MB9]: Piskulic D, Olver JS, Norman TR, Maruff P.

Related Articles, Links Behavioural studies of spatial working memory dysfunction in schizophrenia: a quantitative literature review. Psychiatry Res. 2007 Mar 30;150(2):111-21.

Cannon and colleagues (2000) proposed that SWM deficits constitute an effective endophenotype for schizophrenia (Cannon *et al.,* 2000). They found that healthy monozygotic (MZ) co-twins of affected individuals performed worse than did healthy dizygotic (DZ) co-twins of healthy individuals, who in turn

performed worse than the control twins without a family history of schizophrenia, on a spatial span task of the Wechsler-Memory Scale-Revised (Wechsler *et al.,* 1981). Glahn et al. (2000) also provided further confirmation of the validity of this observation in a subsequent investigation using a spatial delayed response task paradigm (Glahn *et al.,* 2003).These results indicate that performance on the spatial span test is genetically predetermined (Cannon *et al.,* 2000) and can be used as an endophenotype for schizophrenia.

Comment [MB10]: Wechsler D: Wechsler Memory Scale-Revised Manual. New York, Psychological Corp, 1987

Several other endophenotypes have been identified in schizophrenia; these include impairments of executive dysfunctions and impaired verbal memory. These endophenotypes, in addition to providing a measurable phenotype for genetic studies of schizophrenia, have additional value in psychiatry. These include more accurate diagnosis, classification of the disorder into homogeneous subtypes and providing measurable phenotypes in animals that can be used to model human illness.

1.1.5. Pathogenesis

1.1.5.1 The Genetic basis for Schizophrenia

As the search for genetic components of any disease should be preceded by proof of the existence of such components, the following sections will describe such evidence for schizophrena.

1.1.5.1.1. Family Studies

Between 1920 and 1987, as many as 40 independent European family studies, that were similar in diagnostic and ascertainment criteria, were undertaken to investigate the possible role of genetic factors in schizophrenia (reviewed by Shih *et al.,* 2004). From these studies, the risk to first-degree relatives of developing schizophrenia was estimated at 6% for parents, 9% for siblings, 13% for offspring of one schizophrenic parent and 46% for offspring of two schizophrenic parents (Figure 1.2) (Gottesman *et al.,* 1991). From data generated from these studies, it is clear that the risk of schizophrenia in different classes of relatives does not conform to those predicted by a simple Mendelian pattern of inheritance. Some families do contain multiple affected individuals; however, these cases are rather rare (McGuffen *et al.,* 1995). In fact, in a long-term follow up study, Bleuler found that over 60% of schizophrenic patients had no history of the disorder in first or second degree relatives (Bleuler, 1978). Thus, with the mixed evidence from family studies, the question still arises: is the familiality of schizophrenia the result of genetic influences or can it be explained, even in part, by shared environmental effects? In order to answer this question, several investigators have gathered information from twin and adoption studies.

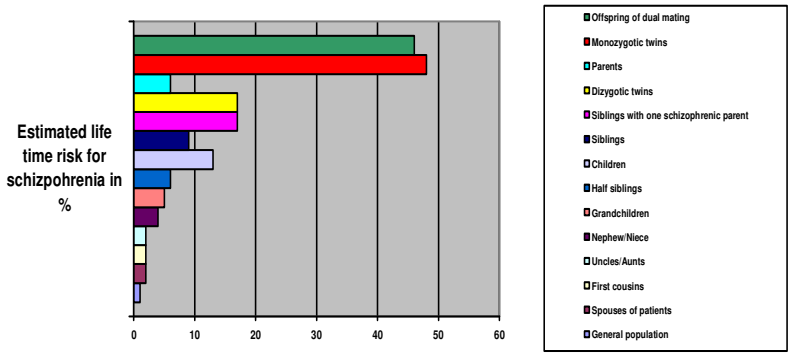
Comment [MB11]: Gottesman, I. I. (1991) *Schizophrenia genesis: The origins of madness*. New York: Freeman.

Comment [MB12]: Bleuler M. (1978). The long-term course of schizophrenic psychosis. In L Wynne, R Cromwell & S Matthysse (Eds.). **The nature of schizophrenia**. New York: Wiley.

1.1.5.1.2. Twin studies

A systematic review of the results of twin studies found the rate of concordance of approximately 53% for MZ monozygotic twins and 15% for DZ twins (Kendler, 1983). In a similar review, Gottesman found a concordance rate of 48% for MZ twin pairs and 17% for DZ twin pairs (Gottesman, 1991). Taken together, these reviews show that MZ twins are approximately three times more likely to exhibit concordance than are DZ twin pairs, which provides persuasive evidence of a genetic component for schizophrenia. This conclusion is further strengthened by research concerning 12 pairs of MZ twins who were reared apart and were

systematically evaluated for schizophrenia, which showed a 58% concordance (Gottesman, 1991; Prescott and Gottesman, 1993).



.

Fig. 1.2: Estimated lifetime risk of schizophrenia in relatives of schizophrenia probands (Adapted from from Gottesman, 1991).

Gottesman and Bernstein showed that children of unaffected co-twins displayed a morbid risk of 17.4%, which is similar to that of the affected twin's offspring (16.8%) (Gottesman and Bernstein, 1989). By contrast, the risk for a child of an unaffected DZ co-twin was much lower (2.1%). This finding further emphasizes that genetic factors underlie at least part of the pathogenesis of the disorder.

1.1.5.1.3. Adoption Studies

One of the first adoption studies in schizophrenia was performed by Heston (Heston, 1966). This study compared, at maturity, 47 adoptees who had been separated from their schizophrenic mothers within three days of birth with a control group of about 50 adoptees who were separated from non-schizophrenic mothers. This study found that five of the experimental group developed schizophrenia compared to none of the control group (Heston, 1966). Similarly, Rosenthal and colleagues also found an excess of schizophrenia spectrum disorders in children of schizophrenia patients raised by normal parents, as compared to a group of adoptees whose parents had no history of schizophrenia (Rosenthal *et al.,* 1988).

Kety and co-workers studied 5483 Danish children between 1923 and 1947. Here again, the study showed that more adopted children separated from a schizophrenic biological parent developed schizophrenia or a related disorder than did the control adoptees (32% versus 18%, respectively) (Kety *et al.,* 1968). These authors also determined the prevalence of schizophrenia and related disorders among the biological relatives of schizophrenic adoptees. They found that 21% of the biological relatives of 33 schizophrenic adoptees were diagnosed with schizophrenia or a related disorder. By contrast, only 11% of the biological relatives of non-schizophrenic adoptees suffered from any schizophrenia-related disorder. (Kety *et al.,* 1968). Moreover, no differences were observed in the rates of schizophrenia in adoptive relatives of schizophrenic and non-schizophrenic individuals, indicating that the adoptive environment was not likely to be responsible for the

increase in schizophrenia rates in the blood-relatives of schizophrenia patients. Furthermore, children born to non-schizophrenic parents but raised by a schizophrenic parent did not show rates of schizophrenia higher than predicted in the general population (Kety *et al.,* 1968).

1.1.5.1.4. Mode of inheritance

Thus, there is ample evidence for a role for genetics in schizophrenia pathogenesis, but it is clear that the recurrence risk decreases rapidly with increasing genetic distance from the proband (Fig 1.2). Therefore, schizophrenia cannot be a single-gene disorder or even a collection of single-gene disorders, even when taking incomplete penetrance into account (Owen *et al.,* 2004; Crow, 2007). Studies of the segregation of the disorder in families have been undertaken to predict the most likely disease model. Using the lambda risk ratio, Risch reported that three to four interacting loci (a multiplicative model) were most likely involved in determining risk, rather than an additive model (Risch, 1990). In a complex segregation analyis of schizophrenia in Chile, a mixed model with a high environmental component was proposed (Ruiz *et al.,* 1997). Freedman and co-workers proposed a multigenic model, in which alleles associated with schizophrenia are relatively common in the general population and individuals inherit schizophrenia risk through at least two loci (Freedman *et al.,* 2001).

Comment [MB13]: Crow TJ.

Related Articles, Links How and why genetic linkage has not solved the problem of psychosis: review and hypothesis.
Am J Psychiatry. 2007 Jan;164(1):13-21

Thus, the number of genes proposed to impact on schizophrenia pathogenesis are small. However, to better predict the type of genes that may be involved in schizophrenia, and thus in its overlapping disorders, it is necessary to discuss possible etiologies of schizophrenia, which include neuropathology, neurochemistry and neurodevelopment.

1.1.5.2. Neuropathology

1.1.5.2.1. Macroscopic neuropathology

Over the past 100 years, many neuropathological investigations of schizophrenia have been undertaken. Despite this, no infallible diagnostic neuropathology has been identified, although a number of interesting findings should be highlighted.

Numerous post-mortem studies of schizophrenia show a decreased brain weight, increase in ventricular volume and a decrease in cortical grey matter volume. Relatives of schizophrenia patients have also been reported to have a decreased cortical volume and enlarged ventricles (Cannon *et al.,* 1993); Lawrie *et al.,* 1999; Honer *et al.,* 1994; Silverman *et al.,* 1998). Moreover, in MZ twin who are discordant for schizophrenia, the affected twin tends to have decreased cortical volume and larger ventricles compared to the unaffected co-twin (Suddath *et al.,* 1990). These studies further suggest that the temporal lobe (Fig 1.3) and the corresponding temporal horn are the most affected (Bogerts *et al.,* 1985; Pakkenberg, 1987). It should, however be noted that other studies found no significant differences in brain size and cortical volume in post-mortem brain sections of schizophrenia subjects compared to control specimens (Rosenthal *et al.,* 1972; Heckers *et al.,* 1990; Pakkenberg *et al.,* 1990; Dwork *et al.,* 1997).

There have also been a large number of studies conducted that employed computed tomography (CT) and magnetic resonance imaging (MRI) of the brains of schizophrenic individuals. These studies have largely confirmed widespread cortical grey matter deficits in schizophrenia compared to control subjects (eg. Johnstone *et al.,* 1976; Cannon *et al.,* 1998). Computed tomography and MRI findings have also confirmed post-mortem findings of increased ventricular volume and degree of cortical volume loss (Ward *et al.,* 1996).

1.1.5.2.2. Histopathology of schizophrenia

Several interesting studies investigating the histological pathology of schizophrenia have been completed, providing much insight into possible pathogenic mechanisms that may be involved in schizophrenia, and hence possibly in overlapping disorders.

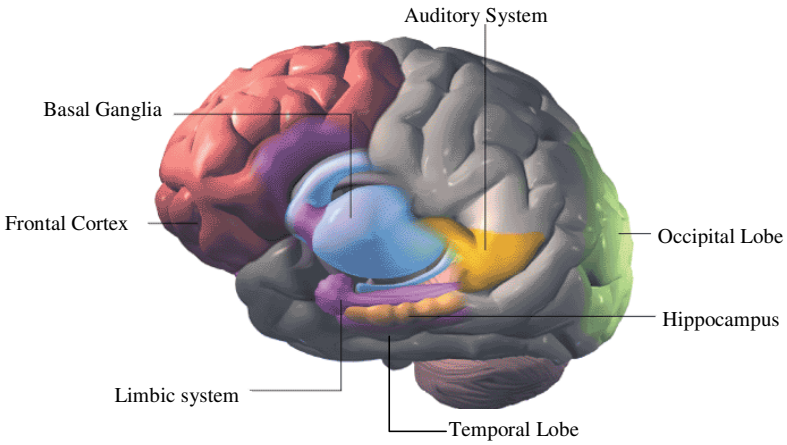


Fig 1.3: **Brain regions implicated in the pathogenesis of schizophrenia.** The basal ganglia are involved in integrating sensory information and emotion. Abnormal functioning of the basal ganglia is thought to contribute to paranoia and hallucinations experienced by patients with schizophrenia. Frontal lobe disturbances in schizophrenic patients are hypothesised to be responsible for difficulty in planning and organising thoughts, while limbic system disturbances are thought to contribute to the agitation frequently observed in these patients. Auditory and visual hallucinations are caused by disturbances in the auditory system and the occipital lobe, respectively, while hippocampal pathology leads to impaired learning and memory in schizophrenic patients (adapted from Alfre Kamajian, http://www.schizophrenia.com).

A number of studies have found slight reductions in hippocampal formation grey matter volume which is attributable to reduced size and number of hippocampal neurons in schizophrenia patients (Benes *et al.,* 1991; Arnold *et al.,* 1995; Zaidel *et al.,* 1997). These findings were further supported by studies showing decreased expression of presynaptic and dendritic markers such as synaptosomal associated protein of 25kDa (SNAP-25) (Young *et al.,* 1998; Mukaetova-Ladinska *et al.,* 2002); complex II (Harrison *et al.,* 1998), synaptophysin (Eastwood *et al.,* 2000) and microtubule-associated protein 2 (MAP2) (Cotter *et al.,* 1997) in schizophrenia post-mortem brain specimens. Furthermore, *in vivo* proton spectroscopy studies have found that the expression of N-acetyl aspartate, a putative marker for neuronal integrity, is reduced in schizophrenic patients (Bertolino *et al.,* 1998). Results from these studies have given rise to the hypothesis that genes encoding proteins

involved in the development and maintenance of the hippocampal formation may be involved in the pathogenesis of at least some aspects of schizophrenia.

The number of neurons has also been shown to be reduced in the dorsolateral prefrontal cortex (DLPFC) and hippocampus of schizophrenia patients. There have also been reports of a 5-10% reduction in cortical thickness and an increase in neuronal cell packing density and decrease in neuronal size in the DLPFC of schizophrenia subjects compared to control individuals (Selemon *et al.,* 1995, 1998, 2003; Rajkowska, 1997). These observations may reflect a decrease in the number of PFC neurons (Rajkowska *et al.,* 1995) or a decrease in the number of axon terminals, distal dendrites and dendritic spines that represent the principal components of the cortical synapse (Lewis and Lieberman, 2000). In addition, as in the hippocampal formation, expression of N-acetyl aspartate and synaptophysin have been shown to be reduced in the PFC of schizophrenia patients (Bertolino *et al.,* 1999 Karson *et al.,* 1999). A similar reduction in the volume of the thalamus has also been reported in schizophrenic patients (Andreasen *et al.,* 1994; Frazier *et al.,* 1996; Gur *et al.,* 1998), which may reflect fewer axonal projections into the PFC (Portas *et al.,* 1998). Backing these observations are studies that show an up to 30% reduction in neuronal number in the mediodorsal (MD) thalamic nucleus, which is the main source of neuronal projections from the thalamus into the PFC; as well as in the anterior nuclei, which project to the PFC and anterior cingulated cortex (Pakkenberg *et al.,* 1990; Popken *et al.,* 2000; Young *et al.,* 2000).

Investigations have also provided some evidence of cyto-architectural abnormalities in the cortex of schizophrenia patients. Jakob and Beckmann (1986) reported abnormalities in the cyto-architecture and lamination of the entorhinal cortex in schizophrenia (Jakob and Beckmann, 1986). Even though Jakob and Beckmann extended their work and their study was partially replicated by others (Arnold *et al.,* 1991), their results were still questionable because of a small number of subjects, the absence of a suitable control group and the lack of objective criteria for the cyto-architectural disturbance. These deficiencies were later overcome by the work of Arnold and colleagues (1995, 1997), who provided further evidence for a disturbance in the location, clustering and size of entorhinal cortical neurons (Arnold *et al.,* 1995; 1997). Disarray in hippocampal pyramidal neurons has also been reported. A number of studies reported disorientation of pyramidal cells in the hippocampus, a decrease in cell density in deeper cortical layers I and II of the rostral entorhinal cortex, incomplete glomerular clustering in layer II and abnormal clustering in deeper cortical layers (Benes *et al.,* 1991, Jakob and Beckman, 1986, Arnold *et al.,* 1991; Conrad *et al.,* 1991). Abnormalities of cyto-architecture were subsequently also noted in an animal model which recapitulates some behavioural aspects of schizophrenia, and which was subsequently shown to be due to defects in the gene encoding the reelin protein (Costa *et al*., 2002).

The data described above provide persuasive evidence implicating a compromised DLPFC, as well as the hippocampal formation in schizophrenia pathogenesis, which may be due to neurodegenerative, neurodevelopmental or neurochemical mechanisms, which, in turn, could have environmental or genetic underpinnings.

1.1.5.3. The neurodevelopmental hypothesis of schizophrenia

The neurodevelopmental hypothesis of schizophrenia suggests that subtle disease processes affecting cortical brain circuits during early development reaches full-blown consequences during adolescence or early adulthood (Marenco and Weinberger, 2000). Orginally, schizophrenia was described as an adult-onset brain disorder similar to other disorders of dementia such as Alzheimer's disease (Bleuler, 1902). Thus, for a great part of the 20th century, schizophrenia was thought to be a neurodegenerative disorder and many studies of the time focused on proving this (Reviewed by Marenco and Weinberger, 2000).

Comment [IT14]: Bleuler E. Dementia praecox.
Journal of Mental Pathology 1902/3;3:113–20.
Get these refs

However, the first retrospective studies of schizophrenia performed in the 1970's (Watt *et al.,* 1972) revealed a pattern of abnormalities in neurological and behavioural parameters that dated back to childhood. Furthermore, seminal longitudinal studies indicated that some degree of recovery was possible in schizophrenia, thereby undermining the concept that schizophrenia is a neurodegenerative disorder (Tsuang *et al* , 1979).

In the late 1970's and 1980's, further evidence for a neurodevelopmental component to schizophrenia pathophysiology, in the form of several underlying macroscopic changes in the schizophrenic brain, had emerged. Firstly, many studies indicated an increased ventricle size and loss of cortical volume in post-mortem schizophrenic brains (Johnstone *et al.,* 1976; Weinberger, 1979; 1982) (section 1.1.5.2.1). Moreover, the fact that the enlarged ventricles were present at the onset of disease and that this enlargement did not progress with the illness provided further evidence against the involvement of neurodegenerative processes in the pathogenesis of schizophrenia (Illowsky *et al.,* 1988). Secondly, cortical cyto-architecture was found to be altered, with neurons being incorrectly positioned and of abnormal size (Harrison, 1997) (section 1.1.5.2.2). Since brain neurons reach their optimal size and position during neurodevelopment, after which they remain static, it suggests that the abnormal cyto-architecture observed in schizophrenia is due to neurodevelopmental insults and not neurodegenerative processes. Thirdly, the majority of post-mortem studies failed to provide any evidence of gliosis in the brains of schizophrenic subjects (Harrison, 1997). Gliosis is a marker for past inflammation and is considered an indicator of damage after the second trimester of gestation (Kreutzberg *et al.,* 1997). The absence of gliosis in schizophrenia would suggest that the changes within the brain would have occurred prior to the third trimester. Since a neurodevelopmental insult before the second trimester would result in overt abnormalitites in the cerebral cortex, some investigators believe that the neurodevelopmental abnormalities in schizophrenia occur during the second trimester (Roberts *et al.,* 1991, Bloom *et al.,* 1993). This would suggest that genes that are expressed in the developing brain *in utero* would be strong candidates for genetic studies of schizophrenia.

Comment [MB15]: REF: Kreutzberg GW, Blakemore WF and Graeber MB. Cellular pathology of the central nervous system. In: Graham DI, Lantos PL., editors, Greenfields neuropathology. London: Edward Arnold; 1997

This paradigm shift in the thinking about schizophrenia as a neurodevelopmental, rather than a neurodegenerative illness, has led to a large number of epidemiological studies focusing on prenatal, behavioural and developmental factors associated with the disorder. These studies provide some of the most compelling evidence to bolster the credibility of the neurodevelopmental hypothesis of schizophrenia and by the end of the 1980's, a comprehensive neurodevelopmental hypothesis emerged (Weinberger, 1986, 1987)

based on the idea that a "brain lesion can remain clinically silent until normal developmental processes bring the structures affected by the lesion 'on-line'" (Marenco and Weinberger, 2000).

Epidemiological studies, longitudinal patient follow-up, brain MRI and neuropathological investigations over the last decade have further supported this hypothesis.

1.1.5.3.1. Neuropathological evidence

As mentioned earlier, cerebral vertricular enlargement is perhaps the most frequently reported neuropathological finding in schizophrenia, demonstrated at all phases of illness (Degreef *et al.,* 1992; De Lisi *et al.,* 1991; Gur, 1998, Wienberger, 1982), have been interpreted to reflect a static neuropathology that predates onset of overt illness (Marenco and Weinberger, 2000). Several longitudinal CT and MRI studies have confirmed the lack of progression of ventricular enlargement during the course of illness (Jaskiw *et al.,* 1994, Illowsky *et al.,* 1988; Degreef *et al.,* 1991, De Lisi *et al.,* 1992). There were, however, investigations that found, in a subset of patients, that ventricular enlargement was progressive (Davis *et al.,* 1998; Vita *et al.,* 1991). These inconsistent findings may indicate that, in a subset of schizophrenic patients, the pathogenesis may be attributed to factors other than neurodevelopmental insults.

Cytoarchitechtural abnormalities have also been reported in schizophrenia (section 1.1.5.2.2). These include neuronal disarray, heterotopias and neuronal malpositioning which may be a consequence of disrupted neuronal proliferation or migration during the gestational period. These findings have been supported by reduction of the reelin protein by up to 50%. This protein is an extracellular matrix glycoprotein for which one function is to act as a "stop signal" for neuronal migration during development (D'Arcangelo and Curran, 1998). Reelin is one of the main focuses of the present study and will therefore be discussed in detail in section 1.4.9.1.1.

Other studies have shown low neutrophil levels and abnormalities in synaptic, dendritic, axonal and white matter tract organisation (Lin *et al.,* 1999). Furthermore, the abnormalities in glutamatergic neurotransmission are consistent with impaired connectivity between various brain regions including the mid-brain, nucleus acumbens, temporo-limbic and prefrontal cortices (Ohrmann *et al.,* 2005, Ghose *et al.,* 2004; Dracheva *et al.,* 2004; Miyamoto *et al.,* 2003).

In summary, several lines of clinical and neuropathological evidence suggest a neurodevelopmental component to the aetiology of schizophrenia. While there is substantial evidence for a role for an early neuropathological insult, occurring during the second trimester of gestation, there has been no evidence that rules out factors operating later, during infancy or adolescence, as other possible causes of the disease (Marenco and Weinberger, 2000). The neurodevelopmental hypothesis remains one of the most widely accepted and best supported theories regarding the pathogenesis of schizophrenia and has opened the door to investigating a variety of novel potential candidate genes for schizophrenia pathogenesis.

1.1.5.4. Neurochemical pathology

1.1.5.4.1. Dopamine

Traditionally, the dysregulation of the dopamine system has been the dominant neurochemical hypothesis of schizophrenia (Di Forti *et al.*, 2007). The role of the dopamine system has been suggested by the antipsychotic effects of dopamine receptor 3 (DRD3) receptor blockers (Carlsson and Lindqvist, 1963) and by the psychotic symptoms induced by indirect dopamine agonist such as amphetamine and cocaine (Randrup and Munkvad, 1987). Recently, the over-activity of dopaminergic neurons in the limbic areas of the brain has also been implicated in the development of positive symptoms, while under-activity in the frontal cortex is thought to be responsible for negative symptoms and cognitive impairments in schizophrenia (Kerwin, 2000).

Comment [MB16]: Di Forti M, Lappin JM, Murray RM.

Related Articles, Links Risk factors for schizophrenia--all roads lead to dopamine. Eur Neuropsychopharmacol. 2007 Mar:17 Suppl 2:S101-7.

Evidence from positron-emission tomography (PET) studies has shown an increase in dopaminergic transmission in schizophrenics (Laruelle *et al.,* 1998, Breier *et al.,* 1997). Elkashef and colleagues further showed abnormal dopamine metabolism in drug-free schizophrenia patients using PET (Elkashef *et al.,* 2000). Increased dopamine receptor densities have also been reported in schizophrenics. For example, both post-mortem and functional MRI studies showed evidence of an increase in the number of dopamine receptor 2 (DRD2) in the brains of schizophrenic patients compared to control individuals (Kestler *et al.,* 2001). Other dopamine transmission abnormalities reported in schizophrenia include increased amphetamine-induced release of dopamine in the striatum, elevated DOPA-decarboxylase activity and increased DRD2 density in the striatum (Laruelle *et al.,* 1996; Breier *et al.,* 1997; Abi-Dargham *et al.,* 2000; Ginovart *et al.,* 1999).

1.1.5.4.2. Serotonin

The idea that the serotonergic system may be involved in schizophrenia has long been advocated based on pharmacological evidence. The serotonergic system is a major therapeutic target for several of the newer antipsychotic agents and, furthermore, the hallucinogen, lysergic acid diethylamide (LSD), is a serotonin (5-hydroxy-tryptophan [5-HT]) agonist (Harrison, 1999). Moreover, the 5-HT2A receptor (5-HT2A) density was found to be significantly reduced in post-mortem brain sections of schizophrenic patients without any prior drug treatment (Mita *et al.,* 1986). Serotonin dysfunction has also been implicated in the pathogenesis of impulsive behaviour such as violence and suicide. Furthermore, there is a constant association between decreased levels of 5-hydroxy-indoleacetic acid (5HIAA), a serotonin metabolite, and suicidal behaviour in schizophrenia (Åsberg, 1997).

Many neurochemical studies have provided further evidence for the connection between serotonin system dyregulation and schizophrenia pathogenesis. The 5-HT2A receptor expression in the frontal cortex of schizophrenia patients was reported to be lowered compared to control subjects (Harrison, 1999). In addition, an elevated number of 5-HT1A receptors have been reported in the schizophrenic frontal cortex (Burnet *et al.,* 1997).

1.1.5.4.3. Glutamate

Glutamate is the primary excitatory neurotransmitter and binds two main types of receptors, the ionotropic and metabotropic (Newcomer and Krystal, 2001). The excitatory effect of glutamate is mediated by three ionotropic receptors ie. the N-methyl-D-aspartate (NMDA), the amino-hydroxy-5-methyl-4-isoxazole (AMPA) and the kainic acid receptors (Newcomer and Krystal, 2001). The glutamatergic dysfunction hypothesis of schizophrenia was born out of observations that phencyclidine (PCP) and ketamine, both potent non-competitive antagonists of the NMDA subtype of glutamate receptor, induce schizophrenia-like symptoms in healthy individuals and worsen some symptoms in schizophrenic patients (Javitt and Zukin, 1991; Coyle, 1996; Jentsch and Roth, 1999). Therefore, decreased NMDA receptor (NMDA-R) function may play a crucial role in schizophrenia pathogenesis (Stahl, 2007). Moreover, post-mortem expression studies of schizophrenics indicate abnormalities in the pre- and pos-synaptic glutamatergic indices, which include decreased expression of non-NMDA-R in the temporal cortex and hippocampus, increased cortical expression of some NMDA receptor subunits, increased glutamate reuptake in frontal cortex and decreased cortical glutamate release (Miyamoto *et al.,* 2003).

Comment [MB17]: Stahl SM.

Related Articles, Links Novel therapeutics for schizophrenia: targeting glycine modulation of NMDA glutamate receptors.
CNS Spectr. 2007 Jun;12(6):423-7.

NMDA-Rs play critical roles in excitatory synaptic transmission and plasticity in the CNS. These receptors exhibit an array of unique features, which include voltage-block by extracellular Mg^{2+}, a high permeability to Ca^{2+} and unusually slow "activation/deactivation" kinetics, as well as sensitivity to a variety of endogenous modulators present in the vicinity of the synapse (Martucci *et al.,* 2003). Functional characteristics of NMDA-Rs are determined by their subunit composition. To date, three distinct NMDA-R subunits have been identified: the NR1 subunit that is ubiquitously expressed, a family of four distinct NR2 subunits (A, B, C and D) and two NR3 subunits (Moriyoshi *et al.,* 1991; Sugihara *et al.,* 1992; Das *et al.,* 1998; Hollmann *et al.,* 1999).

The involvement of glutamate in schizophrenia is in keeping with the dopamine hypothesis because of the anatomical and functional interrelationship between these two systems. There are reciprocal connections between the fore-brain dopamine connections and systems that use glutamate (Walker *et al.,* 2004). Morphological studies have shown that dopamine terminals can be in close apposition to glutamate terminals in the prefrontal cortex (PFC) (Goldman-Rakic *et al.,* 1999). This observation led to the suggestion of local modulation of dopamine release by glutamate. Several *in vivo* studies have supported this theory by showing that the local stimulation and blockade of glutamate ionotropic receptors change the basal and stimulated levels of dopamine release (Feenstra *et al.,* 1995, 2002; Jadema and Moghaddam, 1996; Takahata and Moghaddam, 1998; Wu *et al.,* 2002; Lorrain *et al.,* 2003). Del Arco and Mora (2001) showed that stimulation of NMDA receptors produces a dose-dependant release of dopamine and dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), an effect that was blocked by specific NMDA receptor antagonists (del Arco and Mora, 2001).

Thus, dysregulation in the glutamatergic system would be expected to alter neurotransmission in the dopaminergic system (Walker *et al.,* 2004). It has therefore been suggested that inhibition of NMDA-Rs would

influence dopamine neurotransmission (Miyamoto *et al.,* 2003; Zheng *et al* , 1999). Indeed, positron emission topography (PET) studies of dopamine receptor occupancy, after acute administration of the NMDA-R antagonist ketamine, shows an increase in dopamine release from the striatum, while chronic administration of ketamine elicits hypoactivity of dopamine in the prefrontal cortex (Breier *et al.,* 1998; Smith *et al.,* 1998; Jentsch and Roth, 1999).

Given the possible role of glutamate dysregulation in schizophrenia, the expression of glutamate receptors has been studied in the post-mortem schizophrenic brain. Results of these studies have not been entirely consistent from study to study; however one consistent feature appears to be the under-expression of the NR1 subunit of NMDA-R in the cortex of schizophrenics (Meador-Woodruff and Healy, 2000).

Recently, the glutamatergic dysfunction hypothesis of schizophrenia was expanded to include metabotropic glutamate receptors. Moghaddam and Adams (1998) showed that PCP induced psychosis in rats is reversed by agonists specific to the metabotropic glutamate receptors group II, mGlu2 and mGlu3 (Moghaddam and Adams, 1998). This implies that mGlu2 and mGlu3 may be involved in the aetiology of schizophrenia.

1.1.5.4.4. GABA

Alterations in γ-aminobuteric acid (GABA), the major inhibitory neurotransmitter in the CNS have indirectly been implicated in schizophrenia pathogenesis. Several studies, utilising either immunohistochemical (IHC) or mRNA expression analysis, have produced convincing evidence for reduced expression of pre-synaptic GABA markers in subpopulations of GABAergic interneurons in the frontal cortex of schizophrenic patients (Benes and Berretta, 2001; Lewis *et al.,* 2003; Roberts, 2007). For example, mRNA levels of the 67kDA isoform of glutamate decarboxylase (GAD_{67}), the rate-limiting enzyme responsible for the conversion of glutamate to GABA, have been shown to be decreased in the prefrontal cortex and hippocampus of schizophrenic patients in several studies (Akbarain *et al.,* 1995; Volk *et al.,* 2000) .

Comment [MB18]: Roberts E.

Related Articles, Links GABAergic malfunction in the limbic system resulting from an aboriginal genetic defect in voltage-gated Na+-channel SCN5A is proposed to give rise to susceptibility to schizophrenia. Adv Pharmacol. 2006;54:119-45.

γ-Aminobuteric acid has also been shown to facilitate the release of dopamine in the PFC. When the concentrations of GABAergic compounds are low and dopaminergic cells are at a state of rest, dopamine levels are increased, while under conditions of high GABA concentrations and active dopaminergic cells, dopamine levels are decreased (Cheramy *et al.,* 1977). Therefore, GABA has a modulatory effect, reducing cortical dopamine when levels are high and increasing dopamine levels when concentrations are low (Wassef *et al.,* 2003). Therefore, the involvement of GABA in schizophrenia is also in line with the dopamine hypothesis.

Two distinct receptor subtypes that GABA interacts with to achieve its inhibitory effects have been identified. These are the ionotropic $GABA_A$ and $GABA_C$ receptors that are involved with fast inhibitory synaptic transmission and the metabotropic $GABA_B$ receptors that are involved in slow, prolonged synaptic transmission (Bowery, 2000).The $GABA_A$ receptors are comprised of different combinations of α, β, γ, ε and π subunits with the $\alpha_1/\beta_2/\gamma_2$–containing heteropentamer being the dominant subtype in the mammalian brain

(Benke *et al.,* 1994; Nusser *et al.,* 1998). The $GABA_B$ receptors are highly expressed in the brain where they comprise a heterodimer of two related subunits, $GABA_{B1}$ and $GABA_{B2}$ (Bowery, 2000).

Comment [MB19]: Bowery N. (2000) $GABA_B$ receptors: structure and function. In: Martin, D., Olsen R (eds). Lippencott Williams and Wilkins, Philadelphia, pp 233-244

Having set the scene for the role, and types, of genes that may be involved in schizophrenia development, we can now consider similarities and differences between schizophrenia and obsessive compulsive disorder, the disorder under investigation in this study.

1.2. OBSESSIVE-COMPULSIVE DISORDER

Obsessive-compulsive disorder has been described as a disabling psychiatric disorder (Murray and Lopez., 1996) which significantly impairs the sufferer's social functioning. It is characterised by recurrent obsessions or compulsions that are time consuming, ie., take more than one hour per day, or are severe enough to cause marked distress or significant social and work impairment (Julien *et al*., 2007).

Comment [IT20]: **Murray CJL, Lopez AD, eds. 1996** Global Burden of Disease: A Comprehensive Assessment of Mortality and Morbidity from Diseases, Injuries and Risk Factors in 1990 and Projected to 2020. Vol I. Harvard: World Health Organization

Comment [MB21]: Julien D, O'Connor KP, Aardema F.

Related Articles, Links Intrusive thoughts, obsessions, and appraisals in obsessive-compulsive disorder: a critical review.
Clin Psychol Rev. 2007 Apr;27(3):366-83.

Obsessions are persistent ideas, thoughts, impulses or images that are experienced as intrusive and inappropriate and that cause marked anxiety and distress. The most common obsessions are: i) repeated thoughts about contamination, eg., being contaminated by shaking hands with another person, ii) repeated doubts, eg., wondering whether one has performed some act, such as having hurt someone in a traffic accident or not having turned off the stove at home, iii) a need to have things arranged in a particular order, eg., intense distress when objects are not ordered or symmetrical, iv) aggressive impulses, eg., recurrent thoughts of hurting one's own child and v) sexual imagery, eg., a recurrent pornographic thought.

Compulsions are repetitive behaviours or mental acts, which a sufferer is driven to perform to reduce or prevent anxiety and distress often caused by obsessions. Adults suffering from OCD, at some point during their illness, recognise that their obsessions or compulsions are excessive or unreasonable. This, however, does not apply to children, since they lack significant cognitive awareness to make this judgement.

A number of clinical syndromes and OCD overlap in terms of phenomenological factors, clinical course and treatment. These disorders form a distinct category of inter-related disorders referred to as obsessive-compulsive spectrum disorders and that may share a common pathophysiologic and genetic basis with OCD (Hollander *et al.,* 1996; Rasmussen, 1994; McElroy *et al.,* 1994), hence they will be discussed below.

1.2.1. Obsessive-compulsive spectrum disorders

Obsessive-compulsive spectrum disorders include impulse control disorders (eg., trichotillomania [TTM], pathological gambling and compulsive buying), somatoform disorders (eg., body dimorphic disorder), eating disorders (eg., anorexia and binge eating), compulsive sexual disorders, Tourette's syndrome (TS) and other movement disorders (reviewed by Ravindran, 1999; Dell'Osso *et al*., 2007).

Comment [MB22]: Dell'Osso B, Altamura AC, Mundo E, Marazziti D, Hollander E.

Related Articles, Links Diagnosis and treatment of obsessive-compulsive disorder and related disorders.
Int J Clin Pract. 2007 Jan;61(1):98-104.

The phenomenological similarities between OCD and obsessive-compulsive spectrum disorders have been well documented (McElroy *et al.,* 1994; Hollander and Benzaquen, 1997). These similarities include the nature and content of thoughts, the associated distress caused by these thoughts, the frequent self-evaluation and the demand for reassurance (reviewed by Rivindran, 1999).

Obsessive-compulsive spectrum disorders, like OCD, often have onset in childhood or early adult life. Comorbidity data is perhaps the strongest evidence for a link between OCD and obsessive-compulsive spectrum disorders (Barsky *et al.,* 1986; Hollander, 1993; Rubenstein *et al.,* 1993) and there have been reports of high rates of obsessive-compulsive spectrum disorders in patients with OCD (Rasmussen and Tsuang, 1987; Tamburrino *et al.,* 1994). Further support for this comes from the reports of high rates of OCD in family members of probands with obsessive-compulsive spectrum disorders (Pauls *et al.,* 1995).

The pathophysiology of obsessive-compulsive spectrum disorders still remains unclear, and it has been proposed that they form an overlapping, but heterogeneous group, of disorders with multifactorial aetiology (Rasmussen, 1994).

1.2.2. Epidemiology of OCD

A number of studies have attempted to determine the prevalence of OCD. These studies can be divided into the earlier studies in which diagnosis of OCD was solely based on clinical judgement and the modern studies, in which structured or semi-structured instruments were used in the diagnosis. Early studies revealed that approximately 0.3-1% of the population had OCD (Roth and Luton, 1942; Brunetti, 1977). The more recent surveys can be divided into three groups: the 14 worldwide studies based on the Present State Examination (PSE) (reviewed by Wing *et al.,* 1974); studies based on the Diagnostic Interview Schedule (DIS), of which the most significant are the Epidemiological Catchments Area (ECA) surveys carried out in the U.S in the 1980's (Robbins and Reiger., 1991) and the British National Survey of Psychiatric Morbidity (Jenkins *et al.,* 1997, Meltzer *et al.,* 1995). Overall results from these epidemiological surveys suggest that OCD is prevalent in approximately 2-3% of the population, which makes it more common than schizophrenia (1%) and less prevalent than depressive disorders (Karno *et al.,* 1988)

1.2.3 Pathogenesis

Although the pathogenesis of OCD remains largely unknown, several contributors have been put forward. These include 1) genetic factors, 2) dysfunction of certain brain regions and 3) immunological factors. The following section describes each of these three models.

1.2.3.1. Genetic aetiology of OCD

1.2.3.1.1. Family Studies

Several investigations in the past have indicated that OCD is familial. Several twin studies have found that concordance for OCD is substantially greater in MZ twins (53%-87%) than DZ twins (22%-47%) (Rasmussen and Tsuang, 1986).

Comment [IT23]: **Hollander E, Benzaquen SD. 1997.** The obsessive-compulsive spectrum disorder. In: den Boer JA, Westenberg HGM (editors). Focus on obsessive compulsive spectrum disorders. Amsterdam (The Netherlands): Syn-Thesis Publishers;. p. 33-44

Comment [IT24]: **Rubenstein CS, Pigott TA, Altemus M, L'Heureux F, Murphy DL. 1993.** High rates of comorbid OCD in patients with bulimia nervosa. Eating Dis J Treat Prevent. **1**:147-55.

Comment [IT25]: **Roth WF, Luton FH. 1942.** The mental health program in Tennessee. Am J Psychiatry. **99**: 662-675

Comment [IT26]: **Brunetti PM. 1977.** Rural Vaucluse: two surveys on the prevalence of mental disorders: summary of data. Acta Psychiatrica Scandinavica **263**:15-15

Comment [IT27]: **Wing JK, Cooper JE, Surtorius N. 1974.** The measurement and classification of psychiatric symptoms. Cambridge: Cambridge University Press.

Comment [IT28]: **Robbins LN, Reiger DA. 1991.** Psychiatric disorders in America: the Epidemiological Catchment Area Study. New York: The Free Press

Comment [IT29]: **Meltzer H, Gill B, Petticrew M. 1995.** OPCS Surveys of psychiatric morbidity in Great Britain. Report No. 1. The prevalence of psychiatric morbidity among adults living in private households. London: Office of population censuses and surveys

Some studies found rates of OCD as high as 35% (Lenane *et al.,* 1990) among first-degree relatives of OCD probands, whereas other studies found no increase in rates (Rosenberg 1967; Insel *et al.,* 1983). There are many reasons for these discordant results, including differences in diagnostic criteria and methods of assessment. Most of the earlier studies did not directly interview relatives of OCD sufferers, while others failed to include control groups.

Since the early 1990's, seven studies addressed the shortcomings of the earlier studies (Lenane *et al.,* 1990; Riddle *et al.,* 1990, Bellodi *et al.,* 1992; Black *et al.,* 1992; Leonard *et al.,* 1992; Pauls *et al.,* 1995; Nestadt *et al.,* 2000). Findings from these studies estimated the risk for OCD in first degree relatives of OCD probands at between 10.3% to 35.9%. In the most recent reports (Pauls *et al.,* 1995; Nestadt *et al.,* 2000), the evidence for the familial nature of OCD was considered so strong that the authors speculated on the presence of a gene of major effect which contributes to the pathogenesis of OCD. Hence, the evidence for a genetic component to the etiology of OCD, is at least as strong as that existing for schizophrenia.

1.2.3.1.2. Mode of inheritance

The mode of inheritance of OCD has been investigated by means of segregation analysis in four independent studies (Nicolini *et al.,* 1993; Cavallini *et al.,* 2000; Nestadt *et al.,* 2000). Two of these studies (Nicolini *et al.,* 1993; Cavallini *et al.,* 2000) suggested a gene of major effect, but failed to show any distinct mode of inheritance. The study by Nestadt *et al.,* (2000), investigating 80 case families and 73 control families, showed strong evidence supporting a Mendelian dominant or codominant mode of inheritance.

Additional support for the involvement of specific genes in OCD came from twin and family studies of TS. Tourette's Syndrome is a disorder that typically develops in childhood and is characterised by tics manifested as involuntary movements or vocalisations. Just like compulsions, these tics tend to be exacerbated in times of stress and can be suppressed for a short while, during which time a patient's anxiety escalates. Many patients with TS experience concurrent symptoms of OCD. Also, there is a disproportionately higher incidence of OCD in the family members of TS sufferers (Lenane *et al.,* 1990). Similarly, OCD patients are more likely to suffer from tics or have family members who have tics than control individuals (Pittman, 1989)., As TS appears to have a substantial genetic basis (Pauls, 1992, Simonic *et al.,* 1998), the higher rate of OCD among the relatives of TS probands suggests that certain forms of OCD might be genetically related to TS.

Comment [IT30]: Pittman RK, Green RC, Jenike MA, Mesulam MM. Clinical comparison of Tourette's disorder and obsessivecompulsive disorder. Am J Psychiatry 1989;144:1166-71. (1989)

1.2.3.2. Neuropathology

The neuropathological model of OCD pathogenesis holds that OCD is the consequence of malfunctioning of particular regions of the brain. Currently, there are several brain regions that are prime candidates as possible sites of dysfunction in OCD (Fig 1.4).

1.2.3.2.1. Basal Ganglia

The basal ganglia are subcortical nuclei that comprise the extrapyramidal system and are closely associated with the cortical and limbic structures. They consist of the caudate nucleus, putamen and the globus pallidus

(together, these three make up the corpus striatum) and the amygdaloid complex. Historically, the basal ganlia have been attributed a role in the modulation of corticospinal outflow or movement.

There is some evidence linking basal ganglia dysfunction with TS (Devinsky, 1983) as well as Sydenham's chorea (SC) (Swedo *et al.,* 1989), a disease which is closely related to OCD. Patients with SC, which is a movement disorder that afflicts up to 20% of patients who have suffered from rheumatic fever (RF), have a higher prevalence of OCD symptoms than individuals with RF without SC (Swedo *et al* , 1989).

The basal ganglia are implicated in OCD beyond the above mentioned mutual relationship between movement disorders. The basal ganglia nuclei participate in cognitive aspects of behaviour; whereas the motor functions of the basal ganglia are carried out predominantly by the putamen, the cognitive functions are thought to be carried out by the caudate nucleus (Cote *et al* , 1995) (Fig 1.4a). Thus, the basal ganglia are involved in thought, and abnormalities in these structures could result in obsessions and repetitive patterns of behaviour.
Computerized tomography has suggested structural differences in the brains of OCD patients and control individuals. As in schizophrenia, ventricle/brain ratios have been shown to be enlarged in OCD patients (Behar, *et al* , 1984), and caudate volumes were found to be reduced in OCD patients as compared to normal control individuals (Luxenberg *et al* , 1988).

1.2.3.2.2. Frontal Cortex

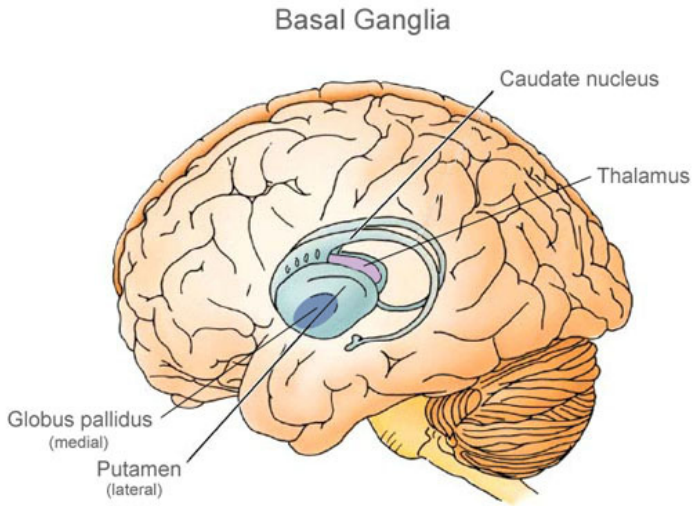
The frontal lobes are associated with neuropsychological functions of programming, regulating, controlling and verifying behaviour. Therefore, frontal lobe pathology correlates with inflexibility, decreased response inhibition, preservation and stereotypy, characteristics which are reminiscent of OCD symptoms (Otto, 1992). Indeed, frontal lobe dysfunction has been implicated in some neuropsychological studies of OCD (Flor-Henry *et al* , 1985; Behar *et al.,* 1984; Malloy *et al.,* 1989).

Positron emission tomography studies have revealed increased metabolic activity of the frontal cortex in the brains of OCD patients compared to control individuals (Fig 1.4b) Positron emission tomography analysis of ten OCD subjects and ten controls demonstrated increased metabolism in the orbital gyrus and orbital gyrus/ipsilateral hemispheric ratio bilaterally in the patient group (Baxter, 1992); these findings have been replicated (Nordahl *et al.,* 1989). Similarly, PET investigation of 18 adult childhood-onset OCD subjects, versus control individuals, revealed increased metabolism of the bilateral prefrontal areas and left orbitofrontal cortex (Swedo *et al.,* 1989). Furthermore, in two separate case reports, OCD patients were found to have local lesions involving the caudate and putamen as determined by MRI (Weilburg *et al* , 1989; Williams *et al* , 1988).

Comment [IT31]: Weilburg J, Mesulam MM, Weintraub S, Buonnano F, Jenike MA, Stakes JW.
Focal striatal abnormalities in a patient with obsessive-compulsive disorder. *Arch Neurol.* 1989;46:233-235.

Comment [IT32]: Williams AC, Owen C, Heath DA. A compulsive movement disorder with cavitation
of caudate nucleus. *J Neurol Neurosurg Psychiatry.* 1988;51:447-448.

A



B

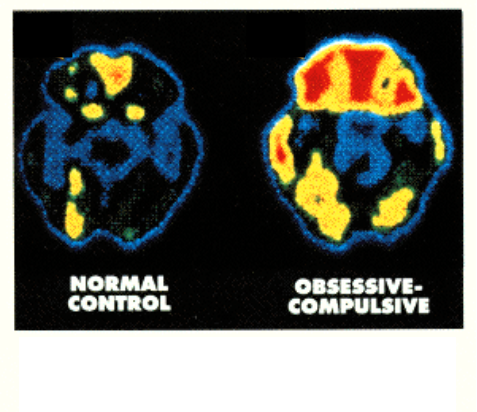


Fig 1.4: Brain regions involved in OCD pathogenesis. A: The structure of the basal ganglia showing the caudate nucleus, which is responsible for cognitive functions, the globus pallidus, the thalamus and the putamen, which is responsible for motor function (taken from http://www.colorado.edu). **B:** PET scan of a control individual's brain (left) and an OCD patient's brain (right). These scans show an increased frontal cortical metabolism in OCD patient compared to the control individual (taken from www.ncbi.nih.gov/publicat/ocdbrain.htm).

Animal models of OCD include bilateral hippocampectomised rats that display repetitive behaviour (discussed in more detail in section 1.4.9.2), invariability, enhanced avoidance and excessive behaviour (Pitman, 1989). One model provides data suggesting that the septohippocampus interacts with cortical outputs to compare sensory information with anticipated conditions, and then modulates behaviour accordingly. This 'comparative' pathway responds to novel or threatening stimuli with behavioural inhibition, enhanced attention and arousal. So, the repetitive behaviour seen in OCD may be due to a malfunction in the septohippocampus.

The cingulum is closely related to the septohippocampal system and the thalamocortical tracts. Positron emission tomography analysis of OCD subjects has shown increased metabolic activity bilaterally in the anterior cingulated cortex (Swedo *et al.,* 1989). Also, it has been shown that cingulate lesions ameliorate OCD symptoms in some patients (Jenike *et al.,* 1991; Kelly *et al.,* 1973; Martuza *et al.,* 1990) and may act by disrupting the thalamocortical tract, as seen in leucotomy[1] (Rees, 1973). It has, therefore, been proposed that cingulum hyperactivity may play a role in compulsive behaviour.

The temporal lobes are thought to be involved in OCD because of the clinical similarities between OCD and temporal lobe epilepsy. Complex partial seizures are characterised by behavioural manifestations which include "forced thinking" that is practically indistinguishable from obsessions experienced in OCD (Bear, 1985). In addition to this, several reports have demonstrated that temporal lobe electroencephalogram (EEG) abnormalities co-exists with OCD (Jenike, 1984).

1.2.3.2.4. OCD functional circuit

Functional imaging studies have suggested that in OCD patients, there is abnormal activity in the orbito-frontal cortex, the anterior cingulate/caudal medial prefrontal cortex and the caudate nucleus (Saxenna *et al.,* 1998). These studies show an increase in metabolic activity at rest in the cortico-basal ganglia network (OCD-circuit) of OCD patients compared to control individuals, an accentuation during provocation of symptoms and a decrease following successful treatment (Saxen *et al.,* 1998; Graybiel and Rauch, 2000). Rauch and colleagues (1997), using PET studies, observed brain activation patterns in OCD patients and compared them to those of control individuals. In their investigation, they demonstrated that OCD patients did not activate the left or right inferior striatum in response to learning tasks as did control individuals (Rauch *et al.,* 1997). Obsessive-compulsive patients, instead, appeared to access the medial temporal regions of the brain, regions of the brain that are more associated with conscious and emotional memory (Rauch *et al.,* 1997).

It is hypothesised that, if the cortico-striatal regions are dysfunctional in OCD patients, these patients may access conscious mechanisms to accomplish tasks that would come automatically to the unimpaired brain. Consequently, inappropriate thoughts repeatedly intrude and the conscious thought process must attempt to suppress them, along with the accompanying behaviour and anxiety (Blier, 2000).

In summary, by using a variety of brain scanning techniques, researchers have shown anatomical and metabolic variations in different brain regions of OCD patients compared to control individuals, thereby implicating these regions in OCD pathogenesis. Furthermore, investigations have identified a functional brain circuit, that encompass these brain regions, which may be involved in the pathogenesis of OCD. Interestingly, several of these regions (eg thalamus, basal ganglia and the amygdala complex) have also beein implicated in the pathogenesis of schizophrenia (Kwon *et al.,* 2003). Therefore, schizophrenia-implicated genes that are expressed in regions of the brain that overlap with the OCD functional circuit, may be particularly good candidates for OCD susceptibility.

Comment [IT33]: Kwon JS, Shin YW, Kim CW, *et al* . Similarity and disparity of obsessive-compulsive disorder and schizophrenia in MR volumetric abnormalities of the hippocampus-amygdala complex. J Neurol Neurosurg Psychiatry 2003; 74:962-964.get ref

[1] surgical interruption of nerve tracts to and from the frontal lobe of the brain

1.2.3.3. Neurochemical pathology

The neurochemical model of pathology of OCD hypothesises that dysregulation in neurotransmitter pathways may involved in its pathogenesis.

1.2.3.3.1. Serotonin

The notion of the involvement of the serotonergic system in the pathogenesis of OCD stems from pharmacological treatment studies. For over a quarter of a century, clomipramine, a potent 5-HT reuptake inhibitor, has been effectively used as a first-line drug against OCD. Other selective serotonin reuptake inhibitors (SSRI) such as fluoxetine, sertraline, fluvoxamine, cepramil and paroxetine have also been shown to be effective in the treatment of OCD and to exert their action by blocking the reuptake of 5-HT (Stahl, 1997; Bloom and Kempfer, 1995).

Comment [MB34]: Stahl S. Essential psychopharmacology: Neuroscientific basis and practical applications. Cambridge University Press, 1997: 217-233

Comment [MB35]: Bloom and Kempfer. Psychphamacology: The forth generation of progress: New York: Raven Press. 1995)

The suggestion that the efficacy of SSRI treatment of OCD is due to blockade of serotonin reuptake is further supported by studies showing a strong positive correlation between improvement in obsessive-compulsive symptoms during clomipramine treatment and drug-induced decreases in the cerebro-spinal fluid 5HIAA and platelet 5-HT concentrations (Baumgarten and Grozdanovic, 1998).

Further evidence for the involvement of 5-HT in OCD comes from studies conducted by Zohar and colleagues (1987, 1988). Their investigations showed that obsessive-compulsive symptoms could be transiently exacerbated in some OCD sufferers by orally administering the 5-HT agonist (m-CCP) and that this effect could be blocked by long-term treatment with clomipramine (Zohar *et al.,* 1987, 1988).

The evidence presented above builds a strong case for the involvement of the serotonergic system in the pathogenesis of OCD. However, serotonergic dysfunction alone cannot account for range of phenotypes observed in OCD. Therefore, other neurotransmitter systems may be implicated, and investigations into such systems have yielded some interesting results.

1.2.3.3.2. Dopamine

As for schizophrenia, neuroanatomical and pharmacological data have provided substantial evidence for the involvement of dopaminergic neurotransmission in the pathogenesis of OCD. Tourette's Syndrome has been shown to be mediated through the dopaminergic system (Pauls *et al.,* 1986, 1991; Pitman *et al.,* 1987). Furthermore, 40%-60% of OCD patients do not respond to SSRI monotherapy; in some of them, considerable improvements in symptoms have been observed following SSRI augmentation with dopamine antagonists (McDougle *et al.,* 1994, 2000). In addition, the emergence of obsessive-compulsive symptoms during treatment of schizophrenia patients with clozapine, a DRD4 antagonist, provides further evidence for the involvement of dopamine dysregulation in the aetiology of OCD (Baker *et al.,* 1992). Dopamine agonists have also been shown, in animal models, to induce stereotypies and repetitive behaviour that are reminiscent of OCD symptomology (Fog *et al.,* 1972).

Furthermore, some clinical evidence points to the possible role of dopamine system genes in the development of OCD, viz., insults to basal ganglia structures of the brain, an area intimately linked to rich dopamine innervations, have been associated with the emergence of obsessive-compulsive behaviour (Carmin *et al.,* 2002).

Based on all the above-mentioned evidence, in can be concluded that genes encoding for dopamine receptors or components of the dopaminergic system may be considered plausible candidate genes for OCD pathogenesis.

1.2.3.3.3. Glutamate and GABA

The possible roles of glutamate and GABA signalling in the aetiology of OCD have also been investigated (Cortese and Phan, 2007). Evidence for the involvement of GABA in OCD has been suggested mainly because of the observation that the augmentation of SSRI treatment of OCD with Gabapentin, a synthetic GABA analog, has been shown to be beneficial in managing OCD in clinical trials (Cora-Locatelli *et al.,* 1998).

Comment [MB36]: CNS Spectr. 2005 Oct;10(10):820-30. ... [1]

Furthermore, McGrath and colleagues (2000) demonstrated, using transgenic mice, that the TS-OCD phenotype may be mediated by cortico-limbic glutamate signalling (McGrath *et al.,* 2000). These investigators created a transgenic mouse model (D1CT-7line) of comorbid OCD and TS by expressing a neuropotentiating cholera toxin (CT) transgene in a subset of dopamine D1-expressing (D1+) neurons thought to be involved in the induction of cortical and amygdalar receptor binding (Campbell *et al.,* 1999), to evaluate the role of glutamate in the TS-OCD. To this end, they tested the effect of glutamate receptor-binding drugs on the behaviour of the D1CT-7 mice. Their results showed that MK-801, a non-competitive NMDA receptor agonist that indirectly stimulates cortical-limbic glutamate output, aggravated the transgene-dependent abnormal behaviour (repetitive climing and leaping) of the D1CT-7 mice (McGrath *et al.,* 2000).

1.2.4. Immunological aetiology of OCD

Immune-based theories of OCD pathogenesis stem from the observed similarities between its symptoms and those of SC. As mentioned previously, SC is a disorder developed in a subset of patients with RF, and is thought to be an immunological illness in which infection with group A β-haemolytic streptococci (GABHS) induces the production of antibodies that cross-react with neuronal proteins (Taranta *et al.,* 1956).

Swedo *et al.,* (1989) showed that patients with SC might show obsessive-compulsive symptoms in up to 75% and clinical OCD in 33% of cases. Furthermore, a symptom pattern of sudden dramatic onset and slow waxing and waning over a period can be found in SC and a subgroup of OCD (Leonard, 1993; Swedo *et al.,* 1989; Swedo *et al.,* 1994). This symptom pattern was described in four paediatric patients by Allen *et al.,* 1995. These observations provided a diagnostic criterion for a subgroup of patients called paediatric, infection-triggered, autoimmune neuropsychiatric disorders (PITANDs) (Allen *et al.,* 1995). Subsequently, many cases of paediatric autoimmune neuropsychiatric disorders associated with streptococcal infection have been described (PANDAS) (Swedo *et al.,* 1997) The diagnostic criteria of PANDAS include evidence of

adventitious movements (eg., mild chorea of motor hyperactivity) (Swedo *et al.,* 1998). The presence of abnormal movements (eg., choreiform) has been reported in OCD patients (Hollander *et al.,* 1989), while tic-like movements have been reported in streptococcal infection and SC (Kiessling *et al.,* 1993), further emphasising the link between OCD and these immune-derived conditions.

The involvement of the basal ganglia in both OCD and SC has been well documented (Berthier *et al.,* 1996, Giedd *et al.,* 1995; Hebebrand *et al.,* 1993; Heye *et al.,* 1993). In a recent study, basal ganglia enlargements were found among patients with PANDAS (Giedd *et al.,* 2000). This result was found to be similar to a report of basal ganglia enlargements in patients with SC (Giedd *et al.,* 1995). Taken together, these studies are consistent with the hypothesis of cross-reactive antibody-mediated inflammation of post-streptococcal OCD or tics in some patients. These studies, however, did not investigate a possible relationship between basal ganglia size and symptom severity, which may indicate that basal ganglia size and pathophysiology are not directly related.

1.3. OCD-SCHIZOPHRENIA OVERLAP

Although OCD and schizophrenia exist as two separate clinical entities, patients having comorbidities of these two disorders are frequently seen in clinical practice. The occurrence of obsessive-compulsive symptoms in schizophrenia patients had been reported well before the introduction of the DSM (Jahrreis, 1926; Gorden, 1926, Parkin, 1966; Bernie and Litman, 1978). The majority of recent reports on schizophrenia-OCD comorbidity utilised rigorous Diagnostic and Statistical Manual on Mental Disorders Third edition Revised (DSM-III-R)/DSM-IV diagnostic criteria for schizophrenia and OCD (Poyurovsky *et al.,* 2004). Several investigations have reported on rates of OCS and OCD among patients with DSM diagnoses of schizophrenia ranging from 3.8% (Craig *et al.,* 2002) to 45% (Lysaker *et al.,* 2000) (see Table 1.2. for summary).

Comment [IT37]: Jahrreis, W.: Obsessions during schizophrenia, *Archieve fur Psychiatrie* 1926, 77: 740-788.

Comment [IT38]: Gordon, A.: Obsessions in their relations to psychosis, *Am J Psychiatry* 1926, 5:647-659.

It has been suggested that the co-expression of these symptoms may reflect an overlap of the structural and functional brain abnormalities associated with schizophrenia and OCD. Indeed, based on structural and functional neuro-imaging studies, abnormalities in the frontal striatal circuits have been identified in both disorders (Cummings *et al.,* 1993). Other brain structures implicated in the pathophysiology of both disorders include the thalamus, basal ganglia and the amygdala complex (Kwon *et al.,* 2003). It should, however, be noted that although similar brain regions have been identified as being functionally and structurally abnormal in both disorders, the abnormalities are not always the same, but are often at opposite ends of the functional spectrum. For example, in OCD, hyper-functionality of the frontal-striatal system has been implicated (Baxter *et al.,* 1988), whereas in schizophrenia, hypo-functionality has been implicated (Kim *et al.,* 2000). Also, studies have shown that in OCD (Kim *et al.,* 2001) the thalamus is enlarged, while in schizophrenia a decrease in thalamic volume has been reported (Andreasen *et al.,* 1994).

Comment [IT39]: Kwon JS, Shin YW, Kim CW, *et al* . Similarity and disparity of obsessive-compulsive disorder and schizophrenia in MR volumetric abnormalities of the hippocampus-amygdala complex. J Neurol Neurosurg Psychiatry 2003; 74:962-964.get ref

Table 1.2. **Rates of obsessive-compulsive symptoms/obsessive-compulsive disorder in schizophrenia patients**

Study	Study Sample	OCD/OCS rates in Schizophrenia	Diagnostic criteria
Eisen *et al.,* 1997	Outpatients: Schizophrenia/ schizoaffective disorder	OCD 6/77 (7.8%)	DSM-IV for OCD and schizophrenia; SCID-P
Porto *et al.,* 1997	Outpatients: Schizophrenia/ schizoaffective disorder	OCD 13/50 (26%)OCS 10/50 (20%)	Structured clinical interview of patients' therapists; DSM-III-R for schizophrenia,; operational criteria fro OCD
Poyurovsky *et al.,* 1998	Inpatients: 1st episode schizophrenia/ schizophreniform disorder	OCD 7/50 (14%)	DSM-IV for OCD and schizophrenia; SCID-P;SANS; ;CGI, SAPS; Y-BOCS
Dominquez *et al.*, 1999	Outpatients: Schizophrenia/ schizoaffective disorder	OCS 17/52 (32.7%)	Chart review, self-rated MOCI for OCS
Tibbo *et al.*, 2000	Outpatients	OCD 13/52 (25%)	SCID-P; DSM-IV for schizophrenia and OCD; PANSS, Y-BOCS; GAF; ESRS
Lysaker *et al.*, 2000	Outpatients: Schizophrenia/ schizoaffective disorder	OCD 21/46 (45%)	Chart review; PANNS; Y_BOCS
Bermanzohn *et a.,l* 2000	Outpatients: chronic schizophrenia	OCD 11/37 (29.7%)	SCID-P; DSM-IV for OCD and schizophrenia; Y-BOCS
Poyurovsky *et a.,l* 1999	Inpatients: chronic schizophrenia	OCD 16/68 (23.5%)	SCID-P; DSM-IV for schizophrenia and OCD; SANS; SAPS; AIMS; CGI HDRS; SBS, Y-BOCS; BAS
Fabisch *et al.,* 2001	Inpatients: acute schizophrenia/ schizo-affective disorder	OCS 10%	DSM-IV for schizophrenia, operational criteria for OCS; PANNS; Y-BOCS
Craig *et al.,* 2002	Inpatients: 1st admissions; schizophrenia/ schizoaffective disorder	OCS 73/450 (16.2%OCD 17/450 (3.8%)	SCID-P; DSM-III-R for schizophrenia and OCD
Ohta *et al.,* 2003	In- and outpatients	OCD 13/71 (18.3%)	SCID-P; DSM-IV for schizophrenia and OCD; Y-BOCS; PANNS; AIMS; BAS;SAS
Nechmad *et al.,* 2003	Inpatients: adolescents	OCD 13/50 (26%)	SCID-P; DSM-IV for schizophrenia and OCD; Y-BOCS; SANS; SAPS; GAF

Abbreviations AIMS, Abnormal Involuntary Movement Scale; BAS, Barnes akathisia scale; CGI Clinical Global Impression scale; DSM-III-R, Diagnostic and Statistical Manual of Mental Disorders (3rd edition, Revised) DSM-IV, Diagnostic and Statistical Manual of Mental Disorders (4th edition); ESRS, Extrapyramidal Symptom Rating Scale; GAF, General Assessment of Functioning; OCD, Obsessive-compulsive disorder; OCS, Obsessive-Compulsive Symptoms; PANNS, Positive and negative syndrome scale for schizophrenia); SANS, Scale for the Assessment of Negative Symptoms; SAPS, Scales for the Assessment of Positive symptoms; SAS, Simpson-Angus Scale (Simpson, and; Angus); SCID-I/P, Structured Clinical Interview for Axis I Disorders - Patient Version; SCID-II/P, Structured Clinical Interview for Axis II Disorders - Patient Version; Y-BOCS, Yale-Brown Obsessive-Compulsive Symptom Severity Scale. Adapted from Poyurovsky *et al.,* 2004).

Comment [IT40]: 26. Dominquez RA, Backman KE, Lugo SC. Demographics, prevalence and clinical features of the schizo-obsessive subtype of schizophrenia. CNS Spectr 1999; 12: 50-6

Comment [IT41]: Fabish K, Fabish H, Langs G, et al. Incidence of obsessive- compulsive phenomena in the course of acute schizophrenia and schizoaffective disorder. Eur Psychiatry 2001; 16: 336-41

More evidence for a pathological overlap between schizophrenia and OCD comes from retrospective pharmacological studies and case reports that suggest that antipsychotic pharmacotherapy may induce OCS or exacerbate existing OCS in schizophrenic patients (Eales and Layeni, 1994; Morrison *et al.,* 1998; de Haan *et al.,* 1999; Mottard and De la Sablonniere, 1999; Tibbo and Warneke, 1999). However, in two prospective longitudinal studies, induction or exacerbation of OCS in schizophrenia patients on antipsychotic medication (olanzapine) was not observed (Baker *et al.,* 1996; de Haan *et al.,* 2002).

In summary, OCD/schizophrenia co-morbidity data, overlapping brain regions and structures from structural and functional studies, and the possible role of antipsychotic medication in mediating obsessive-compulsive symptoms, suggests that these two disorders may share aspects, including genetics, of a pathological pathway. It may be further hypothesized that, although some susceptibility genes may be shared, the functional characteristics of the actual susceptibility variants may be opposed. This idea is exploited and further investigated in the present study of OCD susceptibility genes, and hence a discussion of previously reported genetic studies of both schizophrenia and OCD follows.

1.4. THE SEARCH FOR SCHIZOPHRENIA AND OCD SUSCEPTIBILITY GENES.

The predicted lack of a simple one-to-one relationship between genotype and phenotype in schizophrenia and OCD makes identification of genes involved in their pathophysiology quite a daunting task. However, traditional genetic approaches have yielded some interesting susceptibility genes (Kim and Kim, 2006; Venken and Del-Favero, 2007). The following section will deal with these methods in more detail.

Comment [MB42]: Kim SJ, Kim CH.

Related Articles, Links The genetic studies of obsessive-compulsive disorder and its future directions.
Yonsei Med J. 2006 Aug 31;47(4):443-54.

Comment [MB43]: Hum Mutat. 2007 Jul 20; [Epub ahead of print]

Chasing genes for mood disorders and schizophrenia in genetically isolated populations.

Venken T, Del-Favero J.

1.4.1. Linkage studies

1.4.1.1. Parametric linkage analysis

Linkage analysis is the first step in positional cloning, which is a two-step genetic search strategy to find disease-causing genes. This method is used to identify allelic variations of genes or genetic markers of known chromosomal location that are co-inherited with a disease phenotype, indicating that the disease-causing gene is located in close physical proximity to the marker, in a family setting (reviewed by Keating, 1992). As this technique deals with the comparison of genotype and phenotype, an incorrect or inconsistent diagnosis could lead to incorrect interpretation of linkage data (reviewed by Keating, 1992). Computational linkage programmes analyse the genotype:phenotype data of the family members and calculate the odds that a DNA marker and disease are linked, ie., they are co-inherited more often than would be expected by chance (reviewed by Burmeister, 1999). These odds are represented in a logarithmic form known as the logarithm of odds (LOD) score. A LOD score of +3, which represents odds of 1000:1, is considered as good evidence for linkage, whereas a LOD score of –2, which represents odds of 1:100, means that a locus can effectively be excluded (reviewed by Burmeister, 1999).

Locus heterogeneity, which is when the same clinical disease in different families is caused by mutations in different genes, is a major complication in linkage mapping. Furthermore, for linkage analysis to be effective, the mode of transmission, the definition of phenotype and the degree of penetrance must be known.

Unfortunately, for most complex disorders these parameters are not yet known. Furthermore, in the case of psychiatric disorders, there is a large degree of phenotypic diversity within a specific diagnosis and the definition of the phenotype is not always accurate or consistent between studies. To compensate for these shortcomings, linkage analysis can be performed repeatedly with different values for each set of parameters. The disadvantage of this approach is that positive results must be viewed conservatively as the number of false positive increases with the number of tests performed.

1.4.1.2. Non-parametric linkage analysis

Parametric linkage analysis is most effective in uncovering variations in genes that cause disorders that follow Mendelian patterns of inheritance. It is, however, less effective in identifying genes with small to moderate effect on phenotype, which is the most likely scenario in psychiatric disorders. To avoid some of the problems of selecting a specific mode of inheritance for complex disorders, while still employing some of the power of linkage analysis, non-parametric linkage analysis methods, which are mode-of-inheritance-independent, were developed.

One such method is affected sib pair (ASP) or, more generally, affected pedigree member (APM), analysis. In this method, only sib pairs or other pairs of affected relatives are studied. This, however, means that the power of observing alleles segregating in large families is lost. The basis underlying this strategy is that, independently of whether a disease is dominant, recessive or complex, if there is a disease-causing mutation in a specific chromosomal region, two affected members of a family can be expected to share an allele of a marker in a gene involved in disease development, or adjacent to it, more often than can be expected by chance. With this method, studies can be designed to include very specific diagnoses, eg., only bipolar I disorder with exclusion of bipolar II disorder within a family, thus avoiding unclear phenotypes. Also, no genetic model of the disease needs to be specified and linkage can be detected in the presence of genetic heterogeneity (reviewed by Burmeister 1999). However, the interpretation of levels of significance in these types of linkage studies differs from parametric studies. Lander and Krugylak (1995) suggested guidelines for the interpretation of lod scores in linkage studies where the mode of transmission is unknown. The lod scores in non-parametric analysis depends on the family structure. According to the Lander and Kruglyak guidelines, significant versus suggestive linkage for large pedigrees correspond to lod scores of >3.3 or between 3.3 and 1.9, respectively. For sib-pairs, on the other hand, the corresponding lod scores for significant versus suggestive linkage corresponds to 3.6 or 1.2 respectively (Lander and Kruglyak, 1995; Sham, 2002).

Comment [IT44]: Lander, E. and Kruglyak, L. (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11, 241-247,

Both parametric and non-parametric linkage analyses are based on following the inheritance of marker alleles, that are close to a disease-causing mutation on a chromosome, through a family. They are therefore family-based and rely on a number of affected individuals within a two or multi-generation family. Linkage analysis can be conducted with no knowledge of pathophysiology of the disorder and is therefore not necessarily hypothesis driven. Linkage analysis can, of course, be applied to the analysis of particular candidate causal genes, derived by hypothesis, but the approach is more commonly applied to genome-wide screens, in which sets of markers, roughly evenly spaced throughout the genome (or sometimes through a chromosome of

interest), are tested for linkage to the disease phenotype. However, linkage analysis in this context rarely pin-points the disease gene. Instead, a region surrounding the linked marker is implicated as containing a disease-related gene. When such a chromosomal region is shown to be linked to a phenotype, it may be quite extensive and may contain hundreds of genes. Identifying the particular gene thereafter requires an additional round of screening with more closely spaced markers in the region(s) of interest and subsequent screening of candidate genes within the fine-mapped region for variants associated with the disease. Thus, the ultimate identification of the variants that are contributing to the disease phenotype may be laborious and resource intensive (reviewed by Malhotra and Goldman, 1999).

1.4.2. Association studies

In contrast to linkage analysis, association studies typically allow the identification of susceptibility genes by using knowledge of the disease pathogenesis. These studies rely on the hypotheses of the molecular causes of the disorder being investigated and, unlike linkage analysis, are not necessarily family-based. In association studies, an individual is the basic unit of analysis and no knowledge of the genetic mode of inheritance is needed. Therefore, these studies are well-suited to investigating genetic influences in complex disorders.

Genetic case-control association studies compare the frequency of alternative polymorphic forms (alleles) of a specific candidate gene within a patient group, with that of a control group, with statistical support provided. The generation of a statistically significant difference between allele frequencies and genotype distribution in patient and control groups suggests either a causative role for a functional polymorphism tested or the presence of another functional variant in the same gene, or in a genetically closely-linked one. Since psychiatric disorders are common, and it is assumed that multiple genes play a role in the development of these disorders, the predisposing alleles are expected to be fairly common polymorphisms in the general population rather than rare mutations (Burmeister *et al.,* 1999).

When designing genetic case-control association studies, many factors need to be taken into account. One major pitfall of case-control association studies is the potential for ethnic stratification between subjects. If a particular allele occurs at a different frequency across ethnic groups, and cases and controls are not precisely ethnically matched, differing allele frequencies between the two groups may create the impression of association between one allele and the phenotype of interest, when, in fact, it does not exist (reviewed by Malhotra and Goldman, 1999). One method to control for genetic complexity among population groups is to conduct association studies in populations that are either physically or culturally isolated, as these are frequently also genetically isolated, leading to increased genetic homogeneity (Sheffield *et al.,* 1998). Another way to overcome this population stratification problem in case-control studies is by using family-based approaches such as haplotype relative risk (HRR) and transmission disequilibrium test (TDT).

Candidate genes are generally chosen for analysis based on either their position (as they are located in regions of the genome implicated by linkage analysis) or function (because they encode proteins implicated by biochemical or pharmacological investigations into the pathogenesis of the disease). However, in the case of

complex disorders, if there is moderate or inconclusive evidence for linkage to a number of chromosomal areas, the number of potential candidate genes to be analysed could be extremely large. Additionally, as the complete pathophysiology of the disorder may not be fully understood, genes that encode proteins involved in mechanisms yet-to-be-identified as contributory to disease pathogenesis, may be bypassed.

The selection of an appropriate sequence variant(s) is also crucial in the design of case-control association studies. Polymorphic variants with low frequencies of one allele may provide little statistical power to detect significant associations unless the size of the study group is large enough, or the variant has a strong influence on phenotype. Variants with several relatively frequent alleles provide a number of potential genotypes, thereby increasing the informativeness of the locus, but complicating the statistical analysis (Chang *et al.,* 1996). The functionality of the selected polymorphism must also be considered. Although they may indeed indicate the presence of a closely-linked functional polymorphism in the same or an adjacent gene (linkage disequilibrium), positive findings with non-functional variants may be the result of inadvertent ethnic stratification of cases and controls (reviewed by Malhotra and Goldman, 1996). Of course, this is equally true for functional variants, and furthermore, the functional effects of many variants, non-synonymous as well as variants in non-coding, gene regulatory regions, are still untested. The number of known SNPs has increased exponentially in the last few years (Table 1.4) with completion of the HAPMAP project. Along with that knowledge has come understanding of the extent of LD blocks in various genomic regions; this has impacted on the manner in which association studies have been approached: Whereas, prior to completion of the HAPMAP project, candidate gene analysis typically involved investigation of single polymorphisms in candidate genes, the focus of late has shifted to analysis of HAPMAP derived haplotype tag-SNPs spread throughout the gene of interest. The latter more thorough approach of course comes at a higher cost.

Table 1.3. **Number of *Homo Sapiens* SNPs represented in different builds of dbSNP over the last 6 years**

dbSNP Build	Genome Build	Number of submissions (ss#)	Number of RefSNP Clusters (rs#'s) (# validated)	Number of (rs#'s) in gene	Number of (ss#'s) with genotype	Number of (ss#'s) with frequency
106 (Aug 2002)	30	4,873,188	2,817,196	1,337,870	16,986	47,577
110 (Jan 2003)	31	4,894,587	3,049,569	1,093,014	35,785	199,849
117 (Oct 2003)	34	9,628,832	5,772,564	2,038,150	93,808	287,066
122 (Apr 2004)	35.1	19,950,411	9,839,968 (4,544,754)	3,738,137	235,026	917,990
123 (Nov 2004)	35.1	21,564,104	10,079,771 (5,007,794)	4,007,305	1,822,844	1,045,322
124 (Jan 2005)	35.1	21,581,724	10,054,521 (5,054,675)	3,998,762	2,727,888	488,391
125 (Oct 2005)	35.1	27,189,291	10,430,753 (4,868,126)	4,236,590	2,918,978	662,975
126 (May 2006)	36.1	27,846.394	11,961,761 (5,646,244)	4,116,991	5,546,513	682,608
127 (May 2007)	36.2	31,035,607	11,811,594 (5,689,286)	5,028,168	5,559,898	710,090

Another aspect of association studies that needs to be carefully evaluated, are sample size and the statistical power of the study. In designing this type of study, the sample size needs to be sufficiently large to create enough statistical power to reduce the probability of generating false-positive associations (Type I errors)

(Berry *et al.,* 1998). In cases where association studies may lack sufficient power to prove or disprove an association, a meta-analysis can be performed. A meta-analysis is a statistical procedure that integrates the results of several independent studies of a particular disease provided that the investigators have 1) followed the same diagnostic criteria, 2) followed the same methodology and 3) conducted the study in sufficiently similar population groups as defined by allele frequencies in the control groups. The ability to conduct a successful meta-analysis relies on the availability of all the relevant data. The inclusion of only a subset of all the available data in a meta-analysis may introduce biases and threaten its validity. This is a major disadvantage of meta-analyses, since many studies, especially negative association studies, remain unpublished. Other studies may have limited distribution, or may be published in journals not currently on the bibliographical databases, making their detection and inclusion into a meta-analysis rather difficult (McAuley *et al.,* 2000).

1.4.3. Schizophrenia linkage studies

Several linkage studies of schizophrenia have been completed over the last two decades, yet the numerous loci suspected of carrying the genes predisposing schizophrenia have often not been replicated. Such regions of putative susceptibility vary significantly from study to study and from pedigree to pedigree. The section that follows, will focus on findings that seem promising by virtue of statistical significance or some measure of consistency among studies. These investigations are summarised in table 1.4 and figure 1.5.

1.4.3.1. Chromosome 1

Brzustowicz and co-workers, in their study of 22 schizophrenia-affected Canadian pedigrees, generated a LOD score of 6.5 at chromosomal region 1q21-22 between markers *D1S1653* and *D1S1679*, using a recessive model of inheritance and a narrow disease definition (only individuals with schizophrenia and chronic schizoaffective disorder) (Brzustowicz *et al.,* 2000). Marginal support for linkage to this region has also been reported by Shaw and colleagues (Shaw *et al.,* 1998) in their study of 70 European schizophrenia associated pedigrees (Table 1.4). They observed a LOD of 2.4 at marker *D1S196* using a recessive model and a narrow disease definition (Shaw *et al.,* 1998).

Several other studies have implicated loci on other regions of chromosome (chr) 1q in the development of schizophrenia. Hovatta and others reported a three-stage genome-wide scan in 69 schizophrenia affected families collected from a sub-isolate within the late settlement region of Finland. They observed a maximum LOD score of 3.82 at marker *D1S2891* (1q32.2-q41), under a dominant model and narrow disease definition, with no evidence for locus heterogeneity (Hovatta *et al.,* 1999). This finding was later replicated in a genome-wide scan in a sample of 134 affected sib pairs from Finland (Ekelund *et al.,* 2000). Gurling and co-workers demonstrated a multipoint LOD score of 3.2 at marker *D1S196* on 1q32.2 in 13 British and 5 Icelandic pedigrees using a recessive model and broad disease definition (Gurling *et al.,* 2001).

All these studies provided some compelling evidence for chr1q as a schizophrenia susceptibility locus. This substantial evidence for linkage was evaluated in eight independently collected samples consisting of 779

informative schizophrenia pedigrees containing 984 affected sib pairs (ASP), by genotyping 16 DNA markers across 107 centimorgans (cM) of chr 1 in a large multicentre study (Levinson *et al.,* 2002). No linkage to schizophrenia was identified in any of the eight independent samples. This suggests that while any schizophrenia susceptibility genes present in chr1q may carry a large genetic contribution in the reported linked families (Brzustowicz *et al.,* 2000), they are likely to only have a small genetic effect in the overall population (Levinson *et al.,* 2002).

In a more recent study, Abecacis and collaborators conducted a genome-wide scan using a cohort of 143 pedigrees from the Afrikaner sub-population of South Africa (Abecacis *et al.,* 2004). They reported a LOD score of 2.28 at *D1S2141* using a recessive model of inheritance and broad diagnostic definition (schizophrenia, schizophreniform disorder, delusional disorder, atypical psychosis, and schizoaffective disorder), providing suggestive evidence for a novel schizophrenia susceptibility locus on chr1. The identified region resides on the p arm of the chromosome approximately 148-154cM and 208cM from the loci described by Brzustowitz *et al* (2000) and Hovatta *et al* (1999), respectively (Abercacis *et al.,* 2004). The p-arm of chr1 has also been implicated in schizophrenia susceptibility in a high-density SNP linkage study of 236 Japanese families (Arinami *et al*., 2005). In this study, a LOD score of 3.39 was reported at chr1p21.-p13.2 usnig a narrow diagnostic definition (Arinami *et al*., 2005).

1.4.3.2. Chromosome 2

Loci on chr2 have also been linked to schizophrenia. Blouin and others typed 31 markers on chromosome 2 and reported a maximum non-parametric (NPL) LOD score of 1.26 with the marker *D2S405*, which maps to chr2p22.1 (Blouin *et al.,* 1998). A maximum multipoint NPL score of 2.13 was observed at marker *D2S1337* by Shaw and colleagues in their genome-wide scan (Shaw *et al.,* 1998) (Table 1.4) In a study of a large pedigree from the Micronesian population of Palou, Coon and co-workers reported a maximum LOD score of 2.17 at *D2S441* using a dominant model of inheritance (Coon *et al.,* 1998) and narrow disease definition.

1.4.3.3 Chromosome 5

In a genome-wide scan of 188 pedigrees from Finland, the highest LOD score (3.56) was found on chr5q at *D5S804* using a recessive inheritance model and broad disease definition (Paunio *et al.,* 2001), whereas another study investigating various regions including chr5q in 62 pedigrees from Finland found little evidence to support this region (Hovatta *et al.,* 1998).

Table 1.4. **Summary of selected schizophrenia linkages studies.**

Chromosome	Locus	Marker	Cohort structure	LOD / p-values	Mode of inheritance	Phenotype definition	Reference
1	1q21-22	*D1S1653*	22 Canadian pedigrees	LOD=6.5	Recessive	Narrow	Brzustowicz *et al.,* 2000
		D1S196	70 European pedigrees	LOD=2.4	Recessive	Narrow	Shaw *et al.,* 1998
			13 British and 5 Icelandic pedigrees	LOD=3.2	Recessive	Broad	Gurling *et al.,* 2001
	1q32-44	*D1S2891*	69 Finnish pedigrees	LOD=3.82	Dominant	Broad	Hovatta *et al.,* 1999
		D1S2833	134 Finnish Pedigrees	LOD=2.65	Recessive	broad	Ekelund *et al.,* 2000
	1q32-41	*D1S2141*	20 Finnish pedigrees from small internal isolate	LOD=3.73	Not specified	Broad	Hovatta *et al.,* 1998
			143 South African Afrikaner pedigrees	LOD=2.28	Recessive	Narrow	Abecacis *et al.,* 2004
	1p35-32	*D1S3669*	21 African-American pedigrees	NPL=2.13 (p=0.002)	Not specified	Broad	Garver *et al.,* 1998
	1p-ter-21	*D1S3721*	21 African American pedigrees	NPL=2.18 (p=0.002)	Not specified	Broad	Garver *et al.,* 1998
	1p21.2-13.32		236 Japanese familes	LOD=2.33	Not specified	Narrow	Arinami *et al*., 2005
2	2q12-13	*D2S135-D2S540*	5 Icelandic pedigrees	p=0.000001	Not specified	Narrow	Moises *et al.,* 1995
	2q12	*D2S293*	43 European-American Pedigrees	NPL=2.41 (p=0.008)	Not specified	Narrow	Faraone et al.., 1998
	2q37	*D2S427*	53 Finnish pedigrees (small internal isolate)	LOD=4.43	Recessive	Narrow	Paunio *et al.,* 2001
		D2S1337	70 pedigrees (111 sib-pairs)	NPL=2.13 (p<0.001)	Not specifies	Broad	Shaw *et al.,* 1998
	2p15-14	*D2S441*	Large Micronesian pedigree	LOD=2.17	Dominant	Narrow	Coon *et al.,* 1998
		D2S358	7 Micronesian pedigrees	LOD=4.8	Recessive	Narrow	Camp *et al.,* 2001

Chromosome	Locus	Marker	Cohort structure	LOD / p-values	Mode of inheritance	Phenotype definition	Reference
5	5q	*D5S414*	Finnish late settlement cohort (118)	LOD=3.56	Recessive	Broad	Paunio *et al.,* 2001
	5q22-31	*D5S804*	265 Irish Pedigrees	LOD=3.35	Recessive	Narrow	Straub *et al.,* 1997
	5q32-33	*D5S422*	5 British and 8 Icelandic pedigrees	LOD=3.6	Recessive	Broad	Gurling *et al.,* 2001
			1 large extended Puerto Rican pedigree	LOD=4.37	Dominant	Broad	Silverman *et al.,* 1996
	5p14.1-13.1	*D5S111*		NPL=2.49 (p=0.008)	Not specified	Broad	Sherrington *et al.,* 1988
		D5S426	5 British and 8 Icelandic pedigrees	LOD=2.8	Dominant	Intermediate	Gurling *et al.,* 2001
			21 African-American pedigrees	NPL=2.55 (p=0.009)	Not specified	Broad	Garver *et al.,* 1998
		D5S426	1large Costa Rican Schizophrenia pedigree	LOD=2.7	Not specified	Broad	Cooper-Casey *et al*., 2005
6	6p24-22	*D6S296*	265 Irish pedigrees	LOD=3.55	Additive	Broad	Straub *et al.,* 1995
		MHC region	305 sib-pairs	NPL=3.13 (p=0.0015)	Not specified	Broad	Schwab *et al.,* 1995
	6p25	*D6S253-D6S297*	12 generation, 3400 member pedigree	MLS=7.7			Lindholm *et al.,* 2001
	6q21-22.3	*D6S474*	81 independent sib-pairs from 53 multiplex U.S pedigrees	69% IBD allele sharing	Not specified	Broad	Coa et al., 1997
			141 independent sib-pairs	NPL=3.82 (p=0.000014)	Not specified	Broad	Martinez *et al.,* 1999
	6q23	*D6S1626*	155 subjects from 21 families	NPL=4.6 (p=0.000004)	Not specified	Broad	Lerer *et al.,* 2004
		D6S1626	155 subjects from 21 families	LOD=4.63	Dominant	Broad	Levi *et al*., 2005

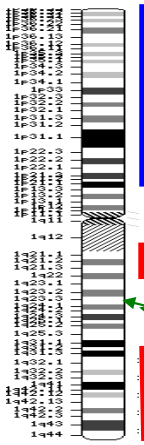
Chromosome	Locus	Marker	Cohort structure	LOD/p-value	Mode of inheritance	Phenotype definition	Reference
7	7q22	*D7S486*	134 Finnish Pedigrees	LOD= 3.18	Dominant	Broad	Ekelund *et al.,* 2000
8	8p22-21	*D8S136*	265 Irish pedigrees	LOD=2.34	Dominant	Broad	Pulver *et al.,* 1995
		D8S1771	54 multiplex North American pedigrees	LOD=4.54 NPL=3.4 (p=0.00001)	Dominant	Narrow	Blouin *et al.,* 1998
		D8S261	Maryland (U.S) pedigrees	LOD=3.06	Recessive	Broad	Levinson *et al.,* 1996
		D8S136	21 Canadian pedigrees	LOD=3.49	Not specified	Narrow	Brzustowicz *et al.,* 1999
		D8S503	5British and 8 Icelandic pedigrees	LOD=3.6	Dominant	Intermediate	Gurling *et al.,* 2001
		D8S1769	40 Korean Families	NPL=1.68	Not specified	Narrow	Kim *et al*., 2006
9	9q32-q34	*D9S175*	30 African-American nucler families	NPL=1.96 p=0.027		Narrow	Kaufman *et al*., 1998
			5 Icelandic Pedigrees, 54 European and European-American and 11 Asian families	P<0.01	Not specified	Narrow	Moises *et al*., 1995

Chromosome	Locus	Marker	Cohort structure	LOD/p-value	Mode of inheritance	Phenotype definition	Reference
10	10p12.31	*D10S582*	146 North American pedigrees	NPL=3.24 (p=0.0006)	Not specified	Broad	Faraone *et al.,* 1998
		D10S2440	265 Irish Pedigrees	LOD=1.91	Recessive	Intermediate	Straub *et al.,* 1998
		D10S582	36 German and 13 Israeli pedigrees	69.5% IBD allele sharing (p=0.0058)	Not specified	Narrow	Schwab *et al.,* 2000
		D10S1423	36 German and 13 Israeli pedigrees	58% IBS allele sharing	Not specified	Narrow	Schwab *et al.,* 2000
	10q24	*D10S189*	155 subjects from 21 families	NPL=3.4 LOD=2.65	Not specified Dominant	Broad	Lerer *et al.,* 2004
	10q22.3	*D10S1744*	29 Multiplex Ashkanazi Jewish families	NPL=4.27	Not specified	Narrow	Fallin *et al*., 2003
		D10S2327	606 Han Chinese Families	NPL=2.88	Not specified	Narrow	Faranone *et al*., 2006
	10p15	*D10S119*	382 Affected sib-pairs	NPL=3.6	Not specified	Narrow	De Lisi *et al.,* 1998
13	13q32	*D13S128*	Maryland (U.S) pedigrees	LOD=2.54	Dominant	Narrow	Antonarakis *et al.,* 1996
		D13S 174	54 North American multiplex pedigrees	NPL=4.18 (p=0.00002)	Not specified	Narrow	Blouin *et al.,* 1998
		D13S1793	21 Canadian pedigrees	HLOD=4.42	Not specified	Narrow	Brzutowicz *et al.,* 1999
		D13S122	10 British and 34 Taiwanese pedigrees	LOD=1.06	Dominant	Broad	Lin *et al.,* 1997
		D13S779	ISHDF pedigrees	LOD=1.36	Recessive	Narrow	Straub *et al.,* 1997

Chromosome	Locus	Marker	Cohort structure	LOD/p-value	Mode of inheritance	Phenotype definition	Reference
13	13q12-13	*D13S1293*	70 pedigrees (111 affected sib-pairs	NPL=1.8 (p<0.001)	Not specified	Narrow	Shaw *et al.,* 1998
20	20p12-p11	D20S171	62 Finninsh pedigrees	LOD=1.22	Not specified	Broad	Ekelund *et al*., 1998
	20q13.3	D20S172	12 African-American pedigrees	NPL=3.4	Not specified	Broad	Garver *et al*., 1998
22	22q12-13.3	*D22S268-D22S307*	39 Maryland (U.S) pedigrees	LOD=2.82	Dominant	Broad	Pulver *et al.,* 1994
		D22S278-D22S276	23 Multiplex pedigrees	LOD=1.5	Recessive	Narrow	Vallada et al., 1996
		D22S776	9 pedigrees	LOD=2.09	Recessive	Narrow	Coon *et al.,* 1994
		D22S278	113 unrelated schizophrenic patients and their 226 parents	P = 0.02	Not specified	Narrow	Moises *et al.,* 1995
	22q11	4cM	353 affected sib-pairs from the U.K, U.S and Sweden	NPL=2.29	Not specified	Narrow	Williams *et al.,* 2003

Abbreviations: HLOD, Heterozygosity logarithm of odds score, IBD, Identity by descent; ISHDF, Icelandic schizophrenia high density families; LOD, Logarithm of odds; MLS, Multipoint logarithm of odds score; NPL, non-parametric logarithm of odds score.

Chromosome 1



Garver *et al.*, 1998

Arinami *et al.*, 2005

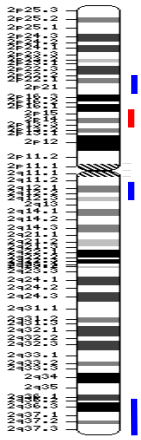
Brzutowicz *et al.*, 2000
Shaw *et al.*, 1998
Gurling *et al.*, 2001

Regulator of G-protein signalling4 (*RGS4*)

Hova ta *et al.*, 1999
Ekelu nd et al., 2000
Hwu t al., 2003
Aber asis et al., 2004

Acetylcholine receptor M3 (*CHRM3*)

Chromosome 2



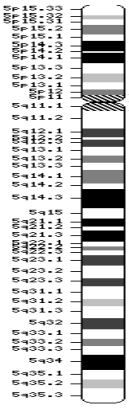
Blouin *et al.*, 1998

Coon *et al.*, 1998
Camp *et al.*, 2001
Shaw *et al.*, 2998

Moisis *et al.*, 1995
Faraone *et al.*, 1998

Paunio *et al.*, 2001

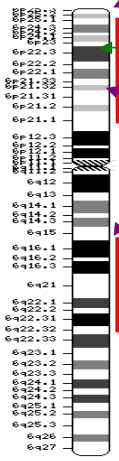
Chromosome 5



Silverman *et al.*, 1996
Sherrington *et al.*, 1998
Gurling *et al.*, 2001
Garver *et al.*, 1998

Straub *et al.*, 1997
Schwab *et al.*, 1997
Paunio *et al.*, 2001
Gurling *et al* 2001
Beyerly *et al.*, 1999
Garner *et al.*, 2001

Chromosome 6



Retinoid X Recpetor β (RXR β)

Lindholm *et al.*, 2001
Straub *et al.*, 1995
Schwab *et al.*, 1995, 2000
Moises et al., 1995

GABAβ receptor 1 (*GBR1*)

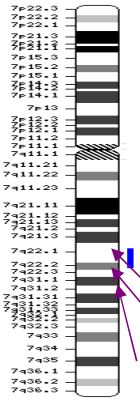
Dysbindin (*DNTBP1*)

Ocatmer binding transcription factor 7 (*POU3F2*)

Coa *et al.*, 1997
Martinez *et al.*, 1999

Lehrer *et al.*, 2004
Levi *et al.*, 2005

Chromosome 7



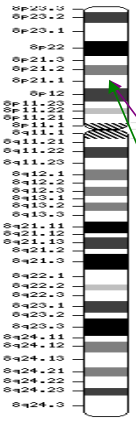
Ekelund *et al.*, 2000

Reelin (*RELN*)

Distal-less likehomeobox 6 (*DLX6*)

Gluatamate receptor, Metabotripic 3 (*GRM3*)

Chromosome 8



Pulver *et al.*, 1995
Blouin *et al.*, 1998
Levinson *et al.*, 1996
Brzustowicz *et al.*, 1999
Stefansson *et al.*, 2002
Kim *et al.*, 2006

Solute carrier family 18, member 1 (*SLC18A1*)

Neuregulin 1 (*NRG1*)

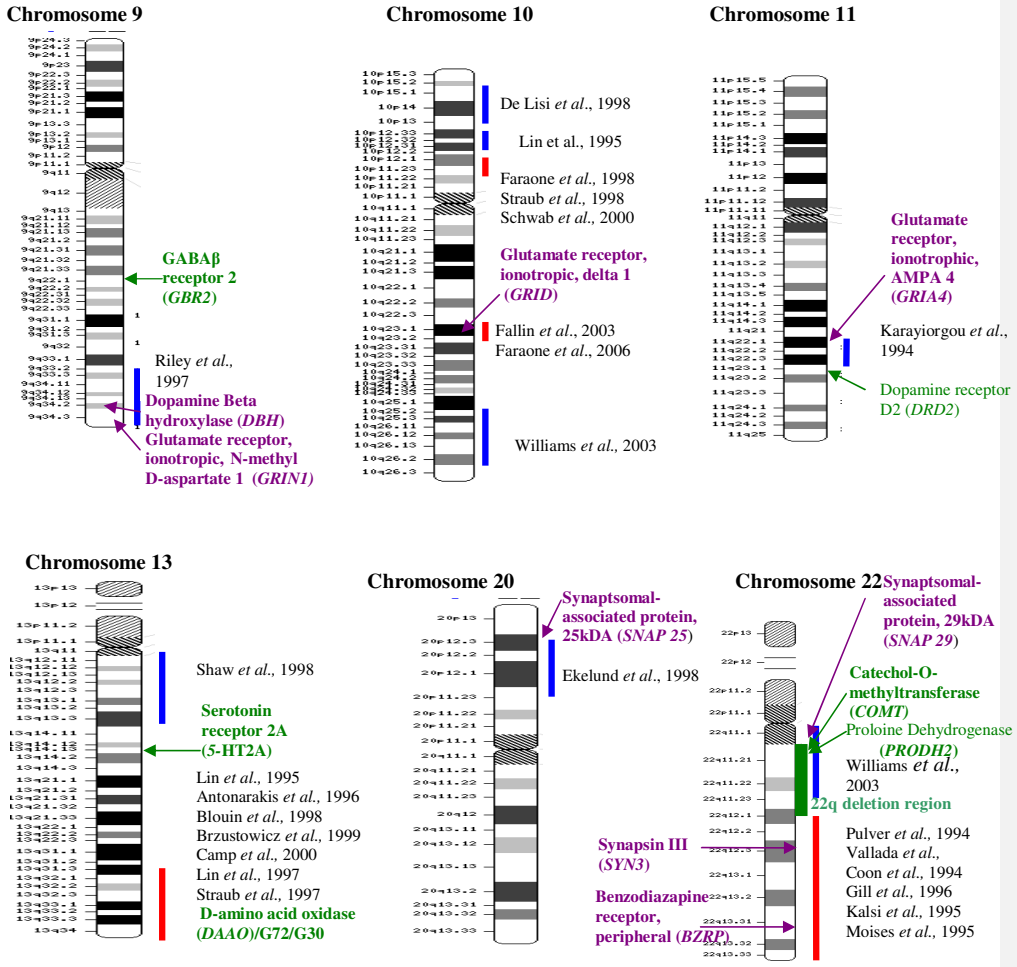


Fig 1.5: **Chromosomal regions implicated in schizophrenia susceptibility** Size of implicated regions are indicated by vertical bars. Red bars indicate regions that have been replicated in independent studies; blue bars indicate loci that have not been replicated in independent samples and the green bar shows the position of the chromosmome 22q deletion syndrome locus. The positions of the candidate genes investigated in the present study are also indicated by purple arrows, while functional and positional candidate genes reviewed in section 1.4.6.1 are indicated by green arrows.

Straub and colleagues reported a maximum heterogeneity LOD (HLOD) score of 3.35, in their cohort of 265 Irish pedigrees, at marker *D5S804* (mapping to chr5q22-q31) under a narrow phenotypic definition and a recessive genetic model (Straub *et al.,* 1997). This result was, however, not replicated in a large multi-centre study (The schizophrenia Collaborative Group III), which included the above-mentioned Irish cohort (Levinson *et al.,* 2000). However, additional support for linkage to this region was reported by Schwab and collaborators (1997) in German and Israeli families (Schwab *et al.,* 1997).

There has also been some evidence for a susceptibility locus on chr5p. In a study of one large Puerto Rican pedigree, Silverman and co-workers reported a maximum LOD score of 4.37 at *D5S111*, which maps to 5p13-14, under a dominant model of inheritance and a broad description of disease (Silverman *et al.,* 1996). Following from the afore-mentioned study, Gurling and others observed a five-point HLOD of 2.8 at *D5S462* (which also maps to chr5p13-14) in a large British and Icelandic cohort. Their study made use of a combined diagnostic system and a dominant model of inheritance.

Chr5p has also been implicated in schizophrenia susceptibility in one large, schizophrenia pedigree from the central valley of Costa Rica. A whole genome scan analysis of this pedigree, which included 11 cases of schizophrenia and schizoaffective disorder, identified a number of markers on chr5p that appear to co-segregate with the disease with a maximum lod score of 2.70 at marker *D5S426* situated at chr5p13 (Cooper-Casey *et al*., 2005).

1.4.3.4. Chromosome 6

One of the best supported regions for linkage in schizophrenia resides on chr6. The chr6p24-22 locus has been implicated in a number of studies. Straub and colleagues obtained a maximum LOD score of 3.55, 0.4 centimorgans (cM) away from marker *D6S296*, using a broad disease definition, with 15-30% of the families linked (Straub *et al.,* 1995). Evidence for linkage declined substantially when a narrow disease definition was used.

Lindholm and colleagues (2001) reported evidence for a schizophrenia susceptibility locus at chr6p25. These researchers completed a genome-scan of 3400 members of a single 12 generation pedigree with schizophrenia. A maximum LOD score of 6.6 was observed with marker *D6S253*, as well as with a 6cM haplotype stretching across markers *D6S253* and *D6S264* that segregated (after 12 generations) with the majority of affected individuals. This study also performed multipoint analysis with markers in the chr6p25 region and a maximum LOD score of 7.7 was obtained (Lindholm et a., 2001). Additional evidence for linkage to this region was reported by Schwab *et al* . (1995 and 2000) and Moises *et al* . (1995) (Table 1.4).

Regions on chr6q have also been linked to schizophrenia. Cao and colleagues reported possible linkage to chr6q21-22.3 in 81 independent sib-pairs from 53 multiplex North American families. (Cao *et al.,* 1997). This study was unique in that a second independent sample of families held by the same researchers was used to replicate the original finding (Riley and McGuffen, 2000, Cao *et al.,* 1997), which provided stronger evidence

for a schizophrenia locus in this area of chr6. A follow-up study, also by the above-mentioned group of researchers, found positive, but less significant, identity-by-descent (IBD) allele sharing using yet a third independent sample (Martinez *et al.,* 1999). In an independent study, data from the African-American pedigrees in the "National Institute of Mental Health (NIMH)/Millennium" schizophrenia genome screen also provided support for the presence of a susceptibility locus at chr6q21-22.3 (NPLZ=1.89 at *D61009* and NPLZ=1.56 at *D6S2056*) (Kaufmann *et al.,* 1998).

Significant evidence for linkage at the adjacent chromosomal region, chr6q23, was reported in an autosomal scan using 350 microsatellite markers in two Israeli-Arab families (Lerer *et al.,* 2003). This study reported a non-parametric LOD score of 3.3 using a dominant model and a broad disease definition (Table 1.4). In a follow-up study, 42 additional microsatellite markers, located wihin the linked region reported in the afore-mentioned study, were genotyped. This follow-up study reported a peak multipoint parametric LOD score of 4.63 at *D6S1626* under a dominant genetic model and narrow disease definition (Levi *et al*., 2005)

1.4.3.5. Chromosome 7

In a four-stage genome-wide scan in a study sample comprising 134 schizophrenia affected sib-pairs from Finland, Ekeland and co-workers found evidence of linkage for schizophrenia to chr7q22. Their most significant finding was a two-point lod score of 3.18 with marker *D7S486* using a dominant model of inheritance (Ekelund *et al.,* 2000). This finding has however not been replicated in independent studies.

1.4.3.6. Chromosome 8

Pulver and co-workers first found preliminary evidence for linkage to chr8p21-22 in a genome wide search, with a maximum LOD score of 2.35 at *D8S136* under a dominant model, and 2.20 under a recessive model of inheritance (Pulver *et al.,* 1995). Blouin and others following up on this study, using a different set of markers within the same family sample, reported a LOD score of 3.64 (Blouin *et al.,* 1998) (Table 1.4). In another independent follow-up study, Kim and co-workers genotyped seven microsatellite markers across this region in 40 Korean families with schizophrenia and reported suggestive evidence for linkage with a NPL LOD score of 1.68 at *D8S1769* under a narrow disease definition (Kim *et al.,* 2006).

The 8p21-22 region was also implicated in the study of 21 narrowly defined Canadian pedigrees (Brzustowicz *et al.,* 1999). Data from the above-mentioned study gave a LOD score of 3.49 at *D8S136*, but this decreased to 2.13 in multipoint analysis (Brzustowicz *et al.,* 1999).

Additional support for the presence of a schizophrenia susceptibility gene within chr8p21-22 was reported in a genome scan in 5 British and 8 Icelandic families (Gurling *et al.,* 2001) (Table1.4). Futhermore, Steffansson and colleagues reported suggestive evidence for linkage to chr8p12-p22 in a large Icelandic population (Steffansson *et al*., 2002).

1.4.3.7. Chromosome 9

The q32-34 region of chromosome 9 has been implicated in the development of schizophrenia in a linkage study by Kaufman and co-workers (Kaufman *et al*., 1998). In their study these investigators made use of 30 African-American nuclear families with 79 DSM-III-R diagnosed schizophrenia probands. Using a narrow disease definition, a NPL score of 1.96 between *D9S930* and *D9S1818* peaking at D9S175 (Kaufman *et al*., 1998).

In another study, Moises and colleagues implicated a region extending from D9S175 to D9S160 in five large Icelandic pedigrees ($p<0.01$). D9S175 was also implicated in an international follow-up study comprising 54 European and European-American and 11 Asian families (Moises *et al*., 1995)..

1.4.3.8. Chromosome 10

A number of regions on chr10 have been linked to schizophrenia, although only the chr10p11-15 and chr10q22 loci have been convincingly replicated. The chr10p11-15 locus was first implicated in the NIMH/Millennium genome screen (Faraone *et al.,* 1998) of 146 North American families of European descent. These researchers observed statistically suggestive evidence for linkage at *D10S1423* ($p=0.0004$) on chr10p13 and the nearby *D10S582* ($p=0.0006$) on chr10p12.31. A consortium that included the above-mentioned NIMH data set and seven other groups (Levinson *et al.,* 2000) also provided some evidence of excess allele sharing in this region.

Supportive evidence was further provided by a number of subsequent investigations. Straub and colleagues, using their cohort of 265 Irish pedigrees, reported a maximum pair-wise HLOD score of 1.91 at *D10S2440* (also situated on chr10p12.31) using an intermediate disease phenotype and a recessive model (Straub *et al.,* 1998). Schwab and others demonstrated a non-parametric lod score (NPL) of 3.2 at *D10S1714* in their 36 German and 13 Israeli family cohorts (Schwab *et al.,* 1998). The remarkable aspect of linkage to this locus is the relatively small region (5 megabases [Mb]) around which linkage has been found in these four studies (Faraone *et al.,* 1998; Levinson *et al.,* 2000; Schwab *et al.,* 2000; Straub *et al.,* 1998).

Evidence for linkage chr10q22 was first reported in a genomewide linkage scan for schizophreinia susceptibility among 29 multiplex Ashkenazi Jewish families conducted by Fallin and co-workers (Fallin *et al*., 2003). Their strongest linkage signal was achieved at *D10S168*6 located at chr10q22.3, with a NPL of 3.35 using a norrow diagnostic definition. Upon follow-up with 23 additional markers in the chr10q region, these investigators reported in increased NPL of 4.27 at *D10S1774* (Fallin *et al*., 2003). Linkage to this region was subsequently replicated in an independent genome scan of 606 Han Chinese schizophrenia families comprising 1234 affected members (Faranone *et al* 2006). In their study, Faranone and colleagues reported a NPL of 2.88 for marker *D10S2327* which is located within the 12.2Mb between *D10S1753* and *D10S1677* implicated by Fallin an co-workers (Faraone *et al*, 2006).

1.4.3.8. Chromosome 13

Interest in chr13 was first generated by Lin and colleagues, who investigated eleven moderately large pedigrees from the United Kingdom (U.K) and two from Japan. These researchers reported a maximum LOD score of 1.62 for *D13S119* located on chr13q32 using a narrow disease definition (Lin *et al.,* 1995).

In an attempt to replicate the above-mentioned findings, Lin and others studied another 10 British families as well as 34 Taiwanese families. The British sample showed a maximum LOD score of 1.72 at *D13S128*, using the same diagnostic criteria as the original study, while the Taiwanese sample showed no evidence of linkage to this region (Lin *et al.,* 1997).

Significant linkage to chr13q32 was also obtained from a genome scan of 54 multiplex pedigrees of mixed ethnicity near marker *D13S174* with an NPL score of 4.18 (p=0.00002) using a narrow disease definition (Blouin *et al.,* 1998). Shaw and co-workers (1998) also provided suggestive evidence for linkage to chr13q32 in the genome scan of 70 pedigrees containing multiple affected individuals by the generation of a maximum LOD score of 2.85 at *D13S1293* (Table 1.4)

Weaker support for this locus was found in the Irish sample of Straub and co-workers (1997). This sample showed a multipoint HLOD score of 1.36 at marker *D13S779* (Straub *et al.,* 1997). Brzustowicz and others, in an investigation of 21 Canadian schizophrenia affected pedigrees, reported a maximum multipoint HLOD score of 4.42 0.1cM centromeric to *D13S793* under a recessive model using a broad disease classification (Brzustowicz *et al.,* 1999). Furthermore, the chr13q32 region has been linked to schizophrenia by data generated from a large Veterans Affairs cooperative linkage study of schizophrenia (Faraone *et al.,* 2002) in which linkage between markers *D13S1241* and *D13S159* was detected.

Since these positive findings produced from non-overlapping families cluster together within the same region of chr13q, it was suggested that this region may harbour one or more schizophrenia susceptibility genes (Faraone *et al.,* 2002). Despite this, there are many negative reports of linkage to this region. Most notably is the report from the large multi-centre study by Levinson and collaborators (Levinson *et al.,* 2000). They studied linkage to chr13q in 784 informative pedigrees containing 823 independent sib-pairs collected from eight centres; two of the included centres (Blounin *et al.,* 1998; Shaw *et al.,* 1998) had previously reported linkage to chr13q32. In this multi-centre study, however, the maximum LOD score at chr13q32 was 0.09 which lacked support of evidence for linkage to this region (Levinson *et al.,* 2000). However, since this multicentre study was made up of a combination of cohorts from different ethnic backgrounds, it is possible that genetic heterogeneity may have masked the “signal” from the chr13q32 locus.

1.4.3.10. Chromosome 20

Two regions on chromosome 20 have been implicated in schizophrenia susceptibility. In an investigation by Ekelund and co-workers, reported a maximum lod score of 1.22 at *D20S172* located on 20p11.3 in a set of 62 pedigrees from Finland (Ekelund *et al*., 1999) using a broad disease definition.

In another investigation, Garver and co-workers also reported positive linkage to chromosome 20. In their study of 12 African-American, these investigators reported an maximum NPL of 3.4 at *D20S171* located at 20q13.3 using a braod disease model (Garver *et al*., 199).

1.4.3.9. Chromosome 22

Chr22 has been one of the most extensively studied chromosomes for putative schizophrenia susceptibility loci. In a study of 39 North American pedigrees, a LOD score of 2.82 at marker locus *ILBR2β* (interleukin receptor 2 beta chain) was observed between *D22S268* and *D22S307*, which maps to chr22q12-13 (Pulver *et al.,* 1994). Furthermore, as part of a systematic search for a major schizophrenic genetic locus, Vallada and co-workers conducted a linkage analysis of chr22 using highly polymorphic and evenly spaced genetic markers in 23 multiplex schizophrenia pedigrees (Vallada *et al.,* 1995). These researchers found suggestive evidence for linkage between markers *D22S278* and *D22S283* (which both map to chr22q12-13) (Table 1.4).

Coon and colleagues (1994) observed a LOD score of 1.45 at *D22S4* (chr22q13-qter) under a recessive model (Coon *et al.,* 1994a) in nine clinically narrowly defined families in their first stage genome scan. Further analysis of their sample, using a dense map of markers across chr22q13-qter, yielded a LOD score of 2.09 for *D22S276*, also under a recessive model of inheritance (Coon *et al.,* 1994b) (Table 1.4). Another locus on chr22 that has been implicated in schizophrenia is chr22q11. Williams and colleagues reported a maximum LOD score of 2.29 at chr22q11 in 353 clinically narrowly defined affected sib-pairs (179 from the U.K, 134 from Sweden and 40 from the U.S) (Table 1.4).

A multi-centre collaborative study subsequently attempted to replicate the findings of the original study by Pulver and others (Pulver *et al.,* 1994). by using 217 families, which included the families originally analysed by Pulver *et al* . However, the analysis of the combined sample of 217 families failed to show any evidence for linkage to the chr22q12-q13 region (Gill *et al.,* 1996). Studies by Kalsi and others (1995), as well as, Polymeropoulos and colleagues (1994) also found no evidence for linkage to chr22q11-q13 (Kalsi *et al.,* 1995; Polymeropoulos *et al.,* 1994) (Table 1.4).

1.4.3.10. Linkage of Schizophrenia Endophenotypes

Segregation analysis of sensory motor gating deficits in schizophrenia was found to be consistent with an autosomal dominant model of inheritance (Elston, 1996). In an initial linkage analysis using this model of inheritance, Coon and co-workers used PPI (section 1.1.4) suppression to identify a potential schizophrenia susceptibility locus at chr15q14 (Coon *et al.,* 1993). Subsequent linkage analysis performed by Freedman and colleagues showed linkage between sensory motor gating deficits in schizophrenia to *D15S1360* on 15q14 (Freedman *et al.,* 1997). This marker was identified in a yeast artificial chromosome (YAC) that contained the α-7-nicotinic cholinergic receptor, which has been the focus of numerous association studies in schizophrenia (Freedman *et al.,* 1997). Linkage of schizophrenia to chr5q14 was further supported by ASP analysis performed but the National Institute of Mental Health (NIMH) genetics initiative (Leonard *et al.,* 1998), which investigated 20 nuclear families (84 individuals and 26 affected sibpairs) with at least one sibpair concordant

Comment [IT45]: Elston RC (1996): SIBPAL, Statistical Analysis for Genetic Epidemiology (Case Western Reserve, Cleveland, OH), version 2.7.

for schizophrenia. Sibpair analysis showed that a significant percentage of alleles of the marker *D15S1360* was shared identical by descent ($p<0.0024$).

Gasperoni and colleagues (2003) used four schizophrenia endophenotypes (including choice reaction time, attention deficits, recall intrusions and spatial working memory dysfunctions) to further elucidate the nature and location of the schizophrenia susceptibility locus on chr1q implicated in two previous studies (Hovatta *et al.,* 1999; Ekelund *et al.,* 2000). Using a composite measure of these four endophenotypes, these investigators produced marginal evidence for linkage at the same marker (*D1S2833*) implicated by Ekelund et al. (2000) using twin pairs from Finland (Gasperoni *et al.,* 2003). Of the four variables comprising the composite measure, only impaired spatial working memory (assessed using the visual span subtest of the Wechsler Memory Scale) was linked to *D1S2833*. Thus this study provides evidence that disturbances in the frontal-executive function are central to the pathophysiology of schizophrenia (Weinberger, 1987; Park and Holzman, 1992; Park *et al.,* 1995, 1999; Cannon *et al.,* 2000).

The linkage studies and genome scans discussed in the above section show that several genetic loci have been identified that possibly harbour schizophrenia susceptibility genes. Relatively few of these loci have been confirmed in independent studies. Furthermore, loci that have been replicated in some independent investigations have not been replicated in others. These discrepant results could imply that genetic factors do not influence schizophrenia pathogenesis; however, this is refuted by data from twin and adoption studies. Another possibility for the disparate genetic results centres around schizophrenia as a complex threshold disease where multiple genetic and environmental insults are required to cause disease. The multiple loci generated by linkage analysis could imply genetic heterogeneity in schizophrenia, where the major genetic contributors to disease are different for different families and population groups or are brought into play by different environmental insults.

1.4.4 Obsessive-compulsive disorder linkage studies

As opposed to the vast amount of linkage and genome scan data available for schizophrenia, to date, only one genome-wide scan has been undertaken for OCD (Hanna *et al.,* 2002). These authors initially completed their genome scan using 56 individuals from seven families, including 27 individuals with a definite OCD diagnoses. A maximum multipoint LOD score, prior to fine mapping, of 2.25 was reported for marker *D9S288* on chr9p24, while three other regions had LOD scores of less than 1 (chr16q, chr2q and chr19q) under a dominant model and narrow a phenotype definition. Denser mapping was subsequently performed using the original 56 subjects plus 10 additional individuals (Hanna *et al.,* 2002). This subsequent fine mapping of chr2, chr9 and chr16 showed a peak multipoint LOD score of 1.97 at *D9S288* on 9p24 (Hanna *et al.,* 2002). This study provided the first linkage data on OCD offering suggestive linkage on chr9p24 while identifying other possible susceptibility loci (chr2p, chr6p and chr19q).

In an attempt to replicate the findings of Hanna and co-workers, the Johns Hopkins OCD research group genotyped 50 small nuclear OCD pedigrees using the 13 microsatellite markers spanning chr9p24

(approximately 19cM) (Willour *et al.,* 2004) used in the original OCD genome-wide scan (Hanna *et al.,* 2002; Willour *et al.,* 2004). As in the original study, this replication study also reported that the strongest parametric findings were under a dominant model of inheritance using a narrow phenotype definition (Willour *et al.,* 2004). A maximum parametric LOD score of 2.26 was found at *D9S1792*, while the nonparametric signal peaked at *D9S1813*. These two markers are approximately 1.1cM and 0.8cM, respectively, telomeric to marker *D9S288*, which showed the maximum LOD score in the original study. The replication study made use of small nuclear pedigrees that excluded probands with Tourette's syndrome, while the genome scan used multigenerational multiplex pedigrees. It is important to note that while this replication study varied from the original in important ways, both studies gave suggestive evidence for the involvement of chr9p24 in OCD susceptibility. However, to date, no susceptibility gene residing within this locus has been implicated in increasing susceptibility to OCD. It is also of interest to note that none of these loci overlapped with schizophrenia linkage loci.

1.4.5. Chromosomal Abnormalities

In addition to chromosomal regions implicated by linkage analysis, some chromosomal regions have also been implicated in the development of these disorders based on chromosomal abnormalities such as deletions, translocations and trisomies.

There are few reports of chromosomal abnormalities associated with major psychiatric disorders (Bassett, 1992; Craddock and Owen, 1994, De Lisi *et al.,* 1994). It is speculated that this is likely due to the fact that geneticists usually consult on paediatric cases and seldom follow up into adolescence or adulthood, when many psychiatric disorders first appear. It would also appear that psychiatrists generally do not suspect genetic syndromes in psychiatric patients as a first cause (Bassett *et al.,* 2000).

Despite this, some researchers have provided compelling evidence for increased prevalence of a number of psychiatric illness among patients suffering from syndromes caused by chromosomal abnormalities. Furthermore, there are numerous examples of diverse conditions where a chromosomal aberration has led the way to the identification of possible susceptibility loci (Castermans *et al.,* 2004). The section that follows will deal with these chromosomal abnormalities, how they provide a possible means to help localise causative genes and the roles they play in our understanding of the genetic complexity of mental illness.

1.4.5.1. Chromosome 22q11 deletion syndrome

Chromosome 22 deletion syndrome (22qDS) is the second most common genetic syndrome after Down Syndrome (Gothelf and Lambroso, 2001) that affects approximately one in 4000 individuals worldwide (du Montcel *et al.,* 1996). The syndrome encompasses velocardiofacial syndrome (VFCS), DiGeorge syndrome and conotruncal anomaly face syndrome (CTAFS), all due to chr22q11.2 microdeletions (Fig 1.7) (Demczuk and Aurias *et al.,* 1995), which generally occur *de novo* (Demczuk and Aurias *et al* , 1995; Leana-Cox *et al.,* 1994). Learning disabilities, palatal anomalies, cardiac defects and atypical facial features are common, although the presentation is highly variable (Yamagisgi *et al.,* 1999). It is inferred that the symptoms

Comment [IT46]: 1.**Du Montcel ST, Mendizabal H, Ayme S, Levy A, Philip N, 1996.** Prevalence of 22q11 microdeletions of 22q11.2 in velo-cardio-facial syndrome. J Med Genet **44**: 261-68

associated with the deletions, which vary in extent in different patients, are associated with the loss of particular genes harboured in the missing region. Most interestingly, a review of 22qDS found that psychiatric disorders, of which schizophrenia was the most frequent, were reported in 53% of cases of 22qDS that were not ascertained from psychiatric sources (Papolos *et al.,* 1996; Pulver *et al.,* 1994). Studies have also shown an increased rate of 22qDS among schizophrenia patients. These studies used fluorescence *in situ* hybridization (FISH) and a chr22q11.2 probe to demonstrate that approximately 2% of patients with schizophrenia have the chr22q11.2 microdeletion, compared to 0.0025% in the general population.

The findings above lend support to the likelihood that a meaningful association exists between this chromosomal anomaly and schizophrenia (Propping *et al.,* 1995; Karayiorgou *et al.,* 1996; Bassett *et al.,* 1999). There have been some positive linkage data to markers on chr22q11.2 (section 1.4.3.9), but most linkage findings have tended to cluster around a 4-5 cM region around 22q13, approximately 25Mb telomeric to chr22q11.2. This could suggest that there may be two or more loci on chromosome 22 involved in schizophrenia (Schwab *et al.,* 1999) (Fig 1.7).

Obsessive-compulsive symptoms have also been observed in a number of 22qDS patients (Karayiorgou *et al.,* 1997, Pulver *et al.,* 1994). A follow up study by Papolos and colleagues on behavioural phenotypes of patients with 22qDS observed a high rate of OCS in many of these patients (Papolos *et al.,* 1994). In fact, Gothelf and colleagues noted that, in their clinical experience, not only did many of their VCFS patients suffer from OCS, but the family members of many of these patients reported that the OCS were the most disruptive behavioural symptoms of the patient (Gothelf *et al.,* 2004). Three investigations reported rates of OCS and OCD in VCFS patients of 14% (Pulver *et al.,* 1994), 8% (Papolos *et al.,* 1996) and 12% (Feinstein *et al.,* 2002). These findings provide strong evidence that genes involved in increased OCD susceptibility may be harboured at the chr22q11 region.

1.4.5.2. Other chromosomal aberrations

A balanced (1;11)(q42;q14.3) reciprocal translocation has been found to co-segregate with schizophrenia in a large Scottish family (Millar et al., 2000; Blackwood *et al.,* 2001; St Clair *et al.,* 1990). In a linkage study of schizophrenia in this family, the translocation generated a LOD score of 3.6 when the phenotype was restricted to schizophrenia. The LOD score increased to 7.0 when the disease definition was expanded to include major depression and bipolar disorder (Blackwood *et al.,* 2001). Furthermore, the Disrupted in Schizophrenia (DISC) genes (Sawamura and Sawa, 2006), *DISC1* and *DISC2*, are disrupted by this translocation that segregates within the family (Blackwood *et al.,* 2001). Interestingly, in an earlier study by Ekelund and colleagues, the strongest evidence for linkage in their combined sample was obtained for marker *D1S2709*, which is an intragenic marker of the *DISC1* gene (Ekelund *et al.,* 2001) (Table 1.4)

Comment [MB47]: Sawamura N, Sawa A. Disrupted-in-schizophrenia-1 (DISC1): a key susceptibility factor for major mental illnesses. Ann N Y Acad Sci. 2006 Nov;1086:126-33.

In their cytogenetic analysis of an extended pedigree, Calzolari and colleagues identified two individuals carrying a balanced translocation with a breakpoint at chr15q13-14. One was diagnosed with psychotic disorder at 15 years of age, while the other was diagnosed with schizoaffective disorder at 16 years of age,

neither of these two disorders were otherwise present within the pedigree (Calzolari *et al.,* 1996). Other genetic illnesses linked to chr15q have been reported to co-express psychosis resembling schizophrenia, namely Prader-Willi syndrome, a disease involving imprinting of genes at chr15q11-12 (Clarke, 1993), Marfan syndrome, which generally involves mutations of the fibrillin gene at chr15q21 (Sirota *et al.,* 1990) and Andersmann's angenesis of the corpus collosum, which is caused by a deletion of chr15q (Casaubon *et al.,* 1996).

Translocations have also been utilised to identify possible OCD susceptibility loci in various studies. However, many of these studies make use of a wide phenotypic definition, viz., TS/OCD/chronic tic disorder (CTD) phenotypic spectrum (Cuker *et al.,* 2004) (section 1.2.1).

Three cases of this TS/OCD/CTD spectrum phenotype associated with translocations have previously been reported. In 1996, Boghosian-Sell and co-workers reported a familial form of TS associated with obsessive-compulsive sympoms that segregated with a balanced t(7;18)(q22-q31; q22.3) translocation (Boghosian-Sell *et al.,* 1996). Subsequently, State and colleagues reported on a young man with CTD and OCD, who was found to carry a paracentric inversion i(18q21.1-q22.2) (State *et al.,* 2003). These investigators mapped the telomeric end of the inversion to a genomic location that is less than 1Mb from the translocation described by Boghsian-Sell and others (1996) (State *et al.,* 2003). More recently, Cuker and others described a 14 year-old girl with a t(2,18)(p12;q22) translocation with severe OCD (Cuker *et al.,* 2004). Fine mapping of the patient's chr18 breakpoint revealed it to be within 4.7Mb of the previously reported breakpoint (Bogh-Sell *et al.,* 1996); thus these studies implicate a locus on chr18q in the development of OCD.

Moreover, two chromosomal breakpoints associated with TS/OCD/CTD has been shown to disrupt particular genes. Petek and co-workers described a 13-year-old boy with a *de novo* duplication of a region of chr7 [dup (7)(q22.1-q31.1)] who developed TS/OCD/CTD without any signs of overt mental retardation. These researchers further showed that this duplication was inverted and disrupted *IMMP2L*, the human homologue to the yeast mitochondrial inner membrane peptidase subunit 2 (Petek *et al.,* 2001). Whether it is this gene that plays a role in OCD or whether inversion of, or the presence of, genes on the duplicated region are involved, has not yet been investigated (Cuker *et al.,* 2004).

In another study, the contactin-associated protein (CNTNAP2) gene (*CNTCAP2*) was found to be disrupted in a TS/OCD/CTD family with a complex translocation involving chr2 and chr7 (Verkerk *et al.,* 2003). Three of the family members investigated (a father and two children) share a chr2q21-p23 insertion on chr7q35-q36, which disrupts the *CNTNAP2* gene. This gene encodes a membrane protein located in a specific compartment at the nodes of Ranvier of axons. The authors speculate that the disruption of *CNTNAP2* could lead to a disturbed distribution of potassium (K) channels in neurons, thereby affecting repolarisation of action potentials and causing the TS/OCD/CTD syndrome.

1.4.6. Association studies in Schizophrenia

To further lay the ground for prioritising novel OCD candidate genes, the genes that have already been implicated in both schizophrenia and OCD by association studies will be reviewed in the following sections.

To date, hundreds of schizophrenia association studies, primarily focused on functional candidate genes have been undertaken (Owen *et al.,* 2004), and typically involved genes chosen based on neuro-psychopharmacological data. For this reason, genes of the dopamine and serotonin systems have been extensively studied in the past; however, given the evidence for other mechanisms in schizophrenia etiology, it can be questioned whether those are the only valid candidate categories. Details of functional candidate genes reviewed in this section are summarised in Table 1.5.

1.4.6.1. Functional candidate genes

1.4.6.1.1. Dopaminergic system

The dopamine D2 (DRD2) and D3 (DRD3) receptors have been extensively studied. Several studies have indicated that the density of DRD2 receptors is abnormally elevated in post-mortem striatum samples from schizophrenic patients (Lee *et al.,* 1978, Owen *et al.,* 1978). Although the results of these investigations were often dismissed as only reflecting the effects of antipsychotic pharmacotherapy (Mackay *et al.,* 1980; 1982), many drug-naïve patients, as well as patients who have not been under antipsychotic treatment for over one year, have also shown increased numbers of DRD2 receptors over that of unaffected subjects (Crow *et al.,* 1978; Lee and Seeman, 1980).

In 1997, Arinami and colleagues ascribed functional relevance to a polymorphism in the promoter region of the DRD2 gene (*DRD2)* designated -141C *Ins/Del* (Arinami *et al.,* 1997). Using a luciferase enzymatic activity assay, they demonstrated that expression from the -141C *Del* allele was 21-42% of that from the -141C *Ins* allele. Furthermore, the -141C *Ins* allele frequency was higher in schizophrenia patients that in the control subjects (Arinami *et al.,* 1997). Subsequently, a number of studies have demonstrated an association between *DRD2* and schizophrenia (Ohara *et al.,* 1998; Jönsson *et al.,* 1999, Breen *et al.,* 1999). The studies by Ohara and colleagues in a Japanese sample, and Jönsson and colleagues in a Swedish population, both found an association with the -141 *Ins* and schizophrenia (Ohara *et al.,* 1998; Jönsson *et al.,* 1999), but, in a study of a British sample, association was demonstrated with the -141 C *Del* allele (Breen *et al.,* 1999).

The *Cys311* allele of the S*er311Cys* variant of *DRD2* (Cravchik and Goldman, 2000), located on the third cytoplasmic loop of DRD2, has been shown to be less effective in inhibiting the synthesis of cyclic AMP (Cravchik *et al.,* 1996). Association studies of this polymorphism have yielded inconsistent results; however, in a recent meta-analysis of *DRD2 Cys311* schizophrenia association studies conducted before June 2002, the data supported the involvement of *DRD2* in schizophrenia (Jönsson *et al.,* 2003).

Table 1.5. **Summary of association studies of functional candidate genes in schizophrenia discussed in this review.**

Candi-date gene	Polymorphism	Sample	Findings	Referrence
DRD2	*-141Cins/del*	Case-control study of 260 unrelated Japanese schizophrenic patients and 312 unrelated Japanese controls	-141C*ins* allele frequency higher in schizophrenic patients	Arinami *et al.,* 1997
		Case-control study 170 unrelated Japanese schizophrenic patients and 121 unrelated Japanese controls	-141C*ins* allele frequency higher in schizophrenic patients (p=0.042);	Ohara *et al.,* 1998
		Case-control study of 129 unrelated Swedish schizophrenic patients and 179 unrelated Swedish controls	-141C*del* allele frequency lower in schizophrenic patients	Jönsson *et al.,* 1999
		Case-control study of 439 Caucasian schizophrenic patients and 437 unrelated Caucasian controls (patients and controls of British and Scottish descent)	-141C*del* allele frequency higher in schizophrenic patients (p=0.02)	Breen *et al.,* 1999
	Ser-311-Cys	Case-control study of 153 Japanese schizophrenic patients and 300 unrelated Japanese controls	No association	Arinami *et al.,* 1994
		Case-control study of 78 Okinawan schizophrenic patients and 112 unrelated controls	No association	Kaneshima *et al.,* 1997
		Case-control study of 366 European schizophrenic patients and 267 unrelated controls	No association	Serretti *et al.,* 2000
DRD3	*Ser-9-Gly*	Meta-analysis of 29 independent case-control samples from 24 association studies (2619 schizophrenic patients and 2517 controls)	Excess homozygosity for allele 1 in African and Caucasian group (p=0.05)	Dubertret *et al.,* 1998
		Meta-analysis of 30 schizophrenia case-control association studies and 57 parent/proband trios	association between DRD3 Ser9Gly homozygosity and schizophrenia (p=0.0009)	Williams *et al.,* 1998
		Case-control study of 311 schizophrenic patients and 306 unrelated controls in European Multi-centre Association Study of Schizophrenia	Excess homozygotes of allele 1(p=0.003)	Spurlock et al, 1998
5-HT2A	*T102C*	Case-control study of 62 Japanese schizophrenia patients and 96 unrelated Japanese controls	*102C* allele found in excess in schizophrenic patients	Inayama *et al.,* 1994
		Case-control study of 278 German schizophrenia patients and 207 unrelated German controls	*102C* allele found in excess in schizophrenia patients (p=0.041)	Erdman *et al.,* 1996
		Case-control study of 571 Caucasian schizophrenia patients and 639 unrelated Caucasian controls	*102C* allele found in excess in schizophrenia patients	Willams *et al.,* 1996
		TDT analysis of 63 schizophrenic parent/offspring trios	Excess of 102C allele transmission from parents to affected offspring (p=0.001)	Spurlock *et al.,* 1998

Candi-date gene	Polymorphism	Sample	Findings	Referrence
5-HT2A	*T102C*	101 Chinese male unrelated schizophrenic patients and 103 Chinese male control subjects.	Excess of 102T allele in schizophrenia patients	Tay *et al.,* 1997
		Case-control study of 97 Chinese schizophrenia patients and 101 unrelated Chinese controls	No association	Lin *et al.,* 1999
		Case-control study of 471 Han Chinese schizophrenia patients and 523 unrelated Han Chinese controls	No association	Chen *et al.,* 2001
		Meta-analysis if 15 studies (1533 schizophrenia patients and 1771 controls	Association found between 102C allele and schizophrenia (p=0.0009)	Williams *et al.,* 1997
		Meta-analysis of 31 case-control association studies (4632 schizophrenia cases and 4410 controls) and 5 family-based studies (473 parent/offspring trios)	Association found between 102C allele and schizophrenia in case-control sample (p=0.015); no evidence for association in family-based studies	Abdolmaleky *et al.,* 2004
		HRR in 67 schizophrenia affected nuclear families and case-control study of 100 schizophrenia patients and 103 controls	No association found in either HRR or case-control studies	Verga *et al.,* 1997
GRIN1	*1719G/A*	Case-control study of 96 Japanese schizophrenia patients and 96 unrelated Japanese controls	No association	Sakurai *et al.,* 2000
	IVS2-22T/C	Case-control study of 96 Japanese schizophrenia patients and 96 unrelated Japanese controls	No association	
	IVS2-11G/A	Case-control study of 96 Japanese schizophrenia patients and 96 unrelated Japanese controls	No association	
	IVS4-34C/T	Case-control study of 94 Japanese schizophrenia patients and 95 unrelated Japanese controls	No association	
	GRIN1/1 (C/G)	HRR of 86 parent/schizophrenic offspring trios and case-control study of 91schizophrenia patients and 91 unrelated controls from Toronto	No association in TDT analysis (p=0.14) No association in case-control analysis (p=0.908)	Martucci *et al.,* 2003

Candi-date gene	Polymorphism	Sample	Findings	Referrence
GRIN1	*GRIN1/10 (A/G)*	TDT of 86 parent/schizophrenic offspring trios and case-control study of 91schizophrenia patients and 91 unrelated controls from Toronto	No association in TDT analysis (p=0.22)No association in case-control analysis (p=0.544)	Martucci *et al.,* 2003
	GRIN1/1 GRIN1/10 haplotype	TDT of 86 parent/schizophrenic offspring trios and case-control study of 91schizophrenia patients and 91 unrelated controls from Toronto	Borderline significant association with *GRIN1/1 G*-allele*/GRIN 1/10 A*-allele haplotype (p=0.049)	
GRM3	*+1131C/T*	Case-control study of 265 German schizophrenic patients and 227 unrelated German controls	Association found with *+1131T* allele (P=0.0022)	Marti *et al.,* 2002
		Second independent case-control study of 288 German schizophrenia patients and 162 unrelated German controls; TDT using 128 parent/schizophrenic offspring trios	Previous association not replicated in either second, independent case-control study or TDT analysis	
	rs146812	Case-control study of 100 Japanese schizophrenia patients and 100 unrelated Japanese controls	Significant difference in allele frequency of rs146812 between groups (p=0.011) Identified 3-marker at-risk haplotype (including rs146812) for schizophrenia (p=0.00083)	Fujii *et al.,* 2003
	Rs229925	Case-control study of 752 Chinese schizophrenic patients and 752 unrelated Chinese controls	Significant difference in allele frequency of rs2299225 between groups (p=0.0297) Identified 3-marker at-risk haplotype for schizophrenia (*rs2237562-C*, *rs1468412-T* and *rs2299225-C*) (p=0.008)	Chen *et al.,* 2005

Abbreviations: *5-HT2A*, Serotonin receptor 2A; *DRD2,* Dopamine receptor 2; *DRD3*, Dopamine receptor 3; *GRIN1*, N-methyl-D-aspartate glutamate receptor NR1 subunit; GRM3, Metabotropic glutamate receptor 3; HRR; Haplotype relative risk; TDT; Transmission disequilibrium test

The DRD3 gene (*DRD3*) is expressed selectively in the brain regions associated with emotional control, motivation and reward and is the target for most antipsychotic agents (Sokoloff *et al.,* 1990). Two meta-analyses of association studies of the *Ser9Gly* polymorphism in *DRD3* (Dubertret *et al.,* 1998; Williams *et al.,* 1998) both provided evidence for association between *DRD3 Ser9Gly* homozygosity and schizophrenia in certain populations. Moreover, very recently, this polymorphism was also found to be associated with diminished parietal function and affected PPI in schizophrenia patients (Mulert *et al*., 2006).

1.4.6.1.2 Serotonergic system

Much of the current focus of research into the role of the serotonergic system in schizophrenia is on serotonin receptor 2A (5-HT2A) (Harrison and Burnet, 1997). The first genetic evidence for the involvement of the serotonin system in schizophrenia came from a report of an association with the *T102C* polymorphism in *5-HT2A* in a Japanese sample, in which the 102C allele was associated with schizophrenia. (Inayama *et al.,* 1994) This association was later replicated by a European multicentre association study (Willams *et al.,* 1996), as well as studies by Erdman and colleagues (1996) and Spurlock and colleagues (1998). However, in a study of 101 Chinese male schizophrenic patients and 103 controls, it was the *102T* allele that was found to be predominant in schizophrenic patients (Tay *et al.,* 1997). This may indicate that the *T102C* polymorphism is in linkage disequilibrium with the real functional variant.

There have also been a number of published studies that failed to replicate these initial *5-HT2A* findings (Verga *et al.,* 1998; Lin *et al.,* 1999; Chen *et al.,* 2001). As a result of these inconsistencies within the literature, several researchers performed meta-analyses on the available data. Williams and co-workers completed a meta-analysis of 15 studies (1533 patients and 1777 controls) that supported the association between *102C* allele (Williams *et al.,* 1997). Six years later, after many more association studies of *5-HT2A T102C* and schizophrenia had been published, Abdolmaleky and colleagues performed another meta-analysis (Abdolmaleky et al, 2003), using the 15 studies from the first meta-analysis as well as an additional 21 new studies, and showed a significant association between the *102C* allele and schizophrenia in the case:control studies. Included in the 21 new studies were five family-based studies which themselves had not show any evidence of association (Abdolmaleky *et al.,* 2003).

1.4.6.1.3. Glutamatergic system

The NR1 (section 1.1.5.3.3) subunit of NMDA-R is encoded by the *GRIN1* gene located on chr9q34.3. In studies done by Rice and colleagues and by Sakurai and colleagues, none of a number of novel identified sequence variations showed any significant association with schizophrenia in a case-control setting either (Sakurai *et al.,* 2000; Rice *et al.,* 2001).

Martucci and colleagues used both TDT (86 nuclear families) and case-control analysis (91 ethnically matched case-control pairs) of *GRIN1* and showed a borderline significant association for a *GRIN1* haplotype in schizophrenia susceptibility consisting of two SNPs (Martucci *et al.,* 2003). Zhao and co-workers also investigated five SNPs within *GRIN1* in a cohort of 707 unrelated Han Chinese schizophrenia patients and 689

control individuals, and found highly significant association with schizophrenia with the SNP rs11146020 (Zhao *et al*., 2006).

The gene encoding the metabotropic glutamate receptor 3 (*GRM3*) has been mapped to chr7q22-31, a region previously implicated in schizophrenia pathogenesis by linkage analysis (Enkelund *et al.,* 2000). Marti and colleagues (2002) identified a synonymous C to T substitution at nucleotide position 1131 (*+1131C/T*) in *GRM3* and tested it for association with schizophrenia in a sample of German descent. Their data showed a significant over-representation of the *GRM3 +1131T* allele in schizophrenic patients compared to ethnically matched control individuals (Marti *et al.,* 2002); however, this was not replicated in a follow-up study by the same authors using an independent German schizophrenic patient and matched controls sample (Marti *et al.,* 2002). These authors concluded that *GRM3* may, in fact, not play a crucial role in the development of schizophrenia, at least not in the German population (Marti *et al.,* 2002).

Fujii and co-workers (2003) reported a significant difference in the distribution of alleles of SNP *rs1468412* in *GRM3* in a Japanese cohort (Fujii *et al.,* 2003). Furthermore, they identified an at-risk haplotype, constructed from three SNPs, including *rs146812*, that showed a significant association with schizophrenia (Fujii *et al.,* 2003). In an attempt to replicate this data, Chen and colleagues (2005) investigated *GRM3* SNPs (Fig 1.6) used in the two previous reports (Marti *et al.,* 2002; Jujii *et al.,* 2003). This investigation was unable to replicate the previous association with *rs1468412* and the three-marker at-risk haplotype (Fujii *et al.,* 2003). Their data, however, revealed an association between SNP *rs2299225* and schizophrenia. Furthermore, another 3-marker at risk haplotype that includes *rs2299225* was identified (Chen *et al.,* 2005). However, in an investigation of the potential role of GRM3 in the development of schizophrenia, conducted using a cohort of 674 unrealated Caucasian schizophrenia patients and 716 unrealted Caucasian control individuals from the UK, Norton and co-workers did not find any evidence for association (Norton *et al*., 2005).

Taken together, these results seem to indicate that *GRM3* may play an important role in the aetiology of schizophrenia in some population groups, the Japanese for example, while in others, like the Caucasian population, the role of *GRM3* may not be that significant, if it plays a role at all.

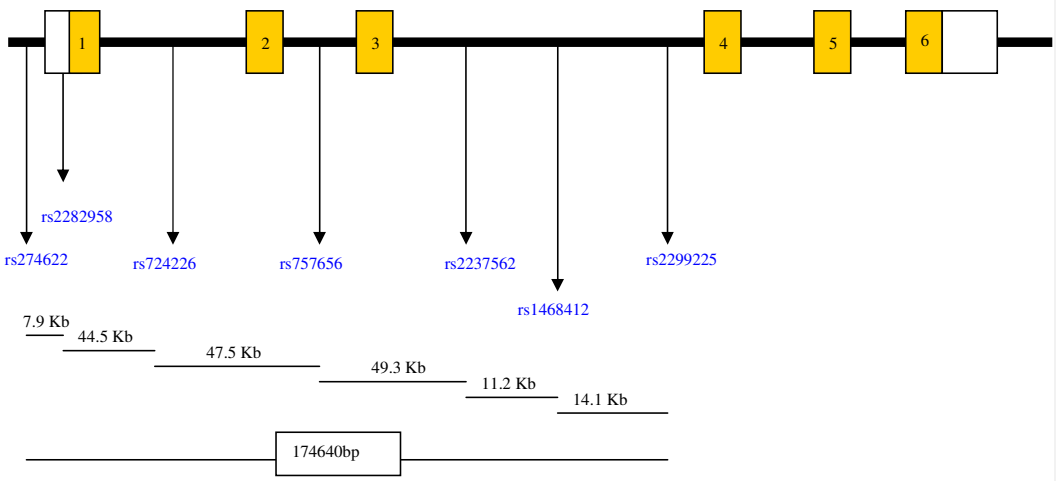


Fig1.6: Schematic representation of spread of SNPs across *GRM3* used in the study by Chen *et al.*, 2005. Exons are represented by solid yellow blocks introns are represented by solid black lines and the untranslated regions are represented by open blocks.

1.4.6.1.4. GABAergic system

The $GABA_A$ and $GABA_B$ receptors have been investigated as possible schizophrenia susceptibility genes in a number of investigations. Papadimitriou and colleagues tested a dinucleotide $(CA)_n$ repeat marker at the promotor region of the $GABA_A$ α_5 subunit gene for association with schizophrenia in a case-control association and found that the frequency of the 282-bp allele was 43.3% in the patient group with late age of onset compared to 23.9% in control individuals ($p<0.005$) (Papidimitriou *et al.,* 2001). This increased frequency was not observed in the unstratified case group. A more recent study investigated the possible association of 19 SNPs in the genes encoding the α_1 (2SNPs), β_2 (10SNPs), γ_2 (4SNPs), ε (1SNP) and π (2SNPs) $GABA_A$ subunits with schizophrenia in a Han Chinese case-control cohort (Lo *et al.,* 2004). None of the SNPs investigated in the α_1, γ_2, ε or π subunit genes were found to be associated with schizophrenia, however, five of the ten SNPs in the β_2 encoding gene (*GBRβ2*) showed a significant association at both genotype and allele levels. The five disease associated SNPS, *B217G1584T, rs1816071, rs194072, rs252944 and rs187269*, are all located in either intron 7 or 8 of *GBRβ2* (Fig 1.7). In an attempt to confirm the results obtained by Lo and colleagues, Liu and co-workers applied TDT analysis to these five SNP genotypes as well as SNP *rs1816072* in 352 Han Chinese trio families (Liu *et al*., 2005). They found no significant association with any of the six investigated SNP and schizophrenia, but did provide strong evidence for association with haplotypes consisting of combinations of these SNPs (Liu *et al*., 2005)

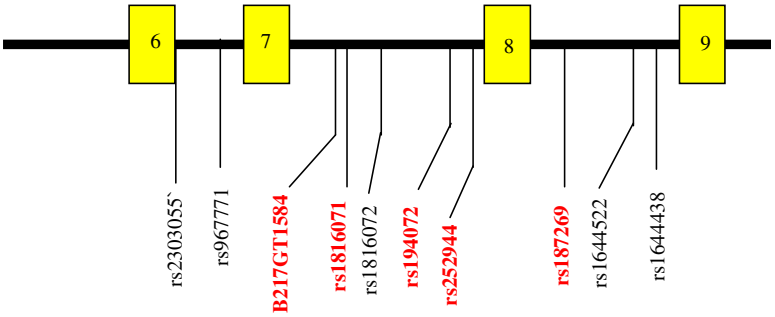


Fig 1.7: Schematic representation of a portion of *GBRβ2* showing the locations of the 10 SNPs analysed in the study by Lo and co-workers (2004) (Adapted from Lo *et al.,* 2004). The five SNPs that were found to be significantly associated with schizophrenia in the study by Lo and co-workers (2004) are shown in red font.

Imai and colleagues analysed a dinucleotide repeat $(AC)_n$ polymorphism, located approximately 1.6kb upstream from the $GABA_B$ receptor 1 gene (*GBR1*) but found no significant differences between their Japanese schizophrenic patient and control groups at either genotypic or allelic levels (Imai *et al.,* 2002).

In a more recent investigation, Zai and co-workers investigated a possible association between five SNPs (*rs29218, rs29220, rs29225, rs29230 and rs3095273*) in *GBR1* and schizophrenia (Zai *et al.,* 2005) (Fig 1.8). In their study, these researchers genotyped a sample of 110 small nuclear families amd 150 case-control pairs matched for age, ethnicity and gender. When analysing the family sample, they did not observe preferential transmission of alleles or haplotypes in a TDT analysis. However, in their case-control analysis, they reported an association between the rs29218 polymorphism, which is an A to G substitution at position -7265 of the promoter region of *GBR1*, and schizophrenia (Zai *et al.,* 2005).

To date, the two above-mentioned studies are the only two studies that specifically focus on the role of *GBR1* polymorphisms and schizophrenia and do not lend strong evidence supporting a role for *GBR1* in the development of schizophrenia. However, based on the important roles of the GABA system in the development of schizophrenia (section1.1.5.3.4), further studies of this gene's possible role in schizophrenia susceptibility are warranted.

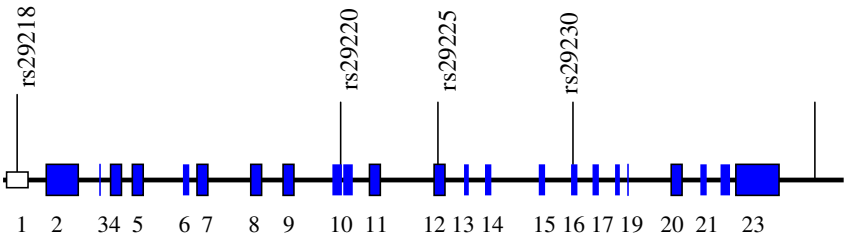


Fig 1.8: Schematic representation of a portion of *GBR1* showing the locations of the 5 SNPs analysed in the study by Zai and co-workers (2005). Solid black lines represent introns, while solid blue blocks represent exons. Open blocks represent untranslated regions.

The evidence presented in the sections above illustrates how many of the studies investigating candidate genes for schizophrenia have focused mainly on neurotransmitter systems (dopamine, serotonin, glutamate and GABA) that have been implicated by pharmacological evidence. The section that follows will focus on association studies of genes that are candidates by both position, from linkage data, and function.

1.4.6.2. Positional candidate genes

1.4.6.2.1. Dysbindin 1 (*DNTBP1*)

In an attempt to identify the susceptibility genes responsible for the linkage they previously found to chromosome 6p, Straub and co-workers applied a systematic linkage disequilibrium (LD) mapping approach using their original 270 Irish pedigrees (Straub *et al.,* 1996) (section 1.4.3.4) (Table 1.4). These investigators performed a family-based association analysis of simple sequence length polymorphism (SSLP) markers and analysis of SNP haplotyping in 6p22. They found significant evidence for association with SNPs within the dysbindin (DNTBP1) gene (*DNTBP1)* with p-values <0.01 for a number of individual SNP markers and p-values of between 0.001-0.08 for multiple 3-marker haplotypes (Straub *et al.,* 2002).

The human *DNTPB1* encodes a 40kDA coiled-coil-containing protein that binds to α-and β-dystrobrevin in muscle and brain tissue to form the dystrobrevin-associated protein complex (Benson *et al* 2001). This protein complex plays an integral structural role in synapse formation and maintenance and is also thought to be involved in NMDA and GABA receptor signalling (Benson *et al.,* 2001).

Since the original association between *DNTBP1* and schizophrenia, several follow-up investigations have been undertaken. In view of the fact that *DNTBP1* is located in the centre of their previously reported linkage peak on 6p (Schwab *et al.,* 1995, 2000), Schwab and colleagues analysed the six most positive SNPs from an earlier study by Straub et al. (1996) in a cohort of 78 German and Israeli families, as well as 127 parent-proband trios in an attempt to replicate the finding by Straub and co-workers (Schwab *et al.,* 2003). Evidence for association was observed in the two samples separately, as well as when they were combined (Schwab *et al.,* 2003).

However, a second attempt at replication in 219 Irish cases and 231 control individuals failed to support the involvement of *DNTBP1* in schizophrenia (Morris *et al.,* 2003), as did a separate study of 708 DSM-IV diagnosed schizophrenia cases and 711 control subjects from the UK and Ireland (Williams *et al.,* 2004). However, investigators involved in the later study screened all exons and the promoter region of *DNTBP1* and identified novel SNP markers. When these novel SNPs were included in the study, together with markers from the original study, highly significant evidence for association with an identified common risk haplotype was obtained (Williams *et al.,* 2004). These same markers were then examined in the Irish cohort (Morris *et al.,* 2003) and the observed risk haplotype reported in the afore-mentioned study was found to be significantly more common in affected individuals within this group (Williams *et al.,* 2004).

The discrepant results in these studies is quite interesting in view of the fact that all these studies were conducted exclusively in European populations, yet the data suggests differences in LD between these populations.

1.4.6.2.2.Catechol-O-methyl transferase (*COMT*)

The gene encoding catechol-O-methyltansferase (COMT), *COMT*, has been localised to chr22q11.1-q11.2 by Grossman *et al.,* (1985), at a genomic locus that has been implicated in schizophrenia by linkage studies (Williams *et al.,* 2003) and chromosomal abberations. COMT catalyses the transfer of the methyl group from S-adenosyl-L-methionine to a phenolic hydroxyl group of catechol neurotransmitters, catechol steroids and catechol drugs (Axelrod, 1966; Campbell *et al.,* 1984). In the brain, COMT degrades catechol amines such as norephinepherine, epinephrine and dopamine into O-methyl esters (Guldberg and Marsden, 1975).

Cell fractionation and immunological studies have shown that the COMT enzyme occurs as two distinct forms in mammals: in the cytoplasm as a soluble form (S-COMT) and associated with membranes as a membrane-bound form (MB-COMT) (Assicot and Bohuon, 1971). S-COMT activity is the more prevalent form in all tissues, while MB-COMT generally represents less than 5% of the total COMT activity (Guldberg and Marsden, 1975; Jeffery and Roth, 1984; Grossman *et al.,* 1985). However, in the brain, MB-COMT activity has been reported to be higher than in other tissues (Rivett *et al.,* 1982).

A functional polymorphism in *COMT,* in which the high (*H*) and low (*L*) activity alleles encode a valine or methionine amino acid residue, respectively, at codon 158 of the MB-COMT and codon 108 of S-COMT, has been described (*Val/Met* polymorphism). There is a three- to four-fold reduction in enzyme activity between the variants encoded by the *H/H* and *L/L* genotypes, with heterozygotes (*H/L*) showing intermediate enzyme activity. This polymorphism is represented by a G to A (Lachman *et al.,* 1996) and has been the focus of numerous association studies in schizophrenia (Table 1.5), but have yielded ambiguous results (Li *et al.,* 1996 Chen *et al.,* 1997; Kunigi *et al.,* 1997; Strous *et al.,* 1997; Ohmori *et al.,* 1998; de Chaldee *et al.,* 2001; Egan *et al.,* 2001; Liou *et al.,* 2001; Norton *et al.,* 2002, Williams *et al*., 2005; Tsai *et al*., 2006). Whereas studies by Ohmori and co-workers (150 schizophrenia patients and 150 control individuals) and Kremer and co-workers (276 cases and 77control individuals) found significant evidence for association between the *L* allele and schizophrenia, several other studies failed to find any association (Strous *et al.,* 1997; Chen *et al.,* 1997; Norton *et al.,* 2002; Williams *et al*., 2005; Tsai *et al*., 2006). Moreover, a number of family-based association studies have provided weak and inconsistent evidence that the *H* allele may be involved (Table 1.6).

The largest reported case-control association study investigating the role of COMT in schizophrenia was performed using over 700 patients and more than 7000 control individuals (Shifman *et al.,* 2002). These investigators not only tested the *Val/Met* polymorphism for association, but also several other SNPs across the *COMT* gene in an Ashkenazi Jewish cohort (Shifman *et al.,* 2002). Interestingly, the *Val/Met* polymorphism by itself only showed modest evidence for association, however, when it was analysed as part of a haplotype study that included two non-coding SNPs, a high level of significance was achieved ($p=9.5x10^{-8}$). These two

non-coding SNPs (one in intron 1 of *MB-COMT* [rs737865] and the other near the 3' UTR [*rs165599*]) were themselves significantly associated with schizophrenia, with *rs165599* showing the highest levels of significance (Shifman *et al.,* 2002) (Fig 1.9). Even though this study reached statistical significance, it remains unreplicated. The data generated in this study suggests that if an association exists between the *COMT* locus and schizophrenia, it cannot wholly be explained by the *Val/Met* polymorphism (Owen *et al.,* 2004).

Given that COMT degrades dopamine, that most treatments for schizophrenia are dopamine blockers and that a deletion of chr22q11, which includes *COMT,* are all associated with increased schizophrenia risk, Bray and colleagues hypothesized that the *COMT* haplotypes associated with schizophrenia in the study by Shifman *et al.,* (2002) would also be associated with lowered COMT mRNA expression levels (Bray *et al.,* 2003). These investigators made use of SNPs within an expressed sequence as a tag for mRNA transcribed from each chromosomal allele and applied quantitative methods of allele discrimination to mRNA from individuals who are heterozygous for the marker polymorphism to measure relative allelic expression. They applied this principle to investigate the possible cis-acting mechanisms that affect expression of *COMT* in the human brain using 23 heterozygous individuals. Their data showed that the *COMT* haplotype implicated in schizophrenia is indeed associated with lowered *COMT* expression. Furthermore, they showed that the SNP *rs165599* (3' UTR SNP), which gave the best evidence for association in the study by Shifman and co-workers, is transcribed in the human brain and exhibits allelic expression differences, with lower expression of the schizophrenia-associated allele (Bray *et al.,* 2003). These results support the hypothesis that the *COMT* haplotype implicated in schizophrenia susceptibility may exert its effect by the down-regulation of COMT and is also compatible with the hyperdopaminergic hypothesis of schizophrenia.

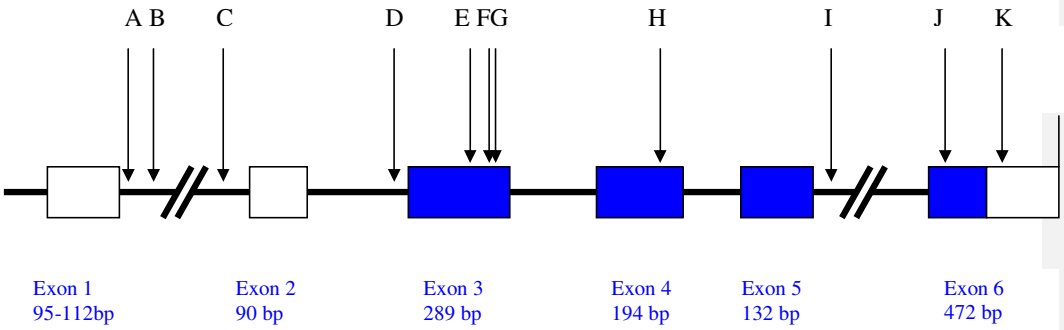


Fig 1.9: Location of SNPs in *COMT* investigated in the study by Shifman *et al.,* 2002. The capital letters represent each of the SNPs as follows: A(*rs737685)*; B(*rs174686*); C(*rs740603*); D(*rs6269*); E(*rs6270*); F(*rs4633*); G(*rs6267*); H(*rs165688*); I(*rs174689*); J(*rs362204*); K(*rs165599*). Solid blue rectangles represent transcribed regions, whereas open rectangles represent untranslated regions.

Table 1.6: **Association studies of COMT *Val/Met* polymorphism in schizophrenia**

Study reference	Study Design	Findings	Statistical values
Li *et al.,* 1996	TDT of 178 Han Chinese parent/schizophrenic offspring trios consisting of schizophrenic patients and their parents	*Val-108* allele predominantly transmitted to affected offspring	p=0.005
Daniels *et al.,* 1996	Case-control association study of 78 unrelated schizophrenia patients and unrelated controls	No association	χ^2 =0.12, 1df, p=0.81 (allele) χ^2 =0.32, 2df, p=0.83 (genotype)
Chen *et al.,* 1997	Case-control association study of 177 unrelated Chinese schizophrenic patients and 99 unrelated Chinese controls	No association	χ^2 =0.01, 2df, p=0.99 (genotype) χ^2 =0.000, 1df, p=1.00 (allele)
Strous *et al.,* 1997b	Case-control association study of 42 unrelated Caucasian schizophrenia patients and 87 unrelated Caucasian controls from the U.S	No association	p=0.07 (genotype) p=0,15 (allele)
Ohmori *et al.,* 1998	Case-control association study of 150 unrelated Japanese schizophrenia patients and 150 unrelated Japanese controls	*Val-108* allele associated with schizophrenia	χ^2 =4.83, 1df, p=0.028 (allele) χ^2 =7.26, 2df, p=0.026 (genotype)
Egan *et al.,* 2001	TDT of 104 Caucasian parent/schizophrenic offspring trios	*Val-108* allele transmitted more frequently to affected offspring	χ^2 =4.57, p=0.03
Liou *et al.,* 2001	Case-control association study of 198 unrelated Chinese schizophrenia patients and 188 unrelated Chinese controls	No association between *COMT* and schizophrenia, but significant differences in age of onset among the different genotypes	χ^2=5.501; p=0.005
Kremer *et al.,* 2002	TDT of 194 Palestinian Arab parent/ schizophrenic offspring trios	*Val-108* allele associated with schizophrenia in case-control study. Association stronger in females.	χ^2=3.935; 1df, p=0.047 χ^2=5.89; 1df, p=0.015 (females)
	Case-control association study of 276 unrelated Palestinian Arab schizophrenia patients and 77 unrelated Palestinian Arab controls	No preferential transmission of either allele in TDT	χ^2= 0.14, p>0.05
Norton *et al.,* 2002	Case-control association study of 346 unrelated Caucasian schizophrenia patients and 334 unrelated schizophrenia controls	No association	χ^2=0.73; 1df; p=0.55
Glatt *et al.,* 2003	Meta-analysis of 14 case-control association studies (2205 cases, 2236 controls and 5 family-based studies (584 parent/offspring trios)	No association found in case-control studies No preferential transmission of either allele found in family-based studies	p=0.57 p=0.13
Sazci *et al.,* 2004	Case-control association study of 297 unrelated Turkish schizophrenia patients and 341 unrelated Turkish controls	*Val-108* allele and *Val/Val* genotype associated with schizophrenia.	χ^2 =13.03, p=0.001 (allele) χ^2 =4.048, p=0.020 (genotype)
Williams *et al*., 2005	Case-control association study of 709 unrealated Caucasian schizophrenia patients and 710 unrelated Caucasian controls and TDT analysis of 488 parent/offspring trios	No association found in case-control studies No preferential transmission of either allele found in family-based studies	p=0.9 p=0.75

Abbreviations: *COMT,* Catechol-O-methyltransferase; TDT, Transmission disequilibrium test; *Val,* Valine

1.4.6.2.3.Proline dehydrogenase 2 (*PRODH2*)

The high frequency of schizophrenia in patients with 22q11DS has led to the hypothesis that sequence variation within one or several genes in the deleted region of chr22q11 (Fig 1.5) might contribute to schizophrenia in the general population (Egan *et al.,* 2001). The overwhelming majority of chr22q11 deletions are 3Mb in size whereas approximately 8% involve a smaller 1.5Mb deletion. Karayiourgou *et al.* reported a schizophrenic patient carrying this smaller 1.5Mb deletion and thus defined the "schizophrenia critical region" on chr22q11 (Karayiourgou *et al.,* 1995). Linkage disequilibrium mapping of this 1.5Mb region in schizophrenic patients identified a segment containing the gene encoding proline dehydrogenase (*PRODH*) (Lui *et al.,* 2002). PRODH is a mitochondrial enzyme that converts proline to Δ-1-pyrroline-5-carboxylate and is involved in the transfer of redox potential across the mitochondrial membrane (Gogos *et al.,* 1999). Δ-1-Pyrroline-5-carboxylate can be converted to glutamate and GABA, both of which are candidate neurotransmitter systems implicated in schizophrenia (Pearlson 2000). Additionally, mice with an inactivated *PRODH* gene have abnormal sensorimotor abnormalities (discussed in section 1.1.4.1.) similar to schizophrenic patients (Gogos *et al.,* 1999).

Liu and colleagues analysed polymorphisms in *PRODH* and found an association between the *PRODH*1945T/C* SNP and schizophrenia using TDT in 107 independent North American triads. These investigators subsequently revealed an association with a two marker haplotype, *PRODH*1945/1766*, which was significantly more associated with schizophrenia than either SNP individually, when analysed using the North American triads and a case-control sample of 109 unrelated Afrikaner schizophrenic patients and 75 unrelated Afrikaner control individuals) (Liu *et al.,* 2002). However, Fan and colleagues found no evidence of preferential transfer of *PRODH*1945T/C* alleles to affected offspring using both TDT and HRR methods in 166 family trios from east China. This study (Fan *et al.,* 2003).

Williams and colleagues undertook a detailed analysis of *PRODH* using a large case-control cohort (368 unrelated Caucasian schizophrenic patients from the U.K and Ireland and 368 unrelated matched Caucasian control individuals, a sample of VCFS probands with and without schizophrenia and a sample of 55 proband trios with juvenile-onset schizophrenia) (Williams *et al.,* 2003). They found none of the SNPS employed in the Liu *et al.* study, nor nine newly identified cDNA varients, to be associated with schizophrenia susceptibility (Williams *et al.,* 2003).

Thus, despite several lines of evidence suggesting that *PRODH* is likely to be a promising candidate gene for schizophrenia, given the lack of replication of the original findings in studies employing 95% power to replicate, *PRODH,* as other schizophrenia implicated genes, may not be ubiquitously associated with increased schizophrenia risk (Owen *et al.,* 2004).

1.4.6.2.4. Neuregulin 1 (*NRG1*)

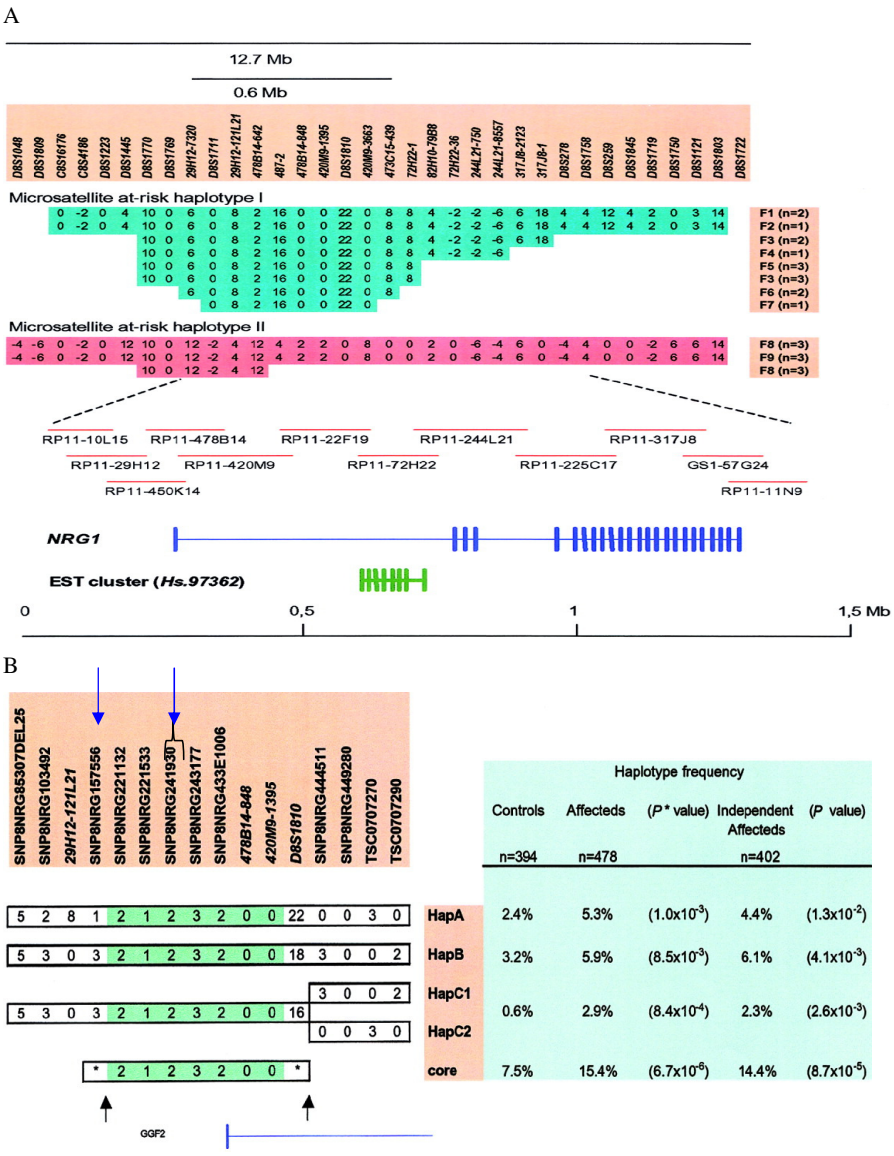
Neuregulin 1 (NRG1), a member of a family of four growth factors, the neuregulins, plays multiple roles in a number of organs including the nervous system, heart and breast (Lemke *et al.,* 1996; Ozaki *et al.,* 2000; Falls *et al.,* 2003). In the brain, NRG1 is involved in synapse formation, activity-dependent synaptic plasticity and

regulation of NMDA, $GABA_A$ and acetylcholine receptor subunit expression (Ozaki *et al.,* 1997; Yang *et al.,* 1998; Rieff *et al.,* 1999; Liu *et al.,* 2001, Liu Stefansson *et al.,* 2002). Neuregulin 1 also regulates the proliferation and migration of Schwann cells (Dong *et al.,* 1995; Lemke, 1996; Gassmann and Lemke,1997) and neurons within the brain (Rieff *et al.,* 1999; Schmid *et al.,* 2003). It has been suggested that NRG1 may mediate schizophrenia by changing expression profiles of a wide variety of neuroreceptors, particularly NMDA and glutametergic receptors (Stefansson *et al.,* 2002).

The neuregulin1 gene (*NRG1*) has been localised to chr8p12-22, which has been implicated in schizophrenia susceptibility in a number of independent studies in varying population groups (Pulver *et al.,* 1995; Kender *et al.,* 1996; Levinson *et al.,* 1998, Brustowicz *et al.,* 1999; Gurling *et al.,* 2001). Stefansson and colleagues conducted a genome-wide scan for schizophrenia linkage in a large Icelandic population and detected suggestive evidence for linkage to chr8p12-21 (Stefansson *et al.,* 2002). This was followed up with systematic LD analysis around the linked region on 8p and three "at-risk" haplotypes (HapA, HapB and HapC) were identified. Each "at-risk" haplotype shared a 290kb core haplotype of seven markers (5 SNPs and 2 microsatellites), upstream from the first 5' exon of *NRG1*, that showed an estimated frequency of 7.5% in the general population and 15.4% among schizophrenic patients (Stefansson *et al.,* 2002) (Fig 1.10). This core "at-risk" haplotype is defined by a minimum haplotype of one SNP (*SNP8NRG221533*) and two microsatellites (*478B14-848* and *420M9-1395*). Following the identification of the "core at-risk haplotype" in an Icelandic population, Stefansson and co-workers replicated this finding in a Scottish cohort of 609 schizophrenic patients and 618 control individuals, suggesting that the "at-risk" haplotype is not specific to the Icelandic population (Stefansson *et al.,* 2003)

Since these initial studies, several investigations of NRG1 involvement in schizophrenia have been completed. Several of these studies provided further evidence for the association of NRG1 and schizophrenia risk. Williams and colleagues genotyped *SNP8NRG221533*, *478B14-848* and *420M9-1395* (defined by Stefansson *et al.,* [2002] as the minimum "core at-risk" haplotype) in their sample of 709 unrelated Caucasian schizophrenic patients and matched control individuals born in the U.K. and Ireland (Williams *et al.,* 2003). None of the three analysed markers achieved significant allelic association with schizophrenia by themselves, however, when testing for haplotype association, the previously described "at-risk" haplotype was significantly more common in the patients than in the control group (Williams *et al.,* 2003).

Corvin and colleagues provided additional support for a susceptibility locus for schizophrenia on chr8p12-22 by replicating (Corvin *et al.,* 2004) the two above-mentioned studies (Stefansson *et al.,* 2002; Williams *et al.,* 2003) in an Irish case-control sample. They identified an 'at-risk' haplotype that overlaps one of the "at-risk" haplotypes (HapB) reported in the Icelandic population (Corvin *et al.,* 2004; Stefansson *et al.,* 2002). This refined haplotype ($HapB_{IRE}$) was found in significant excess in the Irish schizophrenia cases versus control individuals (Corvin *et al.,* 2004). These results were also confirmed in the Scottish schizophrenia cohort described by Stefansson *et al* . Moreover, this study suggests that the expressed sequence tag (EST) cluster Hs97362 may potentially be a susceptibility gene at the NRG1 locus (Corvin *et al.,* 2004). However,



	Haplotype frequency				
	Controls n=394	Affecteds n=478	(*P* * value)	Independent Affecteds n=402	(*P* value)
HapA	2.4%	5.3%	$(1.0x10^{-3})$	4.4%	$(1.3x10^{-2})$
HapB	3.2%	5.9%	$(8.5x10^{-3})$	6.1%	$(4.1x10^{-3})$
HapC1	0.6%	2.9%	$(8.4x10^{-4})$	2.3%	$(2.6x10^{-3})$
HapC2					
core	7.5%	15.4%	$(6.7x10^{-6})$	14.4%	$(8.7x10^{-5})$

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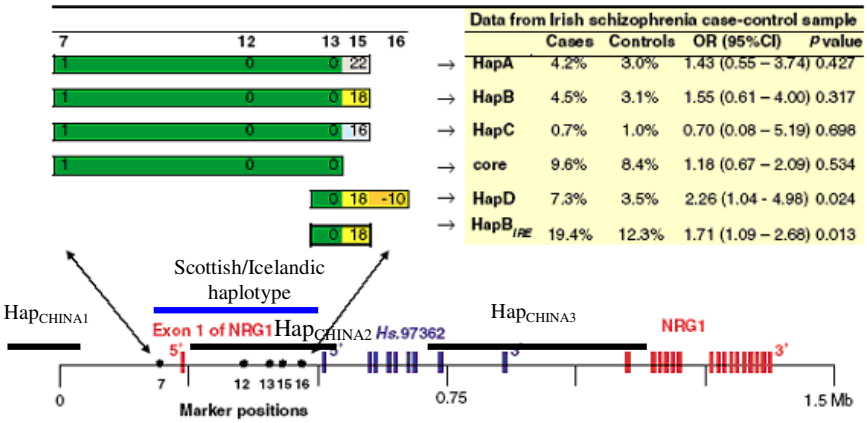


Fig 1.10: Schematic representation of markers used in the study by Stefansson and colleagues (2002). A:. Microsatellite markers on chromosome 8p. The position of NRG1 is schematically shown (taken from Stefansson *et al.,* 2002). **B: Microsatellite markers and SNPs at the 5' end of NRG1.** The 4 haplotypes, HapA-HapC2, were individually found in excess in schizophrenia patients. The blue arrows indicate the three markers that minimally define the core haplotype. The panel on the right indicates the frequencies of each of the four "at-risk" haplotypes in all affected individuals (taken from Stefansson *et al.,* 2002). **C:** Schematic representation of the truncated forms of the 'at-risk' haplotypes. Haplotype frequency of $HapB_{IRE}$ and HapD (the haplotypes that were found to be associated with schizophrenia in the Irish cohort in the study by Corvin *et al.,* 2004) is shown in the panel on the right. The relative physical position of each of the markers on NRG1 is indicated by the black arrows. The positions of the Chinese haplotypes are indicated by solid black lines, while the position of the Scottish/Icelandic haplotype is indicated by the solid blue line (taken from Corvin *et al.,* 2004).

independent studies conducted in the Irish study of high-density schizophrenia families (ISHDSF) (Thiselton *et al.,* 2004) and in a Japanese cohort (Iwata *et al.,* 2004) found no evidence for linkage or association with the previously described "at-risk" haplotypes (Stefansson *et al.,* 2002; Williams *et al.,* 2003; Corvin *et al.,* 2004) at the NRG1 locus.

But, a novel "at-risk" haplotype was identified at this locus in a Han Chinese sample (Li *et al.,* 2004). In this study, five of the seven markers used in the Icelandic sample were genotyped and used for haplotype analysis in family-based (184 parent-offspring trios and 138 affected siblings with at least one parent) and case-control association studies (298 unrelated Han Chinese schizophrenic patients and 336 unrelated Han Chinese control individuals) (Li *et al.,* 2004). Neither the haplotype nor alleles associating with the Icelandic "at-risk" haplotype were found in excess in the Chinese schizophrenia cases. Three interesting haplotypes where, however, identified, ie. Hap_{CHINA1}, Hap_{CHINA2} and $Hap_{CHINA3.}$. Hap_{CHINA1}, situated immediately upstream of the Icelandic "at-risk" haplotype was found in excess in schizophrenic patients versus control individuals. However, this association was lost when family-based methods were used. Hap_{CHINA2} was associated with schizophrenia in both case-control and family-based studies, while Hap_{CHINA3} was only associated in family-based studies (Li *et al.,* 2004).

Recently, Kim an co-workers genotyped the seven markers used in the Icelandic sample (Stefansson *et al*., 2002) in a cohort of 242 unrelated Korean schizophrenia patients and 242 matched controls (Kim *et al*., 2006). In the above-mentioned study, only *SNP8NRG241930* showed a tendacy toward significance in the schizophrenia sample and a significant association was only observed when the schizophrenia group was sub-stratified into patients with auditory hallucinations (section 1.1.1). However, as with the study by Li *et al*., 2004, this study also did not replicate the haplotype association findings of Stefannson and colleagues. Instead, their results showed that another haploytype containing the opposite alleles reported by Stefansson and co-workers was significantly increased in the control group (Kim *et al*., 2006).

The failure of the studies by Li and co-workers and Kim and co-workers to replicate the findings in the Icelandic, Scottish and Irish studies (Stefansson *et al.,* 2002, Williams *et al.,* 2003; Corvin *et al.,* 2004) is not surprising as the origins of Northern European and Chinese populations are separated by tens of thousands of years and different relationships between risk haplotypes and disease susceptibility variants may have evolved during this time (Owen *et al.,* 2004). The same unidentified disease-predisposing genetic variant or variants may be present in both European and Chinese populations but are associated with different overlying haplotypes (Li *et al.,* 2004).

There have also been reports of association studies between a non-synonymous *NRG1* polymorphism (*Arg38Gln*), located in exon 2 of *NRG1*, and schizophrenia. Yang *et al.*, in a study of 246 Han Chinese trios, reported a significant association between this polymorphism and schizophrenia (Yang *et al* . 2003). In an attempt to replicate this finding, Hong and co-workers conducted case-control and family-based association studies of *NRG1-Arg38Gln* in 228 schizophrenic patients and 269 control individuals (Hong *et al.,* 2004). The family-based analysis showed that the *38Gln* allele was transmitted in excess to affected offspring, however no association was detected in their case-control analysis. The discrepancy between results from family-based and case-control studies can be explained in various ways. Firstly, the *NRG1* polymorphism may be a disease locus of small effect and, therefore, the failure of these investigators to detect any association in their case-control study may be a false negative result due to low statistical power for detecting such effects. Secondly, the *NRG1* polymorphism may have no involvement in schizophrenia pathogenesis and the positive finding from the family-based study could be a false positive finding. Thirdly, since the case-control and family-based samples were from different sources (ie. different enthic origins, different diagnostic criteria), the inconsistency of association results between the two samples may be because of differences in disease severity or subtypes of the patients studied or genetic heterogeneity in susceptibility loci.

1.4.6.2.5. G72/G30 and D-amino acid oxidase (*DAAO*)

As a result of significant linkage of schizophrenia to chr13q34, Chumakov and colleagues (2002) conducted an in-depth study of SNPs localized to this region in 213 French-Canadian schizophrenia cases and 241 French-Canadian control subjects. One hundred and ninety one SNPs were identified across the region of interest from pools of 100 unrelated individuals, by sequencing amplicons across the 5Mb segment from chr13q34. Disease association analysis in the schizophrenia affected patients revealed two smaller regions

containing markers that reached nominal significance ($p<0.05$), which these investigators referred to as Bin A (65.9Kb containing 11 SNPs) and Bin B (1.380Kb containing 5 SNPs) (Chumakov *et al.,* 2002). Bin A was shown to harbour two possible schizophrenia susceptibility genes, G72 and G30, which are overlapping but located on opposite DNA strands. G30 did not produce a translation product *in vitro*, thus only G72 was used in subsequent studies (Chumakov *et al.,* 2002). Polymorphisms in and around these genes were reported to be associated with schizophrenia in the French/Canadian sample, as well as a Russian sample (183 cases and 183 controls) (Chumakov *et al.,* 2004) (Tables 1.7 and 1.8).

No homologues for G72 were present in any databases at the time, nor did sequence analysis of putative open reading frame (ORF) yield any likely function. Consequently, Chumakov and colleagues used yeast two-hybrid (Y2H) analysis in order to identify putative G72 interactors in an attempt to assign function to G72. Their analysis showed that G72 interacts with D-amino acid oxidase (DAAO), a detoxifying enzyme against exogenous D-amino acids (Owen *et al.,* 2004) and a potent activator of NMDA and glutamate receptors (Stevens *et al.,* 2003), implicating G72 in schizophrenia via the glutamatergic theory (Konradi and Heckers, 2003). Subsequently, assuming that the interactor(s) of a putative schizophrenia susceptibility gene may themselves also constitute schizophrenia susceptibility factors, eight SNPs covering the *DAAO* gene on chr12q24 were genotyped and tested for association in the same French-Canadian sample that led to the discovery of G72 (Chumakov *et al.,* 2002). Four intronic SNPs in partial LD (MDAAO-4, MDAAO-5, MDAAO-6 and MDAAO-7) were significantly associated with schizophrenia.

The G72/G30 genes were also reported to be associated with schizophrenia in an Ashkanazi Jewish cohort of 60 patients and 130 unrelated controls (Korostishevsky *et al.,* 2004). Moreover, the expression of G72 was shown to be decreased in the post-mortem dorsolateral prefrontal cortex (DLPFC) of schizophrenics, compared to control subjects (Korostishevsky *et al.,* 2004). Association with these two genes has also been reported in a Chinese schizophrenia sample (Wang *et al.,* 2004)., while the association of DAAO with schizophrenia in the French/Candian sample (Chumakov *et al.,* 2002) has recently been replicated in an independent study of 547 Chinese schizophrenia patients and 536 matched control individuals (Liu *et al.,* 2004).

The discovery of the interaction of G72 with DAAO (Chumakov *et al.,* 2002), coupled with the reported association of both these two genes with schizophrenia, represents the first time it was demonstrated that the interaction between two genes, by means of physical interaction of their products, can potentially account for increased risk of developing schizophrenia (Cloninger *et al* , 2002). This, together with the fact that associations of G72 and DAAO with schizophrenia has been replicated in both European and non-European populations, will have important implications in our understanding of schizophrenia pathogenesis. Furthermore, the success of this approach in identifying novel candidate susceptibility genes emphasize the need for a mindshift from the investigation of single genes, to the contemplation of pathways and interactomes in the search for genes involved in complex disorders.

Table 1.7: **Statistical analysis of SNPs in and close to Bin A in French Canadian and Russian cohorts from the study by Chumakov *et al.*, 2002.**

French-Canadian Sample (213 schizophrenia cases and 241 control subjects.)																			
SNPs	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22	M23	M24
BinA position																			
Gene position (G72)																			
Polymorphism	A/G	A/G	A/G	C/T	A/G	C/T	A/G	A/C	A/G	A/G	A/G	A/G	A/C	C/T	A/G	C/T	A/G	C/T	A/T
Allele frequency (cases)	0.50	0.65	0.32	0.30	0.69	0.44	0.64	0.63	0.64	0.65	0.61	0.69	0.44	0.31	0.40	0.63	0.69	0.57	0.55
Allele frequency (controls)	0.49	0.62	0.29	0.27	0.66	0.37	0.55	0.57	0.58	0.58	0.57	0.67	0.40	0.28	0.39	0.58	0.60	0.49	0.47
p-value for allelic tests	>0.01	>0.01	>0.01	>0.01	>0.01	0.062	**0.007**	0.071	**0.038**	**0.032**	>0.01	>0.01	>0.01	>0.01	>0.01	0.069	**0.003**	**0.019**	**0.019**
Russian Sample (183 schizophrenia cases and 183 control subjects.)																			
Allele frequency (cases)	not done	not done	not done	0.27	0.71	0.40	0.57	0.56	0.53	not done	not done	0.72	0.41	not done	0.41	0.62	0.65	0.60	0.54
Allele frequency (controls)	not done	not done	not done	0.29	0.71	0.37	0.59	0.58	0.53	not done	not done	0.76	0.43	not done	0.39	0.61	0.63	0.51	0.48
p-value for allelic tests	not done	not done	not done	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01	not done	not done	>0.01	>0.01	not done	>0.01	>0.01	>0.01	**0.017**	>0.01

Abbreviations: DAOO, D- amino acid oxidase

Twenty markers were selected in and around Bin A and analysed for association with schizophrenia. The results shown in this Table represents univariate analysis. The position of Bin A in reference to the SNP markers is shown by the solid yellow block, while the position of G72 is shown by the solid orange block. The polymorphism studied is indicated and the allele with increased frequency in French Canadian cases compared to French Canadian controls is indicated by blue font. Significant p-values lower than 0.05 are highlighted in red (Adapted from Chumakov *et al.*, 2002)

Table 1.8. **Analysis of DAAO SNPs in French-Canadian samples**

SNPs	MDAAO-1	MDAAO-2	MDAAO-3	MDAAO-4	MDAAO-5	MDAAO-6	MDAAO-7	MDAAO-8
DAOO gene location								
Polymorphism	C/T	C/T	A/C	A/C	C/T	G/T	A/G	A/G
Allele frequency in cases (N=213)	0.76	0.44	0.50	0.36	0.28	0.59	0.33	0.63
Allele frequency in controls (N=241)	0.75	0.42	0.47	0.28	0.21	0.49	0.25	0.61
p-value for allelic tests	>0.01	>0.01	>0.01	**0.017**	**0.007**	**0.0001**	**0.01**	>0.01

Abbreviations: DAOO, D- amino acid oxidase

Eight SNPs analysed in and around DAAO: 24-1443/126 (MDAAO-1), 27-81/180 (MDAAO-2), 24-1457/52 (MDAAO-3), 27-29/224 (MDAAO-4), 27-93/181 (MDAAO-5), 27-2/106 (MDAAO-6), 24-1461/256 (MDAAO-7) and 27-1/61(MDAAO-8). Data for markers and results from univariate analysis are shown. The DAAO gene's relative position in reference to SNPs is indicated by the solid green block. The allele with increased frequency in French Canadian cases ,compared to French Canadian controls, is indicated in blue font. P- values (lower than 0.05) are shown in red (Adapted from Chumakov *et al.,* 2002).

1.4.6.2.6. Regulator of G-Protein signalling 4 (*RGS4*)

The regulator of G-protein signalling 4 (RGS4) is a member of a GTPase-activating protein family that modulate many heterotrimeric G-protein-coupled receptors (GPCR). They shorten the duration of the intracellular signaling of many G-protein-coupled receptors belonging to the dopamine, GABA, glutamate and other neurotransmitter systems (Chowdari *et al.,* 2002).

cDNA microarray analysis of global gene expression showed that RGS4 was most consistently and significantly down-regulated in the prefrontal cortex (PFC) of schizophrenic patients, compared to control subjects (Mirnics *et al.,* 2001). Interestingly, expression levels of other members of the RGS family remained unchanged (Mirnics *et al.,* 2001). RGS4 maps to chr1q21-22, which has been linked to schizophrenia susceptibility in a number of independent linkage studies (Brzustowicz *et al.,* 2000; Shaw *et al.,* 1998). RGS4 is also highly expressed in brain regions implicated in the pathophysiology of schizophrenia (Ni *et al.,* 1999).

Comment [IT48]: Ni, Y.G., Gold, S.J., Iredale, P.A., Terwilliger, R.Z., Duman, R.S. and Nestler, E.J. (1999) Region-specific regulation of RGS4 (regulator of G-protein-signaling protein type 4) in brain by stress and glucocorticoids: *in vivo* and *in vitro* studies. *J. Neurosci.*, **19**, 3674–3680

Chowdari and co-workers used a combination of family-based and case-control association strategies to determine whether altered RGS4 expression reflects a primary inherited anomaly in schizophrenia (Chowdari *et al.,* 2002). They investigated two parent-proband trio samples sets from the North American cohorts, comprising 55 Caucasian parent-offspring trios from Pittsburg and 25 parent-offspring trios from the National Institute of Mental Health Collaborative Genetics sample. The study further investigated the possible role of RGS4 in schizophrenia using 269 parent-offspring trios from India, as well as a Caucasian cohort from the U.S comprising 55 schizophrenia cases and 85 control individuals for case-control analysis. These investigators reported significant associations that were independently obtained in the North American trio samples for three SNPs as well as a haplotype encompassing four SNPs (designated SNP 1, 4, 7 and 18) in the 5' flanking region and first intron of RGS4 (Chowdari *et al.,* 2002) (Fig 1.11). No evidence for association was detected in the Indian sample at the SNP or haplotype level. In the two U.S samples (two trio cohorts and the case control

cohort), different alleles and haplotypes were associated with disease, which raises the possibility that the results of the TDT analysis may reflect independent risk factors between the two samples. Although significant TDT results were not obtained for the Indian cohort, the overall probability for biased parental transmission of haplotypes bearing SNPs1, 4, 7 and 18 was significant when accumulated over the three cohorts.

RGS4 involvement in schizophrenia was also evaluated in the ISHDF sample from Ireland. Single marker TDT for the four core SNPs implicated in the original study (Chowdri *et al.,* 2002) showed modest association with SNP 4 and 18, while for SNPs 1 and 7, the overtransmitted alleles to schizophrenia subjects were the same as previously reported (Chen *et al.,* 2004).

Williams and co-workers was able to replicate the evidence for two of the four SNPs (SNPs 4 and 18) that had previously been associated. Furthermore, their global tests for association using two, three and four marker haplotypes provided strong evidence for association with a haplotype contructed from these two markers (Williams *et al.,* 2004).

These three reports represent the body of evidence collected, thus far, implicating *RGS4* as a schizophrenia susceptibility gene. However, no studies have yet provided a link between particular *RGS4* genotypes or haplotypes and decreased gene expression in the PFC of schizophrenic patients. Furthermore, in a more recent family-based association study, Liu and co-workers failed to show an association with any of the four SNPs used in the afore-mentioned investigations (Chowdari *et al.,* 2002; Chen *et al*., 2004; Liu *et al*., 2004) at single locus or haplotype level in 218 schizhophrenia pedigrees from Taiwan (Liu *et al*., 2006).

1.4.6.3. Association studies of schizophrenia endophenotypes

Schizophrenia endophenotypes (section 1.1.4) have also been used in candidate gene association studies. Specifically, the *Val158* allele of *COMT* has been linked to impaired working memory and executive dysfunction as assessed by the Wisconsin Card Sorting Test (WCST) and N-back test (Egan *et al.,* 2001; Joober *et al.,* 2002; Goldberg *et al.,* 2003). On the other hand, Bilder *et al*., (2002) reported that schizophrenic patients carrying the *Val158* allele performed worse on tasks testing attention and speed of information processing, but not on executive functions

Tsai and colleagues (2003) provided support for these findings. They considered performance on the WCST and the auditory P300 ERP, a PPI test, to test for association with *COMT,* using a cohort of 120 healthy young Chinese females. They failed to find an association with *COMT* and performance on the WCST, but showed that subjects with the *Val158* showed prolonged latency to the P300 ERP (Tsai *et al.,* 2003). Moreover, in a recent study of 49 unrelated schizophrenia sufferers of German descent and 170 unaffected control individiuals (also of German descent), these findings were replicated (Gallinat *et al.,* 2003).

Antal and co-workers (2004) recently reported an association between the *DRD3 ser9* allele and impaired executive function. These investigators demonstrated that schizophrenic patients carrying the *ser9* allele of *DRD3* exhibited worse performances on the WCST compared to those carrying the *gly9* allele (Antal *et al.,* 2004). Interestingly, the *COMT Val158* and *DRD3 ser9* alleles have both been associated with EMD in schizophrenia (Rybakowski *et al.,* 2001; 2002).

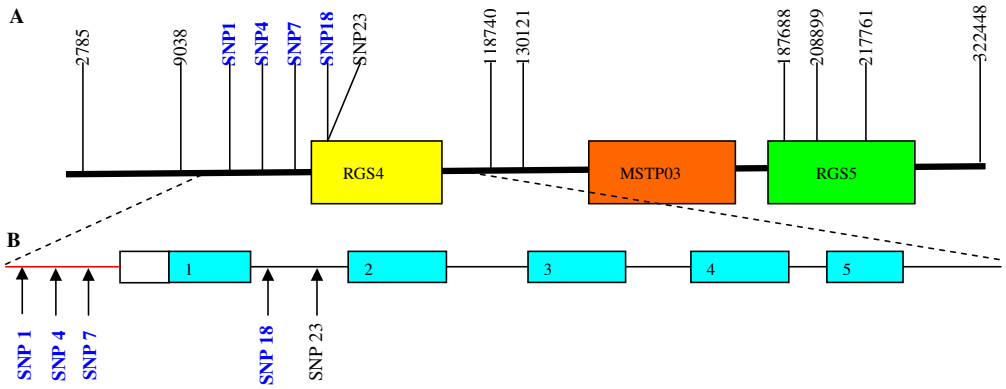


Fig 1.11: Schematic representation of the genomic organization of *RGS4* and flanking regions. The *RGS4* gene is indicated by a yellow block in panel A. Exons are indicated by blue blocks, while introns are represented by a black line and the promoter by a red line in panel B. All SNPs are indicated on the diagram, with SNPs comprising the four marker haplotype indicated by the blue font (Adapted from Chowdri *et al.,* 2002).

1.4.7. Association Studies in OCD

To date, association studies in OCD have focused on functional candidate genes. Most of the candidates analysed are involved in the metabolism of CNS neurotransmitters. Recently, however, genes involved in CNS development have also been targeted. As these results also have bearing on choosing novel candidate genes, the following section will review the result of studies of some of the most promising candidate genes for OCD.

1.4.7.1. Serotonin system genes

The efficacy of SSRIs, and the absence of improvement of OCD symptoms after treatment with norepinephrine-reuptake inhibitors and dopamine antagonists, presents strong evidence for the involvement of 5-HT in the pathophysiology of OCD (Barr *et al.,* 1992; Goodman *et al.,* 1990; Murphy *et al.,* 1989). Thus, genes in the serotonergic pathway, such as the 5-HT transporter (*SLC6A4*) and serotonin receptors (*5-HTR*), are good candidate genes for conferring susceptibility to OCD. Neurotransmission mediated by 5-HT contributes to many physiological functions such as motor activity, food intake, sleep and reproductive activity. It also contributes to cognition and emotional states including mood and anxiety (Ramboz *et al.,* 1998).

1.4.7.1.1. Serotonin Transporter (*5-HTT*)

Serotonergic neurotransmission is terminated by the active transport of 5-HT back into the presynaptic neuron by the serotonin transporter protein (5-HTT). The latter regulates the magnitude and duration of serotonergic

responses by mediating the sodium-dependent re-uptake of 5-HT into presynaptic nerves, and, in so doing, it is central to the fine-tuning of brain serotonergic neurotransmission and the peripheral actions of 5-HT (Lesch *et al.,* 1996). The expression of 5-HTT in the brain is notably abundant in the cortical and limbic areas involved in the emotional aspects of behaviour.

The 5-HTT protein is one of several structurally similar sodium-dependent transporter proteins that contain 12 putative membrane-spanning regions (Amara and Kahar, 1993), and is encoded by a single gen e, *SCL6A4* (solute carrier family 6 [neurotransmitter serotonin], member 4),on chromosome 17q12 (Ramamoorthy *et al.,* 1993) and has the official gene symbol. A unique GC-rich repetitive sequence is located in the proximal 5' regulatory region of *SCL6A4*. Heils *et al.,* (1996) revealed a common polymorphism in this region (*5-HTTLPR*) generated by a 44bp insertion/deletion sequence (*l*/*s*) This polymorphism has been shown to have a significant effect on blood 5-HT level (Hanna *et al.,* 1998), and has been the subject of numerous OCD association studies, summarised in Table 1.9.

In an association study between *SCL6A4* and OCD, McDougle *et al.,* (1998) employed a TDT design in 34 European-American triads and found that the *l* allele of *5-HTTLPR* was significantly more commonly transmitted by heterozygous parents to their OCD affected offspring. In a population-based association study of 75 Caucasian OCD patients and 397 ethnically matched control individuals, Bengel *et al.,* (1999) demonstrated that OCD patients were more likely to be homozygous for the *5-HTTLPR l* allele than control individuals. In a meta-analysis of association studies of *5-HTTLPR* and OCD (129 Caucasian OCD patients and 479 Caucasian control individuals), however, Kinnear and colleagues found no association (p=0.108) (Kinnear *et al.,* 2000). Moreover, another study found that the *5-HTTLPR* polymorphism did not appear to influence response to pharmacotherapy in OCD, thus raising some doubt about the functional importance of the above-mentioned findings (Billett *et al.,* 1997).

1.4.7.1.2. Serotonin receptors

The serotonin receptors $1D_{\beta}$, (5-$HT1D_{\beta}$), 2A (5-HT2A) and 2C (5-HT2C) have also been extensively investigated as possible OCD susceptibility genes (Table 1.10). The 5-$HT1D_{\beta}$ gene (*5-HT1D$_{\beta}$*) is of particular interest: it encodes a terminal auto-receptor and challenge studies of non-selective ligands of this receptor (eg. *meta*-chlorophenyl piperazine, *m*-CPP) showed an acute worsening of OCS in OCD patients (Gross *et al.,* 1998). Worsening of OCS has also been reported following acute administration of sumatriptan, a selective agonist to 5-$HT1D_{\beta}$ (Gross *et al.,* 1998). However, some OCD patients who do not respond to conventional pharmacotherapy have been reported to respond to chronic administration of sumatriptan (Stern *et al.,* 1998).

Comment [IT49]: Gross R, Sasson Y, Chopra M, Zohar J: Biological models of obsessive-compulsive disorder: the serotonin hypothesis, in Obsessive-Compulsive Disorder: Theory, Research, and Treatment. Edited by Swinson RP, Antony MM, Rachman S, Richter MA. New York, Guilford, 1998, pp 147–148

Table 1.9: **Association studies of *5-HTTLPR* and OCD**

Study	Cohort	Method	Findings	p-value
McDougle *et al.,* 1998	• 34 European-American parent-offspring trios	TDT	The "*l*" allele was found to be transmitted more frequently to patients	χ^2 =4.83, p=0.03
Bengel *et al.,* 1999	• 75 Caucasian OCD patients and 397 ethnically matched controls	Case-control association	Patients were more likely to carry two copies of the "*l*" allele	χ^2 =5.19, p=0.023
Frisch *et al.,* 2000	• 39 Ashkanazi Jewish OCD patients and 112 ethnically matched controls	Case-control association	No association	p>0.05
	• 34 non-Ashkanazi Jewish OCD patients and 60 ethnically matched controls	Case-control association	No association	p>0.05
Kinnear *et al.,* 2000	• Meta-analysis of 129 Caucasian OCD patients and 379 matched controls	Case-control association	No association	p>0.05
Camarena *et al.,* 2001	• 115 Mexican OCD patients and 136 ethnically matched controls	Case-control association	No assocaition	χ^2 =1.54, p=0.21
	43 Mexican parent-offspring OCD trios	TDT	No association	p>0.05
Meira-Lima *et al.,* 2001	• 79 Brazilian OCD patients and 202 controls	Case-control association	No association	p>0.05
Chabane *et al.,* 2004	• 106 French OCD patients and 171 ethnically matched controls	Case-control association	No association	χ^2 =0.84, p=0.36
	• 116 parent-offspring OCD trios	TDT	No Association	p>0.05
Walitza *et al.,* 2004	• 64 parent-offspring OCD trios	TDT	No association	p=0.16
Denys *et al*., 2006	• 156 Caucasian OCD patients and 134 ethnically matched controls	Case-control association	Female patients were more likely to carry the S allele compared to female controls	χ^2 =6.0, p=0.014

Abbreviations: OCD, Obsessive-compulsive disorder; TDT, Transmission disequilibrium test

Mundo and colleagues reported an association between a silent G-to-C substitution at nucleotide 861 (*G861T*) of *5-HT1D$_\beta$* using a family-based study design (Mundo *et al.,* 2000). Their investigation showed the preferential transmission of the G allele to affected individuals (Mundo *et al.,* 2000) (Table 1.9). This finding was followed up in an extended sample of 121 OCD families in whom the *G861C* polymorphism, as well as a T-to-G substitution at nucleotide 371 (T371G), were used in a TDT analysis (Mundo *et al.,* 2002) (Table 1.9), which confirmed the findings of the original investigation (Mundo *et al.,* 2000; Mundo *et al.,* 2002). However, two independent studies found no association of the *5-HT1D$_\beta$* G861C polymorphism with OCD (Di Bella *et al.,* 2002, Hemmings *et al.,* 2003) (Table 1.10).

The 5-HT2A receptor gene (*5-HT2A*) encodes a G-protein-coupled receptor that controls signal transduction pathways by activating phospholipase C (Berg *et al.,* 1998). Furthermore, the enhancement of serotonergic neurotransmission by activation of 5-HT2A is a common feature of many serotonin-reuptake inhibitors (SRI) (Greenberg *et al.,* 1998). Two *5-HT2A* SNPs, in complete LD with each other, have been investigated for their potential role in OCD pathogenesis: a T-to-C transition at nucleotide 102 (*T102C*), that does not alter the amino acid sequence of the encoded protein, and a G-to-A transition at position -1438 (*-1438G/*A) in the promoter region. The *-1438G/*A polymorphism was reported to be associated with TS with co-morbid OCD but not with pure TS in both case-control and TDT analyses of a cohort of Chinese individuals (Huang *et al.,* 2001). From these results, it can be speculated that while the *5-HT2A -1438G/*A polymorphism may not be involved in the pathogenesis of TS, it may play a role in the manifestation of OCS. However, several other investigations failed to show any association between *5-HT2C* and OCD (Table 1.10).

Serotonin 2C receptors are the most abundant 5-HT receptors in the basal ganglia, particularly in the globus pallidus and the substantia nigra, which are involved in the neural circuit invoked for OCD (Rapoport *et al.,* 1990). This, coupled with the fact that the effects of *m*-CCP can be inhibited by a variety of agonists and antagonists that specifically act on the 5-HT2C receptor, makes this receptor an attractive OCD candidate. A polymorphism causes an amino acid change at position 23 (*Cys23Ser)* (Lappalainen *et al.* 1995), although the variant has no clear functional significance (Cavallini *et al.,* 1998). Association studies have been undertaken of this 5-HT2A polymorphism in OCD, however, none have as yet reported an association (Table 1.10).

1.4.7.2.Dopamine system genes

1.4.7.2.1. Dopamine receptor 4 (*DRD4*)

Augmentation of SSRI treatment with dopamine agonists appears to be useful in a subset of OCD patients (McDougle *et al.,* 1994), implicating the dopaminergic pathways in OCD pathogenesis. Of all the dopamine receptors, the dopamine receptor 4 (DRD4) has received most attention as possible role player in OCD. For this reason, this section of the review will only focus on DRD4. The DRD4 gene (*DRD4)* encodes a receptor with seven hydrophobic trans-membrane regions joined by three extra-cellular peptide loops (Baldessarini and Turazi, 1996). The 48-bp variable number of tandem repeats (VNTR) polymorphism in exon 3 (van Tol *et al.,* 1992) has been the *DRD4* variant most frequently investigated.

Table 1.10: **Summary of association studies of 5-HT receptors and OCD.**

Receptor	Polymor-phism	Cohort	Method	Findings	P-value	Reference
5-HT1D$_\beta$	*G861C*	• 22 OCD trios where 10 were sib-ships of one affected and one unaffected sib	Combination of TDT and ASP	Preferential transmission of the *G* allele to affected individual	p<0.006	Mundo *et al.*, 2002
		• 121 OCD trios	TDT	Preferential transmission of the *G* allele to affected individual	p=0.02	
		79 OCD parent-offspring trios	TDT	No association	p=0.13	Di Bella *et al.*, 2002
		• 71unrelated Afrikaner OCD patients and 129 ethnically matched controls	Case-control association	No association	p=0.621	Hemmings *et al.*, 2003
5-HT2A	*-1438G/A*	• 62 Caucasian OCD patients and 144 Caucasian controls	Case-control association	Increase frequency of the *A* allele in OCD patients compared to controls	p<0.05	Enoch *et al.*, 2001
		• 101 Caucasian OCD patients and 138 Caucasian controls	Case-control association	Increase frequency of the *A* allele in OCD patients compared to controls	p=0.015	
		• 58 Turkish unrelated OCD patients and ethnically matched controls	Case-control association	No association	p>0.05	Tot *et al.*, 2003
		• 156 Caucasian OCD patients and 134 ethnically matched controls	Case-control	Patients with family istory of OCD had a significant perponderance of *GG* genotype	p=0.015	Denys *et al.*, 2006
	T102C	• 75 unrelated Jewish OCD patients and 172 ethnically matched controls	Case-control association	No association	p>0.05	Frisch *et al.*, 2000

Receptor	Polymor-phism	Cohort	Method	Findings	P-value	Reference
5-HT2A	*T102C*	• 67 Mexican OCD patients and 54 ethnically matched controls	Case-control association	No association	p>0.05	Nicolini *et al.,* 1996
		• 157 Chinese Tourette's syndrome trios	TDT	Association found only in Tourette's patients with co-morbid OCD	p=0.02	Huang *et al.,* 2001
		• 157 Chinese Tourette's patients and 120 Chinese controls	Case-control association	Association found only in Tourette's patients with co-morbid OCD	p=0.004	
		• 58 Turkish unrelated OCD patients and ethnically matched controls	Case-control association	No association	p>0.05	Tot *et al.,* 2003
		• 71unrelated Afrikaner OCD patients and 129 ethnically matched controls	Case-control association	No association	p=0.24	Hemmings *et al.,* 2003
5-HT2C	*Cys23Ser*	• 109 Italian OCD patients and 171 ethnically matched controls	Case-control association	No association	p=0.224	Cavallini *et al.,* 1998
		• 75 unrelated Jewish OCD patients and 172 ethnically matched controls	Case-control association	No association	p>0.05	Frisch *et al.,* 2000

Abbreviations: *5-HT1D*$_{\beta}$, Serotonin receptor 1D β subunit; *5-HT2A*, serotonin receptor 2A; 5-HT2C, serotonin recptor 2C; OCD, Obsessive-compulsive disorder; TDT, Transmission disequilibrium test

While examining the role of *DRD4* in OCD in a case-control setting, Billett and colleagues (1998) observed a decrease in frequency of the exon 3 VNTR 2-4 repeat alleles among the patient group (Table 1.10). This observation was, however, found to be non-significant following correction for multiple testing (Billett *et al.,* 1998). Frisch and co-workers (2000) also failed to provide compelling evidence for the involvement of this gene in OCD (Frisch *et al.,* 2000) (Table 1.10). Recently, however, Millet *et al.,* (2003) reported an association between the 2-repeat allele and OCD in both population-and family-based association studies (Millet *et al.,* 2003) (Table 1.11). Interestingly, in an investigation into the possible role of *DRD4* in phenotypic variance among OCD patients, Cruz and co-workers observed a high frequency of the 7-repeat allele among the group with comorbid chronic motor and vocal tics (Cruz *et al.,* 1997). This finding was later replicated by the same group of researchers in an independent patient sample (Nicolini *et al.,* 1998) (Table 1.11). However, the study by Millet *et al* . (2003) did not find any association between any *DRD4* VNTR alleles and the presence of comorbid tics (Millet *et al.,* 2003).

1.4.7.2.2. Catechol-O methyltransferase (*COMT*)

The *COMT Val/Met* polymorphism (section 1.1.3.1.1) was used in two North American studies to determine the role of *COMT* in OCD. Both of these studies showed an association between OCD and the *Met* allele of *COMT* (Karayiorgou *et al.,* 1997; Karayiorgou *et al.,* 1999) (Table 1.11). However, in a subsequent study on a Japanese population, no association between functional variants of COMT and anxiety disorders, including OCD, was detected (Ohara *et al.,* 1998b). More recently, Niehaus and co-workers (2001) reported association between the *Val/Met* heterozygous genotype and OCD in a cohort of Afrikaner subjects (Niehaus *et al.,* 2000), while Alsobrook found an association with the *Met* allele in female OCD patients. The latter confirmed a previous report supporting a sexual dimorphic effect of the *COMT Met* allele and OCD (Karayiorgou *et al.,* 1999; Alsobrook *et al.,* 2002) (Table 1.11). In 2003, Azzam and Mathews published a meta-analysis of three case-control and five family-based association studies of *COMT* and OCD (Azzam and Mathews., 2003). Overall, the findings from their meta-analysis provided no evidence for an association between *COMT* and OCD.

These disparate results may be a reflection of the genetic differences between population groups used in the various studies; thus, it could be speculated that the impact of *COMT* on disease susceptibility may vary from population to population. One also has to consider the varying diagnostic criteria used in the different studies. Earlier studies (eg., Karayiourgou *et al.,* 1997, Ohara *et al.,* 1998) used DSM-III-R diagnostic criteria, while the more recent studies (Karayiourgou *et al.,* 1999, Niehaus *et al.,* 2001, Alsobrook *et al.,* 2002) used DSM-IV criteria. The clinical heterogeneity of OCD further confounds association studies. Several reports provide evidence for distinctive OCD subtypes eg., age of onset (Hemmings et al, 2004), symptom dimensions (Lochner *et al.,* 2005) and gender (Lochner *et al.,* 2004), therefore, the variability between studies may be the result of the particular combination of OCD subtypes, presumably driven by diverse combinations of genetic and environmental susceptibility factors, used in each study. Thus, it is likely that the complex role of *COMT* in OCD may be better elucidated using clinically and genetically homogeneous OCD cohorts.

14.7.2.3. Monoamine oxidase A (*MAOA*)

Monoamine oxidase (MAO) is a flavin-containing enzyme that degrades a variety of biogenic amines, including the neurotransmitters norepinepherine, dopamine and 5-HT (Weyler *et al.,* 1994). Two forms of the enzyme, MAOA and MAOB, have been identified on the basis of the difference in their molecular weight,substrate specificity, substrate affinities, inhibitor sensitivities and immunological properties (Garrick and Murphy, 1982). In the human brain, MAOB is the prominent form (Garrick and Murphy, 1982), expressed at the highest levels in the astrocytes and serotonergic neurons. In contrast, MAOA, although expressed in the serotonergic neurons as well, is expressed at highest levels in the catecholaminergic neurons (Fowler *et al.,* 1987; Thorpe *et al.,* 1987). In *MAOA* knock-out mice, 5-HT levels in the brain have been shown to be increased up to nine-fold, with the mice exhibiting distinct behavioural changes, including trembling, difficulty in righting (getting upright after being inverted), and fearfulness (Cases *et al.,* 1995). In humans, moderate inhibition of MAOA and MAOB activity can lead to mood elevation, loss of 'rapid eye movement' (REM) sleep, motoric hyperactivity, orthosomatic hypertension and hyperflexia without spasticity (Kupfer and Bowers 1972; Murphy *et al.,* 1983).

The two MAO isoform-encoding genes, *MAOA* and *MAOB*, have been mapped to Xp11.23-11.4 (Ozelius *et al.,* 1988; Levy *et al.,* 1989). Abnormal and often overly aggressive behaviour in five male cases of X-linked borderline mental retardation was attributed by Brunner *et al.,* (1993) to a truncating point mutation in exon 8 of *MAOA,* suggesting a correlation between inappropriate emotional responses and the elimination of MAOA activity via a genetic mutation. However, it has also been observed that drugs that inhibit MAOA activity is effective in the treatment of OCD (Erfurth and Schuss, 1993), and, as MAOA plays crucial roles in COMT-controlled pathways, the gene has been investigated for involvement in OCD pathogenesis.

Hotamisligil and Breakefield (1991) described a silent A to G substitution at codon 287 (exon 8) of *MAOA* (Hotamisiligil and Breakefield, 1991). This synonymous substitution has been associated with varying enzyme activity levels (Hotamisligil and Breakefield, 1991), with the *297CGG* allele conferring higher enzyme activity. Karayiorgou and colleagues (1999) used this polymorphism in an association study of OCD. As they previously described a sexual dimorphic effect of *COMT* on OCD, and since *MAOA* is localised to the X chromosome, they tested for homogeneity of transmission disequilibrium between male and female probands, by comparing transmission of alleles from heterozygous mothers. They showed a significant difference between the transmission of alleles between the two genders. They then investigated 110 nuclear OCD families using TDT and HRR analysis, which confirmed a sexual dimorphic effect of *MAOA* on OCD, with preferential transmission of the *297CGG* allele to affected male probands (Karayiorgou *et al.,* 1999). Similarly, Camarena and colleagues, in both a case-control association study and in 51 OCD parent-offspring trios, found that significantly more female OCD patients carried the *297CGA* allele compared to male OCD patients (Camarena *et al.,* 1998) (Table 1.10). Lochner and co-workers showed a significant association between OCD and another *MAOA* polymorphisms, a C to T substitution at position 1460, with the *C* allele being more prevalent in female OCD patients ($v2=6.763$; $p=0.009$).

Table 1.11: **Association studies of selected Dopamine system genes in OCD.**

Gene	Polymorphism	Cohort	Method	Findings	p-value	Reference
DRD4	*48bd VNTR*	• 12 OCD patients with comorbid tic and 49 OCD patients without comorbid tics	Case-control association	7-repeat allele associated with presence of tics in OCD	p=0.018	Cruz *et al.*, 1997
		• 12 OCD patients with comorbid tic and 66 OCD patients without comorbid tics	Case-control association	7-repeat and 2-repeat allele associated with presence of tics in OCD	p=0.018 (7-repeat) p=0.028 (2-repeat)	Nicolini *et al.*, 1998
		• 118 Caucasian OCD patients and 118 ethnically matched controls	Case-control association	No association after correcting for multiple testing	p>0.05	Billett *et al.*, 1998
		• 75 unrelated Jewish OCD patients and 172 ethnically matched controls • (39 Ashkanazi Jewish OCD, 36 Non-Ashkanazi Jewish OCD; 112 Akanazi Jewish , 60 non-Ashkanazi Jewish controls	Case-control association	No association in Ashkanasi Jewish sample, however the 7-repeat allele was significantly less frequent in non-Askanazi Jewish sample compared to controls	p>0.05 p=0.04 (non-Ashkanazi sample)	Frisch *et al.*, 2000
		• 71unrelated Afrikaner OCD patients and 129 ethnically matched controls	Case-control association	No association	p=0.07	Hemmings *et al.*, 2003
		• 55 OCD parent-offspring trios	Extended TDT	Absence of transmission of allele 2 to OCD patients	p=0.003 (allele) p=0.003 (genotype)	Millet *et al.*, 2003
		• 49 French OCD patients and 63 ethnically matched controls	Case-control association	Significantly lower frequency of the 2-repeat allele in OCD patients	p=0.002	
COMT	*Val/Met*	73 unrelated Caucasian OCD patients and 148 unrelated Caucasian controls	Case-control association	*L* allele significantly associated with OCD, particularly in males	p=0.0002	Karayiorgou *et al.*, 1997
		110 nuclear OCD families	TDT HRR	*L* allele associated with OCD only in males	p=0.0079 (TDT) p=0.00146 (HRR)	Karayiorgou *et al.*, 1999
		• 72 OCD parent-offspring trios	TDT	No association with either allele, however an association with homozygosity	p=0.056 (genotype) p=0.46(allele) p=0.017 (homozygosity)	Schindler *et al.*, 2000
		• 54 unrelated Afrikaner OCD patients and 54 unrelated Afrikaner controls	Case-control association	*H/L* genotype significantly associated with OCD	P=0.017	Niehaus *et al.*, 2001
		• 56 OCD parent-offspring trios	Case-control association	No association with family sample as a whole, however HRR gave a statistically significant association of *L* allele with OCD in females	p=0.174 (whole sample) p=0.0048 (HRR-females)	Alsobrook *et al.*, 2002

Gene	Polymorphism	Cohort	Method	Findings	p-value	Reference
COMT	*Val/Met*	• 59 OCD patients and 114 unrelated controls	Case-control association	No association	P>0.05	Erdal *et al.,* 2003
		• Meta-analysis of 3 case-control association studies and 4 familiy-based studies	Case-control asoociation and TDT	No association	Not available	Azzam and Mathews, 2003
		• 110 nuclear OCD families	TDT and HRR	Preferential transmission of *297CGG* allele to affected males	p=0.018 (TDT) p=0.012 (HRR)9	
MAOA	*A297G*	• 122 Mexican OCD patients and 124 Mexican controls	Case-control association	Association found with *297CGA* allele and OCD in females	p=0.024	Camarena *et al.,* 2001b
		• 51 OCD parent-offspring trios	HRR	Association found with *297CGA* allele and OCD in females	p=0.022	

Abbreviations: COMT, catecol-O-methyltransferase; DRD4, Dopamine receptor 4; HRR, Haplotype relative risk; MAOA, Monoamine oxidase A; OCD, Obsessive-compulsive disorder; TDT, Transmission disequilibrium test

All the results above provide compelling evidence that gender plays a critical role in OCD, and more specifically, that MAOA exerts its effect on OCD in concert with both genetic and physiological mechanisms that are gender-specific.

1.4.8. Interactome analysis of previously identified candidate genes

The interactome refers to the interactions of proteins with one another within the cell. Investigating the interactions of candidate genes that have previously been implicated in disease aetiology can be used as a starting point to identify novel candidate genes for a particular disorder. One way of doing this is by using the yeast two-hybrid (Y2H) system (Fields and Song, 1989). This approach forms an integral part of the present investigation, so in this section, two examples of how the Y2H system (discussed in Chapter 2) has been successfully used in the identification of novel schizophrenia susceptibility genes will be discussed.

Comment [IT50]: Fields S, Song O-K (1989) A novel genetic system to detect protein-protein interactions. Nature 340: 245-246

1.4.8.1. G72 and DAAO

The first example was alluded to in section 1.4.6.2.5 and involves the identification of DAAO as a possible disease susceptibility locus using G72 as a "bait" in a Y2H screen.

In their investigation of the schizophrenia-linked genomic region on chromosome 13 (13q34), Chumakov an co-workers identified *G72* as a potential schizophrenia susceptibility gene (Chumakov et al., 2002). Preliminary functional analysis of the G72 protein indicated that it is localised to the endoplasmic reticulum and Golgi in transfected cells, is able to form multimers and is able to bind carbohydrates. The next question addressed by these investigators was whether the G72 protein could influence a pathway that is implicated in schizophrenia pathogenesis. To this end, they screened 500 000 independent clones from a human brain complementary (cDNA) library using the G72 protein as a bait. Their investigation identified DAAO as a protein that could bind to G72.

D-amino acid oxidase is a protein that oxidises D-serine, an allosteric activator of the NMDA type glutamate receptor (Fields and Song, 1989). Measurements of the ability of DAAO to effectively oxidise D-serine in the presence of increasing concentrations of G72 suggested that the G72 protein acts as an activator of DAAO. These results suggested that this interaction plays an important role in the regulation of NMDA glutamate receptors and that a dysregulation of either G72 or DAAO could result in glutamate receptor hypofunction. Glutamate receptor hypofunction, in turn, is a mechanism that has been implicated in schizophrenia susceptibility (Chumakov *et al.,* 2002). The identification of DAAO as an interactor of G72 has lead to its candidature as a schizophrenia susceptibility factor (section 1.4.6.2.5).

1.4.8.2. Acetylcholine receptor α4 subunit and the chaperone protein 14-3-3η (*YWHA*H)

The second example of how the Y2H system has been used in the identification of novel schizophrenia susceptibility genes focuses on the nicotinic acetylcholine receptor (AChR) α4 subunit. Nicotinic acetylcholine receptors, a family of ligand-gated homo-or heteropentameric ion channels expressed in the peripheral and CNS (Betz, 1990; Bernard, 1992), are made up of different combinations of α1- α9 and β2-β4 subunits

(Deneris *et al.,* 1991; Sargent, 1993). The large cytoplasmic domain present in each subunit is the most variable domain between the different AChR subunits and this variability provides the diversity for each AChR subtype to interact with unique cytosolic proteins with varying function (Jeanclos *et al.,* 2001).

In their investigation, Jeanclos and co-workers aimed to identify the interactors of the large cytoplasmic domain of α2β4 AChR. In order to achieve this, they used the large cytoplasmic domain of the nicotinic AChR α2 subunit as a "bait" to screen a mouse brain cDNA library by Y2H (Jeanclos *et al.,* 2001). They indentified a known protein, 14-3-3ή (YWHAH), which belongs to a family of proteins that primarily function as intracellular regulators or chaperone/scaffolding/adaptor proteins in diverse cellular processes (Wang and Shakes, 1996).

Jeanclos's investigation has some pathophysiological significance to schizophrenia, as it is well established that the chronic intake of nicotine in smokers increases the expression of α2β4 AChRs in their brains (Marks *et al.,* 1983), and that the frequency of smoking among schizophrenics is higher compared to non-schizophrenic individuals. Breese and colleagues (2000) investigated the levels of [^{3} H]-nicotine binding sites in the brains of schizophrenic patients. They showed reduced levels of nicotine binding, particularly to α4β2 AChR (Breese *et al.,* 2000) in schizophrenic smokers compared to control smokers, which could suggest that schizophrenics have deficencies in regulating the expression of α4β2 AChR (Jeanclos *et al.,* 2001).

Interestingly, *YWHAH* had previously been associated with schizophrenia in a Japanese population-based case-control association study (Toyooka *et al.,* 1999). The frequency of the 2-repeat allele of a VNTR polymorphism in the 5'-non-coding region of *YWHAH* occurred more frequently in Japanese schizophrenic patients than control individuals (Toyooka *et al.,* 1999).

1.4.9. Animal Studies

Animal models have generally been extremely useful in the investigation of mechanisms underlying human disease and the design of novel treatment strategies; however, this has not often been the case for psychiatric illnesses: it is impossible to faithfully reproduce cognitive disorders in less-cognitively developed animals (Marcotte *et al.,* 2001). Accordingly, current animal models of psychiatric disorders are not intended to serve as the complete animal equivalent of the human disorder; instead, they are often designed to model specific aspects of the disease and to test specific causative or mechanistic hypotheses.

These models can be validated on the basis of how well the animal's performance in a given test predicts the performance of humans with a specific condition (predictive validity) and on whether the model provides a sound theoretical rationale (construct validity), or whether it faithfully reproduce symptoms of the human condition (face validity). The latter is the most difficult to assess in behavioral disorders, however, a variety of behavioral correlates in animals have been considered to serve as appropriate markers for psychiatric disorders (Marcotte *et al.,* 2001). Thus, although it may not be possible to recreate the diversity and complexity of specific psychiatric disorders in a single animal model, combining insights gained via different models for a

specific condition, eventually leads to a better understanding of the aetiology of the disorder. In the following section, animal models of schizophrenia and OCD will be reviewed and their impact on our present understanding will be discussed.

1.4.9.1. Animal models of schizophrenia

Since a significant portion of the present study is based on the findings derived from the naturally occurring heterozygous reeler mouse model, and its relevance to the neurodevelopmental hypothesis of schizophrenia, particular emphasis will be placed on this animal model of schizophrenia in this review.

1.4.9.1.1. The Heterozygous Reeler Mouse (HRM): Neuropathology and behaviour

The reeler mouse first appeared spontaneously in 1948 in a stock of "snowy-bellied" mice kept at the Institute of Animal Genetics in Edinburgh, Scotland (D'Arcangelo and Curran, 2000). Currently, this strain, the rl^{ed} strain, is being maintained in the Jackson Laboratory in Maine. In 1961, a second spontaneously occurring reeler mouse strain, the rl^{or} strain, appeared in a laboratory in Orleans, France, currently maintained at the Pasteur Institute in Paris.

The reeler locus (*rl*) localized, by linkage analysis of the mutant phenotype in backcross progenies, to the proximal region of mouse chromosome 5 (section 1.4.1). Fine-mapping and exon-trapping eventually led to the discovery of *reelin* as the reeler-phenotype causing gene (D'Arcangelo *et al.,* 1995). Since then, several more reeler mouse strains have been generated spontaneously as well as by deliberate mutagenesis (D'Arcangelo and Curran, 2000). The characteristics of the different reeler strains are summarised in Table 1.12.

Table 1.12: **Characteristics of different reeler mouse strains.**

Origin	Symbol	*Reelin* mutation	mRNA mutation	Protein mutation
Edinburgh	rl^{ed}	Spontaneous ~150 kb genomic DNA deletion	Predicted truncation, no expression	No expression
Orleans	rl^{or}	Spontaneous L1 insertion near 3'	220 bp exon skipping and frameshift	C-terminal truncation
Transgene	rl^{tg}	*supfos* transgene insertion intragenic 7-to 10-kb deletion	No expression	No expression
Albany 1	rl^{alb1}	Chlorambucil-induced	Unknown	Unknown
Albany 2	rl^{alb2}	Chlorambucil-induced IAP insertion	85bp exon skipping and frameshift; reduced expression	Low-level predicted truncated product
Jackson 3J	rl^{3J}	Spontaneous	Reduced levels, higher molecular weight transcript	No expression

(Adapted from D'Arcangelo and Curran, 2000)

In the rl^{or} and rl^{ed} strains, the reeler mutation is autosomal recessive, and, by two weeks postnatally, homozygotes exhibit ataxia, tremors, imbalance and a typical reeling gait. These symptoms are associated with

severe hypoplasia of the cerebellum, as well as malpositioning of the neurons in the laminated brain structures such as the neocortex and hippocampus (Mariani *et al.,* 1977; Caviness and Rakic, 1978; Goffinet, 1984; Caviness *et al.,* 1988) (Fig 1.12)

Comment [IT51]: Caviness VSJ, Crandall JE, Edwards MA (1988) The reeler Malformation. Implications for Neocortical Histogenesis. New York: Plenum Press.

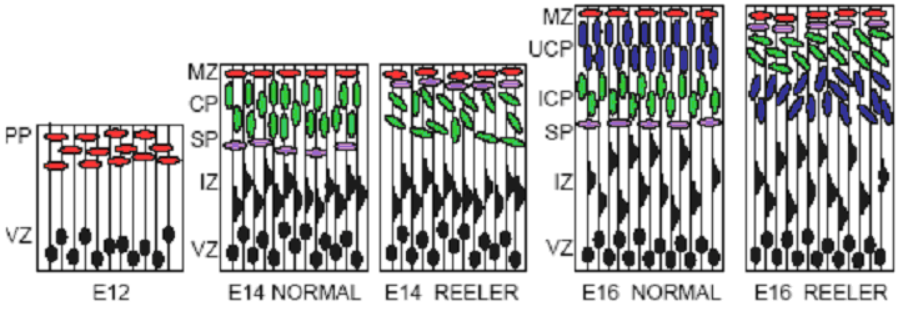


Fig 1.12: Early cortical development in normal and reeler mice. At embryonic day 12, the preplate (PP) appears as a population of horizontal neurons, which include Cajal-Retzius cells (red cells), which secrete reelin. During corticogenesis, proliferation of germinal cells (black) occurs in the ventricular zone (VZ); Post-mitotic neurons exit the VZ, attach to radial glial fibres (fibres that extend the width of the developing neocortex- represented by black lines in the diagram) and migrate through the intermediate zone (IZ) into the developing cortical plate (CP), bypassing the subplate (SP) neurons (purple) and previously born neurons. The CP develops between the subplate and the marginal zone (MZ) that contains Cajal-Retzius cells which secrete reelin. Neurons stop migrating at the top of the CP. Later born neurons migrate past their predecessors in the inner cortical plate (ICP) and settle into ever more superficial positions of the upper CP (UCP)_ resulting in an "inside-out" laminar organization. In normal mammalian neocortical development, the noecortex comprises six layers (as shown in the E16 normal panel). Analysis of the reeler mouse cortex shows that the CP (green) develops beneath the sub-plate (purple). In addition, cellular layering the CP is inverted in an "outside-in" laminar organization (E16 Reeler). Taken from Bar *et al.,* 2000.

Comment [MB52]: Your figure numbering must change – can't first have Fig 1.15 and then Fig 1.14. Please check this.

The heterozygous reeler mice (HRM), in contrast, do not express the aforementioned anatomical and behavioural abnormalities. Instead, they exhibit several other neuropathological and neurochemical abnormalities that are considered typical of schizophrenia (Table 1.13). Moreover, HRM exhibit several behavioural abnormalities that have been linked to schizophrenia. The HRM present with postpubertal appearance of sensorimotor gating deficits, that is reminiscent of that expressed by schizophrenic patients (Tueting *et al.,* 1999), and generally have slow acquisition rates in radial maze as well as olfactory discrimination tasks; these features are comparable to learning deficits experienced by schizophrenia sufferers (Costa *et al.,* 2001; Hoffmann *et al.,* 2001). Furthermore, after approximately five weeks of social isolation, the HRM experience a short-lived depression followed by apathy (Costa *et al.,* 2002). This apathy is reminiscent of schizophrenia social withdrawal (Costa *et al.,* 2002). All in all, the neurochemical, neuroanatomical and behavioural abnormalities common to both HRM and schizophrenia sufferers are strong indicators that the HRM is a valid schizophrenia animal model.

Comment [IT53]: Hoffmann JS, Guidotti A, Costa E, Larson J: **Impaired olfactory discrimination learning in heterozygous reeler mice.** Abstract 245.4 of the 31st Annual Meeting of the Society for Neuroscience, 2001 November 10–15, San Diego.

Table 1.13: **Neurochemical and neuroanatomical similarities between HRM and schizophrenia.**

Physiological cortical measures	Schizophrenia	HRM
GAD_{67} expression	Decreased	Decreased
Reelin expression	Decreased	Decreased
Neurophil expression	Decreased	Decreased
Neuronal cell packing density	Increased	Increased
Dendritic spine density	Reduced	Reduced

Abbreviation: GAD_{67}, glutamate decarboxylase (67kDA); HRM, heterozygous reeler mice
(Taken from Costa *et al.,* 2002)

Since the discovery of the HRM model, several additional lines of evidence suggest that reelin plays a role in the pathogenesis of schizophrenia. The gene encoding human reelin, *RELN,* has been mapped to chr7q22 (DeSilva *et al.,* 1997), a region that has been suggested as a possible schizophrenia susceptibility gene (Ekelund *et al.,* 2000). In addition, several studies have shown a decrease of reelin expression in post-mortem brain sections of schizophrenic patients (Impagnatiello *et al.,* 1998; Fatemi *et al.,* 1999; Guidotti *et al.,* 2000). Accordingly, association studies of reelin in schizophrenia have been undertaken (Akahane *et al.,* 2002; Chen *et al.,* 2002), but have failed to show any association. However, it remains possible that downstream defects in the reelin signaling pathway may have the same effect as defects within reelin itself.

Reelin

Reelin is synthesized in the telencehphalon by Cajal-Retzius cells (Fig 1.12) and in the embryonic cerebellum by external granule cells. The reelin protein has a relative mass of 388 kilo Dalton (kDa) and contains 3461 amino acid residues which are organised into a number of domains (Fig1.13). The carboxy-terminus (C-terminal) of reelin consists of a series of eight tandem repetitive units called reelin repeats, each divided into three further regions, subrepeat A, EGF-like domain and subrepeat B (D'Arcangelo *et al.,* 1995). N-terminal to the reelin repeats, amino acid residues 230-346 of reelin encompass the epitope for the anti-reelin monoclonocal antibody CR-50 (Ogawa *et al.,* 1995; Utsunominiya-Tate *et al.,* 2000). Between the amino-terminal signal peptide and the CR-50 epitope, lies a domain known as the reeler domain, to date found only in one other protein, F-spondin. F-spondin promotes adhesion and outgrowth of commissural axons and inhibits adhesion of neural crest cells (Burstyn-cohen *et al.,* 1998) and plays a critical role in the development of the spinal chord.

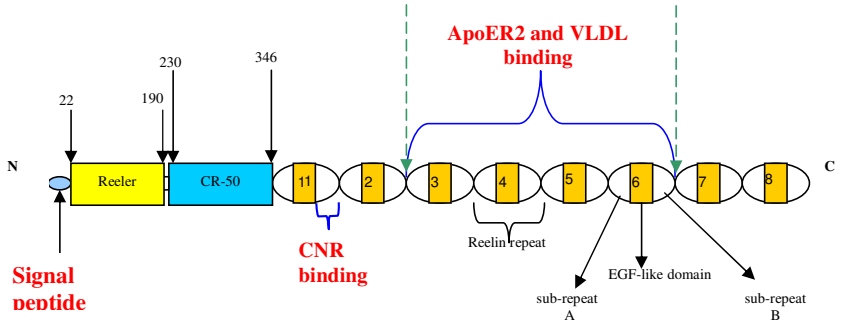


Fig.1.13: Schematic representation of the structure of the reelin protein. Reelin contains a series of eight repeats in which each repeat consists of 2 sub-repeats (sub-repeats A and B) separated by an EGF-like domain. The CR-50 epitope (represented by the blue rectangle) is recognised by the CR-50 antibody that inhibits the function of reelin. The reeler domain stretches from aa 22 to 190 (represented by the yellow rectangle). A cleavable signal peptide (for transport out of the cell) is located at the N-terminus. Binding sites of reelin ligands ApoER2, VLDL and CNR proteins are indicated with blue brackets. Green broken arrows indicate sites at which reelin is cleaved by yet-to-be-identified mettaloproteinases.

Reelin and corticogenesis

During embryonic corticogenesis, reelin has been shown to function as a key regulator of ordered neuronal alignment (D'Arcangelo *et al.,* 1995; Ogawa *et al.,* 1995; Lambert de Rouvroit and Goffinet, 1998). Neurons born in the ventricular zone (VZ) exit the cell cycle and attach to radial glia and migrate radially along these glial fibres into more superficial positions in the developing cortical plate. Studies have shown that the neuronal cells migrate towards the subplate, penetrate this structure and accumulate below the marginal zone (Rakic and Caviness, 1995; reviewed by Gleeson and Walsh, 2000) (Fig.1.12). In the marginal zone, Cajal-Retzius cells secrete reelin into the extracellular matrix (D'Arcangelo *et al.,* 1995; Miyata *et al.,* 1997; Rice and Curran, 1999; Lambert, de Rouvroit and Goffinet, 1998), which in turn directs the organisation of the neurons into the six neuronal layers that will ultimately develop into the adult neocortex. Later-born neurons migrate past their predecessors and settle into ever more superficial positions, forming the cortical plate between the subplate and the marginal zone. This results in an "inside-out" laminar organization, in which earlier born neurons are located in deeper layers than later born ones (Rakic, 1995; Rakic *et al.,* 1996) (Fig1.12)

Reelin subsequently binds to receptors on the surface migrating neuron, specifically apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptors (VLDLR). Binding of reelin to ApoER2 and VLDLR leads to the phosporylation of the adapter molecule Disabled 1 (Dab1), which is bound to the cytoplasmic tails of these receptors (Hiesberber *et al.,* 1999 D'Arcangelo *et al.,* 1999), as well as the internalisation of reelin into the cell (D'Arcangelo *et al.,* 1999).

In vivo, reelin is processed by cleavage by yet-to-be-identifed mettaloproteinases between reelin repeats 2 and 3, and between repeats 6 and 7 (Fig. 1.13), resulting in three final physiologically relevant fragments (Lambert

de Rouvroit *et al.,* 1999). The central fragment, viz. repeats 3 through 6, is both necessary and sufficient for receptor binding to ApoER2 and VLDLR proteins (Jossin *et al.,* 2004). The function(s) of other two peptides remains unknown.

Downstream signaling continues via a PI3-mediated pathway that involves interaction of the PI3 kinase regulatory subunit, p85, with the tyrosine phosphorylated Dab1(Bock *et al.,* 2003). Activation of PI3 kinase leads to the phosphorylation of Akt and GSK3β, resulting in hyperphophorylation of the microtubule-associated protein, tau, and subsequent microtubule rearrangements. This pathway eventually leads to the modulation of the actin cytoskeleton (Heisberger *et al.,* 1999; Beffert *et al.,* 2002) (Fig1.16), a pre-requisite for cellular motility such as neural migration. Additionally, phosphorylated Dab1 has also been found to bind Lis1 (Assadi *et al.,* 2003*)* which interacts with the NUDEL/cytoplasmic dynein complex to regulate lamination of the CNS (Niethammewr *et al.,* 2000; Sweeney *et al.,* 2001; Sasaki *et al.,* 2000).

Anton and colleagues (1999) provided evidence that ECM reelin signal transduction is also initiated when reelin binds to integrin receptor subtypes that include the α3 integrin receptor subunit (Anton *et al.,* 1999; Dulabon *et al.,* 2000); the region of reelin responsible for the binding to integrin α3β1has not yet been identified. These integrin receptors link the ECM signals to the neuronal cytoskeleton proteins via a reelin-mediated receptor clustering. This clustering activates focal adhesion kinase (FAK), which, in turn, phosphorylates Dab1 (Dulabon *et al.,* 2000) (Fig1.14). In addition, a family of cadherin-related neuronal proteins (CNRs) has also been identified as a putative receptor for reelin (Yagi and Takeichi, 2000); sub-repeat B of the first reelin repeat domain is crucial for CNR binding (Senzaki *et al.,* 1999). The cytosolic domain of CNR associates with fyn, which has been shown to phosphorylate Dab1(Costa *et al.,* 2001).

An investigation by Utsunomiya-Tate and colleagues found that the reelin epitope recognised by the CR-50 antibody contributes to the formation of linear, soluble reelin homopolymers at physiological concentrations (Utsunomiya-Tate *et al*., 2000). Deletion of the amino acid residues containing the CR-50 epitope inhibits the homopolymerisation as well as reelin-induced phosphorylation of Dab1. This suggests that the CR-50 domain of reelin plays an important role in the function of reelin.

Not all reelin interactions and functions are known. In particular, the function of the reeler domain remains unknown, but given that this domain only occurs in reelin and in F-spondin, a protein that plays a critical role in the development of the spinal chord, it could be suggested that this domain has an important function in neurodevelopment and warrants further investigation.

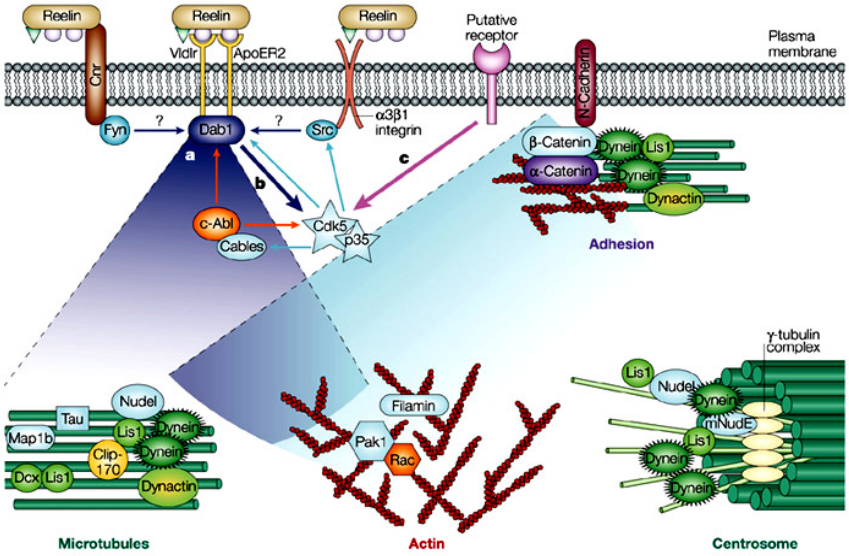


Fig 1.14: The Reelin signaling system. Reelin can bind its receptors ApoER2/VLDLR and α3β1 integrins, directly initiating a signal transduction pathway. Reelin induction of the cascade leads to the clustering of the receptors causing the tyrosine phosphorylation of the adapter protein Dab1 by the Src family of protein kinases. This leads to the activation of downstream signaling pathways that result in cytoskeletal rearrangements and neuronal migration (taken from *et al*., Gupta *et al.,* 2002).

1.4.9.1.2. Pharmacological models

Traditionally, most pharmacological animal models of schizophrenia have focused on phenomena linked to dopamine system dysfunction, since this system has been strongly implicated in the disorder (Kornetsky and Markowitz, 1978, McKinney and Moran, 1981, Costall and Naylor, 1995). Dopamine-based animal models have been found to have some predictive validity (Table. 1.14). Examples of these include dopamine-drug induced stereotypies which represent schizophrenia motor behaviour and apomorphic induced PPI abnormalities which represent information processing deficits (Braff and Geyer, 1990; Costall and Naylor, 1995).

The administration of amphetamine and related psychostimulants to rats reliably simulates some of the schizophrenia behavioural changes, viz. hyperlocomotion and stereotypic movements (Kokkinidis and Anisman, 1980; Sharp *et al.,* 1987). Furthermore, amphetamine-induced stereotypic behaviour can be attenuated by administration of antipsychotic medication, which lend further support for the validity of these dopaminergic animal models (Pijnenberg *et al.,* 1975), as do the disruptive effects of dopamine receptor agonists on PPI (Swerdlow and Geyer, 1998, Swerdlow *et al.,* 1994).

The glutamatergic system has also been the focus of animal studies of schizophrenia pathogenesis. Phencyclidine, a hallucinogen that acts predominantly on the glutametergic NMDA receptor and PCP-like drugs, has been shown to induce altered social behaviour in rats (Steinpreis *et al.,* 1994; Sams-Dodd, 1995).

Comment [IT54]: Kornetsky C, Markowitz R (1978): Animal models of schizophrenia. In Lipton MA, DiMascio A, Killam KF (eds), *Psychopharmacology: A Generation of Progress*. New York, Raven Press, pp 583–593

Comment [IT55]: Costall B, Naylor RJ (1995): Animal neuropharmacology and its prediction of clinical response. In Hirsch SR Weinberger DR (eds), *Schizophrenia*. Oxford, Blackwell Science Ltd, pp. 401–424

Comment [IT56]: Pijnenburg AJ, Honig WM, Van Rossum JM. Inhibition of *d*amphetamine-induced locomotor activity by injection of haloperidol into the nucleus accumbens of the rat. *Psychopharmacologia* 1975;41(2):87-95.

Administration of PCP was also shown to disrupt PPI and startle habituation in rats (Swerdlow and Geyer, 1998; Geyer *et al.,* 1984). Moreover, PCP produces amphetamine-like behavioural alterations in rats that include stereotyped movements, circling and ataxia; these effects are attenuated by antipsychotics (Javitt and Zukin, 1991; Jenysch *et al.,* 1997, Rao *et al.,* 1989).

The serotonin system has also been frequently implicated in the aetiology of schizophrenia (section 1.1.5.3.2). Lysergic acid diethylamide (LSD), a major psychedelic hallucinogenic drug that mediates its effects through the 5-HT2A receptors (Aghajanian and Merek, 1994), has been found to disrupt PPI and startle habituation in humans, as well as rats. Serotonin receptor 3 antagonists have also been shown to attenuate behavioral hyperactivity caused by PCP and amphetamine administration (Costall *et al.,* 1987). The relevance of this model to schizophrenia, is however, flawed since chronic LSD administration in humans and animals leads to behavioral tolerance, unlike the situation in schizophrenia.

1.4.9.1.3.Transgenic models

1.4.9.1.3.1. Models of neurotransmitter systems

The dopamine transporter (DAT) knock-out mouse (DAT-KO) model is an animal model with clear relevance to the dopamine hypothesis of schizophrenia (Giros, 1996). The homozygous (-/-) DAT-KO mice are incapable of reuptake of released dopamine, resulting in increased dopamine levels. These mice show increased stereotypic behaviour (Jones *et al.,* 1998) and hyperlocomotion (Ralph *et al.,* 2001). The hyperlocomotion of these mice is reversed by the administration of dopamine receptor antagonists such as haloperidol and clozapine, which are used in treatment of schizophrenia (Gainetdinov *et al.,* 1999, Spieleway *et al.,* 2000).

However, the DAT-KO heterozygous (+/-) mouse is considered to be a better approximation of the reduced DAT acting in schizophrenia (Lankso *et al.,* 2001). The DAT-KO heterozygous model reproduces several of the features of the amphetamine-administrated animal model (Table1.13). Aside from hyperactivity and stereotypic behaviour, the mutants also show deficits in PPI and spatial cognitive function (Gainetdinov *et al.,* 1999; Ralph *et al.,* 2001). However, as with the amphetamine model, several features of these mice do not correlate well with those of schizophrenic patients. For instance, the mutant mice do not show any deficits in social interactions (Spielewoy *et al.,* 2000). In addition, psychostimulants known to exacerbate psychotic symptoms in schizophrenic patients have been shown to produce a calming effect in these mice. Therefore, although the DAT-KO model may be useful in studying certain aspects of schizophrenia, it does not encompass a full range of schizophrenia-related behaviours.

Table 1.14: **Pharmacological and genetic animal models of schizophrenia with respect to behavioural abnormalities.**

Behaviour	Amphetamine treated	DAT-KO	NMDA-antagonist treated	NR1-KO mice	NR2A-KO Mice
Hyperactivity	Extreme	Extreme	Modest	Modest	Modest
Deficit in social interaction	No	No	Yes	Yes	Not tested
Sensorimotor gaiting deficits	Yes	Yes	Yes	Not tested	Not tested
Cognitive deficits	Yes	Deficits in spatial learning	Yes	Not tested	Not tested
Locomotor responses to antipsychotics	Haloperidol and clozapine effective	Haloperidol and clozapine effective	Haloperidol and clozapine effective	Clozapine effective	Haloperidol and risperidone effective

Abbreviaitions: DAT-KO, Dopamine transporter knockout; NMDA, n-methyl-D-aspartate; NR1-K,O, n-methyl-D-aspartate receptor 1 knock out; NR2A, n-methyl-D-aspartate receptor 2A knock out

Knock-out mouse models of dopamine receptors (Sibley, 1999; Kelly *et al.,* 1998; Xu *et al.,* 1994; 1997; Rubenstein *et al.,* 1997) as well as key enzymes involved in dopamine synthesis and degradation, eg., TH (Zhou and Palmiter, 1995; Kim *et al.,* 2000), COMT (Gogos *et al.,* 1998) and MAOA (Shih *et al.,* 1999) have also been characterized. However, none of these mice were reported to exhibit any dysfunction in social interactions and only the DRD2 knockout mouse showed some PPI dysfunction (Ralph et al., 1999).

Mice deficient in NMDA glutamate receptors have been generated by targeted mutations of the NR1 subunit gene (Mohn *et al.,* 1999). These NR1 knock-down (NR1-KD) mice display similar behaviour to those observed in mice treated with PCP (Table 1.13). Several 5-HT mutant models are also available at present, but have, as yet, not been fully characterised using behavioural tests with relevance to schizophrenia (Murphy *et al.,* 1999).

1.4.9.2. Animal models of OCD

A number of animal models of OCD have been identified and developed. These models can essentially be divided into three distinct classes, ie., ethnological models, pharmacological models and transgenic models. For OCD in humans, information on ritualistic behaviours and obsessions is captured using a battery of rating scales (eg Leyton Obsessional Card Mauldsey Obsessive-Compulsive Inventory, Yale-Brown Obsessive-Compulsive Scale). For animal models, such rating scales, that rely on the subjects' introspection and self-reporting, are obviously not appropriate (Eilam and Szechtman, 2005). Szechtman and colleagues thus developed a spatio-temporal paradigm in which compulsive checking could be evaluated in animals (Szechtman *et al.,* 1998). They reasoned that compulsive checking in animals would present itself to an observer as behaviour that met the following five criteria: in the subject's living space, there would be one or two key areas/objects to which the subject would return more frequently than others; the time taken to return to the particular place/object would be significantly shorter than to other places/objects; excessively few places/objects would be visited in between returns to the key place/object; a characteristic set of rituals would

be performed at the particular place/object; activity would be changed if the key object was removed from the subjects living area or the properties of the environment was changed.

1.4.9.2.1. Ethnological models

Several interesting naturally-occurring, ethnological, animal models of OCD are represented by animals that present with abnormal repetitive, non-functional motor behaviours in veterinary clinics (Overall, 2000). These include acral lick (paw licking) dermatitis (ALD) in dogs, hair pulling in cats and feather picking in birds (Dodman *et al.,* 1997).

Comment [IT57]: Dodman, N.H. et al., "Veterinary Models of OCD **Obsessive-Compulsive Disorders**: Diagnosis, Etiology, Treatment. Eric Hollander, Dan J. Stein. Eds. New York, **Marcel Dekker,** pp. 99-143 (1997)

In particular, ALD in dogs seems to have a high level of similarity to OCD. Just like OCD, ALD also appears to have a strong genetic component as this condition appears to be confined to only a few breeds and even to reside within certain families of particular breeds (Overall, 2000). Furthermore, there is relative homology between the two disorders. Acral lick dermatitis is characterised by repetitive paw licking that may result in severe inflammatory complications, a condition which appears to be very similar to compulsive hand washing, which frequently also presents with complications (Man *et al.,* 2004). Another interesting link between OCD and ALD is that pharmacotherapyknown to be effective in the treatment of OCD, such as clomipramine, is also effective in treating ALD (Rapaport *et al.,* 1992). Unfortunately, the neurobiological mechanisms for ALD remain unknown at present (Man *et al.,* 2004), however, they could be investigated by brain imaging strategies. Indeed, if similar abnormal brain circuits are found to be involved in ALD and OCD, this would represent the best animal model for OCD.

Comment [IT58]: . Man, J; Hudson, A.L; Ashton, D; Nutt, D.J (2004). Current Neuropharmacology, 2 (2) 153-168

1.4.9.2.2. Pharmacological models

One of the most widely investigated pharmacological animal models of OCD is the DRD2/DRD3 agonist, quinpirole (QNP)-treated rat model. Several investigations have shown that rats chronically treated with QNP develop locomotor sensitisation (Culver *et al.,* 2000; Einat *et al.,* 1996; Einat and Szechtman, 1993; Kostrzewa, 1995, Mattingly *et al.,* 1993). Based on their observations, Szechtman and co-workers proposed that QNP-induced behaviour in rats has the form of compulsive checking and may constitute a pharmacological animal model of OCD (Szechtman *et al.,* 1998). Furthermore, motor rituals as a result of chronic administration of QNP have also been reported (Ben Pazi *et al.,* 2001). A follow-up investigation by the same researchers found that QNP-treated rats are able to resist (or interrupt) their obsessive checking (Szechtman *et al.,* 2001). This is reminiscent of OCD patients who, despite having urges to perform rituals, may resist engagement in them for varying amounts of time depending on the circumstances.

Pharmacological animal models based on the serotonergic neurotransmission system have also been described. Yadin *et al.* proposed spontaneous alternation deficits resulting from administration of serotonergic agonists, such as 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT), as an animal model of OCD (Yadin *et al.,* 1991). This model is based on the natural tendency of rodents to enter first one arm and then the other alley of a T-maze in two successive, equally rewarded trials (Ellen and Deloache, 1968). In this serotonergic pharmacological model, rodents persevere in the alley section of the T-maze (Yadin *et al.,* 1991).

Comment [IT59]: Ellen P, Deloache J (1968) Hippocampal lesions and spontaneous alternation behavior in the rat. Physiol Behav 3:857–860

1.4.9.2.3. Transgenic models

An intriguing genetic animal model for OCD is the *HoxB8* knock-out (*HoxB8-KO*) mouse model. *HoxB8* is a member of the mammalian homeobox-containing complex (Hox) group of 39 transcription factors that are best known for their roles during early neurodevelopment in providing positional information along the anteroposterior axis (Capecchi *et al.,* 1997).

Two HoxB8 loss-of-function mutants were engineered that contains nonsense mutations in the first exon of the gene (Greer and Capecchi, 2002). One mutant contained both the exon 1 mutation, as well as a floxed neomycin resistance (*neo*^{r}) cassette in exon 2, while the other contained a *loxP* site in exon 2. Homozygotes of both mutant strains showed, with 100% penetrance, excessive grooming leading to hair loss and deep skin wounds (Greer and Capecchi, 2002). However, the HoxB8^{neo} mutant also demonstrated skeletal abnormalities that were also present in HoxB9 and HoxB6 mutants, thus only the HoxB8^{lox} mutant strain was used in the subsequent analysis.

The excessive grooming behaviour demonstrated by these animals is an interesting finding in the context of OCD research. Grooming is an innate spontaneous behaviour that generally occurs between periods of rest and activity in most animal species, including humans (Fentress, 1988). In rodents, as in many other mammalian species, grooming follows a general pattern. First the head is groomed, followed by the body and finally the anogenital region and tail. This highly ordered pattern of grooming, referred to as the "idealized syntactic grooming chain" (Berridge *et al.,* 1987), was observed in a study of grooming behaviour in rodents, which suggested that pattern-generating signals originating in the CNS organise physical movements required for each bout of grooming (Berridge *et al.,* 1988; Berridge and Whishaw, 1992).

Greer and Cappechi (2002) demonstrated that the excessive grooming behaviour of the HoxB8 transgenic mouse model has a high degree of similarity to OCD in humans. Firstly, OCD is often characterised by excessive behaviours dealing with cleanliness, including grooming. Secondly, the activity of these mutant mice seem to mimic OCD in humans in that, other than the obsessive grooming, their activity is similar, if not identical, to their control littermates, in the same way as the obsessions and compulsions are the only factors separating OCD patients from control individuals. Thirdly. Greer and Capecchi, using *in situ* hybridization assays, showed that HoxB8 is expressed in several brain regions including the ones making up the OCD circuit (section 1.2.3.2.4). Finally, the excessive grooming that leads to loss of hair and formation of lesions is similar to what is observed in TTM, which is an OCS.

Campbell and colleagues generated another transgenic mouse line that expresses an intracellular form of the cholera toxin (D1CT), in order to study the role of DRD1 neurons in behaviour (Campbell *et al.,* 1999). The cholera toxin is a neuropotentiating enzyme that chronically activates stimulatory G-protein signal transduction and cAMP synthesis under the control of the DRD1 promoter (Burton *et al.,* 1991; Zeiger *et al.,* 1997).

They found that in a founder line of these transgenic animals, in which transgene expression was restricted to the areas of the somatosensory cortex and the intercalated nucleus of the amygdala, abnormal psychomotor activity occured. These D1CT mice exhibited a wide range of compulsive behaviours that included episodes of perseverance or repetition of all normal behaviours, repetitive non-aggressive biting during grooming of littermates and repetitive leaping (Campbell *et al.,* 1999). The results of their investigations suggest that chronic potentiation of cortical and limbic DRD1-positive neurons causes behaviours in these mice that are reminiscent of human cortical-limbic induced compulsive disorders, such as OCD (Campbell *et al.,* 1999).

In summary, several different animal models for OCD have either been developed or occur naturally. These animal models have provided researchers with unique opportunities to investigate aspects of OCD pathogenesis in an *in vivo* system. Several of these animal models have good face and predictive value, however, none of them can completely encapsulate the full spectrum of OCD symptoms and sub-types. Bearing this in mind, they do provide clues about genes that may be involved in certain aspects of OCD. For instance, the excessive grooming behaviour of the *HoxB8* mutant mouse could suggest that HoxB8 may play a pivotal role in OCD, particularly in those individuals who have grooming compulsions, while the *D1CT* transgenic mouse model may suggest a role for *DRD1* in OCD. However, investigators have not fully taken advantage of the potential of animal models to provide novel candidate genes for OCD. To date no association studies investigating the possible role of either *HoxB8* or *DRD1* in OCD have been reported, yet the two genetic animal models discussed in this section provide compelling evidence for the involvement of these two genes, or other components within the pathways they direct, in the development of obsessive-compulsive behaviour.

1.5. THE PRESENT STUDY

The literature reviewed in this chapter provides evidence for a genetic component in both schizophrenia and OCD. Moreover, the data presented in section 1.3 as well as Table 1.2 suggest that, although schizophrenia and OCD are considered distinct disorders, clinical overlap between the two exists. This reciprocal comorbidity as well as the polygenic nature of both disorders, suggests at least a partially shared genetic aetiology. Thus, the present investigation hypothesised that different combinations of normally occurring polymorphic variants in a host of distinct genes are conditional for the development of either OCD or schizophrenia, but that variants in some of these genes may contribute to the development of both disorders. Thus, genes that have been implicated in one disease could be considered candidate genes for susceptibility to the other disorder. As schizophrenia has been somewhat more amenable to genetic dissection than OCD, this disease was used as a platform for identifying novel OCD-susceptibility loci.

1.5.1. Bioinformatic identification of novel schizophrenia-linked OCD candidate genes

Comment [MB60]: fix in index

Firstly, schizophrenia-linked genomic regions were combed for plausible candidate genes by bioinformatics means, secondly, interactome analysis of reelin, previously implicated in an animal model of schizophrenia, was used to identify additional novel candidate genes.

Genes used in case-control association studies were chosen and prioritised based on two criteria: they had to reside in schizophrenia susceptibility loci that have been statistically linked to the condition in independent linkage studies were considered, whether or not the particular gene had previously been implicated in schizophrenia or OCD. Secondly, the candidacy of such genes selected purely by position was strengthened, if they were reported to be expressed in the brain and/or show homology to genes encoding enzymes or receptors with roles in the neurotransmitter systems already implicated in OCD pathogenesis.

1.5.2. Interactome-based identification of novel OCD candidate genes

Comment [MB61]: fix in index

Earlier, two examples were given of how the interactors of proteins implicated in schizophrenia have themselves also been implicated in schizophrenia (section 1.4.8). The present study used a similar approach to identify novel schizophrenia and/or OCD candidate genes.

The starting point of this part of the study was the gene encoding reelin (section 1.4.9.1.1). This gene has been considered a candidate gene for schizophrenia based on a number of factors, discussed in section 1.4.9.1.1. We reasoned that, since reelin is implicated in schizophrenia susceptibility, proteins interacting with reelin may also be involved in schizophrenia, and thus also OCD, development.

Several reelin interacting proteins have been indentified and their binding sites on reelin have largely been determined (section 1.4.9.1.1.) However, the functions of the N-terminal repeat region and reeler domain have not yet been determined. The reeler domain was of particular interest, as it has only been identified in one other protein, F-spondin, which is also involved in neurodevelopment. Therefore, since the reeler domain occurs only in proteins essential for neuronal migration, we hypothesised that this domain plays a critical role in neurodevelopment, probably through protein-protein interactions, and that these interactors may themselves be implicated in schizophrenia and thus in OCD. To test this hypothesis, a foetal brain cDNA library was screened, using the reeler domain of reelin as “bait” in Y2H analysis. Putative reeler-interactions identified from the Y2H screen were subjected to co-immunoprecipitation and mammalian-two-hybrid analyses (M2H) as verification.

1.5.3. Case-control association studies of novel candidate genes

Comment [MB62]: fix in index

Genes identified either from bioinformatic analysis of schizophrenia-linked loci, or by interactome analysis of reelin were then investigated for a role in OCD susceptibility. Previously reported polymorphisms in the prioritised candidate genes were retrieved electronically (NCBI.NIH.NLM.GOV/dbSNP), while in the case of *POU3F2* (one of the prioritised candidate genes), polymorphisms were sought by polymerase chain reaction (PCR) single strand conformational polymorphism (SSCP) analysis as, at the time, no validated SNPs were found in the the publically available SNP databases.. Selected polymorphism(s) within these genes were genotyped in a group of unrelated OCD-affected individuals and a group of unrelated, ethnically matched control individuals belonging to the genetically homogeneous Afrikaner sub-population of South Africa, and differences between cases and controls evaluated statistically.

CHAPTER 2: MATERIALS AND METHODS

INDEX **PAGE**

CHAPTER 2 MATERIALS AND METHODS

2.1 STUDY SUBJECTS AND BLOOD COLLECTION

Unrelated OCD patients and controls were recruited through the Medical Research Council (MRC) Unit on Anxiety and Stress Disorders from throughout South Africa by trained clinical psychologists and via media advertisements. These controls were matched to the case subjects according to ethnicity, gender and age. All patients and control subjects participating in this study were of Afrikaner descent, based on Afrikaans being their home language and at least three of their four grandparents being of Afrikaner descent. The University of Stellenbosch Ethics Committee approved the protocol (project number 99/013) and all subjects provided written informed consent.

All OCD patients underwent a structured interview, which included the SCID-I, the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) and the Yale Global Tic Severity Scale (YGTSS) (First *et al.,* 1994; Goodman *et al.,* 1989; Leckman *et al.,* 1989). A trained research clinician conducted each interview. All case subjects selected for inclusion in the present study met DSM-IV criteria for OCD and had no history of psychotic disorders. Importantly, the presence or absence of schizophrenia or schizotypal traits was not used as inclusion or exclusion criteria, and enrolled OCD patients were not substratified according to these traits, since the fundamental overlap between OCD and schizophrenia exists regardless of whether both disorders are present in the same patient. Controls underwent a semi-structured interview (SCID-I screening) and those selected for inclusion in the genetic study had no reported history of an anxiety, mood or psychotic disorder.

Blood samples were drawn from both patients and control individuals by means of venous puncture and collected into 5 ml ethylene-diamine-tetra-acetic acid (EDTA) tubes. The EDTA tubes were immediately processed, if blood was drawn at the MRC Unit on Anxiety and Stress Disorders, while blood drawn from patients from around South Africa was couriered to the research laboratory within 24 hours of sampling.

2.2 DNA PURIFICATION

2.2.1. Extraction of nuclei from whole blood

Blood from three 5ml EDTA tubes per patient was transferred into a 50ml Falcon tube. The tube was then filled to 20 ml with ice-cold lysis buffer (appendix I). After gently inverting the tubes a few times, the sample was incubated on ice for 5-10 min. The sample was then centrifuged at 2500-3000 rpm at room temperature in a Beckman model TJ-6 centrifuge (Scotland, UK). The supernatant was discarded and the pellet was resuspended in 20ml, ice-cold lysis buffer, followed by another round of incubation and centrifugation. The supernatant was discarded and the pellet resuspended in DNA extraction buffer (appendix I), after which the nuclei were either immediately used for DNA extraction, or stored at -70°C until DNA was required.

2.2.2. Extraction of DNA from nuclei.

To the freshly prepared or thawed nuclei, 100µl of proteinase K (10µg/ml) was added and the mixture was incubated overnight at 37°C. After this step, 2ml distilled water, 500µl 3M sodium-acetate (appendix I) and 25µl phenol/chloroform (appendix I) were added to the sample. The tubes were subsequently inverted and

mixed gently for 10 min on a Voss rotator (Voss of Maldon, England) at 4°C. The mixture was then transferred to a glass Corex tube so that the aqueous phase could be clearly distinguished from the organic phase, followed by centrifugation in a Sorvall RC-5B refrigerated super-speed centrifuge (rotor SS 34, Dupont Instruments) at 8000 rpm for 10 min at 4°C.

The upper aqueous phase, containing the DNA, was transferred to a clean Corex tube using a sterile plastic pasteur pipette, while taking care not to disturb the interface or the organic phase. Approximately 25ml chloroform/octanol (appendix I) was added to the aqueous phase after which the tube was closed with a polypropylene stopper and gently inverted for 10 min. This mixture was centrifuged at 4°C, followed by the removal of the upper aqueous phase as described earlier. The DNA was then ethanol precipitated by adding two volumes of ice-cold 96% ethanol and inverting gently until DNA strands appeared as a white precipitate.

The DNA strands were removed using a yellow-tipped Gilson pipette and placed in a clean, 1.5ml Eppendorf microfuge tube. One millilitre 70% ethanol was then added to the DNA and the mixture centrifuged in a Beckman microfuge for 3 min at 13000 rpm. The ethanol was carefully decanted and the 70% ethanol wash repeated one more time in order to remove any excess salts. After careful removal of most of the ethanol, the DNA pellet was air-dried for 30-60 min at room temperature by inverting the Eppendorf microfuge tube on Carlton paper. Two hundred microlitres Tris-EDTA (appendix I) buffer was added and the DNA was resuspended, initially by stationary incubation at 37°C overnight and subsequently by gentle mixing in a Voss rotator at 4°C for a further 3 days. This was followed by stationary incubation at 4°C until the DNA had been fully resuspended.

After 1-2 weeks, when the DNA had completely resuspended in the buffer, the optical density (OD) of the DNA was determined in a Milton Roy series 120i spectrophotometer (USA) at 260nm (OD_{260}). The DNA concentration, in µg/µl, was determined by diluting 10µl of DNA in 500µl of TE and multiplying the measured OD_{260} by a factor of 2.5, while the purity of the DNA was monitored by the OD_{260}/OD_{280} ratio, which should be approximately 1.8 for pure DNA.

2.2.3. Gel purification of PCR-amplified products from agarose gels

Purification of PCR-amplified DNA products from agarose gels was performed to obtain DNA products suitable for sequencing reactions and cloning. The relevant PCR-amplified DNA product was electrophoresed in a 1% agarose gel (section 2.4) and subsequently viewed under ultraviolet (UV) light. The segment of the gel containing the DNA to be purified was excised using a sterile scalpel blade, and the DNA subsequently extracted from the agarose using the GFX® DNA purification kit (AmershamPharmacia Biotech, New Jersey, USA) as per the manufacturer's instructions.

2.2.4.Bacterial plasmid purification

One *Escherichia coli* (*E.coli*) colony containing the plasmid of interest was picked from an appropriate selection plate and inoculated into 10 ml of Luria-Bertani Broth (LB) (appendix I), supplemented with the

correct antibiotic, in a 50ml polypropylene tube. The culture was then incubated at 37°C overnight, while shaking at 250rpm in a YIH DER model LM-530 shaking incubator (SCILAB instrument Co LTD., Taipei, Taiwan).

The following morning, the culture was centrifuged for 10min at 3000rpm in a Beckman model TJ-6 centrifuge (Beckman Coulter, Scotland, UK), after which the supernatant was discarded and the pellet was resuspended gently by pipeting in 1ml of cell suspension solution (Appendix I). Two millilitres of cell lysis solution (Appendix I) was added and the contents were mixed by gentle inversion of the tube which was then incubated at room temperature for 5 min. Two millilitres of neutralisation solution (Appendix I) was added to the tube and the contents were once again mixed by gentle inversion and incubated at room temperature for 5 min. Following this incubation, 5ml of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma, St Louis, MO, USA) was added to the tube and the contents mixed by gentle inversion, followed by centrifugation at 3000 rpm for 15 min at 4°C in a Multex centrifuge (MSE instrumentation, England, UK) in order to allow for phase separation.

The upper clear plasmid-containing top (aqueous) phase was transferred into a new sterile 50ml polypropylene tube and approximately 0.7x volume 100% isopropanol (Merck, Darmstadt Germany) was added to the tube, which was mixed well by gentle inversion. This was followed by centrifugation at 4°C for 45 min in a Multex centrifuge (MSE Instrumentation, England, UK). After centrifugation, the supernatant was discarded and the pellet was washed twice with 2ml ice cold 70% ethanol and then air-dried. The dried pellet was resuspended in 100-200µl ddH_2O and subsequently 3µl of this plasmid preparation was resolved on a 1% T.B.E agarose gel for verification (section 2.5).

2.2.5. Bacterial plasmid purification using Wizard® Purefection Plasmid DNA purification kit

One *E. coli* colony containing the plasmid of interest was picked from an appropriate selection plate and grown overnight, in 20ml of LB with appropriate antibiotic, as described above. The following morning the culture was centrifuged for 10min at 3000rpm in a Beckman model TJ-6 centrifuge, and the supernatant discarded. The plasmid DNA was then extracted from the pellet using the Wizard® Purefection Plasmid DNA purification kit (Promega Corp. Madison Wisconsin, U.S.A). as per manufacturer's instructions.

2.2.6.Yeast plasmid purification

A yeast colony containing the plasmid of interest was inoculated into 1ml synthetic dropout (SD) medium containing the appropriate dropout supplement (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A) and incubated overnight at 30°C in a shaking incubator at 250rpm. The following morning, 4ml YPDA (appendix I) was added to the culture, which was incubated for an additional 4 hours at 30°C. Thereafter, 1.5ml of the culture was transferred into a 2ml Eppendorf microfuge tube, which was benchtop centrifuged at 14000rpm for 30sec in a Beckman Microfuge Lite (Beckman Instruments Inc., CA, USA). The supernatant was discarded and to the pellet was added 200µl yeast lysis buffer (Appendix I), 200µl PCI and 0.3g sterile 450-600µm glass beads. The yeast cells were milled by vortexing this mixture for 2.5min using a Snijders model 34524 press-

to-mix vortex (Snijders Scientific, Tilburg, Holland), followed by benchtop centrifugation at 14000rpm for 5min at room temperature for phase separation. The aqueous phase was transferred to a new, sterile 1.5ml microfuge tube.

Subsequently the DNA was precipitated by adding 20µl of 3M NaAc (pH 6.0) (appendix I) and 500µl 95% ethanol, after which the mixture was incubated at -20°C for 30 min. Following incubation, the mixture was benchtop centrifuged at 14000rpm for 15 min at room temperature. The supernatant was discarded and the pellet washed twice with 1ml 95% ethanol, before being air-dried and resuspended in an appropriate volume of ddH_2O.

2.3. POLYMERASE CHAIN REACTION

2.3.1 Oligonucleotide primer design and synthesis

Oligonucleotide primer sequences were obtained from published data, where possible. When no published data was available, primers were designed using sequence data available either from the Ensembl database (http://www.ensembl.org) or the Genbank database (http://www.ncbi.nlm.nih.gov/Entrez). Before synthesis, each set of primer sequences were analysed for complimentarity (self-complimentarity and primer-primer complimentarity) and compatibility of melting temperatures using DNAman™ version 4 software (Lynnion Biosoft Corp©).

2.3.1.1. Primers for genotyping

Oligonucleotide primers were synthesised according to phosphoramidite methodology at the Department of Molecular and Cell Biology, University of Cape Town (UCT), South Africa. Primer sequences used for each of the polymorphisms genotyped is summarised in Table 2.1.

2.3.1.2. Primers for single strand conformational polymorphism analysis

The sequences of all the primers used to screen the single *POU3F2* exon was obtained using sequence information available in the Ensembl database. Since the *POU3F2* exon is 1331 bp long, primers were designed that generated 7 overlapping amplicons, designated *POU3F2.1A-POU3F2.1G* (Table 2.2), of a size suitable for SSCP analysis. All primers for this part of the study were synthesised at the Department of Molecular and Cell Biology, UCT, Cape Town, South Africa.

Table 2.1. Primer sequences used for genotyping of polymorphisms tested in the present study.

Gene/ Polymorphism	Primer name	Primer sequence	Ta(°C)	Reference
*SNAP25/Dde*I	SNAP25F	5' TTCTCCTCCAAATGCTGTCG 3'	60	Barr *et al.,* 2000
	SNAP25R	5' CCACCGAGGAGAGAAAAT 3'		
*SNAP25/Mnl*I	SNAP25F	5' TTCTCCTCCAAATGCTGTCG 3'	60	
	SNAP25R	5' CCACCGAGGAGAGAAAAT 3'		
SNAP29/C56T	SNAP29F	5' GGAAGGAGTTCGCGCGACGA 3'	68	Saito *et al.,* 2001
	SNAP29R	5' GCGAGTCCACACCAGCCCTG 3'		
SNAP29/G92A	SNAP29F	5' GGAAGGAGTTCGCGCGACGA 3'	68	
	SNAP29R	5' GCGAGTCCACACCAGCCCTG 3'		
GRIA4/rs609239	GRIA4-1F	5' TCCAGTCTAGAAGGCAGGAAA 3'	61	Makino *et al.,* 2003
	GRIIA4-1R	5' AACGTCCACATCACACATTCA 3'		
GRIN1/1	GRIN1/1F	5' GGACGATGCTGCCACTGTAT 3'	60	Martucci *et al.,* 2003
	GRIN1/1R	5' CGGTGATGTTCTCCTTCTCG 3'		
DLX6 IVS1C>T	DLX6F	5' TGGTGCAGCTTCCTTTACCT 3'	60	Nabi *et al.,* 2003
	DLX6R	5' TGCTGCAGACTGATTCTGTG 3'		
BZRP Ala147Thr	PBREx4A	5' TGGGACAGGCACTTGGGTGAAC 3'	60	Kurumaji *et al.,* 2001
	PBREx4B	5' AAGGCACCTGCTGGTGCAGCT 3'		
DBH (I/D)	DBHF	5' GCAAAACTCAGGCACATGCACC 3'	55	Yamamoto *et al.,* 2003
	DBHR	5' CAATAATTTGGCCTCAATCTTG 3'		
SYN3/ -631C>G	SYNP3.1F	5' AGGCATGTACTTGCGTTACC 3'	58	Tsai *et al.,* 2002
	SYN3.11R	5' CCAAATGACTACAAAGATGTACCA 3'		
GBR1.1-C39T	GBR1-EX1F	5' AACCGGCAAGAGGTCGAGTAG 3'	60	Hisama *et al.,* 2001
	GRR1-EX1R	5' CAGGGAAAGGGAAGTGGAGCG 3'		
GBR1.11-T1545C	GBR1-EX11F	5' CACACCCACACACATTCAG 3'	58	
	GBR1-EX11R	5' GAATGCATGTTTGTAGAAGGTG3 '		
*RXRβ/*Val95Ala	RXRβEX2F	5' CGGTGGGGTATTAGAGAATT 3'	60	Present study
	RXRβEX2R	5' CCCATGGAAGAACTGATGACGG 3'		

Gene/ Polymorphism	Primer name	Primer sequence	Ta(°C)	Reference
*CHRM3/Mnl*I	CHRM3-F	5' GCCAATGAGCCTCCCCAATTC 3'	60	Present study
	CHRM3-R	5' CCAGTGACCACTTGGACATG 3'		
SLC18A1/BseRI	18A1F	5' ACT GTT TGT CCT TCG ATT	51	Present study
	18AR	5' GGA CCT TGA AAG GGT TTA AAT TCA		
RELNIVS59C/T	RELNint59C/TF	5' GAAGATACAAGCAGCTTCCAGAATGG 3'	55	Present study
	RELNint59C/TR	5' CCTGGGATTCAGACTTGGAA 3'		
	RELNint59C/T	5' TCCCCTCTCCCAGAGGCTGGAGGCAAGA 3'		
WDR47rs2591000	rs2591000-F	5' TCCTGCAAGGAGGATGTATTG 3'	52	Present study
	rs2591000-R	5' CTCTGCCTCCCAAGTTCAAG 3'		
GRID1 rs10887523	GRID1-F	5' CAACCAGTGACTGCCATGAT 3'	60	Present study
	GRID1-R	5' CCTTCCAAGGTGCTGTGTTT 3'		
ATG16L2rs2282613	rs2282613-F	5' CCCTGGGATGTCTTCGTTT 3'	50	Present study
	rs2282613-R	5' CCAGGGCAGGATGAAAGTTA 3'		

Abbreviations: *BZRP*, peripheral benzodiazepine receptor; *DBH*, Dopamine beta hydroxylase; °C, degrees Celsius; *CHRM3,* Acetylecholine receptor, Muscarinic *3;, DLX6,* Distal-less like homeobox 6; ATG16L2, Hyoptherical protein ATG16L2; *GBR,* GABA receptor 1; *GRIA4,* AMPA receptor subunit GluR4; *GRIN1,* N-methyl-D-aspartate receptor NR1 subunit; *RXRβ,* retinoid X receptor beta; *SLC18A1* Vesicular monoamine transporter*; SNAP25* Synaptosomal-associated protein of 25kDa: *SNAP29*, Synaptosomal-associated protein of 29kDa; *SYN3*, Synapsin III; *RELN,* Reelin; Ta, Annealing temperature used in PCR, WDR47, WD-repeat protein 47

Table 2.2. Primer sequences used in PCR-amplification of the protein-encoding region of *POU3F2*.

Amplicon	Size (bp)	Forward Primer	Reverse Primer	Ta (°C)
POU3F2.1A	251	5' GAGGGAGCCCGAGGCGAAAA 3'	5' GCGTGGCTGAGCGGGTGT 3'	61.6
POU3F2.1B	263	5' CTACCGCGAAGCGCAGAC 3'	5' GCCCGTGCSGCTCGTCTC 3'	63.1
POU3F2.1C	311	5' GGCCAGCCGGACATCAAG 3'	5' CCCAGCATGCCGTTCACC 3'	61.2
POU3F2.D	318	5' ACCTCCCACCCTCCATGG 3'	5' TCCGCCGCTGCTTGAACT 3'	61.7
POU3F2.1E	339	5' CACCATGCCGACCACCAC 3'	5' CGCCTCCTCCAACCACTT 3'	58.8
POU3F2.1F	219	5' ACCACCATCTGCAGGTTT 3'	5' CTTGGGGCATTTGAGGAA 3'	54.4
POU3F2.1G	350	5' CAAGATCGCAGCGCAAGG 3'	5' CCAAGGACCGAAGGGGAG 3'	58.7

Abbreviations: bp, base pairs; °C, degrees Celsius; *POU3F2,* POU domain, class 3, transcription factor 2; Ta: Annealing temperature used in PCR

2.3.1.3 Primers for generation of insert for Y2H cloning

Since the reelin cDNA sequence is 12500bp long, it was decided to use PCR-based exon splicing rather than RT-PCR, to generate the reeler domain-encoding fragment, which is encoded by the first four exons of the reelin gene (Fig 2.1). Primers were designed to amplify each of these exons individually. To facilitate cloning into the appropriate vector, the exon 1 forward primer was designed to contain an *Nde*I restriction sequence at the 5' end, while the exon 4 reverse primer contained an *EcoR*I restriction site and a "stop" codon. In addition, these two primers also included additional "overhang" nucleotides at their 5' ends to facilitate restriction enzyme digestion of the engineered *Nde*I and *Eco*RI sites. The remaining primers were designed so that each contained a "tag" sequence that is complementary to the sequence of the adjacent exon (Fig 2.1). The sequences of the primers used for this part of the study are shown in Table 2.3

2.3.1.4 Primers for Y2H insert screening

In order to amplify inserts cloned into Y2H cloning vectors, primers were designed to vector-specific sequences flanking the multiple cloning site (MCS) of pGBKT7 and pACT2 (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A) Y2H cloning vectors. The specific vector sequences used in the design of these primers were obtained from the Clontech™ Matchmaker™ vector handbook (www.clontech.com). The sequences of these vector-specific primers are shown in Table 2.4.

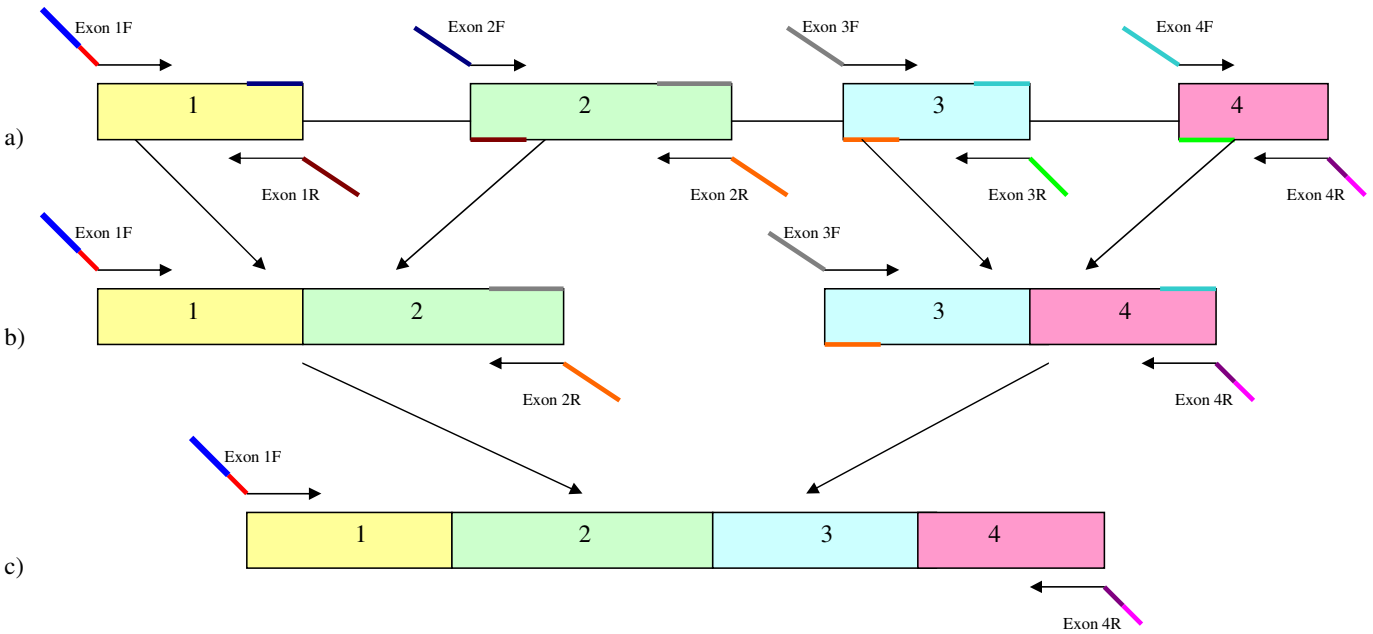


Fig 2.1: Schematic representation of protocol used to generate insert from genomic DNA for cloning into Y2H vector.
a) Briefly, each exons was amplified separately. b) Aliquots of exons 1 and 2 were mixed, and aliquots of exons 3 and 4 were mixed in separate tubes and used in a two-step PCR reaction that effectively joined exons 1 and 2 (using exon1F and exon2R) together and exons 3 and 4 together (using exon 3F and exon 4R). c) The two resulting fragments were then joined in another two-step PCR reaction using exon 1F and exon 4R generating a fragment that comprised the four reeler-encoding exons, as well as appropriate restriction sites for cloning into a Y2H vector, as well as a stop codon for *in vitro* translation.

Table 2.3. **Primer sequences used to amplify each of the first four exons of the reelin gene from genomic DNA**.

Primer Name	Sequence	Ta (°C)	Ta (°C) tag
Reeler Exon 1F	5′ **ACTGCAGAACATATG**CTGTGCACCACACACGGG 3′	62	39
Reeler Exon 1R	5′ **TTGTTGAAATTGTCA**CATGGTATTCTTGTCCCGGAA 3′	66	35
Reeler Exon 2F	5′ **GACAAGAATACCATG**TGACAATTTCAACAAGCACC 3′	56	39
Reeler Exon 2R	5′ **GGTCAGACATGATCC**CAAATCCGAAAGCACTGG 3′	54	43
Reeler Exon 3F	5′ **CTGCTTTCGGATTTG**GGATCATGTCTGACCACCAG 3′	62	41
Reeler Exon 3R	5′ **TGTGTTGCTGTAGCC**ATGAAATTCACACAGCCTGTGC 3′	66	43
Reeler Exon 4F	5′ **CTGTGTGAATTTCAT**GGCTACAGCAACACACCGG 3′	62	37
Reeler Exon 4R	5′ ACTCGA**GAATTCCTA**ATCTTTGAAAATAACCTGGCCC 3′	62	39

Abbreviations: °C, degrees Celsius Ta, Annealing temperature.
Sequences in black font represent the sequence of the primers that anneals to the DNA in the PCR reaction. Sequences in coloured fonts represent the various tags used to effect splicing and cloning (shown in Fig 2.1). **Light blue, "overhang" tag**; **red, *Nde*I restriction site**; **brown, exon 2 5'- tag**; **dark blue, exon 1 3'-tag;** **orange, exon 3 5'-tag**; **grey, exon 2 3'-tag**; **green; exon 4 5'-tag**; **teal, exon 3 3'-tag**; **purple, *EcoR*I restriction site**; **pink, stop codon**.

Table 2.4. **Primer sequences and annealing temperatures used for the amplification of inserts from cloning vectors.**

Name	Sequence	Ta (°C)
pGBKT7-F	5′ TCATCGGAAGAGAGTAG 3′	50
pGBKT7-R	5′ TCACTTTAAAATTTGTATACA 3′	51
pACT2-F	5′ CTATTCGATGATGAAGATACCCCACCAAACC 3′	68
pACT2-R	5′ GTGAACTTGCGGGGTTTTTCAGTATCTACGA 3′	68

Abbreviations: °C, degrees Celsius; Ta, Annealing temperature

2.3.1.5. Primers for *in vitro* transcription and translation

Primers for the generation of products used in *in vitro* transcription and translation experiments were designed using the sequence of the pGBKT7 and pGADT7 vectors obtained from the Clontech™ Matchmaker™ vector handbook (www.clontech.com). The primer sequences and annealing temperatures are shown in Table 2.5.

2.3.1.6. Primers for mammalian two-hybrid analysis (M2H)

Primers were designed in order to amplify the reeler insert from pGBK and clone it into the pM GAL4 DNA-binding domain cloning vector for mammalian two-hybrid (M2H) screening (section 2.17). Separate sets of primers were designed to amplify reeler putative ligands from pACT2 for cloning into the pVP16 GAL4 activation domain M2H vector. The sequence and annealing temperatures of these primers are shown in Table 2.6

Table 2.5. **Primers for the generation of products used in *in vitro* transcription and translation experiments.**

Name	Sequence	Ta (°C)
BK-Myc	5′ AATAAAATTGTAATACGACTCACTATAGGGCGAGCCGCC ACCATGGAGGAGCAGAAGCTGATGTCA 3′	65
BK-R	5′TCACTTTAAAATTTGTATACAC 3′	44
ADHA-F	5′ AATAAAATTGTAATACGACTCACTATAGGGCGAGCCGCC ACCATGTACCCATACGACGTTCCAGAT 3′	61
AD-R	5′ GGGGTTTTTCAGTATCTACGAT 3′	52

Abbreviations: °C, degrees Celsius; Ta, Annealing temperature

Table 2.6. **Primers for the generation of inserts for the creation of constructs to be used in M2H analysis.**

Name	Sequence	Ta (°C)
ReelerF-*Eco*R1	5′ACTGCAGAAGAATTCATGCTGTGCACCACACACGGG 3′	65
ReelerR-*Sal*1	5′ ACTCGAGTCGACCTAATCTTTGAAAATAACCTGGCC 3′	44
WDR47-*BamHI*-F	5′ ACTGCAGAAGGATCCGTTGGCACAACATTTCAT 3′	48
ATG16L2-*Eco*RI-F	5′ ACTGCAGAAGAATTCCGGGCTCAGGATGTGCTG 3′	56
pACT2-*Sal*I-R	5′ ACTGCAGAAGTCGACTATCTACGATTCATACATCT 3′	52

Abbreviations: °C, degrees Celsius; Ta, Annealing temperature
Sequences in black font represent the sequence of the primer that anneals to the DNA in the PCR reaction. The sequence in coloured fonts represents tags for cloning: **Blue, "overhang" tag**; **red, *Eco*RI restriction site**; **purple, *Sal*I restriction site; and green, *Bam*HI restriction site.** Prey constructs were generated using gene-specific forward primers (WDR47-BamH1F; ATG16L2-EcoRI-F) and a the pACT2-SalI-R pACT2 specific primer.

2.3.2. PCR for genotyping and SSCP analysis

DNA amplification was performed in a 50µl reaction containing 0.1µg genomic DNA, 75µM of each of dATP, dCTP, dGTP and dTTP (Promega Corp., Madison Wisconsin USA), 5µl of a 10x Taq DNA polymerase buffer (Bioline UK Ltd, London, UK), 1.5mM magnesium chloride (Bioline UK Ltd, London, UK), 150ng of each oligonucleotide primer, 0.5U Taq DNA polymerase (Bioline UK Ltd, London, UK), 5% glycerol (Sigma chemical company, St Louis, Missouri, USA) and water to a final volume of 50µl. Amplification was performed in a GeneAmp®, PCR system 9700 thermal cycler (P.E Biosystems, Forster, City CA. U.S.A). A typical cycling profile consisted of a single incubation at 94°C for 5 min cycle to allow for the denaturation of the double stranded DNA, followed by 30 cycles of 94°C for 30s, appropriate annealing temperature (Table 2.2) for 30s to allow for the primers to anneal to their target sequences and 72°C for 30s to allow for extension of the PCR product. These 30 cycles were followed by single final incubation at 72°C for 7 min. Following amplification, 5µl aliquots of each sample were electrophoresed on 1-2% agarose gels (section 2.4) for verification.

2.3.3. High fidelity PCR

High fidelity PCR was used to amplify the exons coding for the reeler domain from genomic DNA. These amplified products were subsequently joined together in a PCR-based splicing strategy and the resulting fragment cloned into the pGBKT7 Y2H cloning vector.

For the amplification of the above-mentioned exons, 100ng of human genomic DNA from a control individual was used as a template in a reaction performed in a 50µl volume containing: 150ng of each primer (Table 2.3)

1.5µl of an equimolar dNTP (2.5mM of each, dATP, dCTP, dGTP and dTTP) solution, supplied by the manufacturer (TaKaRa Shuzo Co.Ltd, Shiga, Japan), 5µl ExTaq™ Mg^{2+}-containing 10x reaction buffer supplied by the manufacturer (TaKaRa Shuzo Co.Ltd, Shiga, Japan), 2U ExTaq™ (TaKaRa Shuzo Co.Ltd, Shiga, Japan) and ddH_2O to a final volume of 50µl. Amplification was performed as above in a GeneAmp ®, PCR system 9700 thermal cycler.

2.3.4. PCR-amplification for generation of reeler domain fragment.

The generation of the reeler domain fragment for use in Y2H analysis was performed in a three-stage PCR reaction. First, each of the four reelin gene exons which together encode the reeler domain was amplified individually using the appropriate primer set (Table 2.3) following the protocol described in section 2.3.3. The PCR products were electrophoresed on a 2% agarose gel from which they were subsequently excised and purified (section 2.2.4). Purified products of exon 1 and exon 2 were then mixed in one microfuge tube, while purified products of exon 3 and 4 were mixed in a separate microfuge tube.

A PCR reaction mix was made up consisting of 15µl of an equimolar dNTP (2.5mM of each, dATP, dCTP, dGTP and dTTP) solution, supplied by the manufacturer (TaKaRa Shuzo Co.Ltd, Shiga, Japan), 50µl ExTaq™ Mg^{2+}-containing 10x reaction buffer supplied by the manufacturer and 395µl ddH_2O. Five aliquots of 46µl each of this reaction mix were transferred into 5 sterile microfuge tubes to which 1µl of either the purified exon1-exon2 mix or purified exon3-exon4 mix was added. To each tube, 0.5µl ExTaq™ (TaKaRa Shuzo Co.Ltd, Shiga, Japan) was added. The rest of the reaction mix was stored for further use. Thermo-cycling was performed in a GeneAmp®, PCR system 9700 thermal cycler using the following cycling parameters: 15 cycles of 94°C for 30s, Ta of the "tag" (Table 2.3) for 30s and 72°C for 30s. During this step, and in the absence of any primers, the exonic overlaps provided by the primer tags used in the first amplification reaction allowed exons 1 and 2, as well as exons 3 and 4 to partially anneal to each other. This double-stranded region then primed extension and conversion of a partially annealed exon1-exon2 or exon3-exon4 fragments into full double-stranded fragments of exons 1 through 2 and exons 3 through 4. To the remaining, stored reaction mix, 150ng of each of the appropriate primer set (Exon 1F and Exon2R for exon1-exon2 fragment and Exon 3F and Exon4R for exon3-exon4 fragment) and 25µl formamide were added. Following thermo-cycling, 45µl of the reaction mix and 0.2µl ExTaq were transferred to each of the 5 sample tubes and samples were thermo-cycled again for 15 cycles of 94°C for 30s, Ta of the primers (Table 2.3) for 30s and 72°C for 30s in GeneAmp®, PCR system 9700 thermal cycler. During this step, the two-exon extension products are exponentially amplified. The PCR products were electrophoresed for verification as well as DNA purification (section 2.2.4). After the exon1-exon2 fragment and exon3-exon4 fragments were generated, the two fragments were purified, mixed and the same protocol as above was followed to generate a full exon 1 through exon 4 fragment. A schematic representation of the above protocol is shown in Figure 2.1 and the PCR conditions are summarised in Table 2.7.

Table 2.7. **PCR conditions used to amplify the first 4 exons of reelin from genomic DNA.**

Primer set	[$MgCl_2$] mM	TD ˚C	Time sec	TA ˚C	Time sec	TE ˚C	Time Sec
Exon1F/ Exon1R	1.5	94	30	62	30	72	30
Exon2F/ Exon2R	1.5	94	30	59	30	72	30
Exon3F/ Exon3R	1.5	94	30	63	30	72	30
Exon4F/ Exon4R	1.5	94	30	63	30	72	30

Abbreviations: ˚C, degrees Celcius; mM, millimolar; sec, sec; TA, annealing temperature; TD, denaturing temperature; TE, extension temperature

2.3.5. Bacterial colony PCR

As the vectors used in Y2H and M2H do not support blue-white selection, and in order to rapidly identify bacterial colonies harbouring the desired recombinant plasmid to be used in these analyses, bacterial colony PCRs were performed. In these PCR reactions, instead of using 100ng of genomic DNA as template, a miniscule amount from an individual bacterial colony was picked from an agar plate containing the appropriate antibiotic, and used as template. Y2H vector-specific primers (Table 2.4.) were used in conjunction with PCR reaction mixtures and conditions as described in section 2.3.2 to perform these colony PCR amplifications. PCR amplified products were subsequently electrophoresed on a 1% agarose gel for verification.

2.3.6. Yeast colony PCR

The protocol used for yeast colony PCR was virtually identical to the protocol used for bacterial colony PCR, with the only difference being that a tiny amount of a yeast colony, instead of a bacterial colony was used as template. These reactions were performed as the first step towards generating a restriction enzyme map of each of the putative interactor prey inserts obtained in Y2H library screening.

2.3.7. PCR-amplification for *in vitro* transcription and translation

The Y2H cloning vector containing the 'bait' insert, as well as the clones isolated in the Y2H screen were used as templates for the amplification of inserts for *in vitro* transcription and translation. Amplification was performed as in section 2.3.3, in a 50µl reaction volume containing 0.5µl plasmid preparation (section 2.2.5) as template, and PCR conditions as summarised in Table 2.8.

Table 2.8. **PCR conditions used for generation of templates for *in vitro* transcription/ translation**

Primer set	[$MgCl_2$] mM	TD ˚C	Time sec	TA ˚C	Time sec	TE ˚C	Time sec
BK-Myc/ BK-R	1.5	94	30	49	30	72	150
ADHAF/ AD-R	1.5	94	30	42	30	72	150

Abbreviations: ˚C, degrees Celcius; $MgCl_2$, Magnesium chloride mM, millimolar; sec, sec; TA, annealing temperature; TD, denaturing temperature; TE, extension temperature
The thermocycler was set to perform 30 amplification cycles

2.3.8. PCR-amplification for mammalian two-hybrid analysis

The Y2H cloning vector containing the "bait" insert, as well as the putative reeler-interacting clones isolated in the Y2H screen were used as templates for the PCR preparation of inserts for M2Hconstructs. Amplification was performed as described above, using primers listed in Table 2.6, and PCR conditions described in Table 2.9.

Table 2.9. **PCR conditions used for amplification of inserts for M2H analysis.**

Primer set	[$MgCl_2$] mM	TD ˚C	Time sec	TA ˚C	Time sec	TE ˚C	Time sec
ReelerF-EcoRI/ ReelerR-SalI	1.5	94	30	62	30	72	30
WDR47-BamHI-F/ pACT2-SalI-R	1.5	94	30	50	30	72	150
ATG16L2-EcoRI-F/ pACT2-SalI-R	1.5	94	30	50	30	72	150

Abbreviations: ˚C, degrees Celcius; $MgCl_2$, Magnesium chloride mM, millimolar; sec, sec; TA, annealing temperature; TD, denaturing temperature; TE, extension temperature
The thermocycler was set to perform 30 amplification cycles

2.4. GEL ELECTROPHORESIS

In the present study, agarose gel electrophoresis was used either to visualise PCR-amplified fragments or plasmid preparations for verification, or for excision of DNA fragments for purification (section 2.2.3), while both agarose and non-denaturing polyacrylamide gel electrophoresis (PAGE) were used to visualise restriction enzyme digested PCR-amplified products for allele specific restriction enzyme analysis (Table 2.11). Sodium dodecyl sulphate (SDS) PAGE was used to visualise translated protein products as well as co-immunoprecipitation reactions. The solutions used for making these various types of gels are shown in appendix I.

2.5. SINGLE STRAND CONFORMATIONAL POLYMORPHISM ANALYSIS

Single strand conformation polymorphism (SSCP) analysis was performed on the coding region of *POU3F2* in order to identify polymorphisms that could be used in subsequent case-control association studies. Briefly, 8µl of PCR-amplified product (section 2.3.2.) was mixed with 8µl SSCP loading dye (Appendix I) and incubated at 94°C for 5min in order to denature the DNA. Following the incubation period, the sample was loaded onto a mildy denaturing polyacrylamide gel (appendix I), containing 5% glycerol and electrophoresed at 25W for 16h

at 4°C in 0.5xTBE running buffer. Eight microlitres of a non-denatured control sample and 2µl λ*Pst* (Appendix I) size marker, both of which were mixed with 8µl SSCP loading dye, were co-electrophoresed with the heat-denatured PCR product. After electrophoresis, the bands were visualised by silver staining (section 2.6). For each panel of samples analysed, an 8% as well as a 10%, polyacrylamide gel of 400x300x1mm dimensions (appendix I), which had been covalently bound to a nylon support sheet [Gelbond ® PAG film] (Cambrex Bio Science, Rockland, Inc, Rockland, Maine, U.S.A)] during gel polymerisation, were used in PCR-SSCP mutation screening.

2.6. VISUALISATION OF POLYACRYLAMIDE ELECTROPHORESED PRODUCTS

2.6.1. Silver staining of polyacrylamide gels

After completion of electrophoresis, the gel was immersed in solution B (0.1% $AgNO_3$) and gently shaken for 10 min, then rinsed with water, and subsequently agitated in solution C until stained bands could be seen. The gel was then viewed on a white light illuminator (Lauda Thermostat, Germany) and photographed using a Video Printer (Sony Corporation, Shinagawa-ku, Tokyo, Japan).

2.6.2. Autoradiography of SDS polyacylamide gels

After SDS-PAGE, the electrophoresis apparatus was dismantled and the gel transferred to Whatman 3M paper (Whatman International Ltd, Maidstone, England), and heat- and vacuum-dried in a Drygel Sr™ slab gel drier (Hoeffer Scientific Instruments, San Francisco, C.A., U.S.A) for one hour. After drying, the gel was exposed to autoradiography film [Kodak (Eastman Kodak Company, Rochester, New York, U.S.A)] for 1 day to two weeks (depending on the strength of the radioactive signal and concentration of proteins) after which it was developed in a Hyperprocessor ™ automatic autoradiography film processor (Amersham pharmacia biotech U.K Ltd., Little Chalfont, Bucks, U.K).

2.7. AUTOMATED DNA SEQUENCING

Automated DNA sequencing of PCR-amplified products, as well as cloned inserts, was performed either at the Core Sequencing Facility at the Department of Genetics of the University of Stellenbosch, RSA on an ABI Prism™ 377 or an ABI Prism™ 3100 automated sequencer (P.E. Applied Biosystems, Forster City, CA, U.S.A) or at the Department of Medical Biochemistry at the University of Stellenbosch, RSA on an ABI™ 3100 Avant automated sequencer (P.E. Applied Biosystems, Forster City, CA, U.S.A). The primers used for these sequencing reactions for PCR-amplified products were identical to the original PCR primers, while for the sequencing of Y2H constructs, the vector-specific primers were used.

2.8. SEQUENCE ANALYSIS

2.8.1. DNA sequence analysis

Sequence analysis was done using the ChromasPro computer program (Techelysium Pty Lmt, Helensvale, Queensland, Australia) to verify the sequence integrity of the reeler fragment generated by PCR-amplification (section 2.3.4), as well as to identify Y2H putative interactor prey clones isolated during Y2H library screening. The nucleotide sequence of the spliced reeler domain-encoding exon (section 2.7) was compared to

the reelin reference sequences obtained from the Genbank database (www.ncbi.nlm.nih.gov/Entrez) and the Ensembl database (www.ensembl.org). The Y2H prey constructs were identified by BLASTn comparison of the nucleotide sequences against the GenBank database (www.ncbi.nlm.nih.gov/Entrez) and the Ensembl database (www.ensembl.org). The insert sequence was also translated in the frame dictated by the preceding GAL4AD reading frame (i.e. reading frame 1), and this deduced protein sequence compared against proteins in the Swissprot database by BLASTp analyses.

2.8.2. Protein sequence analysis

Following the identification of the protein encoded by each of the clones obtained from the Y2H screen, the protein sequence was analysed using Proteome Analyst (http://www.cs.ualberta.ca/%7Ebioinfo/PA/Sub/index.html) and ESLpred (http://www.imtech.res.in/raghava/eslpred/.) to determine protein domain structure.

2.9. GENOTYPING BY SINGLE NUCLEOTIDE ddNTP PRIMER EXTENSION (SNaPshot) ANALYSIS OF REELIN INTRON 59 POLYMORPHISM

The SNaPshot genotyping method (Applied Biosystems, Foster City, California, USA) involves the extension of an oligonucleotide probe (that terminates immediately 5' to the SNP of interest) by one of four fluorescently-labelled dideoxynucleotides complementary to the base sequence at the SNP site of interest. The sequences of primers used in the SNaPshot genotyping of *RELNIVS59C/T* polymorphism are shown in Table 2.1.

2.9.1. PCR reaction clean-up

The first step in the SNaPshot reaction entails a PCR-product purification step to remove excess dNTPs and to dephosphorylate unincorporated outer primers that may interfere with the SNaPshot reaction. Here, 5µl of the relevant PCR products were incubated with 0.33U *Exo*I (Amersham, Little Chalfont, Buckinghamshire, UK) and 0.66U shrimp alkaline phosphatase (SAP) (Roche Applied Science, Basel, Switzerland) at 37˚C for one hour, followed by an enzyme deactivation step at 75˚C for 30 min. The purified PCR template was subsequently stored at 4˚C until required.

2.9.2. Primer extension reaction conditions

The internal interrogation primer (*RelnIVS59C/T* Table 2.1) to be used in a singleplex reaction was diluted to a concentration of 0.2µM using ddH_2O. The extension reaction, comprising 3µl of previously cleaned, pooled PCR products, 3µl SNaPshot Multiplex Ready Reaction mix (Applied Biosystems, Foster City, California, USA), which contains differentially fluorescently labelled ddATP, ddCTP, ddGTP and ddTTP, 1µl internal primer and 1µl de-ionised water, was performed by repeating the following cycle 27 times: 96˚C for 10s, 50˚C for 5s, and 60˚C for 30s. Thereafter, a post-extension purification step was employed to avoid further primer extension. This was performed by adding 1U of SAP to the sample, which was subsequently incubated at 37˚C for one hour, and then at 72˚C for 30 min to deactivate the enzyme.

2.9.3. Analysis on ABI Prism 3130 Genetic Analyser

The fluorescently extended probes were separated and detected on an ABI Prism 3130 Genetic Analyser capillary electrophoresis system (Applied Biosystems, Foster City, California, USA). After an appropriate spectral matrix using materials from the matrix standard DS-02 (Applied Biosystems, Foster City, California, USA) was created, the ABI Prism 3130 Genetic Analyser was used with filter set E5 to process the data from the 5 dyes, namely dR110, dR6G, dTAMRA, dROX and LIZ.

Fluorescently labelled extension reactions were prepared for capillary electrophoresis analysis by mixing 9µl of Hi-Di formamide (Applied Biosystems, Foster City, California, USA), 1µl of the SNaPshot product and 0.4µl of GeneScan-120 LIZ internal sizing standard (Applied Biosystems, Foster City, California, USA). The samples were then denatured by placing them at 95˚C for 2 min and thereafter, the prepared samples were then stored on ice until loaded into the capillary electrophoresis system.

A 36cm capillary array filled with denaturing POP4 performance optimised polymer (Applied Biosystems, Foster City, California, USA) was used for DNA fragment separation. Genetic Analyser electrode running buffer with EDTA was used in 1x concentration. Typical run module parameters were: run temperature 60˚C, capillary fill volume 38000 steps, pre-run voltage 15kV, data delay 3600s and run time 14000s.

Comment [MB63]: 4% DMA Homopolymer,8M Urea, 5% 2-pyrrolodine, 100mM N-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid (TAPS) adjusted to pH 8.5 w/ ddH2O. electrode buffer = EDTA

Two negative controls were electrophoresed with each reaction: a PCR template without primers, and the internal pooled primers without template. Allele assignment was subsequently performed using ABI Prism Genotyper software (GeneMapper ID, Ver 3.7 [Applied Biosystems, Foster City, California, USA]).

2.10. GENOTYPING BY TAQMAN® SNP GENOTYPING ASSAYS

Two SNPs in *SYN3* (*rs130753* and *rs130454*) and two SNPs in *DLX6* (*rs1207728* and *rs1004278*) were genotyped using validated Taqman® SNP genotyping assays (Applied Biosystems, Forster City U.S.A), purchased from Applied Biosystems. The assay number for each of the relevant SNPs are summarised in Table 2.10.

The procedure for the assay was as follows. Briefly, 2.5µl Taqman® Universal PCR Mastermix was added to 0.25µl, 20x Taqman® SNP genotyping assay mix and 2.25µl dH_2O. This mixture was then added to 1µl genomic DNA template and PCR-amplified using an ABI 7900HT fast real-time PCR system (Applied Biosystems) at the Centre for Proteomic and Genomic Research (CPGR) at the University of Cape Town. The PCR-cycling parameters were set as follows: An initial hold at 50°C followed by another hold at 95°C for 10min. These two holds were followed by 40 cycles each at 92°C for 15sec and 60°C for 1min30sec. Following the 40 cycles another hold was set at 4°C for 7min. Once amplification was complete, allele assignment was performed using Applied Biosystems Sequence Detection Software (SDS) version 2.3.

Table 2.10. *rs numbers* and Taqman assay numbers of *SNY3* and *DLX6* polymorphisms used.

Gene	Polymorphism	Taqman® Assay number
SYN3	*rs130753*	c___2228023_10
	rs130454	c____898390_1_
DLX6	*rs1207728*	c___887662_10
	rs1004278	c___9507865_10

Abbreviations: *DLX,* Distal-less like homeobox 6; ; *SYN3*, Synapsin III

2.11. RESTRICTION ENZYME DIGESTION

2.11.1. Allele-specific restriction enzyme analysis (ASREA) for genotyping

Allele-specific restriction enzyme analysis (ASREA) was used to screen OCD patients and control individuals for previously described polymorphisms. Generally, 5µl of a PCR-amplified product was added to a reaction mix comprising 1-3U of the relevant enzyme (Table 2.11), 1µl of the relevant restriction enzyme buffer and ddH_2O to a final volume of 10µl. The mixture was subsequently incubated for 2-16 hours at the optimal temperature for the particular enzyme used (Table 2.11).

Following digestion, 8µl of the digested sample was mixed with 1µl bromophenol blue loading dye and loaded either onto a 2% agarose gel or a 12% polyacrylamide gel, depending on the size of the products (Table 2.11), and visualised either on a long wave 3UV transilluminator (UVP, Inc. Upland, CA, U.S.A), for agarose gels, or silver staining, for acylamide gels (section 2.6.1).

2.11.2. Restriction enzyme digestion for cloning

To clone the PCR-generated fragment (section 2.3.4) into the pGBKT7 vector , for Y2H analysis, and pM and pVP16 vectors for M2H both the insert and vector were sequentially double-digested with appropriate restriction enzymes [*Nde*I and *Eco*RI for Y2H and *EcoR*I and *Sal*I for M2H]. The digests were prepared in a 100µl reaction volume as follows: 50µl insert DNA or 20µl vector DNA was mixed with 5µl restriction enzyme , 10µl restriction enzyme buffer and the appropriate volume of ddH_2O (Insert, 35µl; vector, 65µl). The mixtures were incubated at 37°C for 3h. Following this, the samples were purified using the GFX® DNA purification kit, as discussed in section 2.2.3, with the only exception being that instead of gel electrophoresis and excision of DNA from the gel for purification, the samples were purified directly.

The samples were subsequently eluted in 50µl ddH_2O and mixed with 5µl the second restriction enzyme, 10µl restriction buffer and 35µl ddH_2O. These samples were incubated at 37°C for 3 hours, after which they were again purified using the GFX® DNA purification kit

2.11.3. Restriction mapping of Y2H prey clones

Restriction mapping of Y2H prey-inserts was performed in order to group identical prey-plasmids identified by Y2H analysis as interacting with the reeler bait construct. The prey-inserts were PCR amplified by yeast colony PCR (section 2.3.6) and the PCR products digested with *Hae*III restriction enzyme. Five microlitres of PCR product was mixed with 2U *Hae*III, 1µl appropriate restriction enzyme buffer and 3.8µl ddH_2O to a final

reaction volume of 10µl. The samples were then incubated for 2 hours at 37°C and subsequently electrophoresed on a 12% polyacrylamide gel (section 2.4.2.1), and bands visualised by silver staining (section 2.6.1). The restriction pattern of each prey-insert was evaluated and compared to each other. Inserts with the same *Hae*III restriction pattern were then digested with *Rsa*I in a separate reaction, following conditions described above, to verify the similarity of the inserts. Inserts with the same *Hae*III and *Rsa*I restriction enzyme digestion patterns were then considered as identical clones and only one representative of the group was used in further analyses.

Table 2.11. **Restriction enzymes and digestion conditions for genotyping by ASREA**

Polymorphism	Restriction Enzyme	Optimal temperature for enzyme (°C)	Type of gel electrophoresis
*SNAP25/Dde*I	*Dde*I	37	2% agarose
*SNAP25/Mnl*I	*Mnl*I	37	2% agarose
SNAP29/C56T and SNAP29/G92A	*Dde*I	37	12% acrylamide
GRIA4/rs609239	*HSp92*I	37	12% acrylamide
GRIN1/1	*Bse*RI	37	2% agarose
DLX6 IVS1C>T	*Apo*I	50	2% agarose
BZRP Ala147Thr	*Nru*I	37	2% agarose
SYN3/ -631C>G	*Bsr*I	65	3% agarose
GBR1.1-C39T	*Hha*I	37	12% acylamide
GBR1.11-T1545C	*Ear*I	37	2% agarose
GRID1 rs1088753	*Bfa*I	37	2% agarose
*CHRM3/Msl*I	*Msl*I	37	2% agarose
*SLC18A1/Bse*RI	*Bse*RI	37	2% agarose
*RXRβ/*Val95Ala	*Ban*II	37	2% agarose
WDR47rs2591000	*Hinf*I	37	2% agarose
ATG16L2 rs2282613	*Mbo*II	37	20% acrylamide

Abbreviations: *BZRP*, peripheral benzodiazepine receptor; *DBH*, Dopamine beta hydroxylas; °C, degrees Celsius *DLX,* Distal-less like homeobox 6; *GBR,* GABA receptor 1; *GRIA4,* AMPA receptor subunit GluR4; *GRIN1,* N-methyl-D-aspartate receptor NR1 subunit; *RXRβ,* retinoid X receptor beta; *SNAP25* Synaptosomal-associated protein of 25kDa: *SNAP29*, Synaptosomal-associated protein of 29kDa; *SYN3*, Synapsin III

2.12. GENERATION OF CONSTRUCTS

2.12.1. Generation of Y2H and M2H constructs

The Y2H bait-insert was cloned into the pGBKT7 bait-vector and, after verification of the integrity of the sequence and conservation of the GAL4 DNA-BD reading frame by automated sequencing, transformed into the yeast strain AH109. This construct was used to screen a CLONTECH MATCHMAKER pre-transformed foetal brain cDNA library, comprising foetal brain cDNAs cloned into the pACT2 prey-vector and transformed into the yeast strain Y187. For generation of M2H constructs, the reeler bait-insert was cloned into the pM GAL4-DNA binding domain vector, while putative reeler-interacting clones was cloned into the pVP16 GAL-activation domain vector (pVP16). The integrity of the sequence and conservation of the GAL4 DNA-BD and GAL-activation domain reading frame was also verified by automated sequencing.

2.12.2. Alkaline phosphatase treatment of vector

To prevent the vector re-circularising by self-ligation, following the final restriction enzyme digestion step (section 2.10), the ends of the linearised plasmid were CIP-treated to remove the phosphate groups. This was accomplished by mixing 50µl of the digested vector with 1µl CIP (Promega, Madison WI, USA), 10ul CIP buffer and 38µl ddH_2O. The sample was incubated at 37°C for 30 min, after which another 2µl CIP was added and the mixture incubated for a further 30min. Following this, 2µl 0.5M EDTA (Appendix I) was added and the sample was incubated at 65°C for 20 min to inactivate the enzyme. The vector was subsequently purified using the GFX^{TM} DNA purification kit (AmershamPharmacia Biotech, New Jersey, USA) (section 2.2.3).

2.12.3. DNA ligation

DNA ligations were performed in order to generate the Y2H bait constructs to be used in Y2H analysis and M2H bait and prey constructs for M2H analysis (vector maps are supplied in Appendix VI). In general, 2µl of the double-digested insert (section 2.10.4) was added to 1µl of CIP-treated, double-digested vector (section 2.10.4). To this mixture, 5µl 2x T4 DNA ligase buffer (Promega, Madison WI, USA), 5U T4 DNA ligase and ddH_2O, to a final volume of 10µl, were added. The sample was then incubated for 16 hours at 4°C. Following incubation, 5µl of the sample was transformed into the bacterial strain DH5α (section 2.15.1) which was plated onto LB agar plates containing the appropriate antibiotic. After incubation of the plates, successful ligation reactions were confirmed by bacterial colony PCRs (section 2.3.5.).

2.13. BACTERIAL STRAINS, YEAST STRAINS AND CELL LINES

2.13.1. Bacterial strains

To facilitate the selection and purification of Y2H constructs, ligation reactions were transformed into the *E.coli* DH5α strain. Transformed bacterial colonies were selected on the basis of their ability to grown on LB agar plates (Appendix I) containing selection antibiotics, and recombined plasmids identified by colony PCR (section 2.3.5). When selecting for pGBKT7, kanamycin was used as a selection antibiotic, while ampicillin was used when selecting for pACT2.

2.13.2. Yeast strains

The pGBKT7 bait construct was transformed into the yeast strain AH109, while all the clones present in the pre-transformed CLONTECH cDNA library (section 2.16.1) used in the Y2H analysis had been transformed into the yeast strain Y187 by the manufacturer.

2.13.3. Cell lines

The pM and pVP-16 constructs were co-transfected into a HEK293 cell line for M2H analysis together with the pG5SEAP vector.

2.14. GENERATION OF *E.Coli* DH5α COMPETENT CELLS

A scrape of an *E.coli* DH5α frozen (-70°C) glycerol stock was inoculated into 10ml LB-media. The culture was then incubated overnight at 37°C in a YIH DER model LM-530 shaking incubator (SCILAB Instrument

CO. Ltd, Taipei, Taiwan) at approximately 200rpm. Following incubation, a 1ml aliquot of this culture was inoculated into a 2l Ehrlenmeyer flask containing 200ml LB media (Appendix I). This culture was incubated at room temperature for 24 hours, while shaking at 200rpm, to mid-log phase (OD_{600nm}=0.6) on a Labcon orbital shaker (Labcon Pty, Ltd, Maraisburg, RSA). At this point the culture was decanted into 4x 50ml polypropylene tubes, which were centrifuged at 3000rpm for 15 min at 4°C in a Multitex centrifuge (MSE instruments, England). The supernatant was removed and 8ml of ice-cold CAP buffer (Appendix I) was used to resuspend the pellet. The cells were re-pelleted by centrifugation at 3000rpm for 15 min at 4°C in a Multitex centrifuge. The supernatant was discarded and the pellet was resuspended in 4ml of ice-cold CAP buffer. The suspended cells were subsequently transferred into 1.5ml microfuge tubes in 500µl aliquots and snap frozen by immersion in liquid nitrogen. The cells were then stored at -70°C until they were needed.

2.15. CULTURING OF THE HEK293 CELL LINE

2.15.1. Culture of HEK293 cells from frozen stocks

2.15.1.1 Thawing the cells

Frozen HEK293 cells, a kind gift from Prof Janet Hapgood, Dept Biochemistry, University of Stellenbosch, were thawed rapidly by immersing the vial containing the frozen stock in a 37°C waterbath (Memmert®, Schwabach, Germany) for 10min. Once the cells were thawed, the outside of the vial was immediately sterilized with 70% ethanol.

2.15.1.2. Removing DMSO from stocks and culturing cells

As the frozen stocks contained DMSO, it was necessary to remove the DMSO for maximum viability of the cells upon plating, using the following method. One millilitre of growth media (Appendix I), prewarmed to 37°C was added to the thawed stock and mixed by gentle pipetting. The mixture was transferred to a 12ml Greiner tube (Greiner Bio-one, Frickenhausen, Germany) and another 5ml growth media was added. The cells were then pelleted by centrifugation at 10000rpm for 1min using a Sorval® GLC-4 General Laboratory centrifuge (Separations Scientific, Johannesburg, South Africa), followed by removal of the supernatant. The pellet was resuspended in another 5ml growth media and the cells were once again centrifuged at 10000rpm for 1 min. Following this, the cells were resuspended in 10ml growth media and transferred into a T25 culture flask. The flask was gently swirled in order to distribute the cells evenly over the growth surface of the flask. The flask was then incubated at 37°C in a Farma termosteri-cycle 5% carbon dioxide humidified incubator (Farma International, Miami, Florida, U.S.A).

2.15.2. Splitting of cell cultures

Cell cultures were split every 2-4 days when they reached approximately 80%-90% confluency. Briefly, the growth media was removed from the flask as the cells were washed with sterile phosphate buffered saline (PBS) containing no calcium or magnesium. To this, 2ml of trypsin (Highveld Biological, Lyndhurst, South Africa) was added to facilitate the detachment of the cells from the growth surface of the flask. After 3min, 5ml growth media was added and the cells were gently resuspended. The cells were then transferred into 4 flasks each containing 10ml of growth media.

2.16. TRANSFORMATIONS AND TRANSFECTION OF PLASMIDS INTO PROKARYOTIC AND EUKARYOTIC CELLS

2.16.1. Bacterial plasmid transformations

Prior to the transformation, an aliquot of competent *E.coli* DH5α was removed from the -70°C freezer and allowed to thaw in ice for 20-30 min. Once the cells had thawed, 1µl plasmid preparation (section 2.2.4), or 3-5µl of the ligation reaction (section 2.11.2), was added. This mixture was then incubated on ice for 20-30 min after which they were placed in a Lasec 102 circulating water-bath (Lasec Laboratory and Scientific Company Pty Ltd, Cape Town, R.S.A) at 42°C for exactly 45s. The sample was then removed from the water bath and left at room temperature for 2min. Next, 1ml of LB media was added to the mixture and the sample was incubated for 1h at 37°C, while shaking at 200rpm in a YIH DER model LM-530 shaking incubator shaking. Following this incubation step, 200µl of the sample was plated onto LB agar plates containing the appropriate selection antibiotic (Appendix I). The remaining transformation reaction mixture was centrifuged at 13000rpm for 2min in a Beckman Microfuge Lite, the supernatant discarded and the pellet resuspended in 200µl LB media. This was then also plated onto the appropriate LB-agar plates. All the plates were incubated, inverted, for 16h at 37°C in a model 329 stationary CO_2 incubator (Former Scientific, Marieta, Ohio, U.S.A).

2.16.2. Yeast Plasmid transformations

The yeast strain to be transformed was streaked from frozen stocks onto YPDA agar plates (Appendix I). These plates were then incubated at 30°C for 2-3 days in a Sanyo MIR262 stationary ventilated incubator (Sanyo, Electronic Company Ltd, Ora-Gun, Japan). Following incubation, a volume representing 20-50µl of yeast cells was picked and resuspended in 1ml sterile ddH_2O in a sterile 2ml tube. The cells were then re-pelleted by centrifugation at 13000rpm for 30sec in a Beckman Microfuge Lite. The supernatant was removed and the pellet was resuspended in 1ml 100mM lithium acetate (LiAc) (Appendix I) and incubated for 5 min at 30°C in a MIR262 stationary ventilated incubator. The cells were again pelleted by centrifugation at 13000 rpm for 20s in a Beckman Microfuge Lite and all the LiAc was removed. Next, 240µl of 50% polyethylene glycol (PEG) (Appendix I), 36µl 1M LiAc (Appendix I), 25µl of 2mg/ml heat-denatured and snap-cooled sonicated herring sperm DNA (Promega, Madison WI, USA) and 10-20µl plasmid preparation and ddH_2O were added to a final volume of 350µl. The sample was then mixed by vortexing for at least 1 min and incubated at 42°C for 20-30 min in a Lasec 102 circulating water-bath. Following incubation, the cells were pelleted by centrifugation at 13000rpm in a Beckman Microfuge Lite and all the supernatant was removed. The cells were resuspended in 250µl sterile Millipore ddH_2O. One hundred and fifty microlitres of this sample was plated onto the appropriate selection plates (Appendix I) and incubated inverted at 30°C for 2-5 days in a Sanyo MIR262 stationary ventilated incubator.

2.16.3. Transfection of HEK293 cells

Forty-eight hours before transfecting the cells, approximately 1-3 x 10^4 cells per well were plated in complete growth media in a 24-well tissue culture plate (Appendix I) and incubated at 37°C in a 5% Farma- thermosteri-

cycle carbon dioxide humidified incubator (Farma, international, Miami, Florida, U.S.A). Two days later, the cells were visualised under a Nikon TMS light microscope (Nikon, Tokyo, Japan) to determine the level of confluence. Cells were only transfected once they reached approximately 80% confluence. For each transfection performed, 100µl of serum-free medium was aliquoted into a sterile 1.5ml eppendorf tube. Three microlitres GeneJuice® (EMD Biosciences, Darmstadt, Germany) was added to each tube. This mixture was thoroughly vortexed and incubated at room temperature for 5 min. A total of 1µg of the three plasmids combined (pM-reeler, pVP16-putative ligand and pG5SEAP reporter vector [Appendix 1], Table 2.12) was added to the mixture and mixed gently by pipetting. The GeneJuice/DNA/medium was incubated at room temperature for 15min. The entire volume of the mixture was then added drop-wise to the cells in the growth media. The culture plates were gently rocked back and forth in order to evenly distribute the drops across the surface of the plate. The cells were incubated at 37°C, in a 5% carbon dioxide humidified incubator (Farma, International, Miami, Florida) for 48 hours. The cells were subsequently pelleted by centrifugation at 13000rpm in a Beckman Microfuge Lite and 1ml of the supernatant transferred into a sterile Eppendorf tube. The supernatants were stored at -20°C until needed for reporter assays (section 2.20).

Table 2.12 Setup of the transfection experiments used in the present study

Transfection (5:5:1)	GAL4 DNA-Binding plasmid (µl)	VP16 Activation Plasmid(µl)	Promoter(µl)
Experimental 1	**pM-Reeler (2.2)**	**pVP16-WDR47 (2.9)**	**pG5SEAP**
Experimental 2	**pM-Reeler (2.2)**	**pVP16-ATG16L2 (1.9)**	**pG5SEAP**
Untransfected control	**None**	**None**	**None**
GeneJuice control	**None**	**None**	**None**
Basal Control	**pM (1.5)**	**pVP16 (2.8)**	**pG5SEAP**
GAL4 DNA-B control	**pM-reeler (2.2)**	**pVP16 (2.8)**	**pG5SEAP**
VP16 AD control (1)	**pM (1.5)**	**pVP16-WDR47 (2.9)**	**pG5SEAP**
VP16 AD control (2)	**pM (1.5)**	**pVP16-ATG16L2 (1.9)**	**pG5SEAP**
Positive control (1)	**pM3-VP16 (2.8)**	**None**	**pG5SEAP**
Positive control (2)	**pM53 (1.8)**	**pVP16-T (0.9)**	**pG5SEAP**

The ratio of pM: pVP16:pG5SEAP used in the transfections were 5:5:1.

2.17. ASSESSMENT OF Y2H CONSTRUCTS

2.17.1. Phenotypic assessment of yeast strains

Prior to being transformed, each of the yeast strains used in the Y2H analysis was assessed phenotypically. This involved plating strains AH109 and Y187 onto agar plates lacking individual essential amino acids, ie., agar plates SD^{-A}, SD^{-w}, SD^{-H}, SD^{-L} and SD^{-Ura}. Non-transformed yeast cells that were unable to grow on SD^{-A}, SD^{-w}, SD^{-H}, SD^{-L} but able to grow on SD^{-U} were used for transformation with bait vector and subsequent Y2H analysis. Following transformation of the reeler bait contruct into AH109, the transformed yeast was again streaked onto agar plates SD^{-A}, SD^{-w}, SD^{-H}, SD^{-L} and SD^{-U} in order to test whether the AH109 transformed with the bait construct was able to activate transcription of reporter or selection genes autonomously. Yeast containing the bait construct should only be able to grow on SD^{-w} and SD^{-U} plates.

2.17.2. Toxicity tests of transformed yeast cells

In order to proceed with the Y2H assay, it was important to establish whether the bait-construct (section 2.11) had any markedly toxic effect on its host yeast strain, AH109 (section 2.13.2). To achieve this, a growth curve of AH109 transformed with the pGBKT7-bait construct was generated and subsequently compared to a growth curve of AH109 yeast transformed with non-recombinant pGBKT7. These two growth curves were set up concurrently and under the same experimental conditions.

The two growth curves were generated by growing each of the transformed yeast strains to stationary phase in SD^{-W} in a 50ml polypropylene tube at 30°C in a YIH DER model LM-530 shaking incubator shaking at 200rpm. Following this incubation, a 1:10 dilution of each primary culture was made in SD^{-W} and incubated for an additional 24h in a 50ml polypropylene tube at 30°C in a YIH DER model LM-530 shaking incubator shaking at 200rpm. Every 2 hours, over a period of 8 hours during this incubation, a 1ml aliquot of the culture was taken and its OD_{600nm} was measured. An overnight (24h) reading was also taken. A linearised graph of the log of these OD_{600nm} readings versus time was constructed and the slopes of the graphs generated for the recombinant and non-recombinant transformants were compared.

2.17.3 Mating efficiency test

In order to determine the effect that the bait construct (section 2.12.2) had on the mating efficiency of AH109, small scale yeast matings were performed. In these mating experiments, the AH109 transformed with pGBKT7-reeler bait construct was mated with the prey host strain, Y187 transformed with the non-recombinant prey vector pACT2 or the control prey vector, pTD1.1, supplied by the manufacturer (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A). Concurrently, control matings were also performed in which the yeast strain AH109 transformed with non-recombinant pGBKT7 or the control pGBKT7-53 vector supplied by the manufacturer (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A) was mated with the prey host strain, Y187 transformed with the non-recombinant prey vectors pACT2 or the Clontech pTD1.1 control vector. The experimental procedures were as follows:

Each of the yeast strains used in the mating efficiency experiments was plated onto the appropriate nutritional selection plates (AH109 pGBKT7-reeler, AH109 pGBKT7 and AH109 pGBKT7-53 on SD^{-W} plates; Y187 pACT2 and Y187 pTD.1.1 on SD^{-L} plates). These plates were incubated for 2-5 days in a a Sanyo MIR262 stationary gravity-ventilated incubator. A single colony from these agar plates was used for each the test mating experiments; which was performed in 1ml YPDA media (Appendix I) in a 2ml microfuge tube. The matings were incubated overnight at 30°C, shaking at 200rpm, in a YIH DER model LM-530 shaking incubator. Following the overnight incubation, serial dilutions (1:10; 1:100; 1:1000 and 1:10000) of the mating cultures were plated onto SD^{-L}, SD^{-W} and SD^{-L-W} agar plates and incubated for 4-5 days at 30° in a Sanyo MIR262 stationary ventilated incubator. After the incubation period, the colonies on each plate were counted and used to calculate the mating efficiency (Appendix II).

2.18. Y2H ANALYSIS

2.18.1. The foetal brain cDNA library

A pre-transformed human MATCHMAKER foetal brain cDNA Library (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A) consisting of *S.cerevisiae* Y187 transformed with a foetal brain cDNA library, constructed in pACT2, was used in the Y2H library assay.

This library had been constructed from a pool of nine male or female Caucasian foetuses aged between 20-25 weeks. The library was *Xho*I-(dT)15 primed and contains approximately $3.5x10^6$ independent clones inserted into pACT2 through *EcoR*I and *Xho*I sites. The average insert size for this library was reported by the manufacturer as 2.0kb, with a range of between 0.5 and 4.0kb.

2.18.2. Establishment of bait culture

A colony of AH109 transformed with the reeler bait construct was streaked out onto SD^{-W} plates. Four of the resultant yeast colonies were inoculated into four separate 500ml Erlenmeyer flasks, each containing 50ml SD^{-W} media. The reason for producing four bait cultures was to facilitate the pooling of the initial cultures, thereby allowing the generation of a final bait culture with a titre of at least $1x10^{10}$, i.e. 100-fold excess of bait to prey, to facilitate high mating efficiency. The four initial cultures were incubated at 30°C overnight, while shaking at 200rpm in a YIH DER model LM-530 shaking incubator. Following overnight incubation, the cultures were transferred into individual 50ml polypropylene tubes and the cells pelleted by centrifugation at 3000rpm for 10min at room temperature in a Beckman Microfuge Lite. The supernatants were discarded and the four pellets were resuspended together in 50ml SD^{-W} following which, the suspension was transferred to a single 500ml Erlenmeyer flask and the culture was incubated for a further 16h at in a YIH DER model LM-530 shaking incubator, shaking at 200 rpm. After incubation, the titre of the bait culture was estimated by measuring the OD_{600nm} of a 1ml aliquot of the bait culture. This estimation was subsequently confirmed by means of a haemocytometric cell count.

The bait culture was centrifuged at 3000rpm at room temperature for 10min in a Beckman Microfuge Lite to pellet the cells, the supernatant was removed and the pellet resuspended in ml SD^{-W} media. An appropriate number of 10µl aliquots of this culture was removed for control mating experiments.

2.18.4. Library mating

A 1ml aliquot of the pre-transformed foetal brain cDNA library was removed from the -70°C freezer and thawed at room temperature (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A). Once thawed, the library aliquot was vortexed and 10µl aliquoted into a sterile 1.5µl microfuge tube for library titering. The pGBKT7-Reeler transformed AH109 pellet (section 2.17.2.) was then resuspended in 45ml 2x YPDA media (Appendix I) supplemented with 10µg/ml kanamycin (Kan) in a 2L Erlenmeyer flask). Subsequently, the remaining 990µl of the library culture was added to this Erlenmeyer flask. This mating culture was incubated at 30°C overnight, while shaking at 200rpm in a YIH DER model LM-530 shaking.

After the overnight incubation, the entire mating culture was transferred into a sterile 50ml polypropylene centrifuge tube and the cells pelleted by centrifugation in at 3000rpm for 5min in a Multex centrifuge (MSE Instrumentation, England, UK), and the supernatant subsequently removed. The Erlenmeyer flask in which the library mating was performed was rinsed twice with 40ml 2x YPDA containing 10µg/ml Kan. Each time the flask was rinsed, the 2x YPDA medium was used to resuspend the cell pellet and the cells then re-pelleted by centrifugation at 3000rpm for 10min at room temperature in Multex centrifuge . Following the final centrifugation step, the supernatant was removed and the pellet resuspended in 15ml 0.5x YPDA containing 10µg/ml Kan (Appendix I).

Serial dilutions of 100µl aliquots (1:10; 1:100; 1:1000; and 1:10000) of this cell-suspension were plated onto 90mm SD^{-L}, SD^{-W} and SD^{-L-W} agar plates, in order to determine bait:library mating efficiency. Two hundred and fifty microlitres aliquots of the remainder of the culture was plated onto each of 59 140mm diameter TDO (media lacking leucine, trytophan and histidine) plates (Appendix I) (250µl culture/plate for14.9ml culture = 59 plates). The TDO plates were incubated, inverted, at 30°C for 2 weeks in a Sanyo MIR262 stationary ventilated incubator.

2.18.5. Establishing a library titre

The serial dilutions of the mating culture plated onto the 90mm SD^{-L}, SD^{-W} and SD^{-L-W} agar plates were inverted and incubated in a Sanyo MIR262 stationary ventilated incubator for 4 days. Colony counts were performed on the SD^{-L}, SD^{-W} and SD^{-L-W} plates after the 4 day incubation in order to calculate the mating efficiency of the library mating and the number of library plasmids screened (section 2.17.3).

2.18.6. Control matings

Control matings were set up concurrently with library matings, in order to determine whether the recombinant reeler construct (transformed into AH109) had any negative effect on the ability of the transformed AH109 strain to mate with the library strain (Y187). A 10µl aliquot of the bait culture and a single test prey- (yeast strain Y187 transformed with pTD 1.1 control vector) colony were co-inoculated in 1ml 0.5x YPDA containing 10µg/ml kanamycin (Appendix I) in a 2ml centrifuge tube. This culture was subsequently incubated for 24h at 30°C in a YIH DER model LM-530 shaking incubator, shaking at 200 rpm. Following incubation, serial dilutions (1:10; 1:100; 1:1000; 1:10000) were plated onto SD^{-L}, SD^{-W} and SD^{-L-W} agar plates and incubated for 4 days in a Sanyo MIR262 stationary ventilated incubator. Following this, colony counts were done and the mating efficiency was calculated (section 2.17.3). Control preys included non-recombinant pACT2 transformed into Y187 and the pTD1.1 control vector supplied by Clontech.

2.18.7. Detection of activation of nutritional reporter genes

2.18.7.1. Selection of transformant yeast colonies

Yeast transformed with the bait construct to be used in Y2H analysis was plated onto SD^{-W} agar plates. Following incubation of these plates for 4-6 days in a Sanyo MIR262 stationary gravity-ventilated incubator,

transformant yeast colonies were picked and used in small and large scale bait cultures (section 2.16.2) and library matings (2.18.3).

2.18.7.2. Selection of diploid yeast colonies containing putative interactor peptides

In order to identify yeast colonies in which an interaction between the bait- and prey-fusion peptides had taken place, yeast colonies were plated onto TDO plates (Appendix I) as well as QDO (media lacking leucine, histidine, tryptophan and adenine) plates (Appendix I). Growth of the yeast cells on TDO plates signified the transcriptional activation of the *HIS3* nutritional reporter gene, while growth on the QDO plates indicated that both the *HIS3,* as well as the *ADE2* nutritional reporter genes had been transcriptionally activated. The activation of these genes in these diploid yeast cells is indicative of an interaction between the bait and prey peptides.

Briefly, the library mating culture was plated directly onto 59 140mm TDO agar plates (Appendix I) (section 2.18.3) and incubated in a Sanyo MIR262 stationary ventilated incubator for 2 weeks. The growth of these colonies on the TDO plates were monitored every 2 days and colonies were picked and restreaked onto TDO and QDO plates in order to test for the activation of *HIS3* and *ADE2* nutritional reporter genes. These plates were incubated for 3-6 days at 30°. Colonies growing on QDO plates after incubation were picked and plated onto QDO plates containing X-α-galactose, to assess activation of the *MEL1* gene, and incubated at 30°C in a stationary gravity-ventilated incubator for a further 3-5 days.

2.18.8. Detection of activation of colourimetric reporter genes

2.18.8.1. X-α-Galactosidase assay

X-α-galactosidase assays were performed, in order to test for the activation of the *MEL1* reporter gene by the specific interaction between specific bait and prey peptides. Briefly, yeast colonies in which the *HIS3* and *ADE2* reporter genes have been activated, as determined by their growth on QDO agar plates, were replicated from QDO plates onto Hybond N^+ nylon membranes. These membranes were subsequently placed colony-side up onto a QDO plate impregnated with 20mg/ml X-α-Gal solution (BD Biosciences, Clontech, Palo Alto, CA, U.S.A). The plates were subsequently incubated at 30°C in a Sanyo MIR262 stationary ventilated incubator. Following incubation, the intensity of the blue colour of yeast colonies that had activated the *MEL1* reporter gene was assessed.

2.18.9. Rescuing prey plasmids from diploid colonies.

In order to identify the interactor proteins, each individual prey needed to be isolated from the diploid colonies. To this end, plasmid DNA was isolated from each of the diploid cells following the protocol discussed in section 2.2.6 and transformed into *E.coli* strain DH5α as described in section 2.16.1. The transformants were plated onto LBamp plates which only allows for the growth of transformants containing the prey constructs. These prey constructs were subsequently transformed into the yeast strain Y187 (section 2.16.2).

2.18.10. Interaction specificity test

To test whether the interactions detected by Y2H analysis, through the activation of nutritional and colourimetric reporter genes, were specific interactions between the pGBKT7-Reeler bait and a particular prey peptide, interaction-specificity tests were used. Y187 colonies expressing the specific prey peptide were individually mated with the yeast strain AH109, transformed with the pGBKT7-Reeler construct, AH109 transformed with non-recombinant pGBKT7, AH109 transformed with the pGBKT7-53 control bait-plasmid, encoding murine p53, supplied by the manufacturer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) and AH109 transformed with a heterologous bait, encoding a cytosolic cardiac myosin binding protein C (MyBPC) C5 domain. After the resulting diploid clones were selected on SD^{-L-W} plates (section 2.18.6.2), these clones were streaked onto TDO and QDO selection plates to test for the activation of nutritional reporter genes (section 2.18.6), thereby testing whether the prey-peptides were able to interact with these heterologous baits as well as with the reeler bait.

Clones that interacted specifically with the reeler bait, during these interaction specificity tests were, considered putative true interactors. The inserts of these putative interactors were then sequenced to determine their identities.

2.19. CO-IMMUNOPRECIPITATION

Once putative ligands for the reeler domain had been identified by means of the Y2H experiments, the interactions were confirmed using *in vitro* co-immunoprecipitation (Co-IP) analysis.

2.19.1. Creating an RNase-free experimental environment

In order to reduce the chances for RNase contamination during transcription/translation and Co-IP experiments, all surfaces and instrumentation used in these experiments were wiped thoroughly using RNase Zap wipes (Ambion Inc, Austin, TX, U.S.A). Furthermore, only pipette tips and microfuge tubes certified RNase free by the manufacturer (Porex, Fairburn, Georgia, U.S.A) were used.

2.19.2. Transcription and translation of bait and preys

Following the identification of putative preys that interact with the reeler domain, based on their ability to activate both nutritional (section 2.18.6) and colourimetric reporter (section 2.18.7) genes, these prey clones were isolated from Y187 (section 2.2.5). These clones were then PCR amplified using the ADHA-F and AD-R primers (Table 2.5) under conditions described in section 2.3.7, in order to generate a PCR fragment comprising the prey insert linked to the HA-antibody epitope-encoding sequence and a T7 promoter sequence; this promoter sequence is crucial for *in vitro* transcription. The pGBKT7-Reeler construct was also amplified under conditions described in section 2.3.7, using the BK-Myc and BK-R primers (Table 2.5), thereby generating a PCR fragment comprising the reeler domain insert linked to the myc antibody epitope-encoding sequence and a T7 promoter sequence. These two PCR fragments were subsequently transcribed and translated in a one-tube coupled reaction, using the TNT® Quick Coupled Transcription/Translation system (Promega Corporation, Madison, WI., U.S.A) as per manufacturer's instructions. The translated products were

subsequently electrophoresed in a 15% SDS polyacrylamide gel (section 2.4.2.2) and visualised using autoradiography (section 2.6.2).

2.19.3. Co-immunoprecipitation of translated PCR products

Once the PCR products had been translated into the respective bait and prey fusion peptides, these two products were co-immunoprecipitated to assess the interaction identified by Y2H. In brief, 5µl bait and 5µl prey were mixed in a sterile, RNAse-free 1.5ml microfuge tube and incubated at room temperature for 1h, with mixing by gently tapping the tube every 15 min. Following the incubation, 1µl Myc antibody (5µg/ml) (Roche Biosciences, Palo Alto, CA., U.S.A) was added to the mixture. The sample was then incubated at room temperature for 1h. Subsequently, 10µl pre-washed protein G agarose (Appendix I) (Kirkegaard and Perry laboratories, Gaithersburg, ML, U.S.A) and 135µl Co-IP buffer (Appendix I) were added to each mixture. The sample was rotated on a Labnet rotor (Labnet Inc, NJ., U.S.A) at 10rpm at 4°C for 1 hour and was subsequently washed 5 times with TBST (Appendix I).

Single immunoprecipitation experiments with the reeler bait using the Myc antibody (Roche Biosciences, Palo Alto, CA., U.S.A), as well as each of the putative preys using the HA antibody (Roche Biosciences, Palo Alto, CA., U.S.A) were performed in conjunction with the Co-IP experiments to serve as controls. In brief, the 5µl reeler bait was mixed with 1µl Myc antibody in a sterile, RNase-free 1.5ml microfuge tube and incubated at room temperature for 1h. In separate 1.5ml microfuge tubes, 5µl of each of the putative prey ligands were incubated with 5µl HA antibody (Roche Biosciences, Palo Alto, CA., U.S.A). Each of these tubes were also subsequently incubated at room temperature for 1h. Following the incubation, 10µl pre-washed protein G agarose (Appendix I) (Kirkegaard and Perry laboratories, Gaithersburg, ML, U.S.A) and 135µl Co-IP buffer (Appendix I) were added to each tube. The samples were rotated on a Labnet rotor (Labnet Inc, NJ., U.S.A) at 10rpm at 4°C for 1 hour and were subsequently washed 5 times with TBST (Appendix I). 15µl SDS loading dye was added to each samples which were subsequently incubated at 95 °C for 5 min. The samples were then loaded onto a 15% SDS polyacrylamide gel and visualised using autoradiography. To separate peptides of similar size, 20% SDS polyacrylamide gels were used. A schematic representation of the co-immunoprecipitation technique is shown in figure 2.2.

2.20. M2H ANALYSIS

The M2H analysis was performed using the Matchmaker™ Mammalian Assay Kit 2 (BD. Biosciences, Palo Alto, U.S.A.). This kit includes the pM, pVP16 and pG5SEAP vectors (Figs. 2.4, 2.5 and 2.6, respectively). The HEK293 cells used for the M2H analysis were cultured and transfected as described in sections 2.13 and 2.16.3. Expression of the secreted alkaline phosphatase (SEAP) reporter gene (*SEAP*) from the pG5SEAP reporter vector (Appendix VI) occurs when there is an interaction between the fusion proteins generated by the pM and pVP16 constructs that were co-transfected with the reporter vector. Whether or not the *SEAP* reporter gene was activated was determined using the Great EscAPe™ chemiluminescent Detection Kit (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) by measuring the SEAP activity in the culture medium (since this is a secreted alkaline phosphatase) using a chemiluminescent substrate.

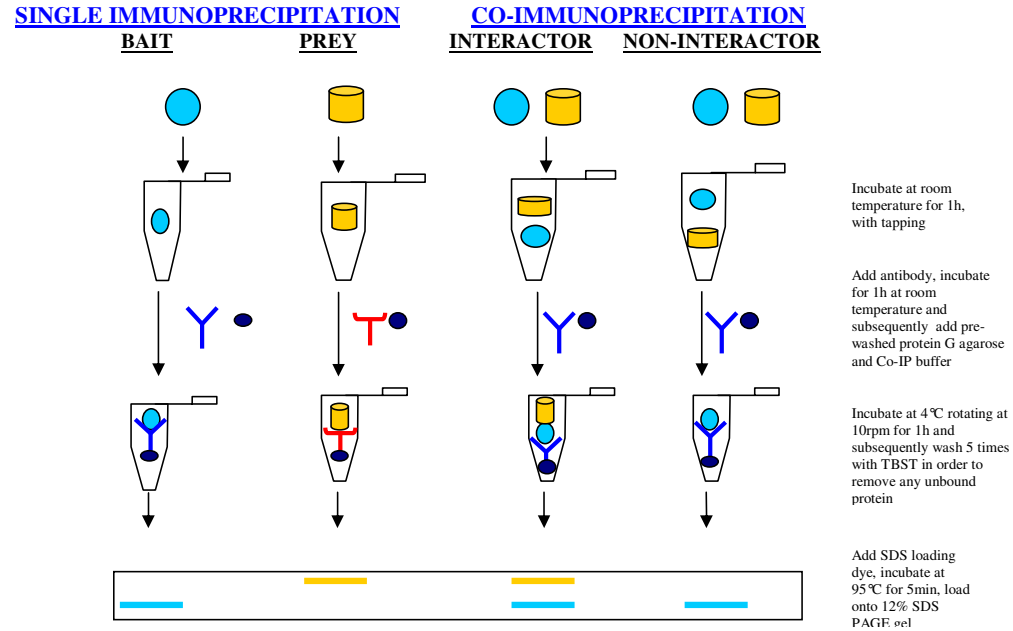


Fig 2.2. **Schematic representation of Co-IP protocol.** Protein A; Protein B; Myc Antibody; HA Antibody; Protein G agarose. Experiments 1 and 2 represent the immunoprecipitation of the proteins using the appropriate antibody. On a 15% SDS-PAGE gel the immunoprcipitation is represented by a single band as seen in lanes 1 and 2 of the gel. Experiment 3 represent Co-IP reactions showing an interaction between the two proteins. Co-IP reactions showing an interaction between proteins A and B is seen as two bands (lane 3);one band for each protein. Experiment 4 shows a Co-IP experiment where protein A and Protein B do not interact with each other. After the 5 TBST washes, unbound proteins are washed away, leaving only proteins bound to the Myc antibody and hence only one band is seen in lane 4.

2.20.1. Secreted alkaline phosphatase (SEAP) reporter gene assay

Fifteen microlitres of each culture medium supernatant from transfected HEK293 cells (see section 2.16.3) to be assayed was aliquoted into separate wells of a white opaque 96-well flat-bottom microtitre plate (PerkinElmer Life And Analytical Sciences, Inc Boston, MA, U.S.A.). To this, 45µl of 1X dilution buffer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) was added and the plate incubated at 65°C for 30 min in a waterbath (Memmert ®, Schwabach, Germany). Following the incubation, the plate was left on ice for 3min to cool down the samples. The samples were then allowed to equilibrate to room temperature before 60µl assay buffer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) was added to each well.

The chemiluminescent substrate (CSPD) was then prepared by making a 1:20 dilution of the CSPD (BD Biosciences, Clontech, Palo Alto, CA, U.S.A) in chemiluminescent enhancer (BD Biosciences, Clontech, Palo Alto, CA, U.S.A). The diluted substrate was then added to each well and the plate was incubated for 10 min at room temperature. The SEAP activity was then determined by reading the chemiluminecent signal every 10 min for a period of 2 ½ hours using a Bio-Tek® Synergy HT plate luminometer (Winooski, Vermont, U.S.A.).

Several control assays were included as comparisons for each experiment as shown in Table 2.12. The reading obtained for the untransfected control represents the background SEAP signal of the HEK293 cells used in the experiments, while the basal control reading corresponds to basal level of SEAP activity in the experiments. The GAL4 DNA-B control and the VP16 AD controls shown in Table 2.12 were included in order to determine whether the reeler construct or each of the putative ligands used in the experiments function autonomously as *SEAP* reporter gene transcriptional activators. Positive control assays were also included in the experiment. The pM3-VP16 positive control plasmid (Table 2.12) encodes a fusion of the GAL4 DNA-binding domain and VP16 activation domain and therefore gives very strong SEAP expression when co-transfected with pG5SEAP. The co-transfection of the pM53, pVP16T and pG5SEAP was also included in the experiment as a positive control. The pM53 expresses a fusion of the GAL4 DNA binding domain to the mouse p53 antigen, while the pVP16T expresses a fusion of the VP16 activation domain to the Simian Virus 40 large T-antigen which is known to interact with p53 and therefore produces strong SEAP expression. Two independent experiments (each assay performed in quadruplicate in each experiment) were conducted and the data from each experiment were analysed separately as well as in a combined data set. The data for each experiment was normalised to the untransfected control so that the data from each separate experiment could be combined and analysed together.

2.21. BIOINFORMATIC SEARCHES OF SCHIZOPHRENIA SUSCEPTIBILITY LOCI FOR PLAUSIBLE OCD CANDIDATE GENES.

2.21.1. Identification of schizophrenia susceptibility loci

Literature searches were conducted using the publicly available PUBMED database (http://www.ncbi.nlm.nih.gov/PubMed), in order to identify all previously reported schizophrenia susceptibility loci. The PUBMED database was searched with the terms “schizophrenia linkage”, “schizophrenia genetic linkage” and “schizophrenia genetics”, and articles reporting on schizophrenia susceptibility loci identified from the resulting list.

2.21.2. Prioritising of genes and polymorphisms within each locus as OCD candidate genes.

The Ensemble (http://www.ensembl.org), the Universitiy of California Santa Cruz (http://www.genome.ucsc.edu) and the NCBI databases provided *in silico* catalogues of all annotated and predicted genes within each of the identified loci. The annotated genes are defined as expressed *in vivo* as identified by experimental analysis (Stein, 2001). By comparison, predicted genes are *in silico* derived sequences that meet the criteria for classification as a gene, but for which no experimental data exists to support that it has a biological function.

Once all the genes within the selected loci were catalogued, they were prioritised based on function and expression profile as plausible OCD candidate genes. For each gene, whose function had been experimentally determined, the OMIM database (http://www.ncbi.nlm.nih.gov/OMIM) was searched to ascertain whether it may be a plausible OCD candidate gene.

Genes that encoded proteins with unknown function were analysed by homology searches to predict a possible function based on their protein domain composition using the pfam (http://www.sanger.ac.uk/Software/Pfam) and prosite (http://www.expasy.org/prosite/) algorithms. The protein sequence encoded by each of these genes were retrieved from the NCBI GENBANK database in FASTA format. Brain tissue expression was a prerequisite in the selection of plausible candidate genes. Therefore, the expression profiles for all genes within the loci were determined using the Unigene EST database (http://www.ncbi.nlm.nih.gov/unigene).

Previously reported polymorphisms within the selected candidate genes which had been targeted in past psychiatric association studies were selected for investigation in the current analysis. Where no investigations of a candidate gene had been reported in the literature, target polymorphisms were selected from a publicly available single nucleotide polymorphism databases (http://www.ncbi.nlm.nih.gov/SNP; http://www.hapmap.org) based on a minor allele frequency of greater than 0.3.

2.22. STATISTICAL ANALYSIS

2.22.1. Calculating allele frequencies

The frequency of alleles of each of the polymorphisms tested in the present study were calculated by dividing the total number of a particular allele by the total number of chromosomes in the study group.

2.22.2. Calculating expected allele frequencies

In order to predict the expected distribution of alleles at each of the loci in the study, the Hardy-Weinberg formula was used. This formula predicts that $p^2+2pq+q^2=1$ (where p= frequency of allele A and q= frequency of allele B), if the genotypes in the population are in Hardy-Weinberg equilibrium. The observed allele frequencies at each locus were inserted into the Hardy-Weinberg equation, while the expected number of genotypes was calculated by multiplying the values of p^2 (AxA), 2pq [2(AxB)] and q^2 (BxB) by the total number of individuals genotyped in the control and OCD patient groups.

2.22.3. χ^2 analysis of the expected versus observed genotype frequencies

To determine whether the genotype frequencies were in Hardy-Weinberg equilibrium, the expected and observed genotype frequencies for OCD patients and control individuals were displayed in a 3X2 contingency Table and χ^2 analysis performed using the Software Programme for Social Science (SPSS).

2.22.4. Assessment of association between OCD and polymorphic loci

In order to best assess the involvement of variants in the development of OCD, with dominant or codominant (additive) effects, while at the same time keeping the number of analyses to a minimum, logistic regression was used to model case-control status as a function each genotype (Lize van der Merwe, Biostatistics Unit, MRC). Each genotype factor was modelled as two variables, one counting the number of *SNAP25/Mnl*I *G, SNAP29 C56T T, GRIA4/rs630567 T, GRIN1/1 G, BZRPAla147Thr, Thr, DBH I/D, I, GBR T1545C C, RXRβVAl95ala, Val, DLX6 IVS1C>T*, *SYN3-639C>G, G, RELNIVS59C>T C, WDR47 rs2591000 C* and the *ATG16L2 C* alleles (0, 1 or 2): the additive effect (add) and another taking the value 0 for any homozygote and

1 for the heterozygotes (the dominance (dom) effect) as described by Cordell and Clayton, 2005. The resulting optimal models are described and summarised in the results. Functions from base R and R packages (R Development Core Team, 2006) were used for all statistical analyses.

CHAPTER 3: RESULTS

INDEX **PAGE**

CHAPTER 3: RESULTS

The sections that follow will describe first the results of the process of identifying plausible novel OCD candidate genes, either bioinformatically or by interactome analysis, and thereafter the results of assessment of particular polymorphisms within those genes in an OCD case:control study.

3.1. BIOINFORMATIC SEARCHES OF SCHIZOPHRENIA-LINKED LOCI FOR NOVEL PLAUSIBLE OCD CANDIDATE GENES.

Eighteen schizophrenia susceptibility loci were searched for plausible OCD candidate genes based on function and expression (Table 3.1). Individual genes within each of the loci were prioritised based on the criteria expounded in section 1.5.1. and 14 functionally and positionally plausible genes, which had not yet been studied in OCD, were selected for proof-of-principle case-control association studies; these genes are listed in Table 3.1. The region of the documented schizophrenia loci that were searched for plausible OCD candidate genes were delineated by markers that defined the linkage area in the relevant reported studies (Table 3.1.)

Table 3.1. **Schizophrenia loci searched for plausible OCD candidate genes**

Locus	Reference	Region	Genes prioritised	Genes screened
1q21-q22	Brustowicz *et al.,* 2000 Shaw et al*., 1998*	D1S1653-D1S1679	Synaptic vesicle glycoprotein 2A (*SV2A)* Regulator of G-protein signalling (*RGS4*)	None
1q32-q41	Hovatta *et al*., 1998	D1S1599-D1S196	None	None
1q41-q44	Hovatta *et al*., 1999	9cM on either side of D1S2891	*Acetylcholine receptor 3, muscarinic (*CHRM3*) Geranylgeranyl pyrophosphate synthetase (*GGPS1*) Kynurenine 3-monooxygenase (*KMO*)	*Acetylcholine receptor 3, muscarinic (*CHRM3*)
1p35-p32	Garver *et al*., 1998	D1S434-D1S1372	Opioid receptor, delta 1 (*OPRD1*) Glycine transporter (*SLC6A9*)	None
2q12-q13	Moises *et al*., 1995	D2S135-D2S2540	None	None
2q37	Paunio *et al*., 2001 Shaw *et al*., 1998	10cM on either side of D2S427	None	None
2p15-p14	Coon *et al*., 1998 Camp *et al*., 2001	D2S337-D2S286	None	None
5q22-q33	Straub *et al.,* 1997 Schwab *et al*., 1997 Paunio *et al*., 2001 Gurling *et al* 2001 Beyerly *et al*., 1999 Garner *et al*., 2001	D5S818-D5S422	Calcium/calmodulin-dependent protein kinase II alpha (*CAMK2A*)	None

Locus	Reference	Region	Genes prioritised	Genes screened
6p25-21	Schwab *et al*., 1995 Lindholm *et al*., 2001 Moises *et al*., 1995	D6S470-D6S264	Retinoid X receptor β (*RXR β*) Dysbindin (*DNTBP*1) GABAβ receptor 1 (*GBR1*)	Retinoid X receptor β (*RXR β*) GABAβ receptor 1 (*GBR1*)
6q16-q23	Coa *et al*., 1997 Martinez *et al*., 1999 Kaufman *et al*., 1998	D6S445-D6S310	Trace amine receptor-3 (*TRAR3*) Trace amine receptor-4 (*TRAR4*) Trace amine receptor-5 (*TRAR5*) Ocatmer binding transcription factor 7 (*POU3F2*)	**Octamer binding transcription factor 7 (*POU3F2*)
7q22	Ekelund *et al*., 2000	D7S477-D7S486	Disital-less like homeobox 6 (*DLX6*) Piccolo (*PCLO*) Reelin (*RELN*)	Distal-less like homeobox 6 (*DLX6*) Reelin (*RELN*)
8p22-21	Pulver *et al.,* 1995 Blouin *et al*., 1998 Levinson *et al*., 1996	D8S503-D8S1171	*Vesicular monoamine transporter 1 (*SLC18A1*) Nociceptin (*PPNOC*) Neuregulin (*NRG1*)	* Vesicular monoamine transporter 1 (*SLC18A1*)
9q34	Riley *et al*., 1997	D9S1825-D9S1818	N-methyl-D-aspartate receptor NR1 subunit (*GRIN1*) Dopamine β hydroxylase (*DBH*)	N-methyl-D-aspartate receptor NR1 subunit (*GRIN1*) Dopamine β hydroxylase (*DBH*)
10q22.3	Fallin *et al*., 2003 Faraone *et al*., 2006	D10S1677-D10S1753	Glutamate receptor, ionotropic, delta 1(*GRID*)	glutamate receptor, ionotropic, delta 1 (*GRID1*)
11q21-q23	Karayiorgou *et al*., 1994	D11S2002-D11S4464	AMPA receptor subunit GluR4 (*GRIA4*)	AMPA receptor subunit GluR4 (*GRIA4*)
13q21-q32	Lin *et al*., 1999 Shaw *et al*., 1998	D13S119-D13S128	Neurobeachin (*NBEA*) Doublecortin and CaM kinase-like 1 (DCAMKL1)	None
20p12-p11	Ekelund *et al*., 1998	5cm on either side of D20S172	Synaptosomal-associated protein of 25kDA (*SNAP25*)	Synaptosomal-associated protein of 25kDA (*SNAP25*)
22q11-q13.3	Williams *et al*., 2003 Pulver *et al*., 1994 Vallada et al., 1995 Moises *et al*, 1995	D22S446-D22S276	Synaptosomal-associated protein of 29kDA (*SNAP29*) Proline dehydrogenase (*PRODH*) Synapsin III (*SYN3*) Benzodiazapine receptor (peripheral) (*BZRP*) Catechol-O-methyltransferase (*COMT*) Proline dehydrogenase (*PRODH2*)	Synaptosomal-associated protein of 29kDA (*SNAP29*) Synapsin III (*SYN3*) Benzodiazapine receptor (peripheral) (*BZRP*)

- *Polymorphism within gene screened was found to be monomorphic in the Afrikaner cohort
- ** No polymorphism identified by SSCP analysis in present study

3.1.1. Synaptosomal-associated protein of 25kDa (*SNAP 25*)

Synaptosomal-associated protein of 25 kDa (SNAP 25), a hydrophilic protein of 206 amino acids, is an integral component of the vesicle docking and membrane fusion machinery that mediates the regulated release of neurotransmitters (Wilson *et al.,* 1996). It is expressed in axonal growth cones and is located on the cytoplasmic face of the plasma membrane in synaptic terminals throughout the axon (Hanson *et al.,* 1997). Developmentally regulated splice variants of SNAP-25 have been identified, with SNAP-25a being expressed early in development, and SNAP-25b expression occurring concurrently with synaptogenesis at which time it functions in axonal growth (Wilson *et al.,* 1996). The gene encoding SNAP-25 resides on chr20p11.2, a locus that has been linked to schizophrenia in a linkage study by Ekelund *et al.,* 1998.

Comment [IT64]: Garver DL, Barnes R, Holcomb J, Filbey F, Wilson R, Bowcock A. 1998. Genome-wide scan and schizophrenia in African-Americans. Am J Med Genet 81:454.

SNAP-25 forms a stable complex with syntaxin and synaptobrevin, to form the soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) complex, which helps attach neurotransmitter-containing vesicles to the inner plasma membrane (Wilson *et al.,* 1996). The formation of this complex is essential for membrane fusion and regulated exocytosis (Chen and Scheller, 2001) of neurotransmitters.

Interestingly, several post-mortem investigations have reported abnormal levels of SNAP-25 expression in various nervous tissues in schizophrenia, compared to unaffected control individuals (Gabriel *et al.,* 1997; Karson *et al.,* 1999). Young and co-workers found that the levels of SNAP-25 protein were reduced in the hippocampus of schizophrenia patients compared to control individuals (Young *et al.,* 1998). This finding was replicated in the study by Thompson and colleagues who found 49% less hippocampal SNAP-25 immuno-intensity of a group of schizophrenia patients compared to control individuals (Thompson *et al.,* 2003). Karson and co-workers demonstrated significantly decreased SNAP-25 mRNA levels in the prefrontal cortex of schizophrenia patients (Karson *et al.,* 1999). In a preliminary study, Thompson and colleagues found elevated levels of SNAP-25 in the CSF of schizophrenia patients compared to controls (Thompson et al., 1999); a finding which they later replicated in a different cohort of subjects (Thompson *et al.,* 2003).

Taken together, the important role of SNAP-25 in regulated exocytosis of neurotransmitters, as well as evidence of abnormal SNAP-25 levels in various brain regions, provide compelling evidence for a role for this protein in the pathogenesis of schizophrenia. Indeed, one genetic association investigation has been undertaken to assess the role of *SNAP-25* in schizophrenia pathogenesis. Tachikawa and co-workers (2001) analysed a polymorphic (TAAA)(n) tandem repeat in the 5'-upstream region of *SNAP-25* for association with schizophrenia in a cohort of 87 unrelated schizophrenia patients and 100 control individuals of Japanese descent. Their results, however, showed no association between the analysed polymorphism in *SNAP-25* and schizophrenia in their study sample.

The possible role of *SNAP-25* in OCD has not yet been investigated. However, SNAP-25 meets the criteria in the present study for analysis as a plausible candidate gene in that it has been implicated in schizophrenia pathogenesis and is located in a schizophrenia linked locus. Additionally, the gene encoding SNAP-25 is

highly expressed in the brain and, furthermore, it plays an important role in the release of neurotransmitters; all factors justifying its candidature for association analysis in OCD.

3.1.2. Synaptosomal-associated protein of 29kDa (*SNAP-29*)

SNAP-29 is a 258 amino acid protein that is proposed to be a ubiquitous soluble N-ethylmaleimide-sensitive factor attachment (SNARE) protein (similar to SNAP-25) involved in membrane trafficking of neurotransmitters (Hohenstein and Roche, 2001). Its role in neurotransmitter release is not yet fully understood; however, data obtained in a recent study by Pan and co-workers suggest that SNAP-29 acts as a negative modulator for neurotransmitter release, possibly by slowing the SNARE-based membrane fusion mechanism and synaptic turnover (Pan *et al.,* 2005).

The gene encoding SNAP-29 (*SNAP*-29) maps to the 22q11DS region (Dunham *et al.,* 1999) (section 1.4.5.2, Fig 1.7) that has been implicated in both schizophrenia and OCD pathogenesis (section 1.4.5.1). *SNAP-29* has previously been tested for association with schizophrenia in an investigation by Saito and colleagues. In their case-control association study of 97 unrelated Caucasian schizophrenic patients and 95 unrelated Caucasian control individuals, they found an association with the *G* allele of an *A* to *G* SNP at position -849 of *SNAP-29* and schizophrenia (p=0.009) (Saito *et al.,* 2001). Therefore, because of its previous association in schizophrenia (Saito *et al.,* 2001), genomic location, and its role in neurotransmitter release (Pan *et al.,* 2005), *SNAP-29* was considered an attractive candidate gene for OCD susceptibility in the present study.

3.1.3. AMPA receptor subunit GluR4 (*GRIA4)*

The glutamate receptor 4 (GluR4), a member of the family of AMPA glutamate receptors (section 1.1.5.3.3) which are the predominant excitatory neurotransmitter receptors in the mammalian brain, is encoded by the GluR4 gene, *GRIA4*, located on chromosome 11q22.3 (Meador-Woodruff and Healy, 2000). Meador-Woodruff and Healy (2000) showed decreased expression of AMPA receptor subunits mRNAs in various brain regions in schizophrenia patients compared to control individuals (Meador-Woodruff and Healy, 2000). Based on this, and the important role of glutamate in mediating schizophrenia pathogenesis (section 1.1.5.3.3), *GRIA4* has been investigated in association studies for possible involvement in schizophrenia susceptibility with varying results. While Makino and colleagues found an association between a 3-marker haplotype (SNP3, SNP4 and SNP 5 – Fig 3.1) in *GRIA4* and schizophrenia in their study of 100 unrelated Japanese patients and 100 unrelated Japanese controls (Makino *et al.,* 2003), Gou and colleagues detected no association of this gene with schizophrenia in their study of 372 unrelated Chinese schizophrenia patients and 392 unrelated Chinese control subjects (Guo *et al.,* 2004). These conflicting results may indicate that the role of *GRIA4* in the pathogenesis of schizophrenia varies between population groups.

However, the fact that *GRIA4* is important in mediating glutamatergic neurotransmission, the dysregulation of which has been implicated in OCD pathogenesis, coupled with its possible role in schizophrenia pathogenesis, was the rationale for evaluating *GRIA4* as a potential role player in the aetology of OCD in the present study.

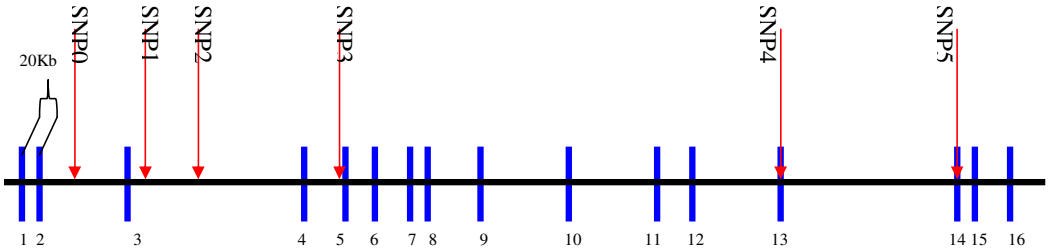


Fig 3.1. **Genomic organization of *GRIA4* and locations of SNPs used for haplotype analysis in the studies by Makino and co-workers (2003) and Gou and co-workers (2004).** Solid blue lines represent exons, while the solid black lines represent the intronic and untranslated regions. The identity of the SNPs used are as follows: SNP0- rs1938960 (C/T); SNP1- rs630567 (A/T); SNP2- rs682708 (A/G), SNP3- rs609239 (A/G); SNP4- rs642574 (A/G) and SNP5- rs659840 (A/G)

3.1.4. *N*-methyl-D-aspartate receptor NR1 subunit (*GRIN 1*)

The NMDA receptor NR1 subunit, encoded by *GRIN1*, is localised to chr9q34.3 (Zimmer *et al.,* 1995), a locus that showed suggestive evidence of linkage in studies by Reily and colleagues, as well as Kaufman and co-workers (Reily *et al.,* 1997; Kaufman *et al.,* 1998). To date, *GRIN1* has not been investigated as a possible OCD susceptibility gene, however, several studies have been undertaken investigating its possible role in mediating schizophrenia pathophysiology (section 1.4.6.1.3). Most of these studies failed to find any association between *GRIN1* and schizophrenia (Rice *et al*., 2001; Sakurai *et al*., 2000), with only one study showing a borderline significant association (Martucci *et al*., 2003). Despite this, inclusion of *GRIN1* into the present study was warranted based on its chromosomal location, as well as its important role in mediating glutamatergic neurotransmission, which has been shown to be important in the aetiology of OCD (section1.2.3.3.3).

3.1.5. Distal-less like homeobox 6 (*DLX6)*

Human distal-less like homeobox 6 (DLX6) is a member of the distal-less like homeobox (DLX) gene family related to the *Drosophila* Distal-less gene (Simeone *et al.,* 1994), genes which are thought to act as transcription factors (Merlo *et al.,* 2000). All DLX genes are expressed in a spatial and temporal pattern in craniofacial primordia, the developing brain and limbs (Robledo *et al.,* 2002).

In the developing brain, the genes encoding DLX5 and DLX6 (*DLX5* and *DLX6*) are detected early in the development of the primordium of the ganglionic eminence and the ventral diencephalons (Simeone *et al.,* 1994). Of particular interest to the present study is evidence that suggests that each *DLX* gene is expressed in the primordium of the basal ganglia, in an overlapping pattern according to the stage of cell differentiation (Liu *et al.,* 1997). This suggests that each member of the *DLX* gene family plays a specific role in the development of the basal ganglia, a brain structure that has been implicated in the pathogenesis of both schizophrenia and OCD (section 1.3).

The *DLX6* gene is located on chr7q21-22 (Simeone *et al.,* 1994), a locus to which suggestive evidence for linkage to schizophrenia has been reported (Ekelund *et al.,* 2000). Therefore, based its role in the developing basal ganglia, its expression profile and genomic location, *DLX6* was included in the present association study.

3.1.6. Benzodiazapine receptor (peripheral) (*BZRP)*

The benzodiazepine (BZD) class of pharmaceuticals are used for their CNS depressant properties, which include sedation, facilitation of sleep, seizure control and general anesthesia (Roy-Burne and Cowley, 1991). Furthermore, benzodiazepines are used in the treatment of various anxiety disorders such as social anxiety disorder and generalised anxiety disorder (reviewed by Davidson, 2004). All benzodiazepines in clinical use bind in a saturable and stereotypic manner to a high affinity neuronal GABA-A/chloride-channel complex known as the central type BZD receptor (BZDR) (Braestrup *et al.,* 1977, Mohler and Okada, 1977).

Comment [MB65]: • . Roy-Byrne, Peter P. & Cowley, Deborah S, (Editor) Benzodiazepines in Clinical Practice - Risks and Benefits (Clinical Practice, No 17) Hardcover 1991 -"This book presents scientific findings regarding the action of benzodiazepines in the brain as well as information about pharmacokinetics and pharmacodynamics."

Comment [IT66]: **Use of benzodiazepines in social anxiety disorder, generalized anxiety disorder, and posttraumatic stress disorder.** **Davidson JR**. J Clin Psychiatry. 2004;65 Suppl 5:29-33.

There is, however, a second type of high affinity BZDR known as the peripheral BZDR, that occurs in non-neuronal brain tissue, as well as in many other peripheral tissues including the heart, kidneys and peripheral blood cells (Gavish *et al.,* 1992; Parola *et al.,* 1993). This receptor, which plays a key role in the rate-limiting step in steroid genesis is localised in the outer mitochondrial membrane of steroid-producing cells such as the astroglial cells in the brain (Itzhak *et al,* 1993). In the brain, the peripheral BZDR is involved in production of neurosteroids. Neurosteroids are steroid hormones, which may potentially change the electrical properties of neuronal membranes (McCauley *et al.,* 1995; Papadopoulos *et al.,* 1992). Therefore, it has been proposed that the peripheral BZDR plays a role in the firing of neurons.

The diazepam-binding inhibitor and its processing products, as well as porphyrins, have been identified as putative endogenous ligands for the peripheral BZDR (Weizman and Gavish, 1993). Overproduction of porphyrins have been shown to give rise to photosensitivity in cutaneous porphyrias, a disease of dysfunctional haeme biosynthesis. Interestingly, some sub-types of this disease has been shown to give rise to a variety of neuropsychiatric symptoms, including anxiety and schizophrenia (Crimlisk, 1997). Furthermore, in a post-mortem study, Kurumaji and colleagues found a decrease in peripheral BZDR density in the superior parietal cortex, primary visual area and putamen of chronic schizophrenic patients compared to control subjects (Kurumaji *et al.,* 1997). In a post-mortem study of thirteen chronic schizophrenic patients and ten control individuals, Kiuchi and co-workers showed an increase in BZDR density in the brains of chronic schizophrenic patients (Kiuchi *et al.,* 1989). Alterations in peripheral BZDR densities have also been reported in platelets of schizophrenic patients (Gavish *et al.,* 1986; Tanne *et al.,* 1987).

The peripheral BZDR gene (*BZRP*) is localised to 22q13.3 (Riond *et al.,* 1991), a locus that has previously been linked to schizophrenia (Coon *et al.,* 1994; Pulver *et al.,* 1994, Moises *et al.,* 1995) (Table 1.3). Kurumaji and coworkers have reported an association between two missense polymorphisms in *BZRP* and schizophrenia in a cohort of 304 unrelated Japanese schizophrenia patients and 369 unrelated Japanese control individuals (Kurumaji *et al.,* 1998). This study, however, has not been replicated.

This gene selected for investigation as it is located in a genomic locus previously linked to schizophrenia and because it is highly expressed in the brain. Moreover, the fact that benzodiazapines are used in the treatment of certain anxiety disorders makes this gene an ideal candidate gene for OCD susceptibility.

3.1.7. Dopamine β-hydroxylase (*DBH*)

Dopamine β-hydroxylase (DβH) is an enzyme localised within the soluble and membrane fractions of secretory vesicles of norepinepherine (NE)- and epinephrine-producing neurons and neurosecretory cells (Cubells *et al.,* 1998). It is responsible for the catalysis of dopamine to NE and is present in both the plasma and cerebrospinal fluid (CSF) (Weinshilboum, 1978; O'Connor *et al.,* 1983).

Several studies have investigated the plasma and CSF levels of DβH in schizophrenia patients and patients exhibiting psychotic symptoms. Fujita and co-workers reported that the serum DβH levels were significantly lower in schizophrenic patients compared to control subjects (Fujita *et al.,* 1978), an observation consistent with a post-mortem study of schizophrenic and control brains by Wise and Stein (Wise and Stein, 1973). Despite this, several studies have found no association between decreased DβH levels and schizophrenia (Meltzer *et al.,* 1976, 1980; Sternberg *et al.,* 1982, 1983).

The gene encoding DβH (*DBH*) has been located on chr9q34 (Craig *et al.,* 1988), a locus that showed evidence of linkage to schizophrenia in studies by Riley and co-workers, as well as Kaufman and co-workers (Riley et al., 1991; Kaufman et al., 1998). Several polymorphisms within this gene have been identified and a number of genetic association studies have been undertaken to investigate the possible role of DβH in schizophrenia, yet no positive associations have been reported. (Meszaros *et al.,* 1996; Wei *et al.,* 1997, Williams *et al.,* 1999; Arrufat *et al.,* 2000, Jonsson *et al.,* 2003, Yamamoto *et al.,* 2003). However, even-though it is not thought to play a major role in schizophrenia pathogenesis, it has been suggested that DβH may act as a modifier of the schizophrenia phenotype (Cubells and Zabetian, 2004). For example, Sternberg and co-workers showed that low CSF DβH activity predicted more severe positive symptoms, but better overall clinical outcome in a small group of schizophrenic patients (Sternberg *et al.,* 1982, 1983). Consistent with these findings, Frecka and colleagues performed a factor analysis of a large number of predictive variables in schizophrenia patients and found that low serum DβH levels had a small but significant degree of predictive value of good neuroleptic response in schizophrenic patients (Frecka *et al.,* 1990).

Therefore, because of the chromosomal location of this gene, its role as a possible modifier of schizophrenia symptomology (Cubells and Zabetian, 2004), together with its high expression in the brain (Cubells *et al.,* 1998) and important role in dopamine catalysis (Weinshilboum, 1978; O'Connor *et al.,* 1983), this gene was used included in the present case-control association study of OCD.

3.1.8. Synapsin III (*SYN3*)

Synapsin III is member of a family of neuron-specific phosphoproteins expressed primarily in the brain (Ferreira *et al.,* 2000). Synapsins are located mainly on the cytoplasmic surface of small synaptic vesicles in

mature nerve terminals (Sudhöf *et al*., 1995; Hosaka and Südhof, 1998; Hilfiker *et al.,* 1999) where they regulate short-term neurotransmitter release, synaptic vesicle function and neural plasticity (Greengard *et al.,* 1993; Südhof *et al.,* 1995). Synapsin III is developmentally regulated and is predominantly expressed during embryogenesis (Ferreira *et al*., 2000), where it helps regulate axonal formation (Ferreira et al, 2000).

The gene encoding synapsin III (*SYN3*), consisting of 13 exons and encoding a 581 amino acid protein (Kao *et al.,* 1998), is located on the long arm of chr22q13 (Kao *et al.,* 1998), a locus previously linked to schizophrenia (Coon *et al.,* 1994; Vallada *et al.,* 1994; Pulver *et al.,* 1994; Moises *et al.,* 1995) (Table 1.1.1). Furthermore, previous studies have demonstrated reduced synapsin immunoactivity in the hippocampus (Browning *et al.,* 1993) and age-specific abnormalities of mRNA encoding synapsin proteins in the temporal cortex (Petersohn *et al.,* 1995) of schizophrenic patients. Several association studies of *SYN3* and schizophrenia have been undertaken. Porton and colleagues identified a rare polymorphism (S470N) in *SYN3* and reported that the S470 allele occurred more frequently in their group of 118 unrelated Caucasian schizophrenic patients than in their group of unrelated Caucasian control individuals (Porton *et al.,* 2004). However, in an independent case-control association study of the S470N *SYN3* polymorphism and schizophrenia in an African-American cohort, Lachman and co-workers found an increase in the frequency of the 470N allele in the schizophrenia group (Lachman *et al.,* 2005). Aside from the two above-mentioned studies, no other positive associations have been reported for any polymorphism in *SYN3* and schizophrenia (Ohmori *et al.,* 2000; Stober *et al.,* 2000; Imai *et al.,* 2001).

Thus, due to its role in neurotransmission, its chromosomal location and its association with schizophrenia, *SYN3* was selected as a potential OCD candidate gene in the present study.

3.1.9. $GABA_B$ receptor 1 (*GBR1*)

Gamma-aminobutyric acid is the major inhibitory neurotransmitter and its relevance to the pathophysiology of schizophrenia is discussed in section 1.1.5.3.4 of this review. The GABA type B receptors are associated with potassium/calcium (K^+/Ca^{2+}) channels and act via guanosine 5'-triphosphate binding (G-) proteins to produce slow, prolonged inhibitory signals (Bowery *et al.,* 2000; Olsen and Homsnics, 2000). They are highly, indeed almost exclusively, expressed in the human brain in regions such as the cerebellum, cerebral cortex, thalamic nuclei and the dorsal horn of the spinal chord (Bowery *et al.,* 1987; Chu *et al.,* 1990).

Comment [IT67]: Olsen, R.W. ,and Homanics, G.E. (2000) Function of GABA-A receptors: insights from mutant and knockout mice. *IN: GABA in the Nervous System: The View at 50 Years. Eds. D.L. Martin, and R.W. Olsen* Lippincott, Williams & Wilkins: Philadelphia 81-96.

The functional GABA B type receptor consists of a heterodimer of two related 7-transmembrane domain subunits $GABA_B$ 1 (GBBR1) and $GABA_B$ 2(GBBR2). The gene encoding GBBR1 (*GBR1*) has been mapped to the q21.3 region on chr6 (Goei *et al.,* 1998), a region previously linked to schizophrenia susceptibility (Cao *et al.,* 1997, Martinez *et al.,* 1999) (Table 1.1.3). Studies have previously been performed to determine whether an association exists between *GBR1* and schizophrenia but they have generated conflicting results(Imai *et al.,* 2002; Zai *et al.,* 2005). Imai and colleagues failed to provide evidence for an association between a dinucleotide repeat polymorphism in *GBR1* and schizophrenia in a cohort of 102 unrelated Japanese schizophrenic patients and 100 unrelated Japanese control individuals (Ismai *et al.,* 2002). More recently,

however, Zai and colleagues evaluated five SNPs in *GBR1* for association with schizophrenia in a study sample compring 150 case/control (gender, age and ethnicity matched) pairs, as well as 101 schizophrenic families (Zai *et al.,* 2005). Their investigation also failed to show any preferential transfer of alleles to affected offspring in the family sample; however, an association was found between the *A-7265* polymorphism and schizophrenia in the case-control sample.

However, due to the gene's chromosomal localisation, its role in mediating GABAergic neurotransmission, as well as its tissue expression pattern, *GBR1* was investigated as a novel OCD candidate gene in the present study.

3.1.10. Retinoid X receptor beta (*RXR* β)

Vitamin A and its derivatives, the retinoids, have been shown to regulate growth, survival and differentiation of a wide variety of cell types by modulating the expression of several genes (Feng *et al.,* 2005). In the developing brain, retinoids have been shown to regulate genes that control neuronal differentiation and migration (Thorogood *et al.,* 1982; Maden and Holder, 1992).

Goodman presented three lines of evidence that suggest that retinoid dysregulation may play a crucial role in the pathogenesis of schizophrenia. Firstly, studies have shown that retinoid toxicity, or deficit, causes mental deficit, enlarged ventricles, microencephaly and an array of major and minor congenital abnormalities that have been shown to occur with increased frequencies in schizophrenia pedigrees compared to control pedigrees (Goodman, 1996). Secondly, genes that have been identified as targets of retinoid transcriptional regulation include those encoding dopamine receptors (Farooqui *et al.,* 1994; Samad *et al.,* 1997), serotonin receptors (Altaba and Jessell, 1991; Ito and Morita, 1995) and glutamate receptors (Ray and Gottlieb, 1993; Hardy *et al.,* 1994), all of which have been shown to play a role in the pathogenesis of schizophrenia (Section 1.1.5.3.3). Finally, several genes of the retinoid cascade are mapped to chromosomal regions that have previously been linked to schizophrenia (Goodman, 1998).

The effects of retinoic acid, the biologically active form of retinoids, are mediated by specific transcription factors. These are the retinoic acid receptor (RAR) α, β and γ and retinoid X receptors (RXR) α, β and γ (Giguere *et al.,* 1987; Petovich *et al.,* 1987; Mangelsdorf *et al.,* 1990). In the presence of retinoic acid, these transcription factors bind to specific DNA motifs (known as retinoic acid response elements or RAREs) within the promoter regions of target genes and drive gene transcription.

The retinoid X receptor β was assessed in the present study, firstly, because of its role in mediating the effects of retinoic acid in regulating genes involved in neurodevelopment and, secondly, because the gene encoding it, *RXRβ,* is located on chromosome 6p21.3 (Hoopes *et al.,* 1992), a genomic locus that has previously been linked to schizophrenia susceptibility (Table 1.3) (Martinez *et al.,* 1999; Lerer *et al.,* 2004).

3.1.11. Glutamate receptor, ionotropic, delta 1 (*GRID1*)

The glutamate receptor, ionotropic, delta1 (GRID1) is encoded by a gene, *GRID1*, located on chr10q22.3. This locus has previously been implicated in schizophrenia susceptibility in studies by Fallin and co-workers (Fallin *et al*., 2003) and Faranoe *et al*., 2006).

Inclusion of this gene in the present study was based on an investigation by Fallin and colleagues, who reported an association between *GRID1* and schizophrenia in a large family-based association study (Fallin *et al*., 2005). In their large study of 227 schizophrenia trios, SNPs in 64 genes were investigated for possible association with schizophrenia, only 6 genes showed association with schizophrenia. Of these, five had been previously associated with schizophrenia, while the association with *GRID1* was novel. Thus, because of its association with schizophrenia, chromosomal location, and role in glutamatergic neurotransmission, GRID1 was considered a novel OCD candidate gene.

3.1.12. Reelin (*RELN*)

Reelin is a secreted glycoprotein involved in the ordered migration of neurons during neurodevelopment (Curran and D'Arcangelo, 1998). The gene encoding reelin has been mapped to chromosome 7q22 (De Silva *et al*., 1997), a region previously linked to schizophrenia susceptibility (Ekelund *et al*., 2000). Furthermore, one of the most robust findings in post-mortem brains of schizophrenia patients is a reduced level of reelin protein and mRNA (Fatemi *et al*., 2000; Guidotti *et al*., 2000; Knable *et al*., 2004).

Moreover, heterozygous reeler mice, mice that are haplo-insufficient for the expression of reelin exhibit several behavioural abnormalities that have been linked to schizophrenia (Tueting *et al.,* 1999, Costa *et al.,* 2001; Hoffmann *et al.,* 2001; Costa *et al.,* 2002). Also they exhibit several neuropathological and neurochemical abnormalities that are considered typical of schizophrenia (see table 1.13). Taken together, the above-mentioned data provides compelling evidence for the involvement of reelin in the pathogenesis of schizophrenia and hence was considered a plausible OCD candidate gene and thus included in the present investigation.

Comment [IT68]: Hoffmann JS, Guidotti A, Costa E, Larson J: **Impaired olfactory discrimination learning in heterozygous reeler mice.** Abstract 245.4 of the 31st Annual Meeting of the Society for Neuroscience, 2001 November 10–15, San Diego.

3.1.13. Acetylecholine receptor, Muscarinic 3 (CHRM3)

The gene encoding acetylecholine receptor, muscarinic 3 mapped to chromosome 1q43 (Bonner *et al*., 1990), a locus previously associated with schizophrenia susceptibility (Hovatta *et al*., 1998). Inclusion of this gene into the current investigation was based on the fact that Acethylcholine receptors have be linked to schizophrenia susceptibility in several investigations (see Singh *et al*., 2004 for review). It should, however be noted, that most studies implicate nicotinic acetylcholine receptors in schizophrenia, however, there has been some evidence that muscarinic receptors may also play a role. Most notably are the post-mortem studies that have shown decreased radioligand ([^{3}H]pirenzepine) binding to muscarinic receptors in the frontal and anterior cortices, caudate-putamen and hippocampus of schizophrenia subjects (Crook *et al*, 2000, 2001; Dean *et al*, 2002; Zavitsanou *et al*., 2004).

3.1.14. Vesicular Monoamine transporter 1 (*SLC18A1*)

Vesicular monoamine transporters (VMATs) are responsible for the mediation of accumulation of monoamines such as serotonin, dopamine and noradrenaline from the cytoplasm into storage oganelles (Edwards, 1992). Two isoforms of VMATs exists, namely VMAT1 and VMAT2 and they are also the first two members of the solute carrier family 18 , hence the genes encoding them have been designated *SLC18A1* and *SLC18A2*, respectively.

The gene encoding VMAT1 is an attractive candidate gene for schizophrenia given its role in modulating monoamines and that it has been mapped to chromosome 8p21.3 (Peter *et al*., 1994), a locus that has been previously implicated in schizophrenia susceptibility (Pulver *et al*., 1994). Furthermore, as monoamines such as dopamine and serotonin has also been implicated in the development of OCD, *SLC18A1* was considered an attractive candidate gene and hence included in the present study.

3.1.15 Octamer binding transcription factor 7 (*POU IIIF2)*

The octamer binding transcription factor 7 (OCT7) encoding gene, *POU3F2*, is mapped to chromosome 6q16 (Atanasoski *et al.,* 1995). This gene was included in the present investigation for a number of reasons. Firstly, it is widely expressed in the developing mammalian CNS (Fujii and Hamada, 1993). Secondly, is a neuronal transcription factor that is necessary for the maintenance of neuronal cell differentiation (Fujii and Hamada, 1993) and therefore may be important in regulating brain development and, thirdly, it contains a RARE (section 3.1.10) in its promoter. The latter point suggests that it is regulated by retinoic acid, which is known to play a crucial role in regulating neuronal differentiation and migration (Thorogood *et al.,* 1982; Maden and Holder, 1992).

3.2. IDENTIFICATION OF PLAUSIBLE OCD CANDIDATE GENES BY INTERACTOME ANALYSIS OF REELIN

3.2.1 YEAST-2-HYBRID ANALYSIS OF REELER DOMAIN OF REELIN

3.2.1.1 Integrity of Y2H constructs

3.2.1.1.1 Sequence analysis of pGBK-reeler bait construct

Following the generation of the pGBK-reeler bait construct, the construct was sequenced in order to verify that the integrity of the coding sequence and reading frame had been maintained. Results of the sequence analysis showed that pGBK-reeler was in the correct reading frame and revealed that the integrity of the nucleotide sequence of the reeler insert had been preserved through the multiple rounds of PCR amplifications used to create the fragment (section 2.3.4).

3.2.1.1.2. Phenotype and toxicity tests

The yeast strain AH109 transformed with the bait construct was able to grow on the appropriate selection media (SD^{-W} and SD^{-U}), but not on selection media lacking other essential amino acids (SD^{-A}, SD^{-L} and SD^{-H}). This confirmed that the phenotype of the AH 109 strain was retained after transformation.

To determine whether the bait construct had any toxic effect on the yeast cells, growth curves comparing AH109 transformed with empty pGBK vector and AH109 transformed with pGBK-reeler (section 2.14.2) were constructed. The slopes of these curves were virtually identical, indicating that the bait construct had no toxic effect on the growth of the yeast (Fig 3.2).

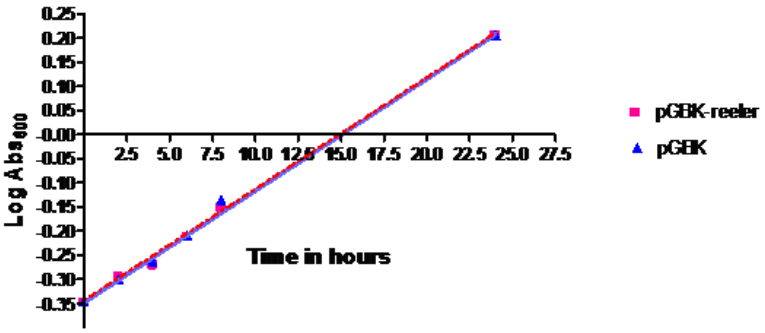


Fig 3.2. Linear growth curves of yeast strain AH109 transformed with non-recombinant pGBK and pGBK-reeler bait construct. In order to determine whether the bait construct had any toxic effect on the AH109, the growth rate of the pGBK-reeler transformant was compared to the pGBKT7 transformant. The growth rate was determined by calculating the slope of each of the curves. The slopes of the two curves were identical indicating that the bait construct had no toxic effect on the growth of the host yeast strain.

3.2.1.1.3. Mating efficiency of AH109 transformed with bait construct

In order to determine whether transformation of the pGBK-reeler construct had significantly affected the mating ability of the AH109 host strain, small scale yeast matings were performed. These mating experiments allowed for the calculation and comparison of mating efficiency of the pGBK-reeler transformant with that of AH109 strains transformed with control plasmids (pGBKT7 and pGBK53) when mated with standard prey transformants (pACT2 and pTD1.1).

The mating efficiency results (Table 3.2) show that pGBK-reeler impeded the mating efficiency of the AH109 slightly. The calculated mating efficiency of the pGBK-reeler transformant (4.1-9.2%) was, however, significantly higher than the minimum of 2% recommended by the manufacturer of the MATCHMAKER Y2H system (BD Bioscience, Clontech, Paulo Alto, CA, U.S.A), and would still, theoretically, result in screening of 10^6 individual clones if mated at a 100-fold excess against the commercial pretransformed library (titre=$3x10^8$).

Table 3.2**. Effect of reeler bait construct on AH109 mating efficiency**

Mating	Mating efficiency (%)
pGBK-Reeler (AH109)and pACT2 (Y187)	**9.2**
pGBKT7 (AH109) and pACT2 (Y187)	30.2
pGBK53 (AH109) and pACT2 (Y187)	10.9
pGBK-Reeler (AH109) and pTD1.1 (Y187)	**4.1**
pGBKT7 (AH109) and pTD1.1 (Y187)	11.8
pGBK53 (AH109) and pTD1.1 (Y187)	25.6

Control matings indicated in normal font, while bold-face font indicates test matings of the pGBK-reeler transformants. Yeast strains used are indicated in brackets

3.2.1.2. Y2H screening of pretransformed foetal brain cDNA library.

Library mating efficiency calculations indicated that approximately 5.9 x 10^6 pretransformed foetal brain cDNA library clones were screened with the pGBK-reeler construct. This screen yielded 92 clones which were able to activate the *HIS3* reporter gene as judged by growth on TDO plates (Table 3.3 column A). Forty-eight of these clones were also able to activate the *ADE2* reporter gene, as they demonstrated growth on QDO plates (Table 3.3 column B). These 48 clones were then analysed for their ability to activate the colourimetric reporter gene *MEL1* (Table 3.3 column C)*;* only 34 clones were found to activate the *MEL1* reporter gene (Table 3.3).

Following restriction digestion of these 34 remaining clones with *Hae*III and *Rsa*I, five clones were represented twice and only single representative clones of these groups of identical clones were analysed further. An additional seven clones were discarded because they contained prey peptides which activated transcription of reporter genes in the presence of heterologous baits (Table 3.4).

Thus, 22 remaining clones were classified as putative interactor clones; these prey constructs were sequenced and their insert identity determined by mining of nucleotide and protein sequence databases. The identities of these clones are shown in Table 3.5. Fifteen of the 22 sequenced clones contained prey insert sequences; that were discarded since their open reading frames (ORFs), fused to the GAL4-AD ORF, did not match the ORF predicted from the gene locus in either NCBI Genbank (http://www.ncbi.nlm.nih.gov) or Ensemble (http://www.ensembl.org) protein databases. This, however, is not unexpected as only one sixth of the clones represented in Matchmaker™ pretransformed cDNA libraries are in the correct reading frame (Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual). Figure 3.4 shows the *in silico* translation of the prey clone insert ORF, in the reading-frame dictated by the GAL4-AD sequence, of two representative examples of clones whose ORFs did not match that predicted from the nucleotide homology, as well as those two whose ORFs did match. Five clones were also discarded as putative interactors because their nuclear localisation (determined using Proteome analyst [http://www.cs.ualberta.ca/~bioinfo/PA/Sub] and ESPpred [http://www.imtech.res.in/raghava/eslpred/]) would place them in a cellular compartment incompatible with reelin's extracellular and cytosolic localisation.

Table 3.3. **Activation of nutritional and colourimetric reporter genes by prey-reeler interaction.**

Colony#	Identical clones[a]	A Growth on TDO (*HIS3 activation*)	B Growth on QDO *ADE2 activation*)	C X-α-galactosidase assay (colour) (*MEL1 activation*)
1	none	++++	-	-
2	none	+++	+++	+ (light blue-green)
3	none	++++	++++	+ (light blue-green)
4	none	+++	+	- (pink)
5	none	++++	+++	+ (light blue-green)
6	none	++	++	++ (light blue-green)
7	none	++	++	++ (light blue-green)
8	none	++++	++++	++ (light blue-green)
9	none	++++	+++	++ (yellow)
10	none	**+++**	**+++**	**+++ (blue-green)**
11	15a	**++++**	**+++**	**++++ (blue)**
12	none	**++**	**++**	**+++ (light blue-green)**
13	none	**+**	**+++**	**+++ (light blue-green)**
14	none	+++	+++	++ (light blue-green)
15	none	+++	-	-
16	none	**++++**	**++++**	**+++ (light blue-green)**
17	none	**+++**	**+++**	**+++ (light blue-green)**
18	none	++++	++++	-
19	none	**+++**	**++++**	**+++ (light blue-green)**
20	none	+	++++	++ (light blue-green)
21	none	**++++**	**+++**	**+++ (blue-green)**
22	none	++++	++	++ (light blue-green)
23	none	++	+	+ (very light blue-green)
24	none	**++**	**++++**	**+++ (light blue-green)**
25	none	++	++	++ (light blue-green)
26	none	++++	+++	+ (light blue-green)
27	none	++++	+++	+ (very light blue-green)
28	none	++	-	-
29	none	+++	++	+ (light blue-green)
30	none	**+++**	**++**	**+++ (blue-green)**
31	none	+++	+	+ (very light blue-green)
32	none	++++	++++	+ (very light blue-green)
33	none	+++	+++	+ (very light blue-green)
34	none	**++++**	**++++**	**+++ (blue-green)**
35	none	+	-	- (pink)
36	none	**++++**	**++++**	**+++ (light blue-green)**
37	none	**++++**	**++++**	**+++ (blue-green)**
38	**57**	**++++**	**+++**	**++++ (blue-green)**
39	none	+++	-	-
40	none	++++	-	-
41	none	+-	-	-
42	none	++++	++	++ (blue-grey)
43	none	+++	+++	+ (very light blue-green)
44	none	**++**	**++++**	**+++ (blue-green)**

Colonies in bold-face activated *HIS3*, *ADE2* and *MEL1* reporter genes. TDO= solid media lacking Leu, Trp and His, QDO= solid media lacking Leu, Trp, His and Ade. Growth of clones on solid media: ++++ = very good; +++ = good; ++ = weak; +=very weak, - = no growth

Colony#	Identical clones	A Growth on TDO *(HIS3 activation)*	B Growth on QDO *ADE2 activation)*	C X-α-galactosidase assay (colour) *(MEL1 activation)*
45	none	+	-	-
46	none	++++	-	-
47	none	++++	+++	+ (very light blue-green)
48	none	**+++**	**++**	**+++(light blue-green)**
49	none	**+++**	**++**	**+++ (blue-green)**
50	none	+++	+++	++ (blue-green)
51	none	+++	++	+++ (blue-green)
52	none	++	-	-
53	none	++++	+++	+ (very light blue-green)
54	none	+++	+++	++ (light blue-green)
55	none	++++	+++	+ (very light blue-green)
56	none	++++	+++	+ (very light blue-green)
57	**38**	**+++**	**++++**	**++++ (blue-green)**
58	none	+	-	-
59	none	+	-	-
60	none	+	-	-
61	none	**++++**	**++++**	**++++ (blue-green)**
62	none	**+++**	**++**	**+++ (light blue-green)**
63	none	++++	++++	++ (light blue-green)
64	none	**++++**	**+++**	**++++ (blue-green)**
65	none	**++++**	**++++**	**+++ (light blue-green)**
66	none	+	-	- (pink)
67	none	++	+++	++ (light blue green)
68	none	+++	-	-
69	none	+	-	-
70	none	+	-	-
71	none	+++	+++	++ (light blue-green)
72	none	**++++**	**++++**	**++++ (blue)**
73	none	+++	-	-
74	none	**++++**	**++++**	**+++ (blue-green)**
75	none	**++++**	**++++**	**+++ (blue-green)**
1a	none	+	-	-
2a	none	+	-	-
3a	none	**++++**	**++++**	**+++(light blue-green)**
4a	none	++++	-	-
5a	none	+	-	-
6a	**7a**	**+++**	**++++**	**+++ (light blue-green)**
7a	**6a**	**++**	**++++**	**+++ (light blue-green)**
8a	none	**++++**	**++++**	**+++ (light blue-green)**
9a	none	++++	+++	+ (yellow)
10a	none	++++	-	-
11a	none	**+++**	**+++**	**+++ (light blue-green)**
12a	none	++	-	-
13a	none	+	-	-
14a	none	**+++**	**+++**	**+++ (light blue-green)**
15a	**11**	**++++**	**+++**	**+++ (light blue-green)**
16a	none	**++++**	**++**	**+++ (light blue-green)**
17a	none	+	-	-

Colonies in bold-face activated *HIS3*, *ADE2* and *MEL1* reporter genes. TDO= solid media lacking Leu, Trp and His, QDO= solid media lacking Leu, Trp, His and Ade. Growth of clones on solid media: ++++ = very good; +++ = good; ++ = weak; +=very weak, - = no growth

As a result of these bioinformatic analyses of the sequences of the putative interacting proteins, the number of clones to be investigated was narrowed down to two: the cytosolic proteins, WDR47 and FLJ00012, also known as ATG16 autophagy-related 16-like 2 (S. cerevisiae) (ATG16L2). The function of neither of these proteins are clear at present. Both proteins contain WD40-repeat domains, which have been implicated in a wide variety of cellular functions that include signal transduction (Li and Roberts, 2001). Moreover, proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins (Smith et al., 1999). Furthermore, the WDR47 protein also contains a LIS1 homology domain, a domain with homology to the LIS 1 protein which has been shown to be involved in neuromigration and neurodevelopment (Gupta *et al*., 2002) as well as a "C-terminal to LIS homoly domain (CTLH), a domain with homology to RAN-binding protein 9, a protein shown to be involved in microtubule assembly (Nishitani *et al*., 2001). ATG16L2 shares homology with the mouse Pre-mRNA-processing factor 17, a protein which associates with the spliceosome and may play a role in the second step of pre-mRNA splicing (http://harvester.fzk.de/harvester/human/IPI00025/IPI00025503.htm).

Table 3.4**. Interaction of preys with heterologous baits in specificity tests as assessed by *ADE2* and *HIS3* activation.**

Clone #	x pGBK-reeler TDO	x pGBK-reeler QDO	x pGBKT7 TDO	x pGBKT7 QDO	x pGBK-53 TDO	x pGBK-53 QDO	x pGBK-C5* TDO	x pGBK-C5* QDO
10	++++	++++	+	+	-	-	-	-
11	++++	++++	+++	+++	+++	+++	++++	++++
12	+++	++++	-	-	-	-	-	-
13	++++	++++	-	+	-	-	-	-
16	+++	++++	-	++	+	+	-	-
17	++++	++++	-	+	-	-	-	-
19	++++	+++	+	+	-	-	-	-
21	++++	++++	+	+	+	-	-	-
24	++++	++++	-	++++	+	+++	++	+++
30	++++	++++	+	++	-	-	-	-
34	+++	+++	++	++	+	+	-	-
37	++++	+++	+	+	-	-	-	+
38	++++	+++	++	-	+	-	-	-
44	+++	++++	++++	+++	+	-	-	-
48	+++	+++	++	+++	++	+++	+	+
49	++++	+++	++++	++++	++++	++++	++++	++++
61	++++	++++	+	-	-	-	-	-
62	+++	+++	+	-	-	-	-	-
64	++++	++++	++	+				
65	+++	+++		+	-	-	-	
72	+++	++++	+	-	-	-	-	-
74	++++	++++	+	+	-	+	-	-
75	++++	++++	++	-	+++	++	-	-
3a	++	-	++	++	+++	++++	-	-
7a	++++	++++	+	+	-	-	-	-
8a	+++	++++	++	+	-	-	-	-
11a	++++	++++	+	+	+	-	-	-
14a	+++	+++	+	-	-	-	-	-
16a	++++	++++	+	-	+	-	-	-

Red font denotes clones that were discarded from further analysis based on their ability to activate reporter genes in the presence of heterologous baits. TDO= solid media lacking Leu, Trp and His, QDO= solid media lacking Leu, Trp, His and Ade. Growth of clones on solid media: ++++ = very good; +++ = good; ++ = weak; +=very weak, - = no growth

Table 3.5. **Identification of putative interactor clones from Y2H screen of foetal brain cDNA library.**

Clone # (identical clones)	**Insert size (bp)**	**BLASTn Acc # (e-value)**	**Identity**	**In-frame ORF BLAST2SEQ (length of ORF)**	**Cellular localisation**
11	801	NM_005883.1 (0.0)	Adenomatous polyposis coli protein	No significant similarity	N/A
72	664	XM_212565.1 ($1x10^{-148}$)	Beta5-tubulin	No significant similarity	N/A
12	1397	NM_003757 (0.0)	eukaryotic translation initiation factor 3, subunit 2 beta	No significant similarity	N/A
64	554	AC098484 ($1x10^{-179}$)	Homo sapiens clone RP 5994D16 (chr1)	Serine/threonine-protein kinase NEK4 [From AA 457-841]	Nucleus
17		AC137783 ($6x10^{-94}$)	Homo sapiens clone RP11-124P23 (chr16)	No significant similarity	N/A
8a	789	AC009364.9 (0.0)	Homo sapiens clone RP11-348A15 (chr7)	No significant similarity	N/A
10	833	AC006332.3 (0.0)	Homo sapiens clone RP11-376O1 (chr7)	No significant similarity	N/A
16a	800	AC100844 (0.0)	Homo sapiens clone RP11-659F24 (chr18)	Tigger transposable element-derived protein 1 [From AA 85-131]	Nucleus
7a	1102	AC058823.4 (0.0)	Homo sapiens clone RP11-810D13 (chr 4)	No significant similarity	N/A
13 (34)	1320	AF330044. (0.0)	Homo sapiens KIAA1008 protein	KIAA1008 [From AA 16-57]	Nucleus
34 (13)	1320	AF330044 (0.0)	Homo sapiens KIAA1008 protein mRNA	KIAA1008[From AA 16-57]	Nucleus
21	555	BC013374. (0.0)	Homo sapiens tubulin, beta polypeptide, mRNA	No significant similarity	N/A
62	455	NM_000431 (0.0)	Mevalonate Kinase	No significant similarity	N/A
37	563	NM_181689.1 (0.0)	Neuronatin (NNAT)	No significant similarity	N/A
16	**1142**	**BC036713. ($2x10^{-90}$)**	**Homo sapiens ATG16L2 protein**	**ATG16L2 NP_203746.1 [from AA 327-619]**	**Cytosolic**
19	**855**	**AB020700. (0.0)**	**Homo sapiens WDR47 protein (KIAA0893)**	**WDR47 NP_055784.2 [from AA 793-920]**	**Cytosolic**

Clone # (identical clones)	**Insert size (bp)**	**BLASTn Acc # e-value**)	**Identity**	**In-frame ORF BLAST2SEQ**	**Cellular localisation**
14a	611	BC_006468.1 (0.0)	Tubulin alpha 3	No significant similarity	N/A
30	292	BC_053521.1 (0.0)	Spectrin Alpha (non-ethrythroid)	No significant similarity	N/A
38 (61)	545	BC_033870.1 ($1x10^{-146}$)	Synaptobrevin 2	No significant similarity	N/A
61 (38)	545	BC_033870.1 ($3x10^{-81}$)	Synaptobrevin 2	No significant similarity	N/A
65	863	AK_125367.1 (0.0)	Tropomyosin 3	No significant similarity	N/A
74	1127	NM_005378 (0.0)	MYCN	MYCN [From AA 275-464]	Nucleus

Clones in bold font were chosen for further analysis by co-immunoprecipitation.
N/A= not applicable, AA= amino acid residue

A.i) Translation of A03_37_pACT2FN_NP_01 (**NNAT**) using Universal code

Number of bases: 1153bp
Total amino acid number: 375, MW=37954
Max ORF: 1-375, 125 AA, MW=13254

```
1          ACTCCGAGACCAGCGGATCTCGGCAAACCCTCTTTCTCGACCACCCACCTACCATTCTTG
1           T  P  R  P  A  D  L  G  K  P  S  F  S  T  T  H  L  P  F  L

61         GAACCATGGCGGCAGTGGCGGCGGCCTCGGCTGAACTGCTCATCATCGGCTGGTACATCT
21          E  P  W  R  Q  W  R  R  P  R  L  N  C  S  S  S  A  G  T  S

121        TCCGCGTGCTGCTGCAGGTGTTCAGGTACTCCCTGCAGAAGCTGGCATACACGGTGTCGC
41          S  A  C  C  C  R  C  S  G  T  P  C  R  S  W  H  T  R  C  R

181        GGACCGGGCGGCAGGTGTTGGGGGAGCGCAGGCAGCGAGCCCCCAACTGAGGCCCCAGCT
61          G  P  G  G  R  C  W  G  S  A  G  S  E  P  P  T  E  A  P  A

241        CCCAGCCCTGGGCGGCCGTATCATCAGGTGCTCCTGTGCATCTCGGCCAGCACGGGAGCC
81          P  S  P  G  R  P  Y  H  Q  V  L  L  C  I  S  A  S  T  G  A

301        AGTGCCGCGCAGGAATGTGGGGTCCCCTGTGTTCCCTCGCCAGAGGAGCACTTGGCAAGG
101         S  A  A  Q  E  C  G  V  P  C  V  P  S  P  E  E  H  L  A  R

361        TCAGTGAGGGGCCAGTAGACCCCCGGAGAAGCAGTACCGACAATGACGAAGATACCAGAT
121         S  V  R  G  Q  *  T  P  G  E  A  V  P  T  M  T  K  I  P  D

421        CCCTTCCCAACCCCTTTGCACCGGTCCCACTAAGGGGCAGGGTCGAGAGAGGAGGGGGGA
141         P  F  P  T  P  L  H  R  S  H  *  G  A  G  S  R  E  E  G  G

481        TAGGGGGAGCAGACCCCTGAGATCTGGGCATAGGCACCGCATTCTGATCTGGACAAAGTC
161         *  G  E  Q  T  P  E  I  W  A  *  A  P  H  S  D  L  D  K  V

541        GGGACAGCACCATCCCAGCCCCGAAGCCAGGGCCATGCCAGCAGGCCCCACCATGGAAAT
181         G  T  A  P  S  Q  P  R  S  Q  G  H  A  S  R  P  H  H  G  N

601        CAAAACACCGCACCAGCCAGCAGAATGGACATTCTGACATCGCCAGCCGACGCCCTGAAT
201         Q  N  T  A  P  A  S  R  M  D  I  L  T  S  P  A  D  A  L  N

661        CTTGGTGCAGCACCNACCGCGTGCCTGTGTGGCGGGACTGGAGGGCACAGTTGAGGAAGG
221         L  G  A  A  P  T  A  C  L  C  G  G  T  G  G  H  S  *  G  R

721        AGGGTGGTTAAGAAATACAGTGGGGCCCTCTCGCTGTCCCTTGCCCAGGGCACTTGCATT
241         R  V  V  K  K  Y  S  G  A  L  S  L  S  L  A  Q  G  T  C  I

781        CCAGCCTCGCTGCATTTGCTCTCTCGATTCCCCTTTCCTCCTCACTGCCTCCCAAGCCCA
261         P  A  S  L  H  L  L  S  R  F  P  F  P  P  H  C  L  P  S  P

841        CCCTACTCCAAAATAATGTGTCACTTGATTTGGAACTATTCAAGCAGTAAAAGTAAATGA
281         P  Y  S  K  I  M  C  H  L  I  W  N  Y  S  S  S  K  S  K  *

901        ATCCACCTTTACTAAAACACTTTCTCTGAACCCCCTTGCCCNTANTGATCTTGCTTTNCC
301         I  H  L  Y  *  N  T  F  S  E  P  P  C  P  X  *  S  C  F  X

961        TGGTCTCATGCAGTTGTGGTCATATNGNGGTATCNCTATTGTACTGATTGTTNAGTGTGC
321         W  S  H  A  V  V  V  I  X  X  Y  X  Y  C  T  D  C  X  V  C

1021       ATAGTTGGGNCTCCCAGCTAGATGGAAGCTCTGGANGAANGGACACTCTACAAAATAAAA
341         I  V  G  X  P  S  *  M  E  A  L  X  E  X  T  L  Y  K  I  K

1081       AGTCCTCCCTGTCTCGAAGTGTCCAGGACCTGGGGGGGATAAGGCCCCCNNNAAAANNAAN
361         S  P  P  C  L  E  V  S  R  T  W  G  D  K  A  P  X  K  X  X

1141       NAANNNNANNNNN
381         X  X  X  X
```

A.ii) Optimal alignment of protein reference sequence for **NNAT** (NP_005377.1) and the above translation of 03_37_pACT2FN_NP_01 (**NNAT**) sequence

Gap_Open_Penalty=10.0 Gap_Extend_Penalty=0.1
Upper line: NNAT NP_005377.1, from 8 to 80
Lower line: A03_37_pACT2FN_NP_01_Translation, from 41 to 112

NNAT NP_005377.1:A03_37_pACT2FN_NP_01_Translation identity= 19%

```
8     SAELLIIGWYIFRVLLQVFLECCIYWVGFAFRNPPGTQPIARSEVFRYSLQKLAYTVSRT
      ||          |              | |   || |   | |    |    |    |
41    SACCCRCSGTPCRSWHTRCRGPGGRCWGSAGSEPP.TEAPAPSPGRPYHQVLLCISASTG

68    GRQVLGERRQRAP
                |
100   ASAAQECGVPCVP
```

B.i) Translation of E02_142_pACT2FN_NP_10 (**TUBA3**) using Universal code
Number of bases: 628bp
Total amino acid number: 204, MW=22836
Max ORF: 232-486, 85 AA, MW=10355

```
1          GTGACGTGGTTCCCAAAGATGTCAATGCTGCCATTGCCACCATCAAGACCAAGCGTACCA
1           V  T  W  F  P  K  M  S  M  L  P  L  P  P  S  R  P  S  V  P

61         TCCAGTTTGTGGATTGGTGCCCCACTGGCTTCAAGGTTGGCATCAACTACCAGCCTCCCA
21          S  S  L  W  I  G  A  P  L  A  S  R  L  A  S  T  T  S  L  P

121        CTGTGGTGCCTGGTGGAGACCTGGCCAAGGTACAGAGAGCTGTGTGCATGCTGAGCAACA
41          L  W  C  L  V  E  T  W  P  R  Y  R  E  L  C  A  C  *  A  T

181        CCACAGCCATTGCTGAGGCCTGGGCTCGCCTGGACCACAAGTTTGACCTGATGTATGCCA
61          P  Q  P  L  L  R  P  G  L  A  W  T  T  S  L  T  *  C  M  P

241        AACGTGCCTTTGTTCACTGGTACGTTGGGGAGGGGATGGAGGAAGGTGAGTTTTCAGAGG
81          N  V  P  L  F  T  G  T  L  G  R  G  W  R  K  V  S  F  Q  R

301        CCCGTGAGGACATGGCTGCCCTTGAGAAGGATTATGAGGAGGTTGGTGTGGATTCTGTTG
101         P  V  R  T  W  L  P  L  R  R  I  M  R  R  L  V  W  I  L  L

361        AAGGAGAGGGTGAGGAAGAAGGAGAGGAATACTAAAGTTAAAACGTCACAAAGGTGCTGC
121         K  E  R  V  R  K  K  E  R  N  T  K  V  K  T  S  Q  R  C  C

421        TTTTACAGGGAAGCTTATTCTGTTTTAAACATTGAAAAGTTGTGGTCTGATCAGTTAATT
141         F  Y  R  E  A  Y  S  V  L  N  I  E  K  L  W  S  D  Q  L  I

481        TGTATGTAGCAGTGTATGCTCTCATATACAATTACTGACCTATGCTCTAAAACATGAATG
161         C  M  *  Q  C  M  L  S  Y  T  I  T  D  L  C  S  K  T  *  M

541        CTTTGTTACAGACCCAAGCTGTCCATTTCTGTGATGGGTTTTGAATAAAGTATTCCCTGT
181         L  C  Y  R  P  K  L  S  I  S  V  M  G  F  E  *  S  I  P  C

601        CTTAAAAAAAAANNNNANAAANNNNNANA
201         L  K  K  X  X  X  X  X  X
```

B.ii) Optimal alignment of protein reference sequence for **TUBA3** (NP_006000.2) and above translation of E02_142_pACT2FN_NP_10 (**TUBA3**) sequence
Upper line: TUBA3 NP_006000.2, from 145 to 315
Lower line: E02_142_pACT2FN_NP_10_Translation, from 2 to 57

TUBA3 NP_006000.2:E02_142_pACT2FN_NP_10_Translation identity= 30%

```
145   TGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTAVVEPYNSILTTHTTLEHSDCAFMV
      |     | |    |              |    |     |  | |   | |
2     TWFPKMSMLPLPPSR.............PSVPSSLWIGAPLASRLASTTSL.........

205   DNEAIYDICRRNLDIERPTYTNLNRLIGQIVSSITASLRFDGALNVDLTEFQTNLVPYPR

40    ............................................................

265   IHFPLATYAPVISAEKAYHEQLSVAEITNACFEPANQMVKCDPRHGKYMAC
                                    |    |   ||     ||
40    ..............................PLWCLVETWPRYRELCAC
```

C.i) Translation of F07_19_pACT2FN_NP_11 (**WDR47**) using Universal code
Number of bases: 855bp
Total amino acid number: 277, MW=27173
Max ORF: 1-384, 128 AA, MW=13720

```
1          GTTGGCACAACATTTCATGGAACTGGCAGTGCAGTGGCATCTGTAGCTGTAGATCCCAGT
1           V  G  T  T  F  H  G  T  G  S  A  V  A  S  V  A  V  D  P  S

61         GGTCGTCTCTTAGCCACAGGTCAAGAAGATTCTAGCTGCATGTTGTATGACATAAGAGGA
21          G  R  L  L  A  T  G  Q  E  D  S  S  C  M  L  Y  D  I  R  G

121        GGAAGAATGGTACAAAGTTATCATCCTCATTCCAGTGATGTTCGCTCTGTTCGATTCTCC
41          G  R  M  V  Q  S  Y  H  P  H  S  S  D  V  R  S  V  R  F  S

181        CCTGGAGCTCACTACTTGCTAACAGGCTCTTATGATATGAAAATAAAGGTGACAGACCTA
61          P  G  A  H  Y  L  L  T  G  S  Y  D  M  K  I  K  V  T  D  L

241        CAAGGGGACCTCACCAAGCAKCTTCCTATCRTGGTGGTGGGGGAGCACAAGGACAAAGTG
81          Q  G  D  L  T  K  X  L  P  I  X  V  V  G  E  H  K  D  K  V

301        ATTCAGTGCAGATGGCACACCCAGGATCTTTGCTTCCTGTCATCCTCTGCAGATAGAACT
101         I  Q  C  R  W  H  T  Q  D  L  C  F  L  S  S  S  A  D  R  T

361        GTCACCCTCTGGACTTACAATGGGTAGAGCACACCGCATGTCAGTCTATGCAGCAAAAGC
121         V  T  L  W  T  Y  N  G  *  S  T  P  H  V  S  L  C  S  K  S

421        ACAGAGACTTAAGACTACTGAGTTGTGAAAATTACAAATCTGAAGAACATAGTGTCCAGG
141         T  E  T  *  D  Y  *  V  V  K  I  T  N  L  K  N  I  V  S  R

481        AKAGTGGTTTAGCACGAAGAGGCCCCTTATTACCATGTATCCCACTGATAGGAGGTGTTG
161         X  V  V  *  H  E  E  A  P  Y  Y  H  V  S  H  *  *  E  V  L

541        GGTGGTGTTATTCCGCAGTGCTTTCAGTCTTCCATGTGAGCTCGTGCTGCTGTGACCTGC
181         G  G  V  I  P  Q  C  F  Q  S  S  M  *  A  R  A  A  V  T  C

601        TATATGTAGTCTCGTTGCCAAAGTCTGCAGAAGAGCTCTTCAGTTGGTTGGYGYGCACTC
201         Y  M  *  S  R  C  Q  S  L  Q  K  S  S  S  V  G  W  X  A  L

661        CAGTCAGGATGGACAATGGGGTTTACNGGTTTAGTATTCAATGCATTCCTTGGTCTTTGC
221         Q  S  G  W  T  M  G  F  T  G  L  V  F  N  A  F  L  G  L  C

721        CTAAATAACAGNTTTTAWATGCCMMATKGAAATGGAATTTWTNCTTCAACTANTATNATT
241         L  N  N  X  F  X  M  P  X  X  N  G  I  X  X  S  T  X  X  I

781        AAANGGNAANGCAACCNARTTNCCTCCCAAANTAAACNTCCCCRGGGGNTCAAAAATTNN
261         K  X  X  X  N  X  X  X  S  Q  X  K  X  P  X  G  X  K  N  X

841        TTTTGCCNCTCCCCM
281         F  C  X  S  P
```

C.ii) Optimal alignment of protein reference sequence for **WDR47 (NP_055784.2)** and above translation of F07_19_pACT2FN_NP_11 (**WDR47**) sequence

Gap_Open_Penalty=10.0 Gap_Extend_Penalty=0.1

Upper line: DNAMAN5, from 793 to 920
Lower line: F07_19_pACT2FN_NP_11_Translation, from 1 to 128

DNAMAN5:F07_19_pACT2FN_NP_11_Translation identity= 97%

```
793   VGTTFHGTGSAVASVAVDPSGRLLATGQEDSSCMLYDIRGGRMVQSYHPHSSDVRSVRFS
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     VGTTFHGTGSAVASVAVDPSGRLLATGQEDSSCMLYDIRGGRMVQSYHPHSSDVRSVRFS

853   PGAHYLLTGSYDMKIKVTDLQGDLTKQLPIMVVGEHKDKVIQCRWHTQDLSFLSSSADRT
      |||||||||||||||||||||||| ||| |||||||||||||||||| |||||||||
61    PGAHYLLTGSYDMKIKVTDLQGDLTKXLPIXVVGEHKDKVIQCRWHTQDLCFLSSSADRT

913   VTLWTYNG
      ||||||||
121   VTLWTYNG
```

D.i) Translation of D07_16_pACT2FN_NP_07 (**ATG16L2**) using Universal code
Number of bases: 1142bp
Total amino acid number: 377, MW=41899
Max ORF: 1-879, 293 AA, MW=32087

```
1          CGGGCTCAGGATGTGCTGGATGCCCACCTCTCTGAGGTCAATGCTGTTCGTTTTGGCCCC
1           R  A  Q  D  V  L  D  A  H  L  S  E  V  N  A  V  R  F  G  P

61         AACAGCAGCCTCCTGGCCACTGGAGGGGCTGACCGCCTGATCCACCTCTGGAATGTTGTG
21          N  S  S  L  L  A  T  G  G  A  D  R  L  I  H  L  W  N  V  V

121        GGAAGTCGCCTGGAGGCCAACCAGACCCTGGAGGGAGCTGGTGGCAGCATCACCAGTGTG
41          G  S  R  L  E  A  N  Q  T  L  E  G  A  G  G  S  I  T  S  V

181        GACTTTGACCCCTCGGGCTACCAGGTTTTAGCAGCAACTTACAACCAGGCTGCCCAGCTC
61          D  F  D  P  S  G  Y  Q  V  L  A  A  T  Y  N  Q  A  A  Q  L

241        TGGAAGGTGGGGGAGGCACAGTCCAAGGAGACACTGTCTGGACACAAGGATAAGGTGACA
81          W  K  V  G  E  A  Q  S  K  E  T  L  S  G  H  K  D  K  V  T

301        GCTGCCAAATTCAAGCTAACGAGGCACCAGGCAGTGACTGGGAGCCGCGACCGGACAGTG
101         A  A  K  F  K  L  T  R  H  Q  A  V  T  G  S  R  D  R  T  V

361        AAGGAGTGGGACCTCGGCCGTGCCTATTGCTCCAGGACCATCAATGTCCTTTCCTACTGT
121         K  E  W  D  L  G  R  A  Y  C  S  R  T  I  N  V  L  S  Y  C

421        AATGACGTGGTGTGTGGGGACCATATCATCATTAGTGGCCACAATGACCAGAAGATCCGG
141         N  D  V  V  C  G  D  H  I  I  I  S  G  H  N  D  Q  K  I  R

481        TTCTGGGACAGCAGGGGGCCCCACTGCACCCAGGTCATCCCTGTGCAGGGCCGGGTCACC
161         F  W  D  S  R  G  P  H  C  T  Q  V  I  P  V  Q  G  R  V  T

541        TCCCTGAGCCTCAGCCACGACCAACTGCACCTGCTCAGCTGTTCCCGAGACAACACACTC
181         S  L  S  L  S  H  D  Q  L  H  L  L  S  C  S  R  D  N  T  L

601        AAGGTCATCGACCTGCGTGTCAGCAACATCCGCCAGGTGTTCAGGGCCGATGGCTTCAAG
201         K  V  I  D  L  R  V  S  N  I  R  Q  V  F  R  A  D  G  F  K

661        TGTGGTTCTGACTGGACCAAAGCTGTGTTCAGCCCGGACAGAAGCTATGCACTGGCAGGC
221         C  G  S  D  W  T  K  A  V  F  S  P  D  R  S  Y  A  L  A  G

721        TCCTGTGATGGGGCCCTTTACATCTGGGATGTGGACACCGGGAAACTGGAGAGCAGACTA
241         S  C  D  G  A  L  Y  I  W  D  V  D  T  G  K  L  E  S  R  L

781        CAGGGACCCCATTGCGCTGCCGTCAACGCCGTGGCCTGGTGCTACTCCGGGAGCCACATG
261         Q  G  P  H  C  A  A  V  N  A  V  A  W  C  Y  S  G  S  H  M

841        GTGAGCGTGGACCAGGGCAGGAAGGTTGTGCTCTGGCAGTAGGGCCACGACCTGCCTGCC
281         V  S  V  D  Q  G  R  K  V  V  L  W  Q  *  G  H  D  L  P  A

901        TGGGCTGGAGCTCTTGCCCGAAGCCTGAAGCTTCCTTCGGCGCCATGCAGGGGTTGGGGT
301         W  A  G  A  L  A  R  S  L  K  L  P  S  A  P  C  R  G  W  G

961        TGGGACTGGAGCTGGCCTTGGGATTTAATGGGGAAGAAGGCCTGGCAGGACCTGGCCTGT
321         W  D  W  S  W  P  W  D  L  M  G  K  K  A  W  Q  D  L  A  C

1021       TTGTTTAAAAATGAAGTATGGGTTGGGGGATTACGCTAGTTTTTCTTTGTATTTTTATCT
341         L  F  K  N  E  V  W  V  G  G  L  R  *  F  F  F  V  F  L  S

1081       CTATCTCCTCACTTTTTCTCCCAAAGTAGAAAAAAATGATATCTGAAAAAAAAAAAAAAA
361         L  S  P  H  F  F  S  Q  S  R  K  K  *  Y  L  K  K  K  K  K

1141       AA
```

D.ii) Optimal alignment of protein reference sequence for **ATG16L2** (NP_203746.1) and D07_16_pACT2FN_NP_07 (**ATG16L2**) sequence

Gap_Open_Penalty=10.0 Gap_Extend_Penalty=0.1

Upper line: ATG16L2 NP_203746.1, from 327 to 619
Lower line: D07_16_pACT2FN_NP_07_Translation, from 1 to 293

ATG16L2 NP_203746.1:D07_16_pACT2FN_NP_07_Translation identity= 100%

```
327   RAQDVLDAHLSEVNAVRFGPNSSLLATGGADRLIHLWNVVGSRLEANQTLEGAGGSITSV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     RAQDVLDAHLSEVNAVRFGPNSSLLATGGADRLIHLWNVVGSRLEANQTLEGAGGSITSV

387   DFDPSGYQVLAATYNQAAQLWKVGEAQSKETLSGHKDKVTAAKFKLTRHQAVTGSRDRTV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61    DFDPSGYQVLAATYNQAAQLWKVGEAQSKETLSGHKDKVTAAKFKLTRHQAVTGSRDRTV

447   KEWDLGRAYCSRTINVLSYCNDVVCGDHIIISGHNDQKIRFWDSRGPHCTQVIPVQGRVT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   KEWDLGRAYCSRTINVLSYCNDVVCGDHIIISGHNDQKIRFWDSRGPHCTQVIPVQGRVT

507   SLSLSHDQLHLLSCSRDNTLKVIDLRVSNIRQVFRADGFKCGSDWTKAVFSPDRSYALAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   SLSLSHDQLHLLSCSRDNTLKVIDLRVSNIRQVFRADGFKCGSDWTKAVFSPDRSYALAG

567   SCDGALYIWDVDTGKLESRLQGPHCAAVNAVAWCYSGSHMVSVDQGRKVVLWQ
      |||||||||||||||||||||||||||||||||||||||||||||||||||||
241   SCDGALYIWDVDTGKLESRLQGPHCAAVNAVAWCYSGSHMVSVDQGRKVVLWQ
```

Fig 3.3. Assessment of match between protein predicted by Blastn of prey clone insert sequence and protein encoded by in-frame ORF of prey clone. A.i-D.i: Translation of in-frame nucleotide sequence of prey clone; A.ii-D.ii: Alignment of these translated ORF sequences with protein reference sequence predicted by identity of prey insert nucleotide sequence. **In A and B,** two representative clones (NNAT and TUBA3) whose translated ORFs did not match the ORFs predicted by their respective gene identity, are shown. In **C and D,** the two clones (WDR47 and ATG16L2) whose translated ORFs matched the ORFs predicted by their respective gene identities, are shown.. Translations of cloned insert DNA sequence as well as protein sequence alignment was performed using the DNAMAN™ software program (Lynnon Biosoft)

3.2.2. CO-IMMUNOPRECIPITATION ANALYSIS

The two prioritised putative reeler ligands (WDR47 and ATG16L2) identified in the Y2H analysis were co-immunoprecipitated, *in vitro,* with the reeler domain to verify these interactions in the absence of the GAL4 domains. Table 3.6 shows the predicted sizes of each of the translated products as well as the predicted sizes and actual sizes of the fusion proteins used in the co-immunoprecipitation reactions. The co-immunoprecipitation analysis revealed a positive interaction between the reeler domain and WDR47 (Fig 3. 4B), but not between the reeler domains and ATG16L2 (Fig 3.4C). Figure 3.5 shows the region of each identified protein encoded by the isolated clones.

Table 3.6. **Predicted molecular weights and approximate molecular weights of fusion proteins used in co-immunoprecipitation analysis.**

Cloned insert	Numberof predicted amino acids	Predicted size (kDa)	Size by electrophoresis (kDa)
Myc-Reeler	191	20.6	≈20
HA-WDR47	155	17.0	≈17
HA-ATG16L2	320	35.15	≈35

Predicted sizes were determined using http://www.basic.northwestern.edu/biotools/proteincalc.html

3.2.3. MAMMALIAN 2-HYBRID ANALYSIS

As post-translational modification and protein folding may not occur appropriately in either the coupled *in vitro* transcription-translation system, or the Y2H system, results of the Y2H screen needed to be verified in a mammalian cell system. Thus, mammalian two-hybrid (M2H) analysis was performed on both putative reeler-interactor clones identified in section 3.2.1.

The SEAP reporter activity was determined for each of the putative interactor constructs co-transfected with the reeler bait construct in HEK293 cells, in two independent experiments, with n=4 replicates per sample in each experiment. A positive interaction between reeler bait and putative interactor was confirmed if the SEAP activity of the interactor construct co-transfected with the bait construct was significantly higher than the basal SEAP level [basal control= background experimental SEAP level in cells transfected with the unrecombined bait (pM) and prey (pVP16) vectors] as well as the sample's two negative controls, viz. the reeler bait co-transfected with empty pVP16 vector (bait control), and the particular prey co-transfected with the empty pM vector (prey control) (Fig 3.6). The ability of the reeler bait construct or each of the putative ligands used in the experiments to function autonomously as *SEAP* reporter gene transcriptional activators was also assessed by the use of the sample controls. Specifically, SEAP activity of the bait control and the prey controls were compared to that of the basal control, which demonstrated that the neither the reeler bait nor any of the putative ligand constructs were able to autonomously activate SEAP gene transcription. Significance was determined using the one-way ANOVA followed by a post-hoc Bonferroni multiple comparison test, where a p-value of less than 0.05 indicated a significant difference. (See appendix VI for Bonferroni matrices which compares each experiment with the appropriate controls).

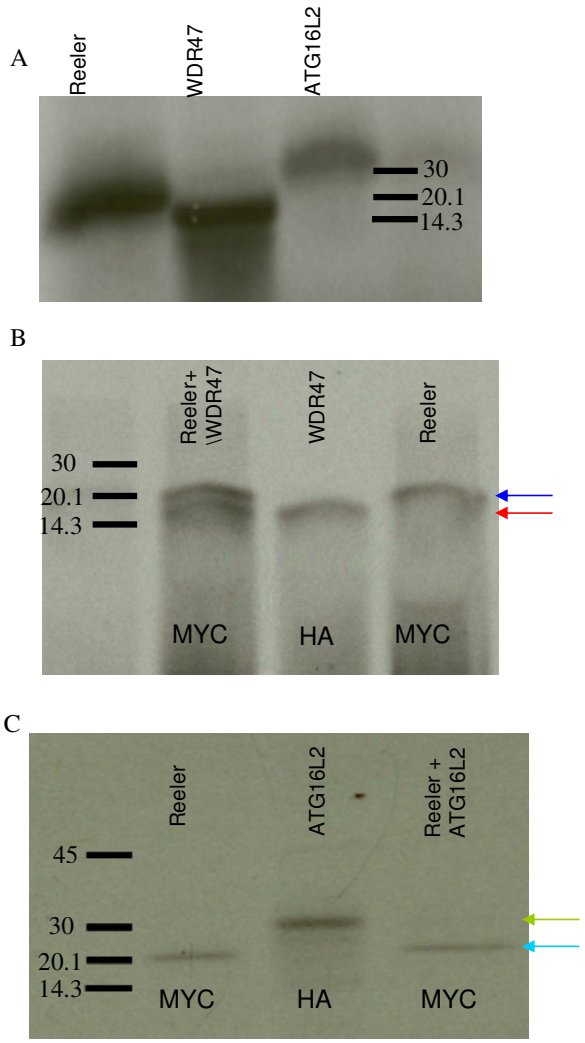


Fig 3.4. Co-Immunoprecipitation of Reeler domain with putative ligands: A. Autoradiograph of radio-actively labeled products from coupled *in vitro* transcription-translation electrophoresed on a 15% SDS-polyacrylamide gel. Black lines indicate positions of the non-radioactive High-Range Rainbow™ molecular weight marker (Amersham Biosciences) bands as transferred from the dried polyacrylamide gel. B. Autoradiograph of Co-IP of Reeler domain and WDR47. Co-IP products were electrophoresed on a 15% SDS-polyacrylamide gel. Arrows indicate the reeler domain (blue arrow) co-immunoprecipitating with the hypothetical protein WDR47 (red arrow). Black lines indicate positions of the non-radioactive High-Range Rainbow™ molecular weight marker (Amersham Biosciences) bands as transferred from the dried polyacrylamide gel. C. Autoradiograph of Co-IP of Reeler domain with ATG16L2 (green arrow) Co-IP products were electrophoresed on a 20% SDS-polyacrylamide gel. Black lines indicate positions of the non-radioactive High-Range Rainbow™ molecular weight marker (Amersham Biosciences) bands as transferred from the dried polyacrylamide gel.

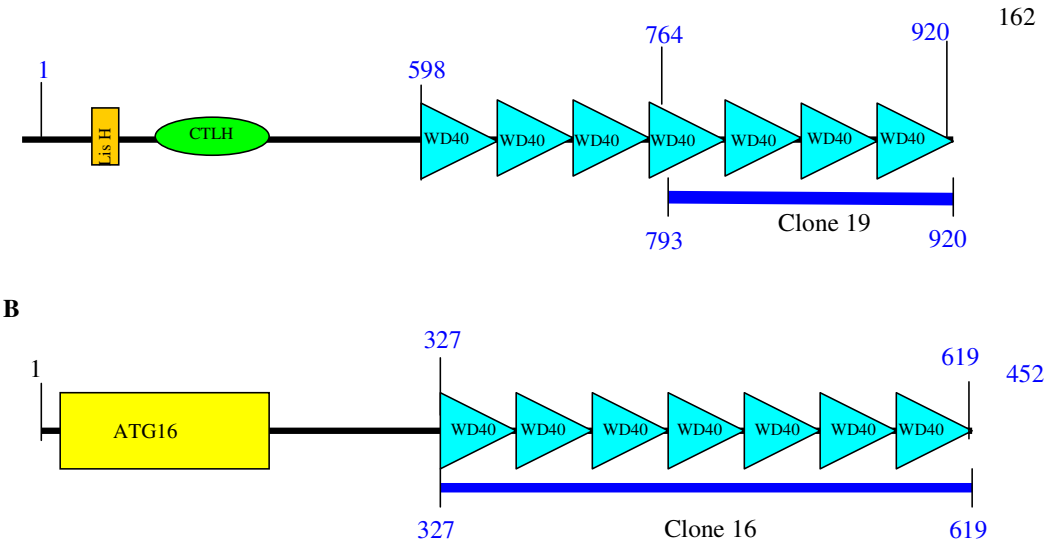


Fig 3.5. Schematic representations of the structures of WDR47 and ATG16L2 .A. WDR47 contains a series of seven WD40 repeat domains (blue triangles). The Lis1 homology domain (LisH) is represented by the yellow rectangle, while the C-terminal of Lis1 domain (CTLH) is represented by the green oval. The blue numbers indicate amino acid numbers, while the blue line below the graphic shows the fragment of WDR47 encoded by clone 19, viz. the last 3.5 WD40 repeats of WDR47. **B.** ATG16L2 also contains seven WD40 domains (blue triangles) and one ATG16 domain (yellow rectangle). The blue numbers indicate amino acid numbers, while the blue line shows the fragment of ATG16L2 encoded by clone 16, viz. the seven WD40 repeats (Table 3.5).

The two independent M2H experiments generally demonstrated results in the same direction, although the magnitude of the luminescence values differed. Thus, in order to compare results between these experiments, luminescence values were normalised to the mean luminescence of the mock-transfected control (HEK293 cells transfected with water, in stead of DNA) of each experiment. The results of the individual experiments, as well as of the combined data-sets, are shown in Figure 3.6. The interaction between the reeler domain and WDR47, already confirmed by the co-immunoprecipitation analysis, was further validated by M2H analysis, as determined by the significantly higher SEAP activity compared to the basal level in each individual experiment (experiment 1: p=0.01126, Fig 3.6A; experiment 2: p=0.00005, Fig 3.6B) as well as the when the data from the independent experiments were combined (p=0.00013, Fig 3.6C). No significant increase in SEAP activity was detected for the reeler-ATG16L2 (experiment 1: p=1.00000, Fig 3.6A; experiment 2: p=1.00000, Fig 3.6B; combined experiments: p=1.00000, Fig 3.6C) co-transfections, indicating that the ATG16L2 peptide did not bind the reeler domain in the HEK293 cells. The Bonferroni matrices for each experiment and combined experiments, as well as the box plots for the WDR47xreeler and ATG16L2xreeler experiments with only their appropriate controls are shown in appendix VI.

The data presented above provides compelling evidence for an interaction between WDR47 and the reeler domain of reelin. Since reelin is a good candidate gene for human schizophrenia, its ligand, WDR47, can also be considered a plausible schizophrenia candidate gene and hence a plausible OCD candidate gene (section 1.5.2). For this reason, the gene encoding WDR47 was included in the case-control association component of the present study. Even though no interaction was detected between ATG16L2 and the reeler domain, the gene

encoding ATG16L2 was investigated as a candidate OCD gene, as spliceosome proteins have been implicated in the consolidation of fear memory, and thus may play a role in anxiety disorders (Najholt *et al*., 2004).

Comment [MB69]: Nijholt et al 2004 Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory
and long-term potentiation

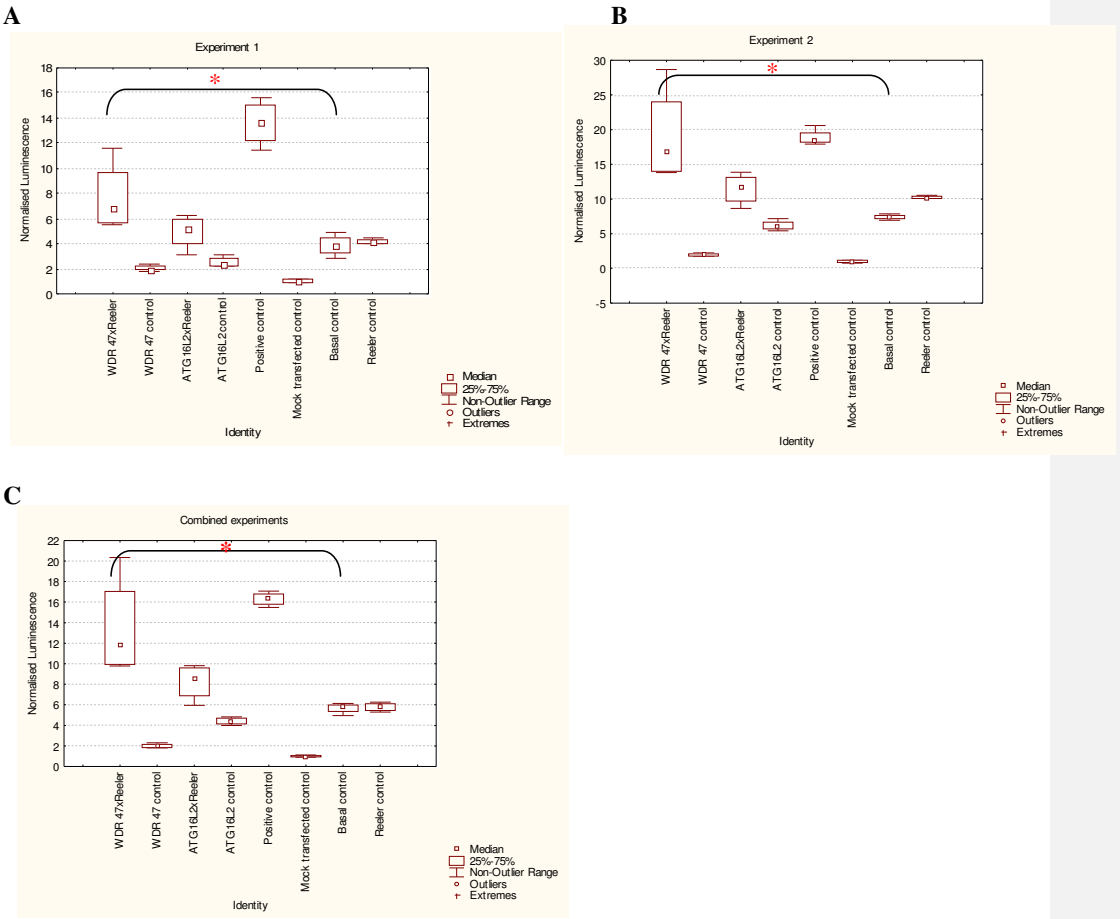


Fig 3.6. Box plots of secreted alkaline phosphatase activity of co-transfected HEK293 cells. Two independent SEAP assays (n=4 samples in each) were performed and the data for each assay was normalised to the mean luminescence value of the mock-transfected control of each experiment. The SEAP activity of each experiment was compared to its corresponding bait and prey control assays as well the basal SEAP activity levels, using ANOVA and post-hoc Bonferroni multiple comparison tests. * indicates a significant difference between experiment and basal control. **A**. * p=0.01126; **B**. p=0.00005. **C**. *p=0.000129

3..3 ASSOCIATION STUDIES

In all, 20 polymorphisms in 14 candidate genes identified either from schizophrenia-linked chromosomal loci (12), or through interactome analysis using the reeler domain of reelin as bait (2), were assessed for their potential role in OCD pathogenesis. In the following sections, where previously investigated polymorphisms were investigated in the present study and had been deposited in dbSNP, rs-identification numbers are given along with the original nomenclature.

Table 3.7. **Number of OCD patients and control individuals genotyped for each polymorphism investigated.**

Polymorphism	OCD			Control		
	Male	Female	Total	Male	Female	Total
*SNAP25/Dde*I (*C/T*)	28	34	**62**	14	48	**62**
*SNAP25/Mnl*I (*T/G*)	39	42	**81**	11	53	**64**
SNAP29/C56T (*C/T*)	27	31	**58**	12	52	**64**
SNAP29/G92A (*G/A*)	27	31	**58**	12	52	**64**
*GRIA4/HSp92*II (*A/G*)	33	35	**68**	20	68	**88**
GRIN1/1 (*G/C*)	38	33	**78**	20	41	**61**
DLX6 IVS1C>T (*C/T*)	20	22	**42**	18	63	**81**
BZRP Ala147Thr (*A/G*)	33	34	**67**	22	69	**91**
DBH (I/D)	38	37	**75**	17	43	**60**
SYN3/ -631C>G (*C/G*)	43	43	**86**	24	93	**81**
GBR1.1-C39T (*C/T*)	16	24	**40**	22	18	**40**
GBR1.11-T1545C (*C/T*)	16	24	**40**	22	18	**40**
*RXRβ/*Val95Ala (*G/A*)	16	24	**40**	22	18	**40**
*CHRM3/Msl*I (*G/A*)	16	24	**40**	22	18	**40**
*SLC18A1/BseR*I	16	24	**40**	22	18	**40**
RELNIVS59C/T (C/T)	18	26	**44**	15	26	**41**
GRID1 rs0887523	24	26	**50**	30	34	**64**
WDR47 rs2591000	25	21	**46**	17	31	**48**
ATG16L2 rs2282613	23	22	**45**	18	28	**45**
POU3F2	16	24	**40**	22	18	**40**

Abbreviations: *BZRP*, peripheral benzodiazepine receptor; *CHRM3*, Acetylcholine receptor, muscarinic 3; *DBH*, Dopamine beta hydroxylase; *DLX,* Distal-less like homeobox 6; *GBR,* GABA receptor 1; *GRIA4,* AMPA receptor subunit GluR4; *GRIN1,* N-methyl-D-aspartate receptor NR1 subunit; *POU3F2,* Octamer*RELN,* Reelin; *RXRβ,* retinoid X receptor beta; *SLC18A1,* Solute carrier family 18 member 1, *SNAP25* Synaptosomal-associated protein of 25kDa: *SNAP29*, Synaptosomal-associated protein of 29kDa; *SYN3*, Synapsin III

Each of the chosen polymorphisms was genotyped in an Afrikaner cohort. Two genes (*SLC18A1* and *CHRM3*) were not investigated further when the polymorphism chosen was uninformative in the Afrikaner population, while a third gene (*POU3F2*) was excluded because SSCP analysis of the coding region of the gene performed in the present study yielded no polymorphisms to test (Table 3.1). This investigation forms part of a larger ongoing investigation in which new patients and controls are constantly being recruited, while other samples were depleted during the study. This accounts for the varying number of individuals genotyped for each polymorphism as shown in Table 3.7.

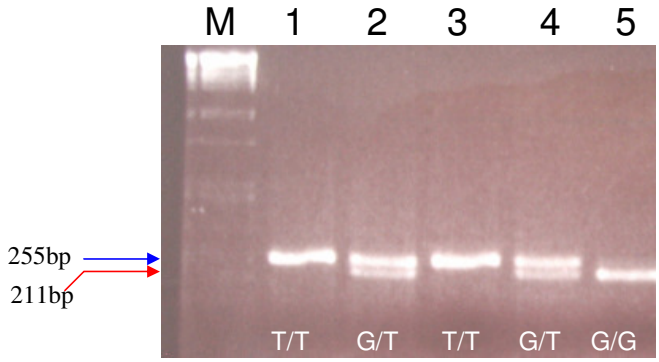
Comparisons of distributions of genotypes and frequencies of alleles between OCD patients and control individuals for each polymorphism tested will be discussed in the following sub-sections; results of logistical regression analysis of each polymorphism is also summarised in Table 3.24).

3.3.1.1. *SNAP25/Dde*I and *Mnl*I polymorphisms

The *SNAP25/*DdeI and *Mnl*I polymorphisms were chosen for analysis as they have previously been used in schizophrenia genetic association studies (Barr *et al.,* 2000). PCR amplification of the 3'UTR of *SNAP25* containing both the *Mnl*I and *Dde*I polymorphisms (section 2.3.2) analysed yielded a 261bp fragment. This fragment contains an invariant *Mnl*I restriction site; *Mnl*I digestion yields two fragments (255bp and 6bp) for the *T* allele, while for the G allele, which introduces another *Mnl*I site the fragment was cleaved into three fragments (211bp, 44bp and 6bp) (Fig 3.7). Genotyping for the *Dde*I polymorphism yielded a 261bp fragment for the *T* allele as no *Dde*I restriction site is present; however, as the *C* allele creates a *Dde*I restriction site, *Dde*I digestion yields two fragments of 228bp and 33bp in size (Fig 3.8). The genotype distributions and allele frequencies for each of these polymorphisms are shown in table 3.8 (*SNAP25/Mnl*I) and table 3.9 (*SNAP25/Dde*I).

Logistic regression analysis, applied to the data to determine whether these *SNAP25* polymorphisms are associated with OCD, revealed no significant association for either polyrphism using the additive model [p=0.484 for *Mnl*I and, p=0.272 for *Dde*I (Table 3.24)] or the dominant model [p=0.763 for *Mnl*I and p=0.838 for *Dde*I (Table 3.24)] between patient and control groups. Both OCD and control groups were in Hardy-Weinberg equilibrium for these two polymorphisms.

A



B

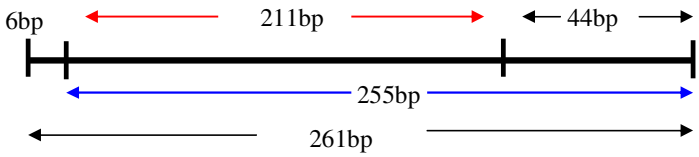


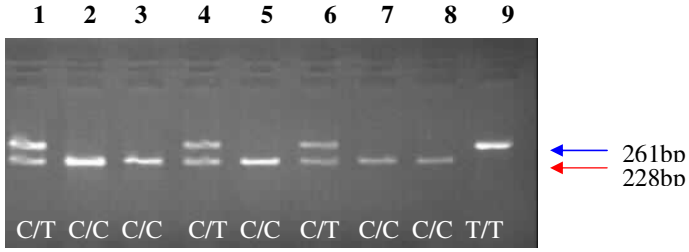
Fig 3.7**. ASREA of the *SNAP25/ MnlI* polymorphism. A.** Representative 2% agarose gel showing the fragment sizes of the *SNAP25* amplicon after digestion with *MnlI*. The 44bp and the 6bp fragments were too small to be resolved on a 2% agarose gel. Lane M: *λPst* molecular weight marker; Lanes1 and 3: T/T homozygous individual; Lane2 and 4: G/T heterozygote individual and Lane 5: G/G homozygote individual. **B**. Schematic representation of the *SNAP25* amplicon, generated by PCR-amplification for genotyping, showing the positions of the non-polymorphic and polymorphic *MnlI* restriction site and the sizes of the fragments generated following *MnlI* digestion.

Table 3.8**. Genotype distribution and allele frequencies of *SNAP25/MnlI* polymorphism in OCD patients and control individuals.**

	GENOTYPES				ALLELES		
SNAP25/MnlI	**TT**	**TG**	**GG**	**Total**	**T**	**G**	**Total**
OCD	31 (32.2%)	42 (51.9%)	8 (9.9%)	81	104 (64.2%)	58 (35.8%)	162
Control	30 (46.9%)	29 (45.3%)	5 (7.8%)	64	87 (69.5%)	39 (30.5%)	128

(Genotype and allele percentages shown in brackets)

A



B

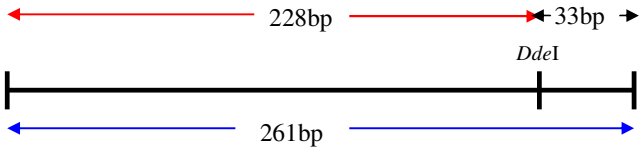


Fig 3.8. **ASREA of the *SNAP25/Dde*I polymorphisms**. **A**. Representative 2% agarose gel showing the fragment sizes of the *SNAP25* amplicon after digestion with *Dde*I. The 33bp fragment was too small to be resolved on a 2% agarose gel. Lane 9: T/T homozygote individual; Lanes 2,3,5,7 and 8: C/C homozygote individuals, and Lanes 1,4 and 6: C/T heterozygote individuals. **B**. Schematic representation of the *SNAP25* amplicon, generated by PCR-amplification for genotyping, showing the position of the *Dde*I restriction sites and the sizes of the fragments generated following *Dde*I.

Table 3.9. **Genotype distribution and allele frequencies of *SNAP25/Dde*I polymorphism in OCD patients and control individuals.**

	GENOTYPES				ALLELES		
***SNAP25/Dde*I**	**TT**	**TC**	**CC**	**Total**	**T**	**C**	**Total**
OCD	31 (50.0%)	25 (41.9%)	6 (8.19%)	62	104 (64.2%)	58 (35.8%)	162
Control	33 (54.5%)	23 (34.4%)	6 (11.5%)	62	87 (69.5%)	39 (30.5%)	128

(Genotype and allele percentages shown in brackets)

3.3.1.2. *SNAP29/C56T* and *SNAP29/ G92A* polymorphisms

The *SNAP29/C56T* (*rs1061063*)and *SNAP29/G92A* (*rs1061064*) polymorphisms have previously been used in a schizophrenia genetic association study and was therefore selected for analysis here (Saito *et al*., 2001). Since these two polymorphisms are located only 36bp from each other, one PCR fragment of 377bp was generated to genotype both polymorphisms (section 2.3.2). Furthermore, the amplicon contains one invariant *Dde*I restriction site and two polymorphic *Dde*I restriction sites that were used for genotyping. Digestion of the amplicon with *Dde*I yields two fragments of 269bp and 108bp as a result of the invariant *Dde*I site; the 269bp fragment contains both polymorphic *Dde*I sites. For the *SNAP29/C56T C* allele, the 269bp fragment remained undigested as no *Dde*I site is present, while the *T* allele creates a *Dde*I site resulting in the digestion of the 269bp fragment into two fragments of 177bp and 92bp (Fig 3.9). For the *SNAP29/G92A A* allele the 269bp fragment remained undigested, while for the *G* allele, which creates another *Dde*I restriction site, the digestion resulted in the 269bp fragment being cleaved into two fragments of 128bp and 141bp. Thus, electrophoresis of the single 377bp *Dde*I digested product resulted in banding patterns that were used to genotype both polymorphisms simultaneously. The genotype distribution and allele frequencies for each of the polymorphisms are shown in table 3.10 (*SNAP29/C56T*) and table 3.11 (*SNAP29/G92A*).

Both the OCD patient group as well as the control group were in Hardy-Weinberg equilibrium for both of the *SNAP29* polymorphisms tested. Logistic regression analysis revealed no significant association between any of these two polymorphisms on either the additive [p= 0.432 for *SNAP29/C56*T (table 3.24) and p= 0.432 for *SNAP29/ G92A* (table 3.24) or the dominant model [p=0.145 for *SNAP29/C56*T (table 3.24) and p=0.145 for *SNAP29/ G92A* (Table 3.24) Because of the data observed in tables 3.10 and 3.11, the level of linkage disequeilibrium (LD) between these two markers were tested using the Haploview^{TM} software package. This analysis confirmed that these two polymorphisms are in complete linkage disequilibrium with each other (Fig 3.10).

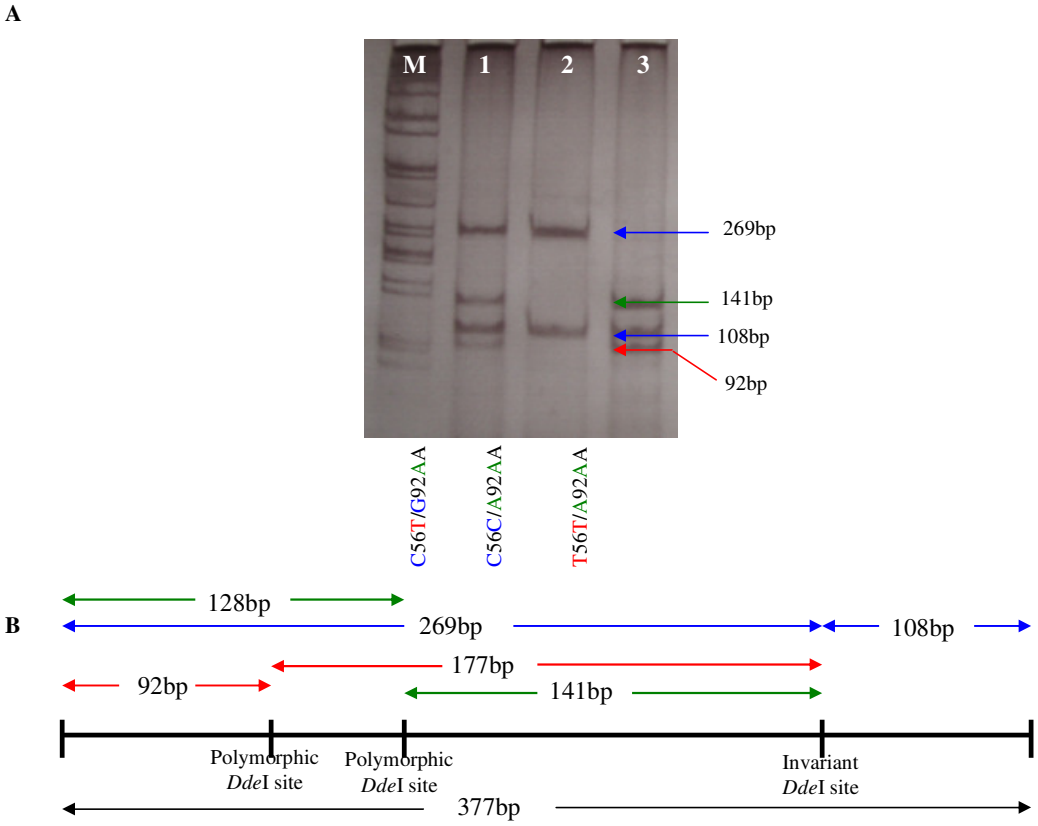


Fig 3.9. **ASREA of the SNAP29/C56T and SNAP29/ G92A polymorphisms**. A. Representative silver-stained 12% acrylamide gel showing the fragment sizes of the *SNAP29* amplicon following digestion with *Dde*I. Lane M: *λPst* molecular weight marker; Lane1: C56T/G92A; Lane 2 C56C/A92A; Lane3: T56T/G92G. B. Schematic representation of the *SNAP25* amplicon, generated by PCR-amplification for genotyping, showing the positions of the non-polymorphic and two polymorphic *Dde*I restriction sites and the sizes of the fragments generated following *Dde*I digestion.

Table 3.10. **Genotype distribution and allele frequencies of *SNAP29 C56T* polymorphism in OCD patients and control individuals.**

	GENOTYPES				ALLELES		
SNAP29-C56T	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	14 (24.1%)	29 (50.0%)	15 (25.9%)	58	57 (49.1%)	59 (50.9%)	116
Controls	9 (14.1%)	40 (62.5%)	15 (23.4%)	64	58 (45.3%)	70 (54.7%)	128

(Genotype and allele percentages shown in brackets)

Table 3.11. **Genotype distribution and allele frequencies of *SNAP29 G92A* polymorphism in OCD patients and control individuals**

	GENOTYPES				ALLELES		
***SNAP29*-G92A**	**GG**	**GA**	**AA**	**Total**	**G**	**A**	**Total**
OCD	14 (24.1%)	29 (50.0%)	15 (25.9%)	58	57 (49.1%)	59 (50.9%)	116
Controls	9 (14.1%)	40 (62.5%)	15 (23.4%)	64	58 (45.3%)	70 (54.7%)	128

(Genotype and allele percentages shown in brackets)

Fig. 3.10. **Linkage disequilibrium plots of SNAP29 SNPs *SNAP29C56T* and SANP29G92A.** From this linkage disequlibrium plot it is clear that these two polymorphisms are in complete LD with one another. Red square represents a D' value of 1.

3.3.1.3. *GRIA4/ rs630567* polymorphism

The *rs630567* polymorphism was selected for analysis as it has previously been used in a schizophrenia genetic association study (Makino *et al.,* 2003). The PCR amplification of the *GRIA4* intron 3 region where *rs630567* resides yielded a 255bp fragment (section 2.3.2) containing a polymorphic *Hsp92*II restriction site. For the *T* allele, the 255bp fragment remained undigested as the *Hsp92*II site is abolished, while for the *C* allele, the fragment was cleaved into two fragments of 145bp and 110bp following *Hsp92*II digestion (Fig 3.11). The genotype distribution and allele frequencies for this polymorphism is shown in table 3.12

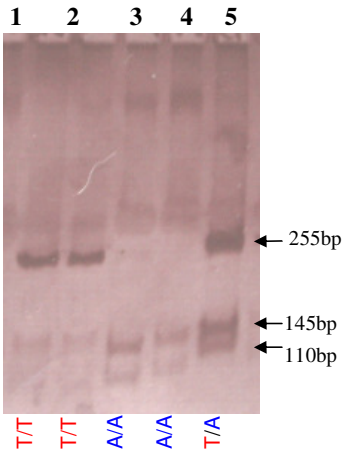
Testing for Hardy-Weinberg equilibrium in both OCD and control groups showed that both groups obeyed the equilibrium rule. Logistic regression analysis of the rs630567 data revealed no association with OCD using either the additive model (p=0.344) or dominant model (p=0.248) (Table 3.24).

3.3.1.4. *GRIN1/1* polymorphism

The *GRIN1/1* (*rs11146020*) polymorphism has previously been used in a genetic association study of schizophrenia (Martucci *et al*., 2003) and hence was included here. The PCR amplification of the region around the *GRIN1/1* polymorphism yielded an amplicon of 308bp (section 2.3.2) containing a polymorphic *Bse*RI restriction site. For the *C* allele, which abolishes the *Bse*RI site, the 308bp fragment remained undigested, while for the *G* allele, the 308 bp fragment was cleaved into two fragments of 275bp and 33bp following *Bse*RI digestion (Fig 3.12). The genotype distribution and allele frequencies for this polymorphism is shown in table 3.13

Logistic regression analysis of the *GRIN1/1* data generated no statistically significant association with OCD under either the additive model (p=0.648) or the dominant model (p=0,455 (table 3.24). Both OCD patient and control groups were in Hardy-Weinberg equilibrium.

A



B

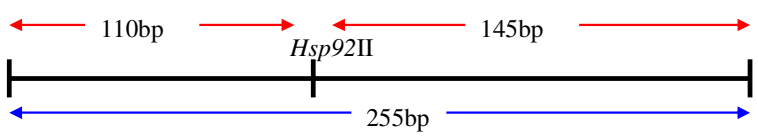


Fig 3.11. **ASREA of *GRIA4/ rs630567* polymorphism. A.** Representative silver stained 12% arcylamide gel showing the fragment sizes of the *GRIA4/rs630567* amplicon following digestion with *Hsp*92II. Lanes 1 and 2: homozygous T/T individuals; Lanes 3 and 4: homozygous A/A individuals; Lane 5: heterozygous T/A individual. **B.** Schematic representation of the *GRIA4 rs630567* amplicon, generated by PCR-amplification for genotyping, showing the positions of the polymorphic *Hsp92*II restriction site and the sizes of the fragments generated following *Hsp92*II digestion.

Table 3.12. **Genotype distribution and allele frequecy of *GRIA4/rs630567* polymorphism in OCD patients and control individuals.**

	GENOTYPES				ALLELE		
***GRIA4/* rs630567**	**AA**	**AT**	**TT**	**Total**	**A**	**T**	**Total**
OCD	23 (33.8%)	41 (60.3%)	4 (5.9%)	68	87 (63.9%)	49 (36.1%)	136
Controls	31 (34.8%)	48 (53.9%)	10 (11.3%)	89	110 (618%)	68 (38.2%)	178

(Genotype and allele percentages shown in brackets)

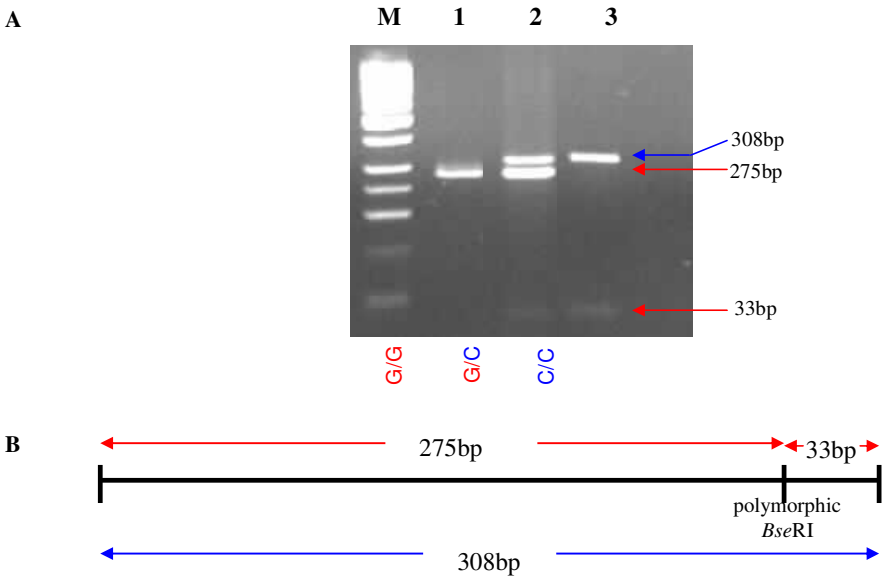


Fig 3.12. **ASREA of *GRIN1/1* polymorphism. A.** Representative 2% agarose gel showing the fragment sizes of the *GRIN1/1* amplicon following digestion with *Bse*RI. Lane M: 100bp molecular weight ladder; Lane 1: homozygous G/G individual; Lane 2: heterozygous G/C individual; Lane 3: homozygous C/C individual. **B.** Schematic representation of the *GRIN1/1* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Bse*RI restriction site and the sizes of the fragments generated following *Bse*RI digestion.

Table 3.13**. Genotype distribution and allele frequencies of *GRIN 1/1* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
GRIN1	**CC**	**CG**	**GG**	**Total**	**C**	**G(M^b)**	**Total**
OCD	12 (15.4%)	21 (26.9%)	45 (57.7%)	78	45 (28.8%)	111 (71.2%)	156
Controls	10 (16.4%)	21 (34.4%)	30 (49.2%)	61	41 (33.6%)	81 (66.4%)	122

(Genotype and allele percentages shown in brackets)

3.3.1.5. *DLX6 IVS1C>T* polymorphism

The *DLX6IVS1C>T* polymorphism was selected for analysis as it has previously been used in an autism association study (Nabi *et al*., 2003). Amplification of the region of intron 1 of the *DLX6* gene where the *DLX6 IVS1C>T* polymorphism resides yielded a 203bp fragment containing a polymorphic *Apo*I restriction site. For the *C* allele, which abolishes the *Apo*I restriction enzyme site, the 203bp fragment remained undigested, while for the *T* allele, the fragment is cleaved into two fragments of 176bp and 27bp following *Apo*I digestion (Fig 3.13). The genotype distribution and allele frequencies for the *DLX6IVS1C>T* polymorphisms is shown in table 3.14.

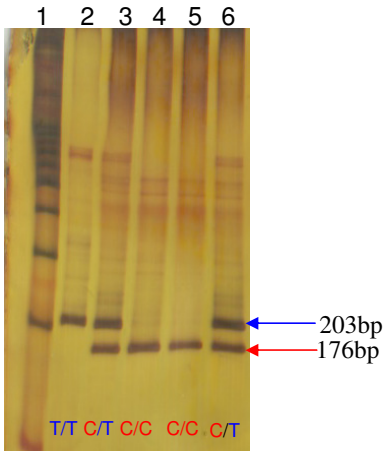
Both OCD and control groups were found to be in Hardy-Weinberg equilibrium. Using logistic regression analysis a statistically significant protective effect of the C*T* genotype of *DLX6IVS1 C/T* was observed under a dominant model (p=0.013, Table 3.24). No statistically significant association was shown under the additive model (Table 3.24).

3.3.1..6. *SYN3/-631C>G* polymorphism

The *SYN3/-631C>G* polymorphism has previously been the focus of a genetic association study in schizophrenia (Ohmori *et al*., 2000) and hence was chosen for analysis here. The PCR-amplification of the promoter region of *SYN3* containing the *-631* polymorphism generated a 105bp fragment with a polymorphic *Bsr*I restriction site. For the *G* allele, which abolishes the *Bsr*I restriction site, the 105bp fragment remains undigested, while for the *C* allele, which creates a *Bsr*I restriction site, the fragment is cleaved into two fragments of 84bp and 21bp following digestion with *Bsr*I (Fig 3.14).

The genotype distribution and allele frequency of this polymorphism is shown in table 3.15. Both OCD and control groups were in Hardy-Weinberg equilibrium. Logistic regression analysis revealed a statistically significant protective effect of the *G* allele of the *SYN3/-631C>G* polymorphism, under an additive model (p=0.009, Table 3.24).

A



B

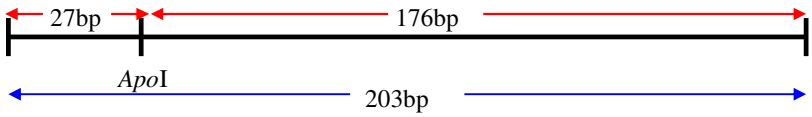


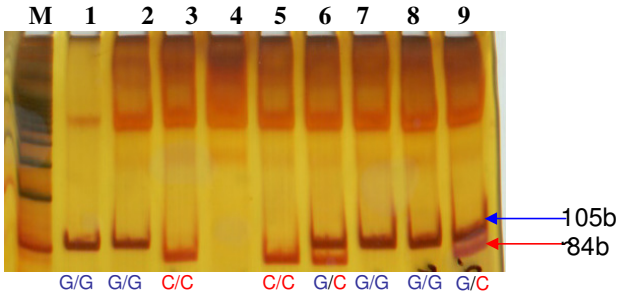
Fig 3.13. **ASREA of the *DLX6 IVS1C>T* polymorphism. A.** Representative 12% acrylamide gel showing the fragment sizes of the *DLX6IVS1C>T* polymorphism following digestion with *Apo*I. Lane M: *λPst* molecular weight marker; Lane 1: T/T homozygous individual ; Lanes 3 and 4: C/C homozygous individuals; Lanes 2 and 5: C/T heterozygous individuals. **B.** Schematic representation of the *DLX6IVS1C>T* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Apo*I restriction site and the sizes of the fragments generated following *Apo*I.

Table 3.14. **Genotype distribution and allele frequencies of *DLX6 IVS1 C/T* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
DLCX6 IVS1C>T	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	14 (33.3%)	16 (38.1%)	12 (28.6%)	42	44 (52.4	40 (47.6%)	84
Controls	10 (13.3%)	47 (58.1%)	24 (34.6%)	81	67 (41.4%)	95 (58.6%)	162

(Genotype and allele percentages shown in brackets)

A



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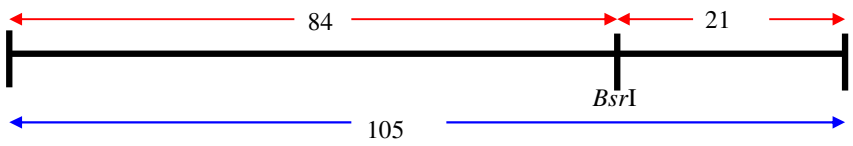


Fig3.14. **ASREA of *SYN3/-631C>G* polymorphism. A.** Representative 12% acrylamide gel showing the fragment sizes of *SYN3/-631C>G* amplicon following digestion with *Bsr*I. Lane M: *λPst* molecular weight marker; Lanes 1,2,7 and 8: G/G homozygous individuals; Lanes 6 and 9: C/G heterozygous individuals; Lanes 3 and 5: C/C homozygous individual. **B.** Schematic representation of the *SYN3/-631C>G* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Bsr*I restriction site and the sizes of the fragments generated following *Bsr*I digestion.

Table 3.15. **Genotype distribution and allele frequencies of *SYN3/ -631C>G* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
SYN3-631C>G	**CC**	**CG**	**GG**	**Total**	**C**	**G**	**Total**
OCD	37 (43.1%)	42 (48.8%)	7 (8.1%)	86	116 (67.4%)	88 (54.3%)	172
Controls	24 (29.6%)	40 (49.3%)	17 (21.1%)	81	56 (32.6%)	74 (45.7%)	240

(Genotype and allele percentages shown in brackets)

3.3.1.7. *BZRP* Ala147Thr polymorphism

The *BZRP* Ala147Thr (*rs6971*) polymorphism was included in the current analysis as it has previously been used in a genetic association study of schizophrenia. Amplification of the exon 4 region of *BZRP,* where the *Ala147Thr* polymorphism resides yielded a 299bp fragment containing a polymorphic *Nru*I restriction site. For the *Ala* allele, which abolishes the *Nru*I restriction site, the 299bp fragment remained undigested, while for the *Thr* allele, the fragment was cleaved into two fragments of 115bp and 184bp following *Nru*I digestion (Fig 3.15). The genotype distribution and allele frequencies are shown in table 3.16. Both patients and control groups were in Hardy-Weinberg equilibrium. Logistic regression analysis showed no association of this polymorphism under either an additive (p=0.362, Table 3.24) or a dominant model (p=0.571, Table 3.24).

3.3.1.8. *DBH (I/D)* polymorphism

The insertion/ deletion polymorphism of *DBH* (*rs34879977*) was chosen for analysis based on the fact that it has previously been the focus of an association study for schizophrenia (Yamamoto *et al.,* 2003). PCR amplification of the region containing the *DBH (I/D)* polymorphism generated an amplicon of 144bp for the *DBH (D)* allele or one of 163bp, for the *DBH (I)* allele was generated (Fig 3.16). The OCD study group and the control group were both found to be in Hardy-Weinberg equilibrium. The genotype distribution and allele frequencies are shown in table 3.17.

Logistic regression analysis showed no statistically significant association between the DBH (*I/D*) polymorphism and OCD using either the dominant (p=0.517, Table 3.24) or the additive model (p=0.395, Table 3.24).

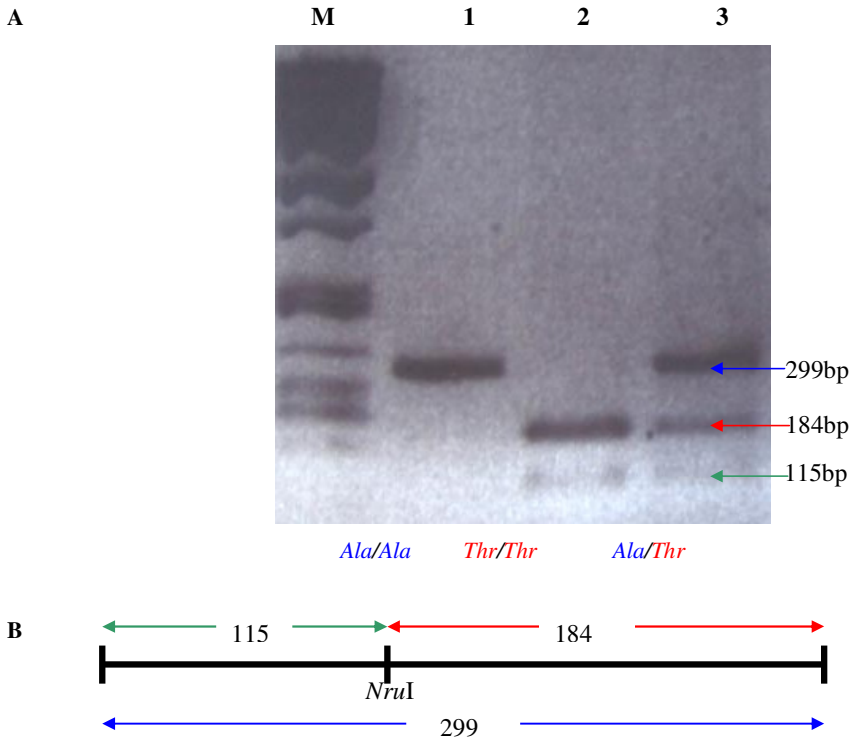


Fig 3.15. **ASREA of the *BZRP* Ala147Thr polymorphism: A.** Representative 2% agarose gel showing the fragment sizes of the *BZRP Ala147Thr* polymorphism following digestion with *Nru*I. Lane M: *λPst* molecular weight marker; Lane 1: *Ala* homozygous individual; Lane 2:*Thr* homozygous individual; Lane 3: *Ala/Thr* heterozygous individual. **B.** Schematic representation of the *BZRP Ala147Thr* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Nru*I restriction site. The sizes of the fragments generated following *Nru*I digestion are also indicated.

Table 3.16**. Genotype distribution and allele frequencies of *BZRP Ala147Thr* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
BZRP Ala/147Thr	**Ala/Ala**	**Ala/Thr**	**Thr/Thr**	**Total**	**Ala**	**Thr**	**Total**
OCD	10 (14.9%)	25 (37.3%)	32 (47.8%)	67	45 (33.6%)	89 (66.4%)	134
Control	9 (9.9%)	36 (39.6%)	46 (50.5%)	91	54 (29.7%)	128 (70.3%)	182

(Genotype and allele percentages shown in brackets)

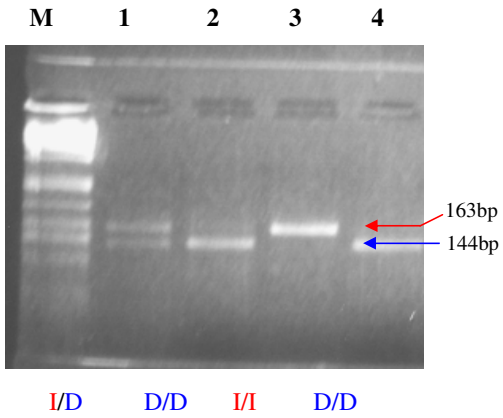


Fig 3.1**. Genotyping of *DBH (I/D)* polymorphism.** Representative 2% agarose gel of PCR fragments generated for genotyping the I/D polymorphism. Lane M: *λPst* molecular weight marker; Lane1: heterozygous I/D individual; Lane 3: homozygous I/I individual Lanes 2 and 4: homozygous D/D individuals.

Table 3.17. **Genotype distribution and allele frequencies of *DBH (I/D)* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
DBH (I/D)	**I**	**I/D**	**D/D**	**Total**	**I**	**D**	**Total**
OCD	20 (26.6%)	40 (53.4%)	15 (20%)	75	80 (53.3%)	70 (46.6%)	150
Control	17 (28.4%)	34 (56.6%)	9 (15%)	60	68 (56.6%)	52 (43.3%)	120

(Genotype and allele percentages shown in brackets)

3.3.1.9. *GBR1.1-C39T* and *GBR1.11-T1545C* polymorphisms

Following the genotyping of 40 OCD patients and 40 control individuals for the *GBR1.1-C39T* polymorphism , it was found that this polymorphism was not informative in the study population used in the present study, as only *TT* homozygotes were observed (Fig 3.17). Thus, no further analysis was performed for *GBR-C39T*.

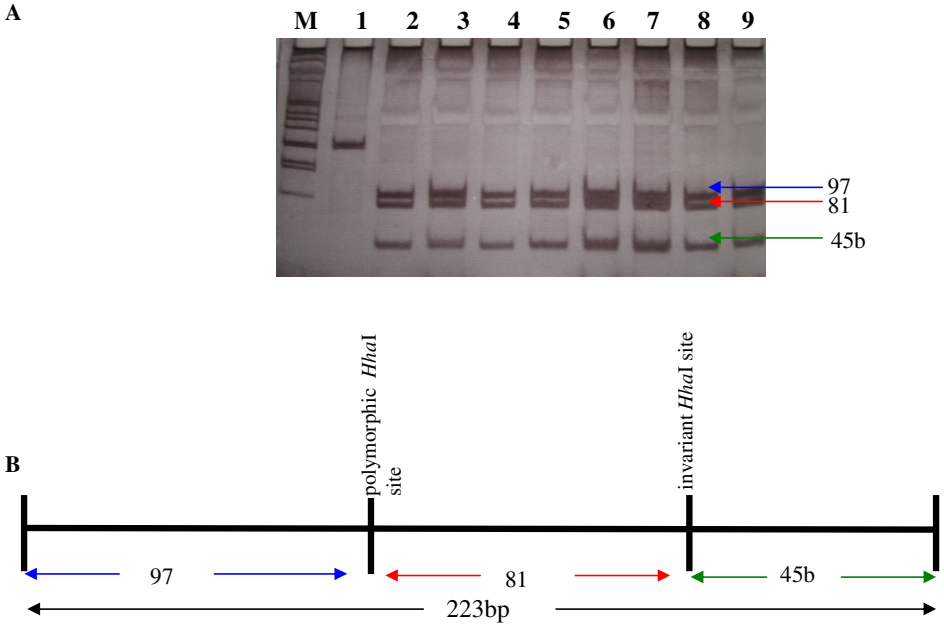


Fig 3.17. **ASREA of *GBR1.1-C39T* polymorphism. A.** Representative 12% acrylamide gel showing the *GBR1.1-C39T* fragment sizes and digestion with *Hha*I. Lane M: *λPst* molecular weight marker; Lane1-9: homozygous C/C individuals. The T allele was not found in the 80 individuals of the population used in the present study. **B.** Schematic representation of the *GBR1.1-C39T* amplicon, generated by PCR-amplification for genotyping, showing the positions of the polymorphic and invariant *Hha*I restriction site and the sizes of the fragments generated following *Hha*I digestion.

Instead, another polymorphism within this *GBR1* gene was targeted. Amplification of exon 11 of *GBR1* where this *T1545C* polymorphism resides yielded an amplicon of 375bp containing a polymorphic *Ear*I restriction site. For the *C* allele, which abolishes the *Ear*I restriction site, the fragment remained undigested, while for the *T* allele, which creates an *Ear*I site, the fragment was digested into two fragments of 271bp and 104bp following *Ear*I digestion (Fig 3.18). The genotype distribution and allele frequencies are shown in Table 3.18.

Both OCD patient and control groups were in Hardy-Weinberg equilibrium. Logistic regression analysis showed no significant association between the *GBR1.11-T1545C* polymorphism and OCD under either the dominant model (p=0.248, Table 3.24) or the additive model (p=0.344, Table 3.24).

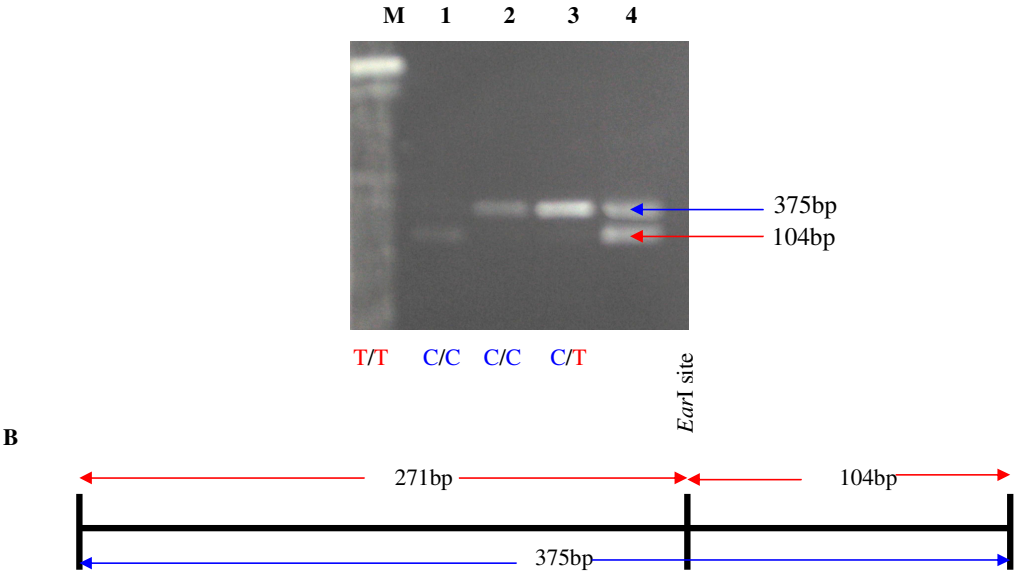


Fig 3.18. **ASREA of the GBR1.*11-T1545C* polymorphism. A.** Representative 2% agarose gel showing the fragment sizes of *GBR1.11-T1545C* amplicon following digestion with *Ear*I. Lane M: *λPst* molecular weight marker; Lane 1: T/T homozygous individual; Lane 2-3: C/C homozygous individuals; Lane 4: C/T heterozygous individual. **B.** Schematic representation of the *GBR1.11-T1545C* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Ear*I restriction site and the sizes of the fragments generated following *Ear*I digestion.

Table 3.18**. Genotype distribution allele allele frequencies of *GBR1.11-T1545C* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
GBR T1545C	**TT**	**TC**	**CC**	**Total**	**T**	**C**	**Total**
OCD	36 (90%)	3 (7.5%)	1 (2.5%)	40	75 (93.8%)	5 (6.2%)	80
Controls	38 (95%)	1 (2.5%)	1 (2.5%)	40	77 (96.3%)	(3.7%)	80

(Genotype and allele percentages shown in brackets)

3.3.1.10. *CHRM3 Msl*I and *SLC18A1* polymorphisms

Following genotyping of 40 OCD cases and 40 control individuals, for the *CHRM3/Msl*I (*rs2067481*) and the *SLC18A1* polymorphism, it was found that both of these polymorphism were not informative in the study population used in the present study, as only *GG* homozygotes were observed for the *CHRM3/Msl*I polymorphism and only *TT* homozygotes (Fig 3.19) were observed for the *SLC18A1* polymorphism (Fig 3.20). Thus, these two polymorphisms were excluded from any further analuysis.

3.3.1.10. *RXRβ Val95Ala* polymorphism

Amplification of the exon 2 region of *RXRβ* where the *Val95Ala* polymorphism resides yielded a 164bp fragment containing a polymorphic *Ban*II restriction site. For the *Val* allele, which abolishes the *Ban*II restriction site, the fragment remains undigested, while for the *Ala* allele, the fragment is cleaved into two fragments of 136bp and 28bp following *Ban*II digestion (Fig 3.21). The genotype distribution and allele frequencies are shown in table 3.19.

Both the OCD patient group and control group were in Hardy-Weinberg equilibrium for the *RXRβ Val95Ala* polymorphism. Logistic regression analysis could however not be performed because of the lack of Val/Val homozygotes in the OCD group (Table 3.19).

3.3.1.11. *GRID1 rs10887523(C/A)* polymorphism

PCR-amplification of the region of *GRID1* containing *rs1088753* resides yielded a 343bp fragment containing a polymorphic *Bfa*I restriction site. Digestion of the 343bp fragment with *Bfa*I yielded two fragments of 239bp and 114bp for the *C* allele. For the *A* allele, which abolishes the *Bfa*I site, the fragment remained undigested (Fig 3.22). The genotype distribution and allele frequencies are shown in Table 3.20.

Both the OCD patient group and control group were in Hardy-Weinberg equilibrium for the *GRID1 rs10887523*(*C/A*) polymorphism. Logistic regression analysis showed no significant association between *GRID1 rs10887523* and OCD under either the dominant model (p=0.838, Table 3.24) or the additive model (p=0.272, Table 3.24).

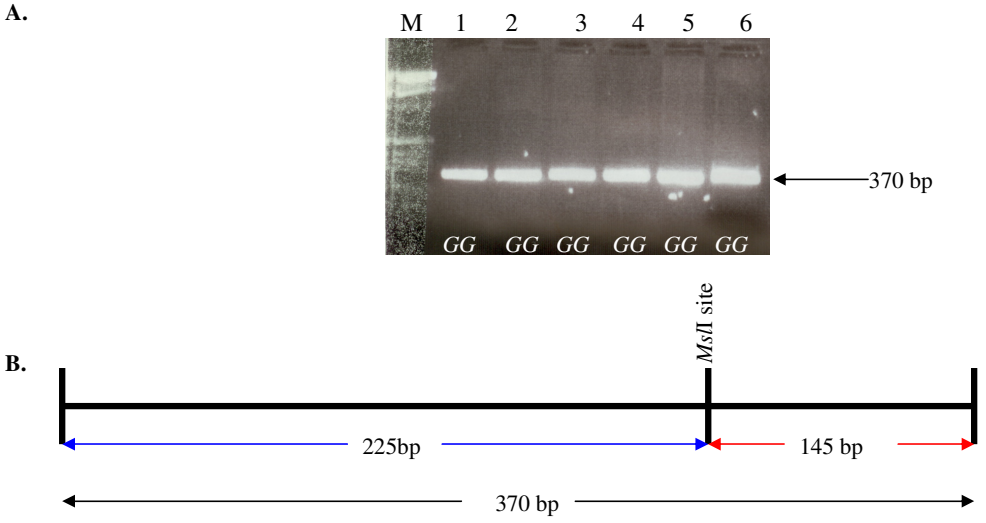


Fig 3.19. **ASREA of *CHRM3Msl*I polymorphism. A.** Representative 2% agarose gel showing the *CHRM3Msl*I fragment sizes and digestion with *Msl*I. Lane M: *λPst* molecular weight marker; Lane1-6: homozygous G/G individuals. The A allele was not found in the 80 individuals of the population used in the present study. **B.** Schematic representation of the *CHRM3Msl*I amplicon, generated by PCR-amplification for genotyping, showing the positions of the polymorphic *Msl*I restriction site and the sizes of the fragments generated following *Msl*I digestion.

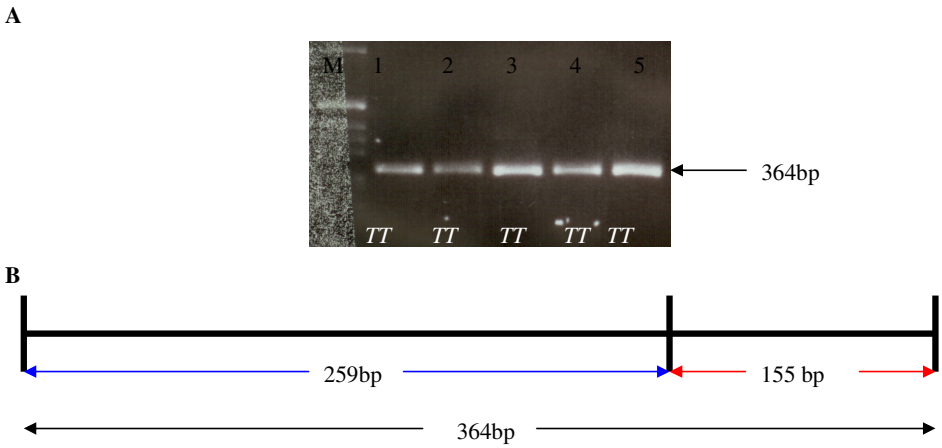


Fig 3.20. **ASREA of *SLC18A1* polymorphism. A.** Representative 2% agarose gel showing the ***SLC18A1*** fragment sizes and digestion with *Msl*I. Lane M: *λPst* molecular weight marker; Lane1-6: homozygous G/G individuals. The A allele was not found in the 80 individuals of the population used in the present study. **B.** Schematic representation of the ***SLC18A1***amplicon, generated by PCR-amplification for genotyping, showing the positions of the polymorphic *Msl*I restriction site and the sizes of the fragments generated following *Msl*I digestion.

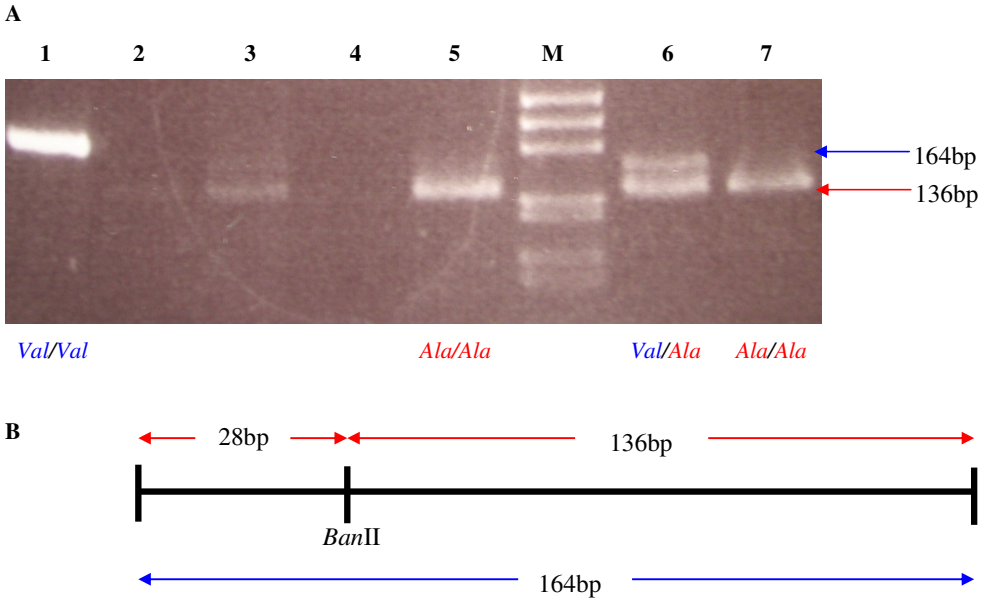


Fig 3.21. **ASREA of *RXRβ Val95Ala* polymorphism: A.** Representative 2% agarose gel showing the fragment sizes of the *RXRβ Val95Ala* amplicon following digestion with *Ban*II. Lane M: *λPst* molecular weight marker; Lane 1: Val/Val homozygous individual; Lanes 5&7: Ala/Ala homozygous individuals; Lane 6: Val/Ala heterozygous individual. Lanes 2-4: genotyping results were ambiguous, and were therefore repeated. **B.** Schematic representation of the *RXRβ Val95Ala* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Ban*II restriction site and the sizes of the fragments generated following *Ban*II digestion.

Table 3.19. **Genotype distribution and allele frequencies of *RXRβ Val95Ala* polymorphism in OCD patients and control individuals**

	GENOTYPE				ALLELE		
RXRβ Val95Ala	**Val/Val**	**Val/Ala**	**Ala/Ala**	**Total**	**Val**	**Ala**	**Total**
OCD	0 (0%)	2 (5%)	38 (95%)	40	2 (2.5%)	78 (97.5%)	80
Controls	1 (2.5%)	2 (5%)	37 (92.5%)	40	6 (7.5%)	74 (92.5%)	80

(Genotype and allele percentages shown in brackets)

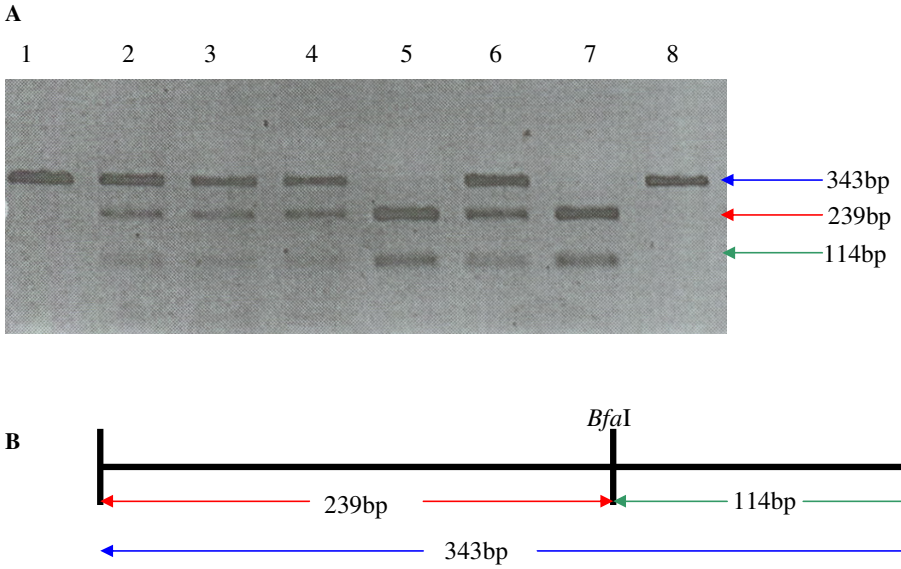


Fig. 3.22. **A. ASREA of *GRID1* rs10887523 polymorphism: A.** Representative 2% agarose gel showing the fragment sizes of the *GRID1* rs10887523 amplicon following digestion with *Bfa*I.. Lanes 1 and 8: A/A homozygous individuals. Lanes 2-4 and 6: C/A heterozygous individuals. Lanes 5 and 7: C/C homozygous individuals. **B.** Schematic representation of the *GRID1* rs10887523 amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic *Bfa*II restriction site and the sizes of the fragments generated following *Bfa*I digestion.

Table 3.20. **Genotype distribution and allel frequencies of *GRID1 rs10887523(C/A)* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
GRID1 rs10887523	**CC**	**CA**	**AA**	**Total**	**C**	**A**	**Total**
OCD	21 (42%)	23 (46%)	6 (12%)	50	65 (65%)	35 (35%)	100
Control	20 (31.3%)	33 (55.1%)	11 (17.2%)	64	73 (57%)	55 (43%)	128

(Genotype and allele percentages shown in brackets)

3.3.1.12. *RELNIVS59C>T* polymorphism

The PCR-amplification of the region of the reelin gene where the intron 59C/T polymorphism is located yielded a 380bp fragment. The PCR fragments generated were then SNaPshot genotyped (section 2.9) (Fig 3.23). The genotype distribution and allele frequencies are shown in Table 3.21..

Both OCD and control groups were found to be in Hardy-Weinberg equilibrium. . Logistic regression analysis showed no significant association between the *RELNIVS59* C>T polymorphism and OCD under either the dominant model (p=0.840) (Table 3.24) or the additive model (p=0.765) (Table 3.24).

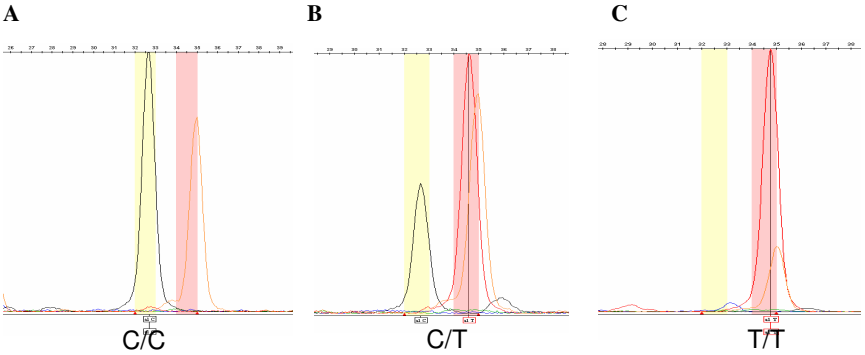


Fig 3.22. **SNaPshot results for the *RELNIVS59C/T* polymorphism. A***:* representative SNaPshot result for the C/C-genotype, **B**: representative SNaPshot result for the C/T-genotype and (**C**): representative SNaPshot result for the T/T-genotype.

Table 3.21**. Genotype distribution and allele frequencies of *RELN IVS59C/T* polymorphism in OCD patients and control individuals**

	GENOTYPE				ALLELE		
RELN IVS59C/T	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	20 (45.5%)	21 (47.5%)	3 (6.8%)	44	27 (30.7%)	61 (69.3%)	88
Control	16 (39%)	17 (41.5%)	8 (19.5%)	41	33 (40.2%)	49 (59.8%)	82

(Genotype and allele percentages shown in brackets)

3.3.1.13. *WDR47 rs2591000* polymorphism

PCR amplification of intron 10 of *WDR47* where *rs2591000* resides yielded a 574bp fragment that contains an invariant *Hinf*III restriction site. Digestion of this fragment with *Hinf*III yielded two fragments of 549bp and 25bp for the *T* allele, while for the *C* allele, which creates another *Hinf*III restriction site, the 549bp fragment was digested into two fragments of 375bp and 174bp (Fig 3.24). The genotype distribution and allele frequencies are shown in table 3.22.

Both OCD and control groups were in Hardy-Weinberg equilibrium. Logistic regression analysis showed no significant association between *rs2591000* and OCD under either the dominant model (p=0.620, Table 3.24) or the additive model (p=0.302, Table 3.24).

3.3.1.14. *ATG16L2 rs2282613* polymorphism

PCR amplification of intron 4 of the gene encoding *ATG16L2* where *rs2282613* resides yielded a 312bp fragment that contains three invariant *Mbo*II restriction sites. Digestion of this fragment with *Mbo*II yielded 4 fragments of 134bp, 113bp, 62bp and 3bp for the *C* allele, while for the *T* allele, which creates another *MboII* restriction site, the 113bp fragment was digested into two framents of 105bp and 8bp (Fig 3.25). The genotype distribution and allele frequencies are shown in table 3.23.

Both OCD and control groups were found to be in Hardy-Weinberg equilibrium. Logistic regression analysis showed no significant association between *rs2282613* and OCD under either the dominant model (p=0.367, Table 3.24) or the additive model (p=0.653, Table 3.24).

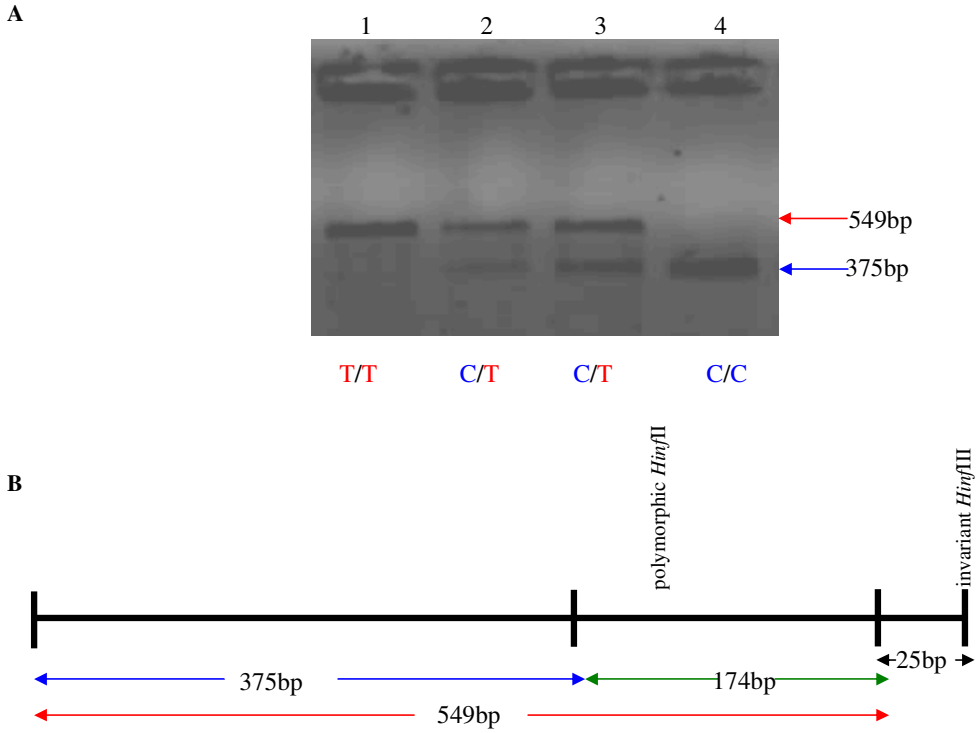


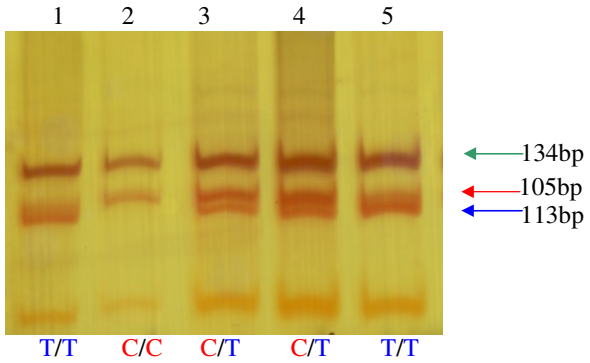
Fig 3.24. **ASREA of *WDR47 rs2591000* polymorphism. A.** Representative 2% agarose gel showing the fragment sizes of *WDR47 rs2591000* amplicon following digestion with *Hinf*III. Lane 1: T/T homozygous individual; Lane 2-3: C/T heterozygous individuals; Lane 4: C/C homozygous individual. The 174bp fragment is not shown. **B.** Schematic representation of the *WDR47 rs2591000* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic and invariant *Hinf*III restriction sites and the sizes of the fragments generated following *Hinf*III digestion.

Table 3.22**. Genotype distribution of *WDR47 rs2591000* polymorphism in OCD patients and control individuals.**

	GENOTYPE				ALLELE		
WDR47 rs2591000	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	17 (36.9%)	17 (36.9%)	12 (26.2%)	46	51 (55.4%)	41 (44.6%)	92
Control	16 (33.3%)	19 (39.6%)	13 (27.1%)	48	51 (53.1%)	45 (46.9%)	96

Genotype and allele percentages shown in brackets)

A



B

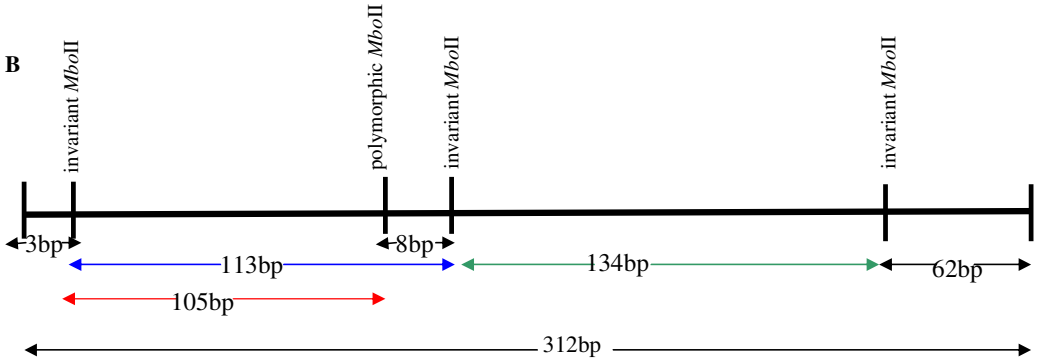


Fig 3.25. **ASREA of *ATG16L2 rs2282613* polymorphism. A.** Representative 20% acrylamide gel showing the fragment sizes of the *ATG16L2 rs2282613* amplicon following digestion with *Mbo*II. Lanes 1 and 5: T/T homozygous individuals; Lane 2: C/C homozygous individual; Lanes 3 and 4: C/T heterozygous individual. The 3bp, 8bp and 62 bp fragments are not shown. **B.** Schematic representation of the of *ATG16L2 rs2282613* amplicon, generated by PCR-amplification for genotyping, showing the position of the polymorphic and invariant *Mbo*III restriction sites and the sizes of the fragments generated following *Mbo*II digestion.

Table 3.23**. Genotype distribution and allele frequencies of *ATG16L2 rs2282613* polymorphism in OCD patients and control individuals**

	GENOTYPE				ALLELE		
ATG16L2 rs2282613	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	19 (42.2%)	19 (42.2%)	7 (15.5%)	45	57 (63.7%)	33 (36.7%)	90
Control	19 (42.2%)	20 (44.4%)	6 (13.3%)	45	58 (64.5%)	32 (35.5%)	90

Genotype and allele percentages shown in brackets)

3.3.1.15.PCR-SSCP analysis of *POU3F2*

At the time of this study, no polymorphisms were available on any of the public databases. Hence, in order to identify sequence variation *ab initio*, the single exon of *POU3F2* was PCR amplified in seven overlapping PCR fragments using the primer sets shown in Table 2.2. and subjected to PCR-SSCP. After screening 40 OCD patients and 40 control individuals under two conditions optimised for high sensitivity (de Lange, 2004) no mobility shifts were detected in any of the fragments indicating that no there is no informative polymorphic sites in the coding region of *POU3F* in the population sample under investigation. Therefore, no association analysis was performed using this gene in the present study.

Table 3.24. **Summary of logistic regression analysis of genotypes of novel OCD candidate genes.**

Polymorphism	OR	95% CI		p-value
***SNAP2 5MnI*I**				
TT	1			
For each *G* allele	1.24	0.68	2.37	0.484
Heterozygote	1.13	0.51	2.43	0.763
***SNAP25/Dde*I**				
CC	1			
For each *T* allele	0.721	0.392	1.28	0.272
Heterozygote	0.921	0.419	2.05	0.838
SNAP29 C56T				
CC	1			0.301
For each *T* allele	0.802	0.457	1.39	0.432
Heterozygote	0.581	0.278	1.2	0.145
SNAP29 G92A				
GG	1			0.301
For each *A* allele	0.802	0.457	1.39	0.432
Heterozygote	0.581	0.278	1.2	0.145
GRIA4.1				
AA	1			
For each *T* allele	0.73	0.73	1.39	0.344
Heterozygote	1.57	1.57	1.48	0.248
GRIN 1.1				
CC	1			
For each *G* allele	1.118	0.687	1.81	0.648
Heterozygote	0.745	0.343	1.61	0.455
DLX6 IVS1 C>T				
CC	1			
For each *T* allele	0.69	0.41	1.15	0.158
Heterozygote	0.38	0.18	0.81	**0.013**
SYN3 -631C>T				
CC	1			
For each *G* allele	0.51	0.30	0.83	**0.009**
Heterozygote	1.36	0.71	2.69	0.362
BZRP *Ala/147Thr*				
Ala/Ala	1			
For each *Thr* allele	0.79	0.47	1.31	0.362
Heterozygote	0.81	0.39	1.66	0.571

Polymorphism	OR	95% CI	p-value	
DBH (I/D)				
D/D	1			
Add	0.73	0.73	1.39	0.344
Heterozygote	1.57	1.57	1.48	0.248
GRID1 rs1088532				
C/C	1			
For each *A* allele	0.721	0.392	1.28	0.272
Heterozygote	0.921	0.419	2.05	0.838
RELN IVS59C/T				
T/T	1			
For each *C* allele	1.10	0.60	2.01	0.765
Heterozygote	0.91	0.38	2.22	0.840
WDR47 rs2591000				
T/T	1			
For each *C* allele	0.776	0.47	1.25	0.302
Heterozygote	0.781	0.29	2.06	0.620
ATG16L2 rs228261				
T/T	1			
For each *C* allele	0.87	0.45	1.62	0.653
Heterozygote	0.66	0.26	1.62	0.367

Significant associations are highlighted in red.

3.3.2 Further exploration of associated loci

Two investigated loci demonstrated association with OCD, viz. the *DLX6IVS1 C>T* and *SYN3-631C>G.* As these results were tantalizing, further investigation of these polymorphisms as well as additional polymorphisms within these genes were undertaken.

In order to further investigate the role of the *DLX6IVS1C>T* polymorphism in the development of OCD, the sample size of the case and control groups were increased and the analysis repeated (Seaman and Műller-Myhok, 2005; Skol *et al*., 2006). The genotype distribution and allele frequencies of the expanded sample is shown in Table 3.25. Logistic regression analysis again showed a dominant protective effect of the *T* allele of *DLX6 IVS1C>T* polymorphism (p=0.038, Table 3.26).

Two more SNPs, *rs127728* and *rs1004278*, residing in the *DLX6* gene were genotyped using Taqman® genotyping assays (Table 3.27). These SNPs were chosen as, at the time of the study, they were the only other known SNPs residing within this gene with a minor allele frequency greater than 0.1 in the CEU population, which is the HAPMAP population closest to the Afrikaner. No association was observed between any of these markers and OCD (Table 3.28). The level of linkage disequilibrium between *DLX6 IVS1C>T, rs127728* and

rs1004278 were also analysed using the HaploView software program. These analyses revealed no linkage disequilibrium between these three markers (Fig 3.26), which may explain why an association was observed with *DLX6 IVS1C>T* but not with the other markers.

As with *DLX6IVS1C>T*, the observed association of the *SYN3/-631C>G* polymorphism with OCD prompted further investigation in an enlarged sample of OCD cases and control individuals. The genotype distribution and allele frequency of the larger cohort is shown in Table 3.25. In this larger cohort, the additive protective effect of the *G* allele was once again observed (p=0.011, Table 3.26).

Following from these results, two more SNPs (*rs13075* and*, rs130454*) within *SYN3* were genotyped using Taqman® genotyping assays and analysed for their potential association with OCD (Table 2.27). These SNPs were chosen as they were spread over the gene and had minor allele frequencies greater than 0.4 in the CEU HAPMAP population. These two SNPs were in Hardy-Weinberg equilibrium, but did not show any association with OCD (Table 3.28). The level of linkage disequilibrium between *SYN3/-631C>G, rs130753* and *rs130454* was also evaluated using the Haploview software program. These analyses revealed no linkage disequilibrium between the three markers (Fig 3.27), which may explain the observed association with *SYN3/-631C>G,* but the absence of association with the other two markers.

Table 3.25 **Genotype distribution and allele frequency of the *DLX6IVS1C>T* and *SYN3-631C>G* polymorphisms in the increased sample of OCD patients and control individuals**

DLCX6 int1C/T	**CC**	**CT**	**TT**	**Total**	**C**	**T**	**Total**
OCD	15 (16.9%)	39 (43.8%)	35 (39.3%)	89	69 (38.8%)	109 (61.2%)	178
Controls	13 (9.7%)	76 (56.7%)	45 (33.6%)	135	102 (31.1%)	166 (61.988	268
SYN3-631C>G	**CC**	**CG**	**GG**	**Total**	**C**	**G**	**Total**
OCD	47 (37.1%)	65 (50%)	17 (12.9%)	127	164 (62.1%)	100 (37.9%)	264
Controls	33 (23.2%)	77 (54.2%)	32 (22.6%)	142	143 (50.4%)	141 (49.6%)	284

Table 3.26. **Summery of logistic regression model for *DLX6 IVS1 C>T* and *SYN3 -631C>G* for case-control status for the increased sample of OCD patients and control individuals**

DLX6 IVS CT	**OR**	**95% CI**		**Estimate**	**SE**	**z-value**	**p-value**
C/C	1						
For each *T allele*	0.82	0.53	1.27	-0.197	0.22	-0.90	0.371
Heterozygous	0.54	0.30	0.96	-0.613	0.296	-2.07	**0.038**
SYN3 -631C>G	**OR**	**95% CI**		**Estimate**	**SE**	**z value**	**p-value**
C/C	1						
For each *G* allele	0.51	0.42	0.89	-0.482	-0.188	-2.56	**0.011**
Heterozygous	1.36	0.59	1.59	-0.035	-0.253	-0.14	0.891

Table 3.27. **Genotype distribution and allele frequencies of additional DLX6 and SYN3 SNPs in OCD patients and control individuals**

DLX6 rs1004278	***AA***	***AG***	***GG***	**Total**	***A***	***G***	**Total**
OCD	11 (17.4%)	26 (41.3%)	26 (41.3%)	63	48 (38.1%)	79 (61.9%)	126
Control	6 (9.8%)	28 (45.9%)	27 (44.3%)	61	40 (32.8%)	82 (67.2%)	122
DLX6 rs1207728	***CC***	***CG***	***GG***	**Total**	***C***	***G***	**Total**
OCD	4 (6.4%)	27 (43.6%)	31 (50.0%)	62	35 (28.2%)	89 (71.8%)	124
Control	3 (5.1%)	19 (32.2%)	37 (45.7%)	59	25 (21.2%)	93 (78.8%)	118
SYN3 rs130753	***CC***	***CT***	***TT***	**Total**	***C***	***T***	**Total**
OCD	28 (44.4%)	24 (38.1%)	11 (17.5%)	63	80 (63.5%)	46 (36.5%)	126
Control	27 (45.8%)	27 (45.8%)	5 (8.5%)	59	81 (68.6%)	37 (31.4%)	118
SYN3 rs130454	***AA***	***AG***	***GG***	**Total**	***A***	***G***	**Total**
OCD	17 (26.5%)	28 (43.8%)	19 (29.7%)	64	62 (48.4%)	66 (51.6%)	128
Control	12 (20.7%)	28 (48.3%)	18 (31.0%)	58	52 (44.8%)	64 (55.2%)	116

Table 3.28 **Summary of logistic regression analysis of additional SNPs genotyped in *DLX6* and *SYN3*.**

Polymorphism	**OR**	**95% CI**		**p-value**
DLX6 rs1004278	**OR**	**95% CI**	**p-value**	
A/A	1			
For every *G* allele	0.73	0.40	1.26	0.265
Heterozygote	0.70	0.32	1.51	0.367
DLX6 rs1207728	**OR**	**95% CI**	**p-value**	
C/C	1			
For every *G* allele	0.79	0.34	1.75	0.562
Heterozygote	1.35	0.49	3.60	0.554
SYN3 rs130454	**OR**	**95% CI**	**p-value**	
A/A	1			
For every *G* allele	0.86	0.53	1.41	0.556
Heterozygote	0.82	0.40	1.68	0.583
SYN3 rs130753				
C/C				
For every *T* allele	1.46	0.82	2.74	0.212
Heterozygote	0.59	0.26	1.30	0.198

Add= additive model, Dom = dominant model, CI = confidence interval

Figure 3.26. **Linkage disequilibrium plots of DLX6 SNPs *DLX6IVS1C>T, rs1207728* and *rs1004278*.** D´ values are indicated in blocks. The low D´ values and a the lack of a clear haplotype block shows that these 3 markers are not in strong linkage disequilibrium with each other.

Figure 3.27. **Linkage disequilibrium plots of *SYN3* SNPs, *SYN3/-631C>G, rs130753* and *rs130454*.** D´ values are indicated in blocks. The low D´ values and a the lack of a haplotype block shows that these 3 markers are not in linkage disequilibrium with each other.

3.3.3. Analysis of epistatic interaction between SNPs associated with OCD development.

As the *DLX6IVS1C>T* and *SYN3-631C>G* demonstrated association with OCD in both the original and the enlarged cohort, further logistic regression analysis was performed in order to assess whether there was indication of a significant interactive effect of these two polymorphisms on the development of OCD. Results from this analysis showed that there was indeed a significant interactive effect between these two

polymorphisms and OCD (p=0.039, Table 3.29), where individuals who were heterozygous *CG* for the *SYN3/-631C>G* polymorphism as well as homozygous *CC* for the *DLX6int1C/T* polymorphism had increased susceptibility to developing OCD, while individuals who were heterozygous at both these loci were protected against OCD (Fig 3.28).

Table 3.29. **Analysis of deviance table for logistic regression model including interaction between *DLX6IVS1C>T and SYN3-631C>G.*** Significant interaction is highlighted in red

	df	Deviance	Residual df	Residual Deviation	p-value
NULL			169	233.3	
SYN3-631C>G	2	2.7	167	230.6	0.300
DLX6IVS1C>T	2	2.8	165	227.8	0.200
SYN3-631C>G: DLX6IVS1C>T	4	10.1	161	217.7	**0.039**

df= degrees of freedom

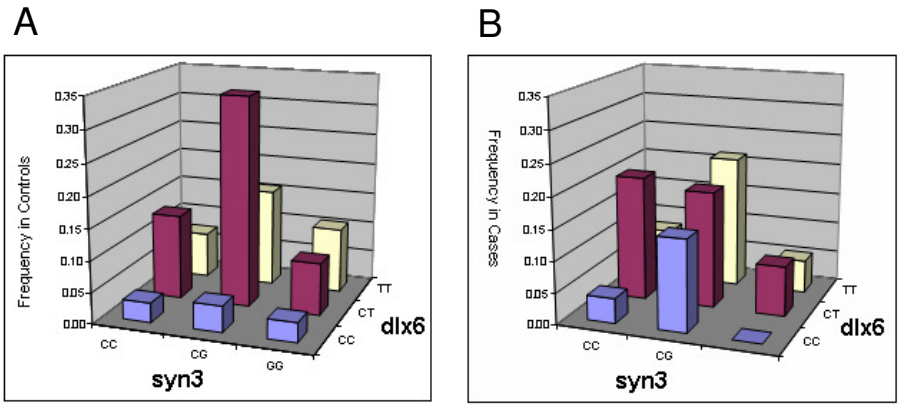


Fig. 3.28**. Bar graphs representing joint *DLX6IVS1C>T* and *SYN3-631C>G* genotype frequencies A** Genotype frequencies for the control panel, B Genotype frequencies for the OCD patients.

CHAPTER 4: DISCUSSION

CHAPTER 4: DISCUSSION

Obsessive-compulsive disorder is a common, disabling psychiatric condition for which the underlying molecular pathophysiology still remains unclear. Currently, it is proposed that this disorder is caused by the complex interplay of various environmental and genetic factors. In order to dissect the intricate relationship between these factors, researchers have adopted several strategies in an attempt to identify genes involved in the pathophysiologies of these disorders. Although no susceptibility gene that plays a major role in OCD has been identified to date, a number of plausible candidate genes that may contribute modest effects have received attention. The involvement of these genes has been investigated in several population and family-based association studies, however, divergent results have generally confounded final interpretation (section 1.4.7). A possible explanation for these inconsistencies is that genes found to be associated with OCD in some populations may only contribute very minor effects to disease susceptibility within those populations while having no effect in others. This could have serious implications for the identification of genetic factors involved OCD, given that the focus of many investigations is the replication of previously reported associations in different populations. A consequence of all these replication attempts is that the same genes are constantly being analysed, hence, no concerted effort is being made to identify novel OCD candidate genes which may in fact play larger contributory roles to the pathogenesis of the disorder in wider populations.

One possible approach to identifying such OCD candidate genes is to utilise existing knowledge of diseases with phenomenological overlap with OCD, which lend themselves to better genetic dissection by linkage analysis and animal models. Genetic loci for such disorders identified through linkage analysis could potentially harbour novel OCD candidate genes, while genes implicated through animal models may lead to the identification of additional susceptibility factors by interactome analysis. One such disorder is schizophrenia, where, in addition to several case-control association study data, linkage data, studies of chromosomal aberrations and animal models have led to the identification of many chromosomal regions that may contain genes involved in its aetiology (reviewed by Owen *et al.,* 2004) and may therefore also harbour plausible OCD candidate genes.

This approach was employed in the present investigation, where schizophrenia susceptibility loci were searched for credible OCD candidate genes, and information regarding a well-characterised schizophrenia animal model, the heterozygous reeler mouse, was used as a gateway for interactome analysis to identify further OCD candidate genes. The identified genes were then assessed for their potential contributory effects to OCD susceptibility in case-control association studies in an Afrikaner study cohort, in a proof-of-principle study.

4.1. BIOINFORMATIC SEARCHES OF SCHIZOPHRENIA LOCI

In order to identify potential candidate genes for OCD, 18 reported schizophrenia susceptibility loci where searched. Each of the loci searched in the present investigation, were chosen as they were regions implicated in previous linkage studies.

One problem using this approach is the sheer number of genes located in each of the identified regions (table 3.1). Therefore searching these 18 loci is a time-consuming and arduous task. Furthermore, during the course of the present investigation, several builds of the human genome (in both NCBI and ensemble) have been released. The data represented in each of these builds tend to differ somewhat resulting in the delineation of some of the schizophrenia susceptibility loci changing as a result of repositioning of markers from build to build. To overcome this dilemma, the databases were regularly monitored during the course of the investigation.

4.2. INTERACTOME ANALYSIS OF REELIN

As described in section 1.4.9.1. reelin is a large secreted glycoprotein involved in the regulation of ordered neuronal alignment during the development of laminar brain structures (D'Arcangelo *et al.,* 1995; Ogawa *et al.,* 1995; Lambert de Rouvroit and Goffinet, 1998). The gene encoding reelin has been considered a good schizophrenia candidate gene for a number of reasons. These include its vital role in neurodevelopment, with several studies showing reduced reelin protein and mRNA levels in post-mortem brains of schizophrenic patients, its chromosomal location and the observed behaviour and neurochemical similarities between the heterozygous reeler mouse and schizophrenia patients.

In the present study, the proposal that reelin can be considered a plausible schizophrenia susceptibility gene was extended to include genes encoding proteins that interact with reelin. This reasoning, as expounded in section, 1.5 can thus be applied to OCD susceptibility gene candidature. To date, several reelin-interacting proteins have been identified and their binding sites on reelin have largely been determined (section 1.5.2.). The reelin region containing reelin repeats 3-6 has been shown to be necessary for the binding of reelin to ApoER2/VLDLR (Jossin *et al.,* 2004), while sub-repeat B of the first reelin repeat domain is crucial for CNR binding (Senzaki *et al.,* 1999) (Fig 1.14). However, the function of the reeler domain of reelin still remained unknown. As mentioned in the first chapter, the reeler domain has only been identified in one other protein, F-spondin, which is also an extracellular matrix protein that is involved in neural crest cell migration. Therefore, since the reeler domain occurs only in proteins essential for neuronal migration, it was hypothesised that, by directing neuronal migration, it plays a critical role in neurodevelopment, probably through protein-protein interactions. To further understand its function, a foetal brain cDNA library was screened, using the reeler domain of reelin as "bait" in Y2H analysis.

4.2.1. Yeast two hybrid analysis to identify ligands for the reeler domain of reelin

4.2.1.1. Number of independent clones screened

Library mating efficiency calculations indicated that approximately 5.9 x 10^6 pre-transformed foetal brain cDNA library clones were screened (section 3.2.1.1.3) Therefore, since the pre-transformed foetal brain cDNA library used contained approximately 3.5x10^6 independent clones, statistically, each clone represented in the library was screened at least once.

4.2.1.2. Preys excluded from further analysis

Following the Y2H mating and subsequent stringency experiments to test for the activation of reporter genes, 22 clones were identified. However, sequence analysis showed that 22 of these clones could be considered unlikely to encode biologically relevant reelin-interacting proteins for a number of reasons discussed in the following section.

4.2.1.2.1. No significant protein matches

The insert sequences of fifteen of the 22 clones had significant DNA matches in both NCBI Genbank (http://www.ncbi.nlm.nih.gov) or Ensemble (http://www.ensembl.org) databases, yet the ORF in-frame with the GAL4 activation domain had no significant protein matches in either of these databases (Table 3.5). One reason for the lack of significant protein matches, in spite of significant DNA matches is due to the fact that in classical two-hybrid library constructions such as the Clontech library used in this study, which are derived from oligo-dT primed cDNA, only one out of six of all cloned inserts are in frame with the transcription factor activation domain (van Criekinge and Beyaert, 1999).

4.2.1.2.2. Incompatible cellular compartments

The identification of the sub-cellular location of proteins is key to understanding their functions. This is especially true in the context of protein-protein interactions as proteins that are localised in separate cellular compartments would not be able to interact with one another. However, for a number of proteins, the sub-cellular localisation has not yet been experimentally determined. To overcome this obstacle, several systems have been developed that support automated prediction of sub-cellular localisation, based on amino acid sequence information. (Nakai and Kanehisa, 1992; Horton and Nakai, 1997; Reinhardt and Hubbard, 1998; Hua and Sun, 2001; Emanuelssen, *et al.,* 2000; Nair and Rost, 2002). There are, however, two limitations to these systems. Firstly, they have limited accuracy for predicting sub-cellular localisation and secondly, they have limited coverage (the number of sub-cellular regions supported by the predictor) (Lu *et al.,* 2003).

The accuracy of the publicly available sub-cellular localisation prediction programs vary quite significantly. For this reason, the present study used two prediction programs, namely “Proteome Analyst” and “ESLpred” which have reported prediction accuracy of 92% (Lu *et al.,* 2003) and 88%, respectively. Although, several other programs are available to predict sub-cellular localisation, these two programs were chosen as they have the highest accuracy and cover nine cellular compartments (mitochondrion, nucleus, endoplasmic reticulum, extracellular, cytoplasm, plasma membrane, golgi, lysosome, peroxisome).

Using Proteome Analyst and ESLpred, five clones were found to encode proteins that localise to the nucleus, a cellular compartment incompatible with reelin's reported (and predicted) extracellular localisation. However, investigations have shown that reelin is internalised into the cell following binding to VLDL receptors via clathrin-dependant enocytosis (section 1.4.9.1.1) (D'Archangelo *et al.,* 1999). For this reason, cytosolic prey proteins were also considered as physiologically plausible interactors. However, as the automated prediction programs are not 100% reliable, it remains possible that some preys identified may have been assigned to incorrect cellular compartments, but due to project constraints, the predicted sub-cellular localisation of each prey was not verified experimentally.

4.2.1.3. Preys identified as putative reelin ligands

Two prey clones, encoding WDR47 and ATG16L2, were identified as putative reelin interacting proteins. Although the functions of each of these proteins are not fully understood, there is evidence, discussed below, to suggest that they may both play a role in neuronal migration and brain development.

4.2.1.3.1. WDR47

Although the function of the WDR47 protein, which has been predicted to localise to the cytosol, is unknown, its domain structure was of considerable interest as it is very similar to that of LIS1, a protein which forms part of the reelin signaling pathway (Fig 4.1). In addition to having seven WD40-repeat domains in common, both WDR47 and LIS1 contain a LISH domain at their N-termini. The high degree of similarity between WDR47 and LIS1 may cause one to speculate that they represent two aliases of the same protein. However, based on the sequence alignment shown in Figure 4.2 it is clear that they are two distinct proteins. WDR47 also contains a C-terminal to LISH domain, that is not present in LIS1. While the CTLH domain is a domain of unknown function, the WD40-repeat and the LIS1 domains both play essential roles in neuronal migration. Furthermore, it is interesting to note that the CTLH domain is also found in RAN-binding protein 9, a protein shown to be involved in microtubule assembly (Nishitani *et al*., 2001). One could therefore deduce, based on their similar domain structure, that since LIS1 plays a vital role in neuronal migration, that WDR47 may also be important in this process.

<u>WD40 repeat Domain</u>

WD40 repeat domains are minimally conserved domains of approximately 44-60 amino acids that typically contains a glycine-histidine (GH) dipeptide 11-24 residues from the N-terminus and with a tryptophan-aspartic acid (WD) dipeptide at the C-terminus. Between the GH and WD dipeptides is a minimally conserved core sequence of approximately 40 amino acids (Smith *et al.,* 1999). This domain type was first recognised in the β subunit of the GTP-binding protein transducin (van der Voorn and Ploegh, 1992) and has since been found in approximately 136 human proteins. WD 40 repeat proteins perform a wide range of cellular functions, which include signal transduction, RNA synthesis and processing, chromatin assembly, cytoskeletal assembly, cell cycle control and apoptosis (Li and Roberts, 2001). The underlying common function of all WD40 proteins, however, is coordinating the assembly of large protein complexes. These repeating units are thought to serve

as a platform for protein interactions and these interactions can occur concurrently with a number of different proteins (Tyers and Williams, 1999).

Of particular interest to the present study is the fact that some of the identified WD40 repeat-containing proteins play crucial roles in signal transduction and cytoskeletal assembly, two processes that are vital for neuronal migration and neurodevelopment. Moreover, mutations in WD-40 domain genes have been identified that result in inherited diseases of impaired neurodevelopment. One such disorder is Joubert Syndrome, a rare, autosomal recessive, neurodevelopmental disorder characterised by hypoplasia of the cerebellar vermis, hypotonia, ocular motor apraxia and global developmental delays (Dixon-Salazar *et al.,* 2004). There is a large degree of clinical heterogeneity within this disorder, which has hampered efforts to decipher its molecular aetiology. Interestingly, mutations in *AHI1,* the gene encoding the WD40-40 domain protein, jouberin, has been shown to result in a specific Joubert syndrome subtype that is characterized by excessive cortical folding and a simplified four-layered or unlayered cortical architecture (Dixon-Salazar *et al.,* 2004) resulting from aberrant neuronal migration.

The lissencephaly-1 (LIS1) gene, *LIS1*, was the first WD40-repeat encoding gene identified as playing a role in human disease. Mutations in *LIS1* have been shown to cause Miller-Dieker lissencephaly, a brain malformation that results in severe mental retardation, epilepsy and an early death. Patients exhibit a smooth cerebral surface that arises from disturbances in neuronal migration (Morris, 2000). The amino acid sequence of LIS1 shows significant homology to that of β subunits of heterotrimeric G-proteins with multiple WD40-repeats in their primary structure, suggesting that LIS1 may play a fundamental role in the signal transduction pathway involved in cerebral development (Reiner *et al.,* 1993).

LIS1 homology domain

The LIS1 homology domain was first described as a novel sequence motif in the products of genes mutated in Miller-Dieker Lissenecephaly, Treacher Collins and oral-digital type 1 syndromes, three disorders associated with defects in cell migration (Emes and Ponting, 2001). As mentioned in the previous section, mutations in *LIS1* are associated with Miller-Dieker lissencephaly, which is a consequence of abnormal neuronal migration. Interestingly, in mice heterozygous for a *LIS1* mutation, that removed the LISH domain, aberrant morphology of the developing cortex was found, which is consistent with defects in neuronal migration (Cahan *et al.,* 2001). Furthermore, in a patient with low severity lissencephaly, a mutation was found within the LISH domain of the LIS1 gene (Cahan *et al.,* 2001). This suggests that the LISH domain may play a vital role in mediating neuronal migration during neurodevelopment. Additionally, LISH domains have also been suggested to contribute to the regulation of microtubule dynamics, either by mediating dimerisation, or by binding cytoplasmic dynein heavy chain or microtubules directly (Emes and Ponting, 2001). Microtubule rearrangement has been shown to play an important role in neuronal migration (reviewed by Jossin, 2004).

Based on the functions of both WD40-repeat domains and LISH domains in neuronal migration, it is quite reasonable to assume that the WDR47 protein, which contains both of these domains, may also play a critical

role in the process of neuronal migration. Therefore, since reelin is a pivotal component of neuronal migration, WDR47 can be considered a plausible putative reelin ligand.

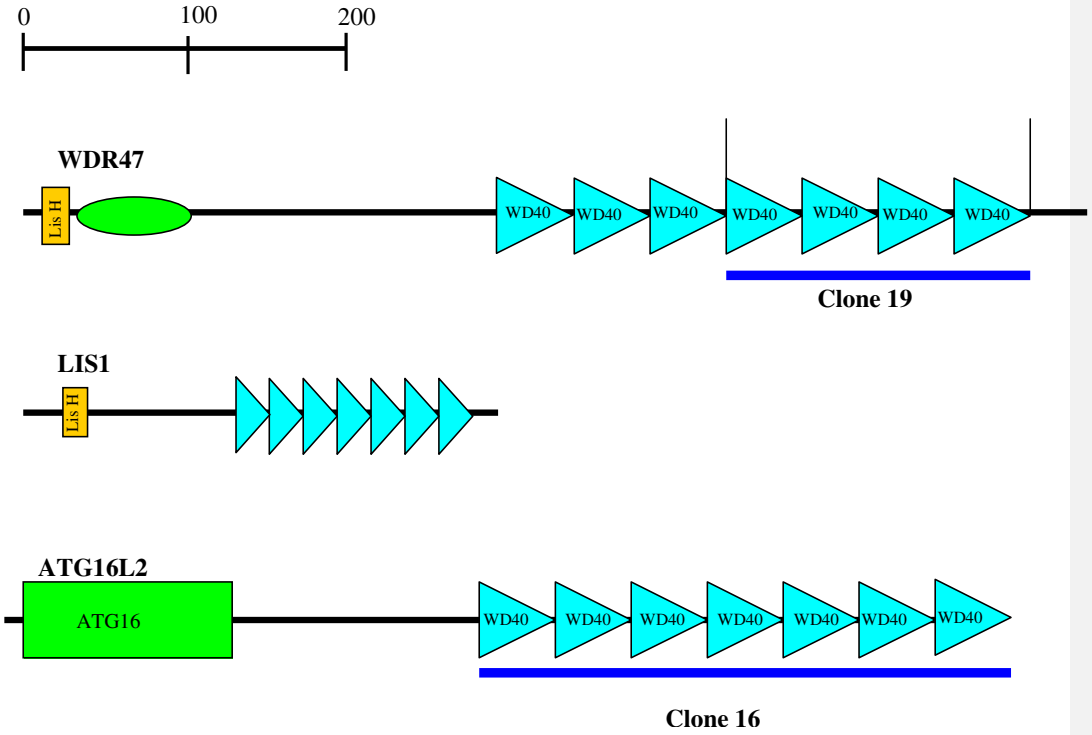


Fig 4.1. Domain structures of WDR47, LIS1 and ATG16L2. A comparison of the domain structures of WDR47, LIS1 and ATG16L2. This comparison shows that WDR47 and LIS1 have a similar domain composition and structure. WD40 repeat domains are represented with blue triangles. The Lis1 homology domain (LisH) is represented by the yellow rectangles, while the C- terminal of Lis1 domain (CTLH) is represented by the green oval. The ATG16 domain of ATG16L2 is represented with the green rectangle. The blue lines represent the fragment of WDR47 and ATG16L2 encoded by clones 19 and 16, respectively. The scale indicates number of amino acids.

```
CLUSTAL W (1.83) multiple sequence alignment

LIS1            ------------------------------------------------------------
ATG16L2         ------------------------------------------------------------
WDR47           MTAEETVNVKEVEIIKLILDFLNSKKLHISMLALEKESGVINGLFSDDMLFLRQLILDGQ 60


LIS1            ------------------------------------------------------------
ATG16L2         ------------------------------------------------------------
WDR47           WDEVLQFIQPLECMEKFDKKFFRYIILKQKFLEALCVNNAMSAEDEPQHLEFTMQEAVQC 120


LIS1            ------------------------------------------------------------
ATG16L2         ------------------------------------------------------------
WDR47           LHALEEYCPSKDDYSKLCLLLTLPRLTNHAEFKDWNPSTARVHCFEEACVMVAEFIPADR 180


LIS1            ------------------------------------------------------------
ATG16L2         ------------------------------------------------------MAGPGVP 7
WDR47           KLSEAGFKASNNRLFQLVMKGLLYECCVEFCQSKATGEEITESEVLLGIDLLCGNGCDDL 240


LIS1            ------------------------------------------------------------
ATG16L2         GAPAARWKRHIVRQLRLRDRTQKALFLELVPAYN-HLLEKAELLDKFSKKLQPEPNSVTP 66
WDR47           DLSLLSWLQNLPSSVFSCAFEQKMLNIHVDKLLKPTKAAYADLLTPLISKLSPYPSSPMR 300


LIS1            ------------------------------------------------------------
ATG16L2         TTHQGPWEESELDSDQVPSLVALRVKWQEEEEGLRLVCGEMAY----------------Q 110
WDR47           RPQSADAYMTRSLNPALDGLTCGLTSHDKRISDLGNKTSPMSHSFANFHYPGVQNLSRSL 360


LIS1            ----------------------------------------MVLSQRQRDELNRAIADYL 19
ATG16L2         VVEKGAALGTLESELQQRQSRLAALEARVAQLREARAQQAQQVEEWRAQNAVQRAAYEAL 170
WDR47           MLENTECHSIYEESPERSDTPVDAQRPIGSEILGQSSVSEKEPANGADNFGPAKQEKNEL 420
                                                 .   .    :   : *

LIS1            RSNG--YEEAYSVFKKEAELDWNEELDK--KYAGLLEKKWTSVIRLQKKVMELESKLNEA 75
ATG16L2         RAHVGLREAALRRLQEEARDLLERLVQR--KARAAAERNLRNERRERAKQARVSQELKKA 228
WDR47           RDSTEQFQEYYRQALRYQQHLEQKEQQRQIYQQMLLEGGVNQEDGPDQQQNLTEQFLNRS 480
                *       :       .  .   :.  ::        *    .      :    .. *:.:

LIS1            KEEFT--SGGP--LGQ-KRDPKEWIPRP-------PEKYALSGHRSPVTRVIFH--PVFS 121
ATG16L2         AKRTVSISEGPDTLGDGMRERRETLALA-------PEPEPLEKEACEKWKRPFRSASATS 281
WDR47           IQKLGELNIGMDGLGNEVSALNQQCNGSKGNGSNGSSVTSFTTPPQDSSQRLTHDASNIH 540
                 :.    . *   **:     .:    .       ..  .:       :   :  .

LIS1            VMVSASEDATIKVWDYETG-------------------------DFERTLKG------- 148
ATG16L2         LTLSHCVDVVKGLLDFKKRRGHSIGGAPEQRYQIIPVCVAARLPTRAQDVLDA------- 334
WDR47           TSTPRNPGSTNHIPFLEESPCGSQISSEHSVIKPPLGDSPGSLSRSKGEEDDKSKKQFVC 600
                   .   . .  :   :                          .

LIS1            -----HTDSVQDISFDHSGKLLASCSADMTIKLWDFQG--FECIRTMHG----------- 190
ATG16L2         -----HLSEVNAVRFGPNSSLLATGGADRLIHLWNVVGSRLEANQTLEG----------- 378
WDR47           INILEDTQAVRAVAFHPAGGLYAVGSNSKTLRVCAYPDVIDPSAHETPKQPVVRFKRNKH 660
                     . . *. : *   . * *  . .  :::    .    . :

LIS1            HDHNVSSVAIMPNGDHIVSASRDKTIKMWEVQTGYCVKT-----FTGHREWVRMVRPNQD 245
ATG16L2         AGGSITSVDFDPSGYQVLAATYNQAAQLWKVGEAQSKET-----LSGHKDKVTAAKFKLT 433
WDR47           HKGSIYCVAWSPCGQLLATGSNDKYVKVLPFNAETCNATGPDLEFSMHDGTIRDLAFMEG 720
                   .: .*   * *  : :.: ::  ::  .    .  *     :: *   :

LIS1            -----GTLIASCSNDQTVRVWVVATKECKAELREHEHVVECIS---------------- 283
ATG16L2         -----RHQAVTGSRDRTVKEWDLGRAYCSRTINVLSYCNDVVCGDH--IIISGHNDQKIR 486
WDR47           PESGGAILISAGAGDCNIYTTDCQRGQGLHALSGHTGHILALYTWSGWMIASGSQDKTVG 780
                          : : * .:             :         :

LIS1            -WAPESSYSSISEATG-------SETKKSGKPGPFLLSGSRDKTIKMWDVSTGMCLMTLV 335
ATG16L2         FWDSRGPHCTQVIPVQGR-----VTSLSLSHDQLHLLSCSRDNTLKVIDLRVSNIRQVFR 541
WDR47           FWDLRVPSCARVVGTTFHGTGSAVASVAVDPSGRLLATGQEDSSCMLYDIRGGRMVQSYH 840
                 *  . . .:    .          :   .     * : ..*.:  : *:  .
```

Comment [MB70]: Why do the LISH domains of Lis and WDR47 not align? This is something Nicky will jump on! Need to find out why and make statement in the legend, or else use another program???

```
LIS1            GH-----DNWVRGVLFHSGGKFILSCADDKTLRVWDYK---NKRCMKTLN-AHEHFVTSL 386
ATG16L2         ADGFKCGSDWTK-AVFSPDRSYALAGSCDGALYIWDVD---TGKLESRLQGPHCAAVNAV 597
WDR47           PH-----SSDVRSVRFSPGAHYLLTGSYDMKIKVTDLQGDLTKQLPIMVVGEHKDKVIQC 895
                  .     .. .: . * ..  : *: : *  : : * .   . :    :   *   *

LIS1            DFHKTAPYVVTGSVDQTVKVWECR- 410
ATG16L2         AWCYSGSHMVSVDQGRKVVLWQ--- 619
WDR47           RWHTQDLSFLSSSADRTVTLWTYNG 920
```

Fig 4.2. Multiple protein sequence alignment of WDR47, ATG16L2 and LIS1. The Clustal-W alignment of the protein sequences of WDR47, ATG16L2 and LIS1 clearly shows that these are three distinct proteins. The positions of the seven WD40 repeat domains in each of the proteins are highlighted in yellow, LIS homology domains are highlighted in green and the CTLH is highlighted in blue. "*****" residues in that column are identical in all sequences in the alignment, "**:**"conserved substitutions have been observed and "**.**"semi-conserved substitutions are observed

4.2.1.3.2. ATG16L2

The ATG16L2 protein, like WDR47, is predicted to be a cytosolic protein of unknown function, and also contains seven WD40-repeat domains (Fig 4.1). Besides the WD40-repeat domains, no other known conserved protein domains are present in ATG16L2. ATG16L2 shares homology with the mouse Pre-mRNA-processing factor 17, a protein which associates with the spliceosome and may play a role in the second step of pre-mRNA splicing (http://harvester.fzk.de/harvester/human/IPI00025/IPI00025503.htm). Interestingly, alternative splicing and spliceosome components have been implicated in neurological responses during fear conditioning.

Comment [MB71]: Add reference:
Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory
and long-term potentiation
I Nijholt1,6, N Farchi2,3,6, M Kye1, EH Sklan2, S Shoham4, B Verbeure5, D Owen5, B Hochner3,7,
J Spiess1, H Soreq2,7 and T Blank1
Molecular Psychiatry (2004) 9, 174–183

4.2.1.4. Limitations of Yeast two-hybrid analysis

Despite the yeast two-hybrid system being a popular method to detect protein-protein interactions, it has its limitations. To begin with, some classes of proteins are not suitable for analysis using this system, for example transcriptional activators that may activate transcription of reporter genes without any interactions with other proteins (auto activation). Another limitation of the Y2H system is the necessity for nuclear localisation of the interacting ligands in order to activate transcription of reporter genes. This is problematic since many proteins, such as membrane-bound proteins and proteins with competing organellar translocation signals, may not be amenable to this recruitment. Furthermore, the nucleus may not be the appropriate organelle for the investigation of certain interactions (Mcalister-Henn *et al.,* 1999).

The use of artificially made fusion proteins is also potentially risky when attempting to identify protein-protein interactions. The fusion might alter the conformation of the bait and/or prey proteins and consequently may alter their functions and binding properties.

Although providing a Eucaryotic environment, the use of the yeast *Saccharomyces cerivisiae* as a host is another potential disadvantage for study of mammalian protein interactions. Some interactions between proteins depend upon post-translational modifications that either do not occur, or occur inappropriately, in yeast. Such modifications occur frequently and include the formation of disulphide bridges, glycosylation and phosphorylation (Walhout *et al.,* 2000). Furthermore, the yeast cells may not be able to correctly fold the constructed fusion protein as the necessary chaperones may be absent (Walhout *et al.,* 2000).

Yeast two-hybrid assays are also notorious for producing false positives, that is, indicating reporter gene activity where no direct bait-prey interaction has taken place. False positive interactions are frequently caused by the bait construct acting as an auto-activator of transcription. In the present study, the ability of the reeler bait construct to act as an auto activator of the reporter genes was evaluated. The results show that the reeler bait construct was unable to activate transcription of reporter genes autonomously. In order to minimise the number of false positives, the present study employed a system that utilises multiple reporter genes, each under slightly altered GAL4-responsive upstream activation sequences. Furthermore, heterologous mating experiments were performed in order to further weed out non-specific protein-protein interactions.

For the reasons of limitation noted above, protein-protein interactions detected using Y2H analysis should be viewed as hypotheses and need to be confirmed using other molecular biochemical tools. The present study made use of two such methods in order to validate the Y2H results, namely M2H analysis and co-immunoprecipitation.

4.2.2. Verification studies

As post-translational modification and protein folding may not occur appropriately in the Y2H system, the two putative interactions identified in the Y2H screen were investigated in a mammalian cell system by M2H analysis. Additionally, to assess the observed interactions between the reeler domain and the two putative reeler interactors in the absence of GAL4 transcription factor domains, *in vitro* co-immunoprecipitation was used.

The interaction between the reeler domain and WDR47, identified by Y2H, was validated by the M2H analysis (section 3.2.3), while no interaction was observed between the reeler domain and ATG16L2. Similarly, co-immunoprecipitation analysis confirmed the interaction between WDR47 and the reeler domain of reelin detected by Y2H analysis (section 3.2.2). The interaction between the reeler domain of reelin and ATG16L2 was not confirmed by co-immunoprecipitation analysis.

4.2.2.1. Limitations of verification studies

A limitation of the M2H system, as for Y2H, is the necessity for nuclear localisation of the interacting ligands in order to activate transcription of reporter genes, and thus proteins with strong targeting signals for particular cellular compartments may not assort to the nucleus. However, in the system used in this study, a nuclear localization signal precedes the GAL4 transcription factor domains, which are also N-terminal to the proteins of interest, and alleviating this potential problem. Additionally, the proteins studied by M2H did not assort to any particular subcellular compartment.

Another potential problem with M2H is that bait or prey fusion constructs autonomously activate transcription of reporter genes. For this reason, several control experiments were included to determine whether any of the constructs were auto-activators of transcription (section 2.19; Table 2.11). These experiments showed that

none of the constructs acted as auto activators of transcription; therefore, any increase in SEAP activity was due to a protein-protein interaction.

A limitation of *in vitro* co-immunoprecipitation systems like the one used in the present study is that the interactions of proteins detected may not represent true physiological interactions that occur *in vivo*, as in this system, proteins that would not normally have the opportunity to interact with each other because of their cellular localisations, may now do so. However, in this study, only proteins that occur in cellular compartments where reelin has been reported to occur, were tested. Another way to overcome this potential problem is to perform *in vivo* co-immunoprecipitation studies in appropriate cell lines. However, the ability to perform these types of analyses is dependent on the availability of either an appropriate cell line or a neuronal primary cell culture expressing reelin. Unfortunately, these options were not available in the laboratory at the time when this investigation was conducted and therefore, it was decided to use the *in vitro* method.

Furthermore, in co-immunoprecipitation studies, some of the peptides being analysed may bind the protein G agarose beads non-specifically and precipitate in this manner, rather than via their binding partner, thereby giving a false positive precipitation result. In the present investigation however, the protein G agarose beads were pre-washed multiple times in order to overcome this problem.

Thus, the results of this study provided compelling evidence for biologically relevant interactions between the reeler domain and WDR47.

4.2.3. Possible mechanisms of action of WDR47 in reelin-dependant neuronal migration

Since the function of the novel reelin-interacting protein, WDR47 has not yet been determined, one can only speculate on its potential roles in reelin-dependant neuronal migration; further investigations into the functions of this protein are warranted if we are to fully understand reelin-dependant neuronal migration. However, given our current state of knowledge, the following models for reelin signalling could be proposed: Certainly, as WDR47 is localised to the cytosol of the cell, they are only able to interact with cytosolic reelin. Therefore, it could be speculated that they could form part of the VLDLR/ApoER2 receptor pathway, as binding of reelin to VLDLR has been shown to result in the internalisation of reelin (D'Arcangelo *et al.,* 1999).

Reelin binds to VLDLR/ApoER3 receptors of the cell surface, which leads to the tyrosine phosphorylation of dab1, as well as to the internalisation of reelin. Cytosolic reelin could then bind the WD40-repeat domains of WDR47, via its reeler domain. WDR47, in turn, could be bound either to microtubules directly (Fig 4.3B) or to cytoplasmic dynein heavy chain (Fig 4.3A) via its LISH domain, thereby regulating microtubule dynamics in a reelin-dependent manner. As LIS1 is known to dimerise via its LISH domain, it is also possible that, in these interactions, WDR47 may be acting as a dimmer.

Comment [MB72]: The Structure of the N-Terminal Domain
of the Product of the Lissencephaly Gene *Lis1*
and Its Functional Implications
Myung Hee Kim,1 David R. Cooper,1 Arkadiusz Oleksy,2 Yancho Devedjiev,1 Urszula Derewenda,1 Orly Reiner,3 Jacek Otlewski,2 and Zygmunt S. Derewenda1,*
Structure, Vol. 12, 987–998, June, 2004

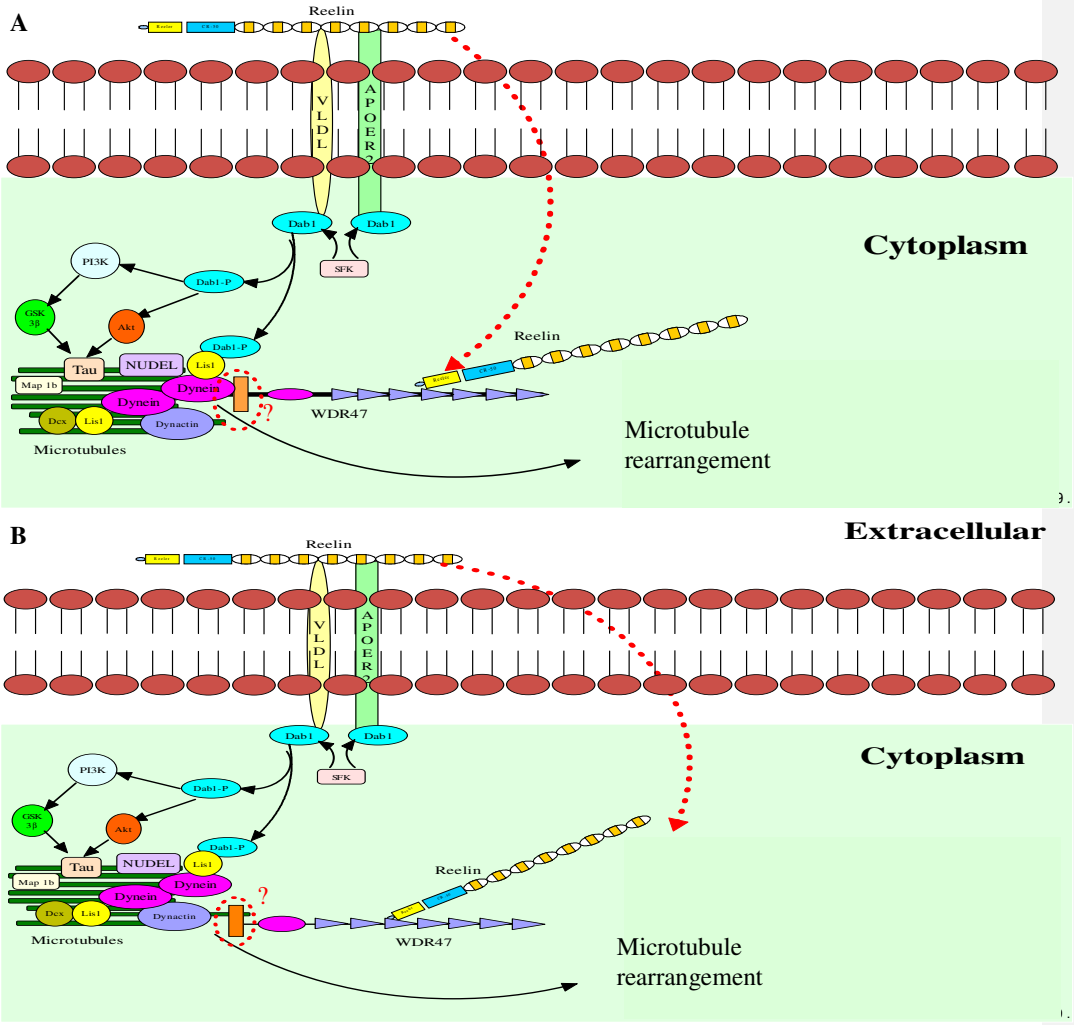


Fig 4.3. A schematic representation of the proposed mechanisms of action of WDR47 in the reelin signalling pathway. Reelin binds to the VLDLR and ApoER2 receptors on the cell surface and is internalised into the cell. Reelin then binds to the WD40-repeat region of WDR47 through its reeler domain. **A.** WDR47 binds dynein heavy chain via its LISH domain, thereby regulating microtubule dynamics in a reelin-dependent manner. **B**. WDR47 could also bind microtubules directly via its LISH domain and influence microtubule dynamics

4.3. CASE-CONTROL ASSOCIATION STUDIES.

In the present study, a total of 20 sequence variants in 14 candidate genes were examined in a panel of unrelated Afrikaner OCD patients and control individuals (Table 3.7). These genes derived from schizophrenia susceptibility loci were chosen for analysis based on the fact that: they were reported to be expressed in the brain, that they either are or show homology to genes encoding enzymes or receptors with roles in the neurotransmitter systems already implicated in OCD pathogenesis and/or are involved in brain development. The genes derived from the interaction study included both WDR47 and ATG16L2; although the latter was not shown to be a reeler domain interactor, it was included as a potential novel OCD candidate gene, due to the involvement of alternative splicing in fear conditioning (Najholt *et al*., 2004). As the pathogenesis of OCD still remains unresolved, with dysregulation of several neurotransmitter and neurodevelopmental systems implicated, it was decided not to focus on one specific neurotransmitter or neurodevelopmental system for the present investigation. Thus other than the criteria expounded above, the genes chosen for analysis have little else in common. Polymorphisms within the selected candidate genes previously targeted in psychiatric association studies were selected for investigation in the current analysis; alternatively, where no investigations of a candidate gene had been reported in the literature, target polymorphisms were selected from a publicly available single nucleotide polymorphism databases (http://www.ncbi.nlm.nih.gov/SNP; http://www.hapmap.org) based on a minor allele frequency of greater than 0.3 in a Caucasian population, to maximise likelihood for informativeness.

Logistic regression analysis of the genotypes, employed to assess the involvement of variants under more than one genetic model simultaneously and so reduce the number or statistical tests (Dr Lize van der Merwe, Biostatistician, MRC Biostatistics unit), generated statistical support to implicate two of the selected candidate genes, namely *DLX6* and *SYN3,* in the development of OCD.

4.3.1. *DLX6IVS1C>T* and *SYN3-631C>G* association with OCD

4.3.1.1. *DLX6IVS1>/T*

Logistic regression analysis revealed that the *CT* heterozygote genotype of the *DLX6IVS1C>T* polymorphism confers protection against the development of OCD, with the odds of developing OCD significantly reduced in heterozygous individuals (Table 3.24). In order to further investigate the observed association, additional OCD patients and control individuals were obtained, genotyped and a joint analysis (as per advice from Dr Lize van der Merwe, Biostatistician, MRC Biostatistics unit; Skol *et al*., 2006)of the original and new subjects performed. In this larger cohort the dominant protective effect of the *T* allele was once again observed (p=0.038, Table 3.26). To the author's knowledge, the present study represents the first report of association between OCD and *DLX6*.

The above-mentioned association prompted further investigation into the role of *DLX6* in OCD by analysis of two further polymorphisms (*rs127728* and *rs1004278*) within *DLX6*. No association was found between either of these two SNPs and OCD. Analysis of linkage disequilibrium between the three analysed *DLX6* markers

(*DLX6IVS1C>T, rs127728* and *rs1004278*) showed that these markers were not in LD with one another (Fig 3.23), which may explain why an association was observed with the original marker and not the later two.

Since *DLX6* is a key regulator of fore-brain development, it has been investigated as a potential autism susceptibility gene in an association study (Nabi *et al.,* 2003). Although the aforementioned study showed no significant association of *DLX6* with autism, it may be possible that it plays a role in the aetiology of a sub-set of autistic patients with increased obsessive-compulsive tendencies, as, in a study by Bolton and colleagues, OCD was found to occur more commonly in family members of autistic probands compared to the general population (Bolton *et al.,* 1998).

Whether the *DLX6IVS1C>T* intronic polymorphism has any functional significance is yet to be determined, hence explanations for the described association are currently speculative. One explanation is that the *DLX6IVS1C>T* polymorphism is in linkage disequilibrium with a functional polymorphism, either within *DLX6* itself or an adjacent gene that is associated with OCD pathogenesis. In fact, *DLX6* forms a bigene cluster with another *DLX* gene, *DLX5*, a gene that has also been shown to be involved in basal ganglia development (section 1.5.1.1.5) (Liu et al., 1997).

Another possibility is that, although this polymorphism resides within an intron, it may indeed be functional. Introns were traditionally considered "genetic waste" (Hill *et al.,* 2006); however, the recent discovery of intron-derived micro RNA (miRNA) completely changed that perception (Ying and Lin, 2006). Micro RNAs are ubiquitous, small, single stranded RNA, distinct from, but related to, small interfering RNA that regulate gene transcription by binding specific sequences within the 3' UTR of target mRNA to which it is significantly complementary (Sevignani *et al.,* 2006). The binding of the miRNA to mRNA results in either the target mRNA remaining untranslated, which reduces the levels of the corresponding protein, or the degradation of the bound mRNA, resulting in reduced transcript levels. Intronic miRNA is a sub-population of miRNAs that are derived from introns (Lin et al., 2003). It is therefore conceivable that polymorphisms within introns from which miRNA are produced could have an effect on the generation of these intronic miRNAs and thus influence levels of gene expression. To date, approximately 1000 miRNAs have been identified that have been catalogued in the miRNA database (http://microrna.sanger.ac.uk/sequences/) and this number is constantly growing. Whether an intronic miRNA derived from intron one of *DLX6* exists remains undetermined, however, if one does exist, it is feasible to consider that *DLX6IVS1C>T* may have a functional influence of the expression levels of *DLX6* or some other gene. It may also be possible that the region of *DLX6* in which *DLX6IVS1C>T* resides contains a recognition sequence for a miRNA.

To investigate this possibility, the *DLX6* intron 1 sequence was analysed for potential miRNA target sequences using the PicTar program (Krek *et al*., 2005). This analysis showed that no known miRNA target sequence resides within intron 1 of *DLX6* (data not shown).

An additional functional effect of an intronic polymorphism is that it may influence gene splicing by affecting a splice donor or acceptor site. In order to investigate this possibility, the intron 1 sequence was analysed using the NetGene2 internet-based neural network (http://www.cbs.dtu.dk/services/NetGene2/). Data from this analysis showed that this polymorphism had no effect on either donor or acceptor splice sites or that it created a cryptic splice site (data not shown).

4.3.1.2. *SYN3-631C>G*

In the present study, the *G*-allele of the *SYN3-631C>G* polymorphism was found to confer protection against OCD, with an additive effect (Table 3.24). This observed association was further investigated by genotyping additional OCD patients and controls and analysing the larger combined cohort, as for *DLX6IVS1C>T*. Using the larger cohort, the additive protective effect of the *G* allele was again observed (p=0.011, Table 3.26).

Again, to further investigate *SYN3* involvement in OCD, two more SNPs (*rs130753* and *rs130454*) within *SYN3* were genotyped. No association was observed between the two markers, *rs130753* and *rs130454*, and OCD. Linkage disequilibrium analysis of these two SNPs and *SYN3/-631C>G* showed no linkage disequilibrium between these SNPS (fig 3.24), which could explain why an association was observed with *SYN/-631C>G* but not any of the other SNPs tested.

SYN3 has been the focus of many association studies in schizophrenia (Ohmori *et al.,* 2000; Ohtsuki *et al.,* 2000; Imai *et* al 2001; Tsai *et al.,* 2002; Porton *et al.,* 2004; Lachman *et al.,* 2005; Lachman *et al.,* 2006). Three of these studies (Ohmori *et al.,* 2000; Ohtsuki *et al.,* 2000; Tsai *et al.,* 2002) specifically investigated the role of the *SYN3-631C>G* polymorphism in this disorder, but failed to show any statistically significant association. A statistically significant association has, however, been reported between schizophrenia and a rare S470N polymorphism in *SYN3* in a group of unrelated Caucasian schizophrenic patients and control individuals (Porton *et al.,* 2004). However, the level of LD between the *SYN3-631C>G* and S470N polymorphisms have not yet been evaluated.

The functional significance of the *SYN/-631C>G* polymorphism still remains undetermined. Since this polymorphism resides in the promoter region of the gene, it is tempting to speculate that it may disrupt binding of transcription factors, thereby altering *SYN3* gene expression. This possibility was investigated by Ohmori and co-workers who scanned the *SYN3* promoter sequence for known transcription factor recognition sites using the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html), which represents the largest repository of experimentally-derived transcription factor binding sites (Fogel *et al.,* 2005). Their analysis showed that *SYN3-631G>C* was not located in any known transcription factor binding site and thus these researchers concluded that it has no effect on gene expression (Ohmori *et al.,* 2000). However, care should be taken when interpreting data generated by transcription binding site recognition programs such as TRANSFAC, since they rely on pre-existing knowledge of experimentally determined recognition sequences

for known transcription factors. Therefore, one cannot rule out the possibility that *SYN3-631C>G* may disrupt a recognition sequence for an unidentified transcription factor.

Another explanation for the observed association between *SYN3* and OCD in the present study is that the *SYN3-631C>G* polymorphism is in linkage disequilibrium with a functional polymorphism, either within *SYN3* or an adjacent gene. Interestingly, the *SYN3-631C>G* polymorphism was found to be in almost complete linkage disequilibrium with another polymorphism, -196G>A, in the promoter region of *SYN3* in a Japanese schizophrenia cohort (Ohmori et al., 2000) and an Italian multiple sclerosis cohort (Liguori *et al.,* 2004). The A allele of the latter polymorphism results in a 6bp base match to the core recognition octamer sequence of the Oct-1 transcription factor (Lachman *et al.,* 2006). The level of linkage disequilibrium between these two polymorphisms in the Afrikaner population has not been determined, however, based on the aforementioned observations, one could speculate that the association of *SYN3-631C>G* polymorphism with OCD observed in the present study may reflect an association with the -196G>A polymorphism.

4.3.1.3. Analysis of epistatic interaction between SNPs associated with OCD development

Another interesting finding emerging from this study was the observed interactive effect of *DLX6IVS1C>T* and *SYN3-631C>G*, where individuals who carried at least one C allele of both variants were more susceptible to OCD than individuals who were *TT* and *GG* homozygotes at the *DLX6IVS1>T* and *SYN3 -631C>G* loci, respectively.

One possibility is that these two proteins may be involved in a common pathway or have similar functions. However when examining *DLX6* and *SYN3* using gene ontology tools (http://amigo.geneontology.org), it showed that while DLX6 functions as a transcription factor during neuronal development, SYN3 is involved in neurotransmitter release. Also, using SMART (http://smart.embl-heidelberg.de/) analysis revealed that these two proteins have no functional motifs in common.

Another other possible explanation for the observed interaction between the *DLX6 IVS1C>T* and *SYN3 -631C>G* polymorphisms would be that the transcription factor DLX6 is involved in regulating the transcription of *SYN3*. However, *in silico* analysis of the *SYN3* promoter region using the Gene2Promoter program (http://www.genomatix.de/products/Gene2Promoter/index.html) did not reveal any *DLX6* binding sites (data not shown). Therefore, the interactive effect between *SYN3* and *DLX6* remains unresolved and warrants further investigation.

4.3.2. Limitations of association studies

The *DLX6* and *SYN3* genes represent two attractive candidate genes for OCD development and the data presented in this investigation provides evidence suggesting proof of the proposed principle. Their vital roles in brain development and modulation of neurotransmitter release respectively, along with the previously reported association of *SYN3* with increased schizophrenia susceptibility further provides *a priori* support for their involvement in OCD.

One should, however, bear in mind that, although the data presented here is intriguing, case-control association studies by nature have several short-comings. Confounding factors such as population stratification, phenotypic heterogeneity, number of markers tested and statistical power and sample size, have limited the success of association studies in general. The relevance of these pitfalls in the present study will be discussed in the sections that follow.

4.3.2.1. Population stratification

Difficulties in confirming positive or negative associations detected between a specific candidate gene and a disease, in different studies, are often ascribed to underlying genetic differences between populations. Our current understanding of complex disorders, such as OCD and schizophrenia, is that several mechanisms (genetic and environmental) may play a role in the presentation of disease phenotype in different individuals (Zohar et al., 1987; Stein 2000). Taking into account the genetic and environmental differences among population groups, it is possible that different combinations of environmental and genetic mechanisms could contribute to disease pathogenesis in each population. Furthermore, in all major population groups, there seems to be some degree of cryptic population substructure, which generally follows ethnic lines (Ziv and Burchard, 2003). Therefore, if the population used in the association study comprises cryptic subpopulations in which allele frequencies for the chosen candidate gene and disease risk differ, it may result in spurious association between the genetic variant and the disease under investigation. The reason for this is that any allele that occurs at a higher frequency in the subpopulation with greater disease risk will be associated with the disease. Similarly, population stratification may also result in a Type II error (not finding an association when one does exist) if the disease is more prevalent amongst the subpopulation with the lower allele frequency.

To circumvent this problem OCD, patients and control individuals of Afrikaner descent were recruited. These study subjects were classified as being Afrikaner if at least three of their grandparents were of Afrikaner descent; however, this may have introduced genetic admixture because of the lack of rigour regarding the ethnicity of the fourth grandparent. This raises the question of whether the study population was sufficiently genetically homogeneous. This question was answered in a concurrent collaborative study, utilising the same cohort as the present investigation (Appendix IX) (Hemmings, 2005; Hemmings *et al.,* 2007). In that study, genotypes from 23 unlinked autosomal polymorphisms, including some genotypes generated in the present study, were analysed using the *Structure* (version 2) program to detect population substructure. The investigation revealed no evidence for a cryptic sub-population in the Afrikaner OCD or control cohorts (Hemmings, 2005; Hemmings *et al.*, 2007), which suggests that the cases and controls used in the present study were genetically matched and that the fourth grandparent did not confound the data (Hemming *et al*., 2007).

4.3.2.2 Phenotypic resolution

The current understanding of OCD is that it is a clinically heterogeneous disorder and that a whole host of environmental and neurobiological factors influence the expression of the phenotype. Indeed, several OCD

subtypes have been identified recently (Leckman *et al.,* 2003; Ball *et al.,* 1996; Mataix-Cols *et al.,* 1999), and it is possible that each subtype may be mediated by different neurobiological, environmental and genetic factors (Miguel *et al.,* 2005). Thus, these OCD subtypes may represent specific phenotypes that are more closely related to a particular genetic mechanism than the higher order construct of OCD (Hemmings et al., 2004). Therefore, classifying OCD patients into clinically defined subtypes in genetic association studies increases the power to detect small effect sizes, as well as reducing the background "noise" created by using a clinically heterogeneous sample. However, even though several genetic association analyses make use of clinically define sub-types of OCD, it should be noted that the formal, categorical OCD diagnosis may still give an indication as to which genes contribute to the overall pathology of OCD.

In the present study, the OCD patient sample was not sub-stratified into clinically defined subtypes because of the relatively small sample size; sub-stratifying the OCD patient sample into various OCD subtypes could have drastically reduced the study power. Moreover, given that the original hypothesis was based on the clinical overlap between OCD and schizophrenia and since there is no evidence to suggest that any particular OCD subtype occurs more frequently than others in schizophrenia affected individuals, there was no rationale to select any particular OCD subtype. Hence, the objective here was only to identify genes involved in general OCD pathology.

4.3.2.3. Statistical power and sample size

Statistical power and sample size are other confounding factors that could account for the inconsistencies between association studies of specific candidate genes and complex diseases. The sample size required to reach statistical significance in genetic case-control association studies is dependent on a number of interacting factors. These include i) the power of the study (ie., the expression of the ability of the study to detect a true effect if one exists and the ability to differentiate between a significant negative association and an inconclusive result) and ii) the specific effect size (ie. the contribution to disease pathogenesis made by the specific allelic variation under investigation) (Berry *et al.,* 1998).

It has been proposed that as a convention, when an investigator has no other basis for choosing the desired power of the study, a value of 80% be used, which represents the ability to detect a 20% difference between two values. (Berry *et al.,* 1998). It is also common practice that the level of significance (ie., the p-value) be set at 0.05, that is with a 95% confidence level. The association between the *DLX6IVS>T* polymorphism and OCD reported was achieved using a cohort of 42 OCD patients and 81 control individuals (Table 3.7). In order for the investigation to have reached the above-mentioned criteria for power, and given the allele frequencies in our population, 239 OCD cases and 497 control individuals should have been analysed (calculated using the "*Power for Association with Error*" program- http://linkage.rockefeller.edu/pawe). Similar analysis performed using the *SYN3* data reported revealed that 175 OCD cases and 350 control individuals should have been analysed to reach 80% power. Therefore, the sample sizes used in the analyses of the *DLX6 and SYN3* polymorphisms, even when enlarged (DLX6: 89 OCD, 135 controls; SYN3:127 OCD, 142 controls) were too small to detect an association at the 95% confidence level and thus may have led to a Type I error (the

probability that the test statistic indicates an association with the disease, when in fact none exists). It is therefore important to re-evaluate the findings of the *DLX6IVS1C>T* and *SYN3/-631C>G* polymorphisms association studies in a follow-up study employing a second, larger group of OCD patients and control individuals of Afrikaner descent. Replication of the association would confirm the involvement of *DLX6* and *SYN3* in OCD pathogenesis in the Afrikaner population.

Likewise, the analyses in which candidate loci were found to have no statistically significant association with OCD may also have been the result of analysing insufficient samples which did not allow the detection of a significant association (Type II error). One is therefore unable to exclude these markers as playing a role in OCD and these results should be viewed as inconclusive. The data, therefore, warrant follow-up studies using larger sample sizes to determine whether these markers can conclusively be excluded as playing a role in OCD pathogenesis.

The small sample size employed in the present study can be attributed to a number of factors. One major contributing factor is the problem of recruiting OCD patients from the community. Patients often feel ashamed of their condition because of the stigma placed on psychiatric disorders and therefore do not seek the appropriate help. This significantly reduces the number of patients willing to participate in association studies.

Another factor limiting the number of samples analysed in the present study is the lack of a renewable source of DNA. When studies are conducted over a period of several years, it is important that a renewable source of patient and control DNA (such as cell lines) is available, especially, as is the case in the present study, when several concurrent investigations using the same sample are being performed. Unfortunately, due to financial constraints, no renewable DNA sources were available for many of the patients, which greatly reduced the amount of good quality DNA. This accounts for the discrepancy in the number of samples genotyped for each of the markers tested.

One way to overcome small sample sizes in genetic association studies is to use meta-analyses, a strategy which applies a set of statistical procedures designed to accumulate experimental and correlational results across independent studies that address a related set of research questions. Thus, in genetic case-control association studies, meta-analyses provide one with the means to increase the study power. In the present study, however, performing meta-analyses was not possible since the markers investigated have been assessed in OCD for the first time in the present study. However, when these results are published, it may encourage other investigators to attempt to replicate them, which may, in turn, make meta-analyses feasible.

4.3.2.4. Number of markers tested

In the present study, the classic case-control analysis comparing allele frequency and genotype distribution differences of a single SNP between case and control groups was employed. Over the last five years, the manner in which genetic association studies are performed has changed exponentially, as the generation of the HAPMAP and similar datasets lead to greater understanding of LD structure and its influence on association

studies. The norm in the early 2000's was to approach association studies by means of single SNPs. However, single SNP-based approaches have their limitations. Firstly, a single SNP generates relatively low information content and, secondly, for a gene with multiple tightly linked SNPs, linkage disequilibrium information contained in flanking markers will be ignored (Niu *et al.,* 2002). This situation is exacerbated in complex disorders such as OCD, where it is currently proposed that several genes act in unison with unknown environmental factors to predispose to disease. The single SNP analysis approach with multiple candidate genes and SNPs may require tens of thousands of samples to detect significant associations (Lee *et al.,* 2005).

The understanding of the need for greater coverage of a gene or genetic region in genetic association studies which flowed from the HAPMAP project lead to other approaches. Once such method involves using haplotype analysis, which combines the information of multiple adjacent SNPs evenly spread across the gene of interest. Not only are haplotypes more informative, but they also capture the regional LD information. These methods have been shown to be much more powerful and robust to detect associations in the study of complex disorders (Akey *et al.,* 2001, Daly *et al.,* 2001; Prichard *et al.,* 2001).

More recently, the use of tagSNPS for association studies have become popular. TagSNPs are SNPs that are in strong LD with other SNPs, therefore genotyping a tagSNP will provide the researcher with information regarding all other SNPs in LD with it. Depending on the extent of LD in the particular genetic region of interest, using tagSNPs may reduce the number of SNPs that would otherwise be typed using SNPs evenly spread across the region (i.e. selected by SNP density approaches). However, in regions with little LD, the number of SNPs, and with it, genotyping costs, remain large.

The more frequent use of tagSNPs in association studies in recent years has certainly been stimulated by the availability of data generated by the international HAPMAP project. This project, launched in October 2002 and first released in 2006, is a multi-country collaborative effort to identify patterns of LD in human population (www.HAPMAP.org). The HAPMAP database provides researchers with information regarding LD patterns across the genomes of four different population groups (Central European from Utah [CEU], Yoruba from Ibadan, Han Chinese and Japanese from Tokyo) and the opportunity to identify tagSNPs, and the SNPs "tagged" by them.

Several studies have, however, shown that patterns of LD vary between populations and ethnic groups (Abecasis *et al*., 2001, Zavattari *et al*., 2000). Therefore it remains controversial whether the data of the four populations sampled by the HAPMAP project is transferable to other populations or whether it would be necessary to construct specific haplotype maps for each of the world's population groups. However, some very recent studies have suggested the transferability of HAPMAP tagSNPS between populations, but a bias towards SNPs with a high minor allele frequency exists.(Ribas *et al*., 2006). However, in other populations, transferability of tagSNPs may not be as clearcut. At the time of the study, and indeed to date, no data on the transferability of the HAPMAP data and tagSNPs to the Afrikaner population was available; for this reason, and for reasons of cost, the original approach of first using single SNPs was not altered to involve the use of

Comment [MB73]: 1: Genet Epidemiol. 2007 Apr;31(3):189-94. FULL TEXT AVAILABLE ONLINE InterScience

Links

Tag SNPs chosen from HapMap perform well in several population isolates.

Service S; International

Comment [MB74]: Genet Epidemiol. 2006 Feb;30(2):180-90. FULL TEXT AVAILABLE ONLINE InterScience

Links

Tag SNP selection for Finnish individuals based on the CEPH Utah HapMap database.

Willer CJ, Scott LJ, Bonnycastle LL, Jackson AU, Chines P, Pruim R, Bark CW, Tsai YY, Pugh EW, Doheny KF, Kinnunen L, Mohlke KL, Valle TT, Bergman RN, Tuomilehto J, Collins FS, Boehnke M.

Comment [MB75]: Ann Hum Genet. 2007 Jul 12; [Epub ahead of print] Full Text at Blackwell Synergy Links

Portability of Tag SNPs Across Isolated Population Groups: An Example from India.

Roy NS, Farheen S, Roy N, Sengupta S, Majumder PP.

HAPMAP tagSNPs or the identification of population-specific SNPs by resequencing. For interest though, the number of tagSNPs, with r^2 of >0.8, available for each of the genes investigated is given in Table 4.1. This table also highlights another problem with using HAPMAP data, viz. genetic variation is not equally covered in all genes under the current HAPMAP data; however, this situation will no doubt improve as additional similar datasets become available.

Table 4.1. **Number of tagSNPs with r^2 threshold of 0.8 and minor allele frequency≥0.2 in the CEU population of the HAPMAP project, identified in July 2007 by the Tagger algorithm in each of the genotyped genes in the present study**

Gene	Number of TagSNPs
SNAP25	19
SNAP29	3
GRIA4	24
GRIN1	1
DLX6	0
BZRP	6
DBH	13
SYN3	98
GBR1	0
RXRβ	3
CHRM3	46
SLC18A1	11
RELN	104
GRID1	79
WDR47	4
ATG16L2	0
POU3F2	0

4.4 FUTURE DIRECTIONS

Novel and very exciting data has been presented that warrants further investigation in order to fully appreciate the significance of these findings in the context of the pathophysiologies of OCD and schizophrenia. The identification of the novel reelin-interacting protein, WDR47 has created an opportunity to further augment our understanding of the reelin signalling pathway. However, defining their role in reelin-dependant neuronal migration is dependent on knowledge of their own particular functions, which are yet to be determined. Therefore, in future it is planned that Y2H screens of foetal brain cDNA libraries will be conducted using WDR47 the bait. The identification of ligands of WDR47, other than reelin, may elucidate their functions and thus clarify their roles in the overall architecture of the reelin-dependant neuronal migration pathway and brain development.

The observed associations of *DLX6* and *SYN3* with OCD will have to be confirmed by replication in an independent study cohort. The present investigation forms part of a larger, ongoing collaborative research endeavour to unravel the intricate pathophysiology OCD in the relatively genetically homogeneous Afrikaner population. Therefore, new OCD patients and controls are constantly being recruited for this larger study which will provide the independent sample for a replication study. Future studies of *DLX6* and *SYN3I* will also have to be extended to include the screening of additional, preferably tag-SNPS across both genes in order to capture as much genotypic information within these genes as possible and dramatically increase the power of the case-control association studies using the single marker approach (Akey *et al.,* 2001, Daly *et al.,* 2001; Prichard *et al.,* 2001). These types of analyses could also be extended to the selected candidate genes analysed in the present investigation in which no association was detected. Also the observed interactive effect observed between DLX6 and SYN3 in the development of OCD needs to be further investigated in order to fully appreciate the significance of the findings of this study.

4.5. CONCLUSION

This investigations had two major outcomes that may contribute to our understanding of the pathophysiologies of OCD and schizophrenia: the novel associations of *SYN3* and *DLX6* with OCD and the identification of a novel reelin-interacting protein, WDR47. The associations of *SYN3* and *DLX6* with OCD are rather exciting as they may point to mechanisms involved in OCD that have not been investigated as yet. The identification of WDR47 as a novel reelin-interacting protein has significant implications to our understanding of reelin-dependant signalling. This investigation is the first to report interaction of reelin with cytosolic proteins and, therefore, may lead to the identification of novel roles for reelin in neurodevelopment. These novel components of the reelin signalling pathway may, based on our initial hypothesis, also be considered OCD and schizophrenia candidate genes. This may, in turn, augment the existing knowledge of the pathophysiologies of OCD, schizophrenia and other neurodevelopmental disorders.

The identification of genetic susceptibility factors to psychiatric disorders such as OCD and schizophrenia remains a daunting task. Nonetheless, the recent advances in technologies and statistical methodologies have provided researchers with valuable tools and novel approaches to identify and assess potential candidate genes for these disorders. With the availability of resources such as gene and protein micro-arrays, the increasing amount of information on the human genome sequence and whole genome association analysis, much of the "guess-work" in identifying potential candidate genes for the psychiatric disorders may be a thing of the past. These technologies, however, may not currently be readily available in most laboratories therefore, many researchers will have to, for now, develop innovative approaches to identify candidate genes for psychiatric disorders.

The present investigation is a good example of how unconventional approaches can be used to identify plausible candidate genes for a complex disorder, such as OCD. By utilising existing knowledge of schizophrenia, a disorder that has been shown to have phenomenological overlap with OCD, the search for novel OCD candidate genes was extended to genetic loci implicated in schizophrenia. The fact that two genes,

DLX6 and *SYN3*, located in schizophrenia susceptibility loci were found to be associated with OCD shows that this is method is valid. In fact, genetic investigation of bipolar disorder, another disorder that has been shown to share pathological mechanisms with schizophrenia, have identified genes such as *COMT, G70/G30* and *BDNF* (reviewed by Maier *et al.,* 2005), that have been associated with both disorders. Therefore, the use of clinically and pathologically overlapping disorders is an tactic that should be explored further and could be utilised more frequently to identify novel candidate genes for many complex disorders.

Furthermore, one does not often consider the Y2H system as an option when designing research strategies to identify novel candidate genes for complex disorders. However, in the present investigation, this method was applied to identify novel OCD candidate genes based on an animal model of schizophrenia. Although preliminary data suggests that genes identified using this method do not play a major role in the aetiology of OCD, the Y2H system has previously been successfully used to identify schizophrenia candidate genes. In their investigation of the schizophrenia-linked genomic region on chromosome 13 (13q34), Chumakov an co-workers identified *G72* as a potential schizophrenia susceptibility gene (Chumakov et al., 2002). Subsequently, these researches used G72 as bait in a Y2H screen in order to elucidate its function and isolated DAAO as a ligand. The gene encoding DAAO was later shown to also be associated with schizophrenia (Chumakov et al., 2002) (section 1.4.8.1). In another study, Jeanclos and co-workers aimed to identify the cytosolic ligand(s) of the large cytoplasmic domain of α2β4 AChR, a receptor whose expression has been shown to be abnormal in the brains of schizophrenic patients, using Y2H. These researchers isolated protein 14-3-3ή (YWHAH) (Jeanclos *et al.,* 2001), which has previously been shown to be associated with schizophrenia susceptibility in a Japanese population (Toyooka *et al.,* 1999). Thus the approach is validated, and WDR47 may yet be proven to be a schizophrenia susceptibility gene, an angle that was not explored in the current investigation.

The data presented in the present study yielded exciting results that warrant future follow up investigation. Ultimately, the goal of studies, like this one, is to get a clearer handle on the aetiologies of complex psychiatric disorders, such as OCD and schizophrenia. The identification of *DLX6* and *SYN3* as novel OCD susceptibility genes, as well as the identification of WDR47 as a reelin-interacting protein, may provide investigators with alternative avenues of research into potential drug targets for OCD and schizophrenia pharmacotherapy.

APPENDIX I

1 DNA EXTRACTION SOLUTIONS

CELL LYSIS BUFFER

Sucrose	0.32M
Triton-X-100	1%
$MgCl_2$	5mM
Tris-HCl	10mM
H_2O	1l

3M NaAc

$NaAc.3H_2O$ (Merck)	40.18g
ddH_2O	50ml

Adjust pH to 5.2 with glacial acetic acid and adjust volume to 100ml with ddH_2O

DNA EXTRACTION BUFFER

NaCl	0.1M
Tris-HCl	0.01M
EDTA (pH8)	0.025M
SDS	0.5%
Proteinase K	0.1mg/ml

T.B.E-BUFFER (10x stock)

Tris-HCl	0.89M
Boric Acid	0.89M
Na_2EDTA (pH8)	20Mm

2. BACTERIAL PLASMID PURIFICATION SOLUTIONS

CELL RESUSPENSION SOLUTION

50mM Tris-HCL, pH 7.5	2.5ml 1M Tris
10mM EDTA	1ml 0.5M EDTA

Make up to 50 ml with H2O

CELL LYSIS SOLUTION

0.2M NaOH	2.5ml 4M NaOH
1% SDS	5ml

Make up to 50ml with H2O

NEUTRALISATION SOLUTION

1.32M KOAc, pH 4.8	13.2ml 5M KOAc

Make up to 50ml with H2O

3. YEAST PLASMID PURIFICATION SOLUTIONS

YEAST LYSIS BUFFER

SDS	1%
Triton X-100	2%
NaCl	100mM
Tris (pH8)	10mM
EDTA (pH8)	1mM

4. ELECTROPHORESIS SOLUTIONS

10% AMMONIUM PERSULPHATE (APS)

APS	10g
ddH_2O	100ml

T.B.E-BUFFER (10x stock)

Tris-HCl	0.89M
Boric Acid	0.89M
Na_2EDTA (pH8)	20mM

T.A.E BUFFER

Tris-HCl	0.89M
Boric Acid	0.89M
Na_2EDTA (pH8)	

SDS-PAGE RESOLVING GEL BUFFER (4X)

Tris base	109.2g
ddH_2O	330ml
10%SDS	24ml
pH to 8.8 using 1N HCL	
Make up to 600ml using ddH_2O	

SDS-PAGE STACKING GEL BUFFER (4X)

Tris base	36.3g
ddH_2O	330ml
10%SDS	24ml
pH to 6.8 using 1N HCL	
Make up to 600ml using ddH_2O	

SDS-PAGE RUNNING BUFFER (10X)

Tris base	30g
Glycine	144g
10%SDS	100ml
Add ddH_2O to a final volume of 1L	

5 GELS

12% POLYACRYLAMIDE GEL

30 acrylamide/0.8% bis-acrylamide stock	4ml
10xTBE	1ml
Distilled H_2O	5ml
APS	80µl
TEMED	30µl

10% MILDLY DENATURING POLYACRYLAMIDE GEL WITH 5% GLYCEROL

30 acrylamide/0.8% bis-acrylamide stock	40.5ml
10xTBE	8ml
Urea	24g
Distilled H_2O	84ml
APS	1000µl
TEMED	160µl

6. LOADING DYES

BROMOPHENOL BLUE

Bromophenol blue	0.2%(w/v)
Glycerol	50%
Tris (pH8)	10mM

SSCP LOADING DYE

Formamide	95%
EDTA	20mM
Bromophenol blue	0.01%
Xylene cyanol	0.05g

SDS LOADING DYE

1M Tris-HCl (pH6.8)	50Mm
DTT	100m
SDS	2%
Bromophenol blue	0.1%
glycerol	10%

6. MOLECULAR SIZE MARKER (LAMBDA *Pst*I)

Bacteriophage Lambda DNA (250µg)	100µl
Buffer M (Boerhinger Mannhein)	15µl
*Pst*I (Boerhinger Mannheim)	11µl
H_2O	32µl

Incubate at 37°C for 2 hours followed by heat inactivation at 65°C for 5 min. Load 2µl onto polyacrylamide gels.

7 SOLUTIONS FOR SILVER STAINING

0.1% $AgNO_3$ (Solution B)

$AgNO_3$	1g
H_2O	1L

DEVELOPING SOLUTION (Solution C)

NaOH	15g
$NaBH_4$	0.1g
Formaldehyde	4ml
H_2O	

8. PCR BUFFER

10x NH_4 PCR BUFFER (BIOLINE U.K.)

Ammonium sulphate	160mM
Tris-HCl (pH 8.8)	670mM
Tween-20	0.1%

9 CO-IMMUNOPRECIPITATION BUFFER

1M Tris base (pH-7.5)	400µl
5M NaCl	60µl
1M DTT	20µl
2ng/ml Aprotinin	50µl
50mM PMSF	200µl
Tween 20	20µl

Make up to 20 ml using ddH_2O

10. SOLUTIONS USED FOR THE ESTABLISHMENT OF BACTERIAL COMPETENT CELLS

CAP BUFFER:

60 mM $CaCl_2$	2.21 g
15% glycerol	37.5ml
10mM PIPES	0.76 g

Make up to 250ml with sterile millipore H_2O. pH to 7.0. Keep in fridge.

11. BACTERIAL MEDIA

LURIA-BERTANI (LB) MEDIA

Bacto tryptone	5g
Yeasy extract	2.5g
NaCl	5g

ddH_2O to final volume of 500ml
Autoclave at 121°C for 20min and add appropriate antibiotic (ampicillin, 25mg/ml; Kan 5mg/ml) to media when a temperature of >55°C is reached.

LB AGAR PLATES

Bacto tryptone	5g
Yeasy extract	2.5g
NaCl	5g
Agar	8g

ddH_2O to final volume of 500ml
Autoclave at 121°C for 20min and add appropriate antibiotic (ampicillin, 25mg/ml; Kan 5mg/ml) to media when a temperature of >55°C is reached, prior to pouring plates.

12 YEAST MEDIA

YPDA MEDIA

Difco peptone	10g
Yeast extract	10g
Glucose	10g
L-adenine hemisulphate (0.2% stock)	7.5ml

Autoclave at for 121°C 15min

YPDA AGAR

Difco peptone	10g
Yeast extract	5g
Glucose	10g
Bacto agar	10g
L-adenine hemisulphate (0.2% stock)	7.5ml

Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

SD^{-W} MEDIA

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-w} amino acid supplement	0.4g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min.

SD^{-W} PLATES

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-w} amino acid supplement	0.4g
Bacto agar	12g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

SD^{-L} MEDIA

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L} amino acid supplement	0.4g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min.

SD^{-L} PLATES

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L} amino acid supplement	0.4g
Bacto agar	12g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

SD^{-L-W} MEDIA

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L-W} amino acid supplement	0.4g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min.

SD^{-L-W} PLATES

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L-W} amino acid supplement	0.4g
Bacto agar	12g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

TDO MEDIA

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L-W-H} amino acid supplement	0.4g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min.

TDO PLATES

Glucose	12g
Yeast nitrogen base without amino acids	4g
SD^{-L-W-H} amino acid supplement	0.4g
Bacto agar	12g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

QDO MEDIA

Glucose	12g
Yeast nitrogen base without amino acids	4g
$SD^{-L-W-H-Ade}$ amino acid supplement	0.4g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min.

QDO PLATES

Glucose	12g
Yeast nitrogen base without amino acids	4g
$SD^{-L-W-H-Ade}$ amino acid supplement	0.4g
Bacto agar	12g
0.2% adenine hemisulphate	9ml

ddH_2O to a final volume of 600ml
Autoclave at 121°C for 15min and allow to cool to approximately 55°C prior to pouring plates.

13. EUKARYOTIC CELL CULTURE MEDIA

COMPLETE GROWTH MEDIA

DMEM	90ml
Hams F12	90ml
Foetal calf serum	20ml
Penstrep	

SERUM-FREE MEDIA

DMEM	100ml
Hams F12	100ml

APPENDIX II

CALCULATING YEAST MATING EFFICIENCIES

Count number of colonies on all plates with 30-300 colonies after 4 days

$$\#\text{colony forming units (cfu)/ml} = \frac{\text{cfu} \times 1000\mu\text{l/ml}}{\text{volume plated } (\mu\text{l}) \times \text{dilution factor}}$$

1. Number of cfu/ml on SD^{-L} plates = viability of prey partner
2. Number of cfu/ml on SD^{-W} plates = viability of bait partner
3. Number of cfu/ml on SD^{-L-W} plates = viability of diploids
4. Lowest Number of cfu/ml of SD^{-L} or SD^{-W} plates indicate limiting partner

5. $$\text{Mating efficiency} = \frac{\#\text{cfu/ml of diploids} \times 100}{\#\text{cfu/ml of limiting partner}}$$

Library titre

Count number of colonies on all plates with 30-300 colonies after 4 days

$$\#\text{cfu/ml} = \frac{\#\text{colonies}}{\text{plating volume(ml)} \times \text{dilution factor}}$$

colonies clones screened= # cfu/ml x final resuspension volume

APPENDIX III

LIST OF SUPPLIERS

*Aci*I	New England Biolabs
Acrylamide	Merck
Adenie hemisulphate	Bio101
Agar	Merck
Agarose	Whitehead Scientific
$AgNO_3$	Merck
Ammonium persulphate	Merck
Ampicillin	Roche
Aptotinin	Roche
Autoradiography film	Kodak
*Bam*HI	Promega
*Ban*II	Promega
β-galactose	Southern Cross
*Bse*RI	New England Biolabs
*Bsr*I	New England Biolabs
Bis-acrylamide	Merck
Boric acid	Merck
Bromophenol blue	Merck
Calf intestinal alkaline phosphatase	Promega
Chloroform/octanol	Sigma
dATP	Boerhinger Mannheim
dCTP	Boerhinger Mannheim
*Dde*I	Promega
dGTP	Boerhinger Mannheim
DMEM	Highveld biological
dNTP mix	TaKaRa
DTT	Roche
dTTP	Boerhinger Mannheim
*Eco*RI	Promega
*EcoR*V	Promega
*Ear*I	Promega
EDTA	Boerhinger Mannheim
Ethanol	Boerhinger Mannheim
Ex Taq™ polymerase	TaKaRa
Ex Taq™ polymerase Mg^{2+}-containing reaction buffer	TaKaRa
FACS Lysing Solution	Beckton Dickenson
Foetal calf serum	Delta Bioproducts
Formamide	Merck
Formaldeyde	Merck
Gelbond	Merck
GFX® DNA purification kit	Amersham Pharmacia
Glucose	Kimix
Glycerol	FMC Promega
Great EScAPe™ chemiluminescence detection kit	BD Biosciences
HA monoclonal antibody	Roche
*Hae*III	Promega
Ham's H12	Highveld Biological
Herring sperm DNA	Promega
*Hsp*92II	Promega
Isopropanol	Merck
K-acetate	Sigma
Kanamycin	Roche
KCl	Merck

Lambda DNA	Promega
LiAc	Sigma
Matchmaker™ Mammalian Assay Kit 2	BD Biosciences
Matchmaker™ Two-hybrid system 3	BD Biosciences
Mineral oil	BDH Chemicals
*Mnl*I	Fermentas
$MgCl_2$	Bioline
Myc monoclonal antibody	Roche
NaAc	Merck
NaCl	BDH Chemicals
$Na_2HPO_4.7H_2O$	Merck
$Na_2HPO_4.H_2O$	Merck
NaOH	Sigma
*Nde*I	Promega
*Nla*III	New England Biolabs
*Nru*I	Promega
Oligonucleotide primers	Department of Molecular and Cell Biology, University of Cape Town (UCT), Cape Town, South Africa.
pACT2	BD Biosciences
PBS	Sigma
PEG4000	Merck
Penicillin/streptomycin	Highveld Biological
Peptone	Difco
pG5SEAP	BD Biosciences
pGBKT7	BD Biosciences
pM	BD Biosciences
PMSF	Roche
Phenol	Merck
Phenol/choloform	Sigma
Phenol/choroform/isoamyl	Sigma
Proteinase K	Sigma
pVP16	BD Biosciences
QDO	BD Biosciences
Qiagen Kit	Stratagene
RNAse wipes	Ambion
SDS	Sigma
SD^{-L}	BD Biosciences
SD^{-w}	BD Biosciences
SD^{-L-w}	BD Biosciences
T4 Ligase	Promega
Taq polymerase	Bioline
TDO	BD Biosciences
TEMED	Sigma
TNT® Quick Coupled transcription/translation system	BD Biosciences
Tris	Merck
Tris-OH	Merck
Tris-HCl	Merck
Trypsin	Highveld Biological
Tryptone	Fluka
Urea	BDH Chemicals
Whatman 3M paper	Whatman international
Wizard® Purefection plasmid purification kit	BD Bioscences
X-α-galactsoe	Southern Cross
Yeast extract	Difco
Yeast nitrogen base (without amino acids)	BD Biosciences

APPENDIX IV

BACTERIAL STRAIN PHENOTYPE

E.coli strain DH5α

Φ 80d *lac*ZΔ*M15* recA1, *end*A1, *Gry* A96 *thi*-1, *hsd*R17 *sup*E44, *rel*A1, *deoR* Δ(*lac*ZYA *argF*)u169

YEAST STRAIN PHENOTYPEs

Yeast strain AH109

*MAT*a, *trp1-901*, *leu2-3, ura3-5, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1*$_{uas}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ* (James *et al.,* 1996)

Yeast strain Y187

*MAT*α*, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ, *met*$^{-}$, *gal80*Δ, *URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ* (Harper *et al.,* 1993)

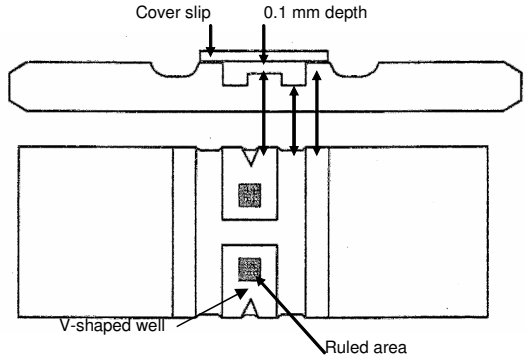
APPENDIX V:

Heamocytrometric cell count protocol

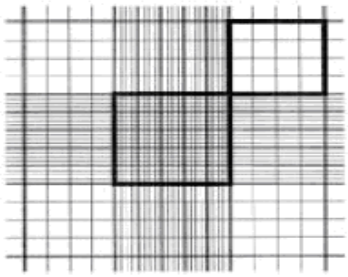
Haemocytometric cell count using a Neubauer haemocytometer (Superior, Berlin, Germany) was performed to determine the titre of bait culture used in the library mating experiment. Prior to aliquoting the sample onto the haemocytometer, a glass coverslip was placed over the counting surface (Fig 2.7). Approximately 50µl of a 1 in 10 dilution of bait culture was then pippeted into one of the V-shaped wells (Fig A). This allowed for the area under the coverslip to be filled with the sample through capillary action. The counting chamber was subsequently placed on a microscope (Nikon TMS, Nikon Instruments inc., New York U.S.A) stage and the counting area was brought into focus under low magnification. The organisation of the counting area is shown in Figure B and Figure C. The number of cells per millilitre was determined using the following formula:

number of cells/ml = number of cells x dilution factor x 10^4 (a constant used because the depth of the haemocytometer is 0.1mm)

A



B



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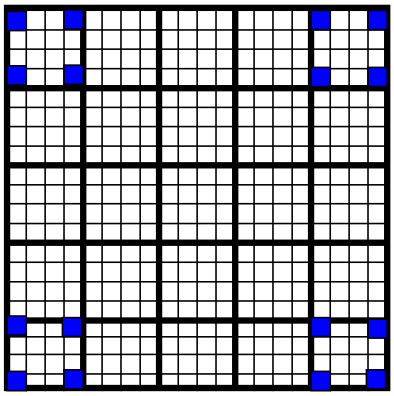


Neubauer heamocytometer, side and top view. The central platforms contain the ruled counting areas and are 0.1 mm under the cover slip, which is suspended on the raised ridges (taken from McNeel and Brown, 1992). b) **Magnified view of the ruled counting area** (taken from McNeel and Brown, 1992).. c) **View of the central quadrant of the haemocyometer that was used to determine the number of cells per milliliter.** The number of cells per milliliter was determined as follows: The number of cells in each of the blue squares within the 4 outer larger squares (in the diagram) were counted and added together. This amount was multiplied by 4 to give an approximate number of cells each of the 4 large outer squares. The amount of cells in each of the 4 larger outer squares was then added together and divided by 4 to give an average number of cells for each of the 25 large squares of the central quadrant of the haemocytometer. This average number was then multiplied by 25 to yield a average number of cells within the large central quadrant. Number of cells per milliliter was then determined using the formula:

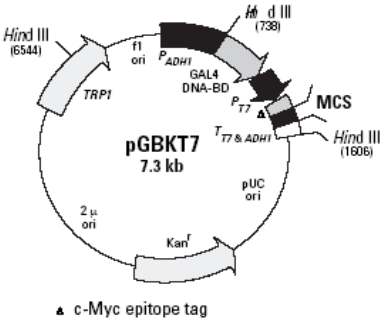
Cells/ml=number of cells x dilution factor x 10^4

Comment [IT76]: McNeely J. C. and Brown D. 1992. Laboratory evaluation of leukocytes. (In Stiene-Martin, E. A., Lotspeich-Steininger, C.A., and Koepke, J. A. (eds.) *Clinical Hematology: Principles, Procedures, Correlations*. 2nd Edition. Copyright © 1998 by Lippencott-Raven Pubishers

APPENDIX VI

Restriction maps of Yeast two-hybrid and Mammalian two-hybrid vectors

A



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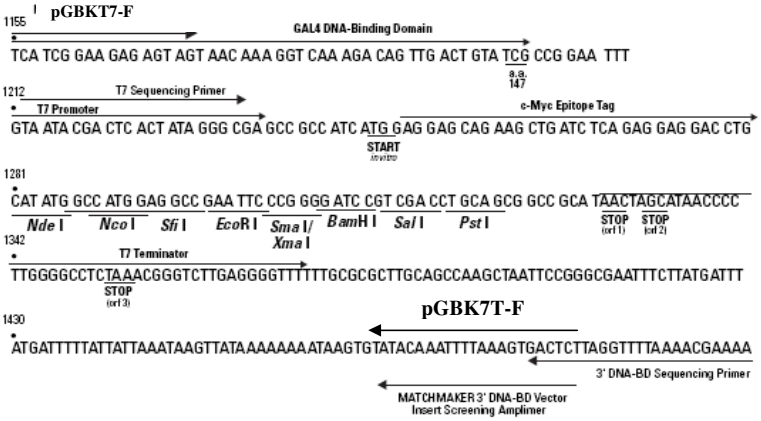
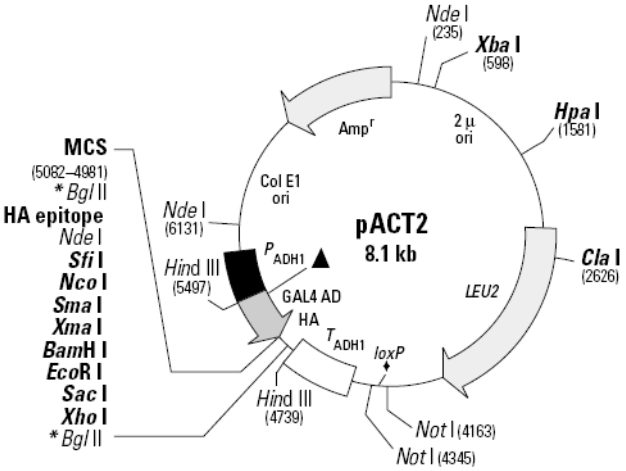


Restriction map and multiple cloning site of pGBKT7 Y2H bait vector.
A) The positions of the kanamycin resistance gene (*kan*), TRP1 and GAL4-BD coding sequences, f1 bacteriophage and pUC plasmid origins of replication, the truncated *S.cerivisiae* ADH1 promotor sequence (P_{ADH1}), the T7 RNA polymerase promoter, the T7 and c- Myc epitope tag are indicated on the map. B) Nucleotide sequence of the pGBKT7 MCS. The positions of all unique restriction enzyme recognition sequences, stop codons in the T7 terminator sequence, the GAL4-BD coding sequence, the T7 promoter sequence, c-Myc epitope tag and the positions of pGBKT7-F and pGBKT7-R screening primers and sequencing primers are indicated on the sequence (taken from Clontech MATCHMAKER vectors handbook).

A



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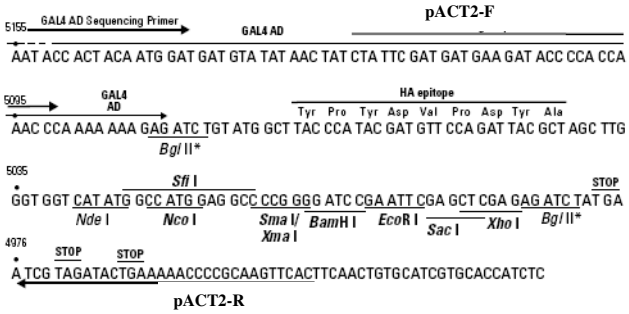
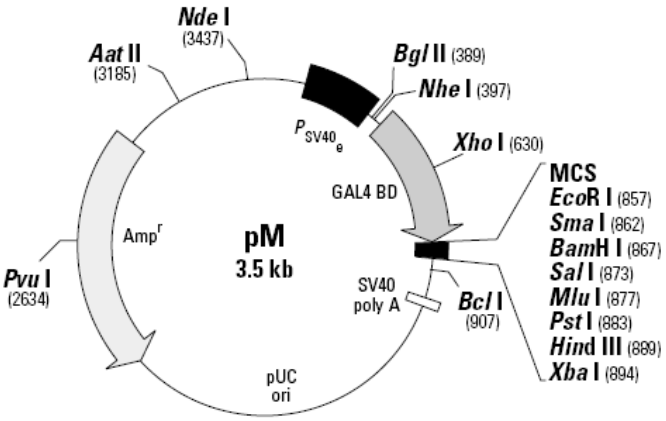


Restriction map and multiple cloning site of pACT2 Y2H prey vector. A) The positions of unique restriction sites are indicated in bold. The position of the ampicillin resistance gene (*Amp*^{r}), LEU2 and GAL4-AD coding sequences, and pBR322 plasmid origins, the *S.cerevisiae* ADH1 promoter, *S.cerevisiae* ADH1 termination sequence, Lox sites (Lox 1 and Lox 2), the heamagglutinin (HA) epitope tag and the MCS are indicated on the map. B) Nucleotide sequence of the pACT2 MCS. The positions of all unique restriction sites, stop codons, the position of the final codon (881) of GAL4-AD coding sequence, the positions of the pACT2-F and pACT2-R primers and the HA epitope tags are all indicated in the map (taken from Clontech MATCHMAKER vectors handbook).

A



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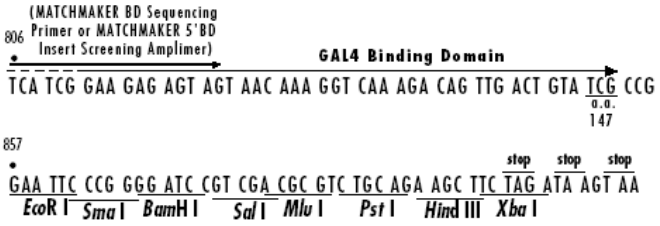
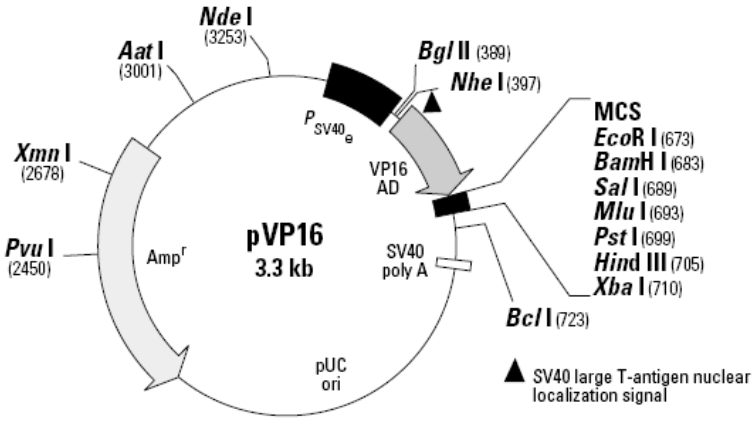


Restriction map and multiple cloning site of pM M2H GAL4 DNA binding vector. A)The positions of unique restriction sites are indicated in bold. The position of the ampicillin resistance gene (*Amp*^{r}), GAL4-BD coding sequences, the SV40 promoter and SV40 polyA transcription termination sequence and the MCS are indicated on the map. B) Nucleotide sequence of the pM MCS. The positions of all unique restriction sites, stop codons, the position of the GAL4-BD coding sequence and the position of the pM sequencing primer are all indicated in the map (taken from Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).

A



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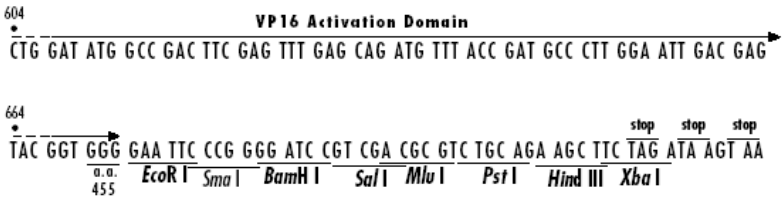
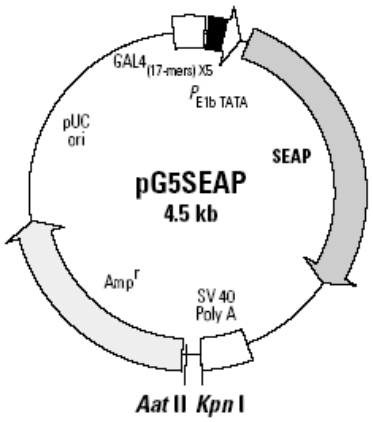


Restriction map and multiple cloning site of pVP M2H VP16 activation domain vector. A) The positions of unique restriction sites are indicated in bold. The position of the ampicillin resistance gene (*Amp*^{r}), GAL4-AD coding sequences, the SV40 promoter and SV40 poly A transcription termination sequence and the MCS are indicated on the map. A) Nucleotide sequence of the pM MCS. The positions of all unique restriction sites, stop codons, the position GAL4-AD coding sequence and the position of the pVP16-F and pVP16-R primers are indicated on the map (taken from Clontech MATCHMAKER Two-Hybrid Assay Kit User Manual).



Map of pG5SEAP reporter vector. This vector contains 5 GAL4 binding sites and an adenovirus E1b minimal promoter sequence upstream of the secreted alkaline phosphatase (SEAP), as indicated on the map.

APPENDIX VII

Bonferroni matrices and box plots for Mammalian two-hybrid assays

Bonferroni test; variable Normalised Lum/E (Experiment 1)
Probabilities for Post Hoc Tests
Error: Between MS = 1.7216, df = 24.000

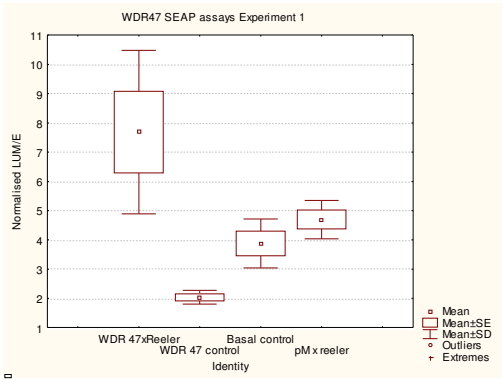
Cell No.	Identity	{1} 3.8759	{2} 7.6861	{3} 2.0365	{4} 4.9197	{5} 2.5620	{6} 4.1953	{7} 1.0000	{8} 13.591
1	Basal control		0.011260	1.000000	1.000000	1.000000	1.000000	0.136928	0.000000
2	WDR 47xReeler	0.011260		0.000077	0.181453	0.000312	0.026819	0.000005	0.000039
3	WDR 47 control	1.000000	0.000077		0.134367	1.000000	0.804749	1.000000	0.000000
4	ATG16L2xReeler	1.000000	0.181453	0.134367		0.501967	1.000000	0.008348	0.000000
5	ATG16L2 control	1.000000	0.000312	1.000000	0.501967		1.000000	1.000000	0.000000
6	Reeler control	1.000000	0.026819	0.804749	1.000000	1.000000		0.059242	0.000000
7	Mock transfected control	0.136928	0.000005	1.000000	0.008348	1.000000	0.059242		0.000000
8	Positive control	0.000000	0.000039	0.000000	0.000000	0.000000	0.000000	0.000000	

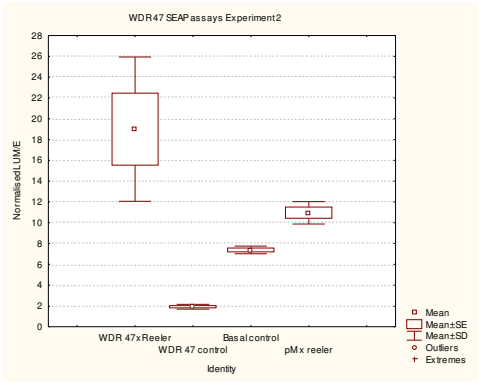
Bonferroni test; variable Normalised Lum/E (Experiment 2)
Probabilities for Post Hoc Tests
Error: Between MS = 6.8892, df = 24.000

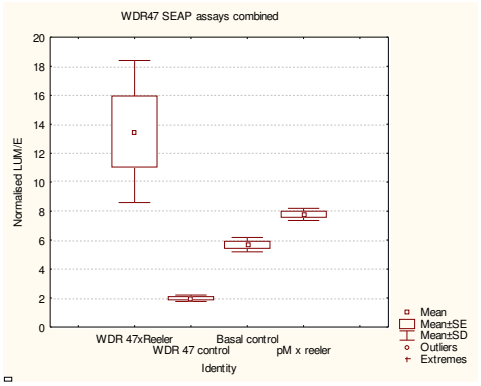
Cell No.	Identity	{1} 7.3958	{2} 19.007	{3} 1.9375	{4} 11.410	{5} 6.1736	{6} 10.222	{7} 1.0000	{8} 18.868
1	Basal control		0.000051	0.199832	1.000000	1.000000	1.000000	0.058930	0.000061
2	WDR 47xReeler	0.000051		0.000000	0.011648	0.000011	0.002291	0.000000	1.000000
3	WDR 47 control	0.199832	0.000000		0.000895	0.885343	0.004547	1.000000	0.000000
4	ATG16L2xReeler	1.000000	0.011648	0.000895		0.264615	1.000000	0.000251	0.014076
5	ATG16L2 control	1.000000	0.000011	0.885343	0.264615		1.000000	0.286155	0.000013
6	Reeler control	1.000000	0.002291	0.004547	1.000000	1.000000		0.001259	0.002771
7	Mock transfected control	0.058930	0.000000	1.000000	0.000251	0.286155	0.001259		0.000000
8	Positive control	0.000061	1.000000	0.000000	0.014076	0.000013	0.002771	0.000000	

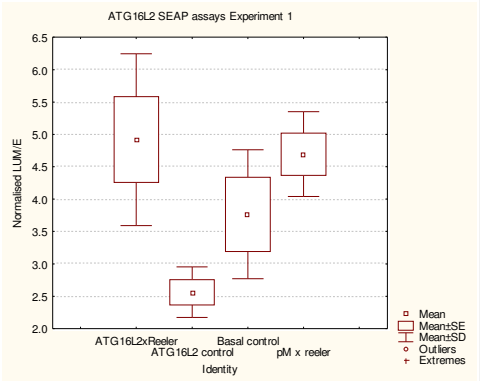
Bonferroni test; variable Norm combined (Combined Experiments)
Probabilities for Post Hoc Tests
Error: Between MS = 3.5275, df = 24.000

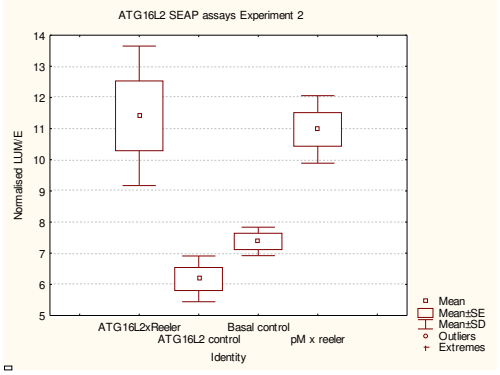
Cell No.	Identity	{1} 5.6797	{2} 13.488	{3} 1.9858	{4} 8.2456	{5} 4.4128	{6} 5.7776	{7} 1.0000	{8} 16.295
1	Basal control		0.000129	0.290233	1.000000	1.000000	1.000000	0.048644	0.000001
2	WDR 47xReeler	0.000129		0.000000	0.016861	0.000013	0.000154	0.000000	1.000000
3	WDR 47 control	0.290233	0.000000		0.002409	1.000000	0.244483	1.000000	0.000000
4	ATG16L2xReeler	1.000000	0.016861	0.002409		0.227457	1.000000	0.000369	0.000082
5	ATG16L2 control	1.000000	0.000013	1.000000	0.227457		1.000000	0.470747	0.000000
6	Reeler control	1.000000	0.000154	0.244483	1.000000	1.000000		0.040513	0.000001
7	Mock transfected control	0.048644	0.000000	1.000000	0.000369	0.470747	0.040513		0.000000
8	Positive control	0.000001	1.000000	0.000000	0.000082	0.000000	0.000001	0.000000	

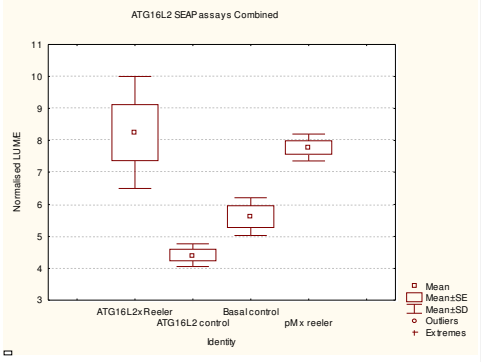












Appendix VIII

PATIENT INFORMATION AND INFORMED CONSENT
Genetics of Anxiety Disorders

PURPOSE:

This study is part of a research project we are conducting to learn more about the genetic causes and symptoms of anxiety disorders (including obsessive-compulsive and spectrum disorders, panic disorder or social phobia). We would like to discuss your life experiences and those of your other family members with you. Doctors and scientists at the MRC Unit on Anxiety and Stress Disorders and the University of Stellenbosch, in collaboration with qualified researchers from other research institutions worldwide, hope to identify the genes that may increase susceptibility to these disorders. This is not a treatment study. Information is being collected for research purposes only.

STUDY PROCEDURE:

If you decide to participate, we shall ask you to attend an interview (which may be videotaped) with a researcher. This interview will include neuropsychological tasks and a number of questions related to your current illness, your prior history of treatment for psychiatric conditions, and particular symptoms you may have experienced as part of your illness. In addition, we may ask to take photographs of your face and hands. This whole procedure will last about 4-5 hours (two 2-hour sessions with a break in-between).

You will also be asked to have your blood drawn. Approximately 48 ml (3 Tablespoons) of blood will be drawn from your arm. We may need to contact you again to get another blood sample should we fail to get a DNA sample from your blood. The blood sample you give may be used to create a cell line. This is done by changing some of your blood cells so that they can grow forever. The cell line is living tissue and it can be used to make more of your DNA at any time in the future. This process will take place at the MRC Centre for Molecular and Cellular Biology and the Division of Medical Biochemistry, Faculty of Health Sciences, at the University of Stellenbosch. The DNA will then be taken from the cell line and saved for scientific analyses which will be performed now, and possibly in the future.

We may contact you later for further information, or request you to complete another interview at a later date, in order to obtain follow-up information that may be of use in our genetic analyses. This may involve an assessment similar to the current assessment, including a series of interviews and/or another blood sample. Your current participation is in no way binding to your future participation.

We would like your permission to contact your relatives in order to get more information about any family history of mental illness. You can still participate in the study even if your relatives do not.

Personal information that could be used to identify you (such as your name, contact information, etc) will not be given out. Your data and DNA is likely to be made available to qualified scientists around the world to study your particular anxiety disorder. Your cell line and DNA will be maintained permanently, unless you request to have it removed. If at any time in the future you wish to have your DNA, cell lines or clinical data removed from the storage site, you may do so by contacting the researchers conducting this study (Christine Lochner at 021 - 938 9179).

The researchers who will have access to your DNA include those who work with private and/or for profit companies. These researchers may be interested in eventually developing commercial medical products using the DNA from you and other participants. They may sell or patent discoveries based on this research and thus benefit financially. Please note that you or your heirs will not receive any compensation if this occurs.

We do not expect to discover any information of direct benefit to your condition, or your treatment, during the next few years. If later on, diagnostic tests or new ways to treat your condition are

discovered, this information will have to be obtained from properly licensed clinical labs, clinics, or your physician, and will not be available from the research team.

If you are hospitalized at a psychiatry facility or have received any treatment from a mental health professional, we would like your permission to review your treatment records, which will be obtained from your doctor.

RISKS:

There are no more than minimal medical or psychological risks associated with this study. If you feel fatigued, tired, uncomforTable, or in any way upset during any part of the session(s), you may ask to stop for a rest break or have the interview discontinued. The research interview does not take the place of a full psychiatric evaluation. You may experience some emotional discomfort when answering some questions. If any particular question makes you feel uncomforTable, you may discuss its importance with the specially trained interviewer. You may choose not to answer any question which you are still uncomforTable with.

You may feel some pain associated with having blood withdrawn from a vein. You may experience discomfort, bruising and/or other bleeding at the site where the needle is inserted. Occasionally, some people experience fleeting dizziness or feel faint when their blood is drawn.

Some insurance companies may mistakenly assume that your participation in this study is an indication that you are at higher risk of a genetic disease, and this could hurt your access to health or other insurance. We will not share any information about you, or your family, with an insurance company. However, if you discuss your participation in this study with your doctor, and he or she records it in your medical record, it is possible that an insurance company may access the information as part of a medical record review. It is the opinion of the investigators that participation in this study does not constitute genetic testing. Although one long-term goal of this research is the development of a genetic test for the anxiety disorders, at the current time, no information from your DNA sample that would be useful in the treatment of your disorder will be obtained. Therefore, participation in this study should not be reported as genetic testing.

Your unidentified DNA and cell line will be available to qualified researchers permanently.

BENEFITS:

There are no direct benefits to you. However, individuals who might develop one or more of these anxiety disorders in the future, their family members, and future generations may benefit if we can locate the genes that lead to such disorders. That knowledge could then lead to the development of methods for prevention and new treatments for curing these diseases.

CONFIDENTIALITY:

If you consent to participate in this study, your identity will be kept confidential. Your answers will not be shared with other family members or anyone else except for staff members involved in this study. All data will be kept in locked file cabinets accessible only to the research staff. All research information obtained will not be associated with your name; research staff will use only a coded number and/or your initials. Blood samples will be safely stored and identified by code number and access will be limited to authorized scientific investigators. Copies of treatment records from hospitals or mental health professionals are kept in locked files and are reviewed by members of the research team only. Any publications resulting from this study will not identify you by name.

VOLUNTARY PARTICIPATION:

Your participation in this study is voluntary and you may refuse to participate or withdraw from the study at any time without any loss of benefits to which you are otherwise entitled. Some members of the team of investigators conducting this study may be responsible for your clinical care. Refusal to participate in this study will not change your clinical care.

RESEARCH QUESTIONS AND CONTACTS:

If you are interested in genetic counseling, you will be given information about where you can receive such counseling and a new blood sample may be required at that time. DNA information about a relative will be released only if the genetic counsellor confirms that the relative in question is deceased or cannot be found and that the information is essential for clinical counseling.

The researchers will answer any questions you might have about the procedures described above, or about the results of the study. If you have any questions, you may call Christine Lochner at (021) 938 9179.

The University of Stellenbosch Research Subcommittee C has approved recruitment and participation of individuals for this study.

You have been given a copy of this consent form to keep.

INFORMED CONSENT:

I have read the above patient information, my questions have been answered, and I consent voluntarily to participate in this study.

Print name: _____ Signature: _____

Date: _____

I have discussed the proposed research with this subject and, in my opinion, this patient understands the benefits, risks, and alternatives (including non-participation) and is capable of consenting to voluntary participation.

Print name: _____ Signature: _____
Study Investigator or Designee

Date: _____

Print name: _____ Signature: _____
Witness (if applicable)

Date: _____

PASIëNTINLIGTING EN INGELIGTE TOESTEMMING
Genetika van Angssteurings

DOELWIT:

Hierdie projek is deel van 'n navorsingsprojek wat tans onderneem word om meer uit te vind oor die genetiese oorsake en simptome van angssteurings (insluitend obsessief-kompulsiewe- en spektrumversteurings, paniek-, of sosiale angssteuring). Ons wil graag oor u lewenservarings en dié van u gesinslede met u gesels. Dokters en wetenskaplikes by die MNR Eenheid vir Angs- en Stressteurings en die Universiteit van Stellenbosch, in samewerking met gekwalifiseerde navorsers van ander navorsingsinstellings wêreldwyd, hoop om die gene wat vatbaarheid vir hierdie angssteurings laat toeneem, te identifiseer.
Dit is nie 'n behandelingstudie nie. Inligting word alleenlik vir navorsingsdoeleindes versamel.

PROJEKPROSEDURE:

Indien u besluit om deel te neem, sal ons u vra om 'n onderhoud (wat moontlik op videoband vasgelê kan word,) met 'n navorser te voer. Hierdie onderhoud sluit neurosielkundige take en 'n aantal vrae in wat met die volgende aspekte verband kan hou: u huidige siekte, u geskiedenis van behandeling vir psigiatriese steurings, en spesifieke simptome wat u dalk kon ervaar as deel van u siekte. Daarmee saam, kan ons u vra om foto's van u hande en gesig te neem. Hierdie hele prosedure sal ongeveer 4-5 ure duur (twee 2-uur sessies met 'n pouse tussen-in).

U sal ook gevra word om toe te laat dat u bloed getrek word. Ons kan dalk weer met u in verbinding moet tree om nog 'n bloedmonster te trek in geval ons nie daarin kon slaag om 'n DNA monster van u bloed te verkry nie. Die bloedmonster wat u gee, kan gebruik word om 'n sellyn te skep. Dit word gedoen deur sommige van u bloedselle te verander sodat dit vir altyd kan groei. Die sellyn is lewende weefsel en dit kan gebruik word om meer van u DNA in die toekoms te maak. Hierdie proses sal plaasvind by die MNR Sentrum vir Molekulêre en Sellulêre Biologie en die Afdeling Geneeskundige Biochemie, Fakulteit Gesondheidswetenskappe, Universiteit van Stellenbosch. Die DNA sal dan van die sellyn geneem en gehou word vir wetenskaplike analise wat nou, en moontlik in die toekoms gedoen sal word.

Ons kan met u in aanraking kom vir verdere inligting, of u vra om nog 'n onderhoud te voltooi op 'n latere stadium, ten einde opvolg-inligting te bekom wat gebruik kan word in ons genetika-analise. Dit kan 'n soortgelyke assessering as die huidige wees, insluitend 'n reeks van onderhoude en/of ander bloedmonsters behels. U huidige deelname verbind u onder geen omstandighede tot toekomstige deelname nie.

Ons wil graag u toestemming hê om met u familielede in aanraking te kom ten einde meer inligting oor enige familiegeskiedenis van geestessiekte te bekom. U kan steeds deelneem aan die projek selfs al is u familielede nie betrokke nie.

Persoonlike inligting wat gebruik kan word om u te identifiseer (soos u naam, kontakbesonderhede, ens.), sal nie uitgegee word nie. U data en DNA sal moontlik aan gekwalifiseerde wetenskaplikes regoor die wêreld beskikbaar gestel word om u betrokke angssteuring te bestudeer. U sellyn en DNA sal permanent gehou word, behalwe wanneer u vereis dat dit verwyder word. Indien u op enige stadium in die toekoms besluit om u DNA, sellyne of kliniese inligting uit die bergingsplek te laat verwyder, kan u dit doen deur die navorsers wat hierdie projek behartig, te vra om dit te doen (Christine Lochner by 021 - 938 9179).

Die navorsers wat tot u DNA toegang het, sluit diegene in wat werk met private en/of winsgeoriënteerde maatskappye. Hierdie navorsers kan ook daarin geïnteresseerd wees om uiteindelik kommersiële mediese produkte te ontwikkel deur van u en die ander deelnemers se DNA gebruik te maak. Hulle kan hierdie uitvindings, wat op hierdie navorsing gebaseer is, verkoop of patenteer en sodoende finansieel daaruit voordeel trek. Let asseblief daarop dat u of u erfgename nie enige kompensasie hiervoor sal ontvang indien dit wel gebeur nie.

Ons verwag nie om enige inligting te bekom wat van direkte nut vir u toestand of u behandeling gedurende die volgende paar jare sal wees nie. Indien daar in die toekoms diagnostiese toetse of nuwe wyses om u toestand te behandel, ontdek word, sal hierdie inligting van behoorlik gelisensieërde kliniese laboratoria, klinieke, of u mediese dokter verkry moet word, en dus nie van die navorsingspan nie.

Indien u by 'n psigiatrie fasiliteit gehospitaliseer word, of behandeling van 'n geestesgesondheidswerker ontvang, wil ons graag u toestemming hê om u behandelingsrekords, wat van u dokter verkry sal word, na te gaan.

RISIKO'S:

Daar is nie meer as die minimum mediese en sielkudige risiko's geassosieer met hierdie projek nie. Indien u uitgeput, ongemaklik, of ontsteld raak tydens enige gedeelte van die sessie(-s), kan u vra om te onderbreek vir 'n ruskansie of om die onderhoud te beëindig. Die onderhoud wat met u gevoer word, neem nie die plek van 'n deeglike psigiatriese evaluasie nie. U kan dalk 'n mate van emosionele ongemak verduur wanneer u sommige van die vrae beantwoord. Indien enige vraag u ongemaklik laat voel, kan u die belang daarvan met die spesiaal opgeleide onderhoudvoerder bespreek. U kan verkies om enige vraag waarmee u steeds ongemaklik voel, nie te beantwoord nie.

U kan moontlik 'n mate van pyn ervaar wanneer die bloed getrek word. U kan ongemak, kneusing en/of bloeding by die plek waar die naald ingesteek word, ervaar. Soms ervaar sommige persone verbygaande duiseligheid of 'n flou gevoel wanneer hulle bloed getrek word.

Sommige versekeringsmaatskappye kan verkeerdelik aanneem dat u deelname aan hierdie projek 'n aanduiding is dat u 'n verhoogde risiko het vir 'n genetiese siekte, en dit kan u toegang tot gesondheid- of ander versekering skaad. Ons sal nie enige inligting oor u, of u familie aan 'n versekeringsmaatskappy bekendmaak nie. Indien u egter u deelname met u dokter bepreek, en hy/sy maak 'n nota daarvan in u mediese rekord, is dit moontlik dat 'n versekeringsmaatskappy hierdie inligting as deel van 'n hersiening van mediese rekords kan bekom. Dit is die mening van die navorsers dat deelname aan hierdie studie nie genetiese toetsing is nie. Alhoewel een langtermyn-doelwit van hierdie navorsing die ontwikkeling van 'n genetiese toets vir die angssteurings is, sal geen inligting van u DNA-monster wat nuttig kan wees in die behandeling van u toestand, tans verkry word nie. Daarom behoort deelname aan hierdie studie nie as genetiese toetsing beskryf te word nie.

U ongeïdentifiseerde DNA en sellyn sal <u>permanent</u> aan gekwalifiseerde navorsers beskikbaar wees.

VOORDELE:

Daar is geen direkte voordele vir u nie. Individue wat egter in die toekoms een of meer van hierdie angssteurings ontwikkel, hulle familielede, en toekomstige generasies, kan voordeel daaruit put as ons die gene wat tot sulke versteurings aanleiding kan gee, kan identifiseer. Hierdie kennis kan dan lei tot die ontwikkeling van metodes vir voorkoming en nuwe behandelingswyses vir genesing van die siektes.

VERTROULIKHEID:

Indien u toestem tot deelname aan die projek, sal u identiteit vertroulik gehou word. U antwoorde sal nie met u familielede of enige iemand anders behalwe die personeellede wat gemoeid is met hierdie projek, gedeel word nie. Alle inligting sal in geslote liasseringskabinette wat slegs vir navorsingspersoneel toeganklik is, gehou word. Alle navorsingsinligting wat verkry word, sal nie met u naam verbind kan word nie; navorsingspersoneel sal bloot 'n kodenommer en/of u voorletters gebruik. Bloedmonsters sal veilig gestoor en geïdentifiseer word deur die kodenommer, en toegang sal tot die gemagtigde wetenskaplike navorsers beperk wees. Kopieë van behandelingsrekords van hospitale of geestesgesondheidswerkers word in geslote lêers gehou en word slegs deur lede van

die navorsingspan deurgegaan. Enige publikasie wat uit hierdie projek voorspruit, sal u nie by name identifiseer nie.

VRYWILLIGE DEELNAME:

U deelname aan hierdie projek is vrywillig en u kan deelname weier of u op enige stadium van die projek onttrek sonder verlies van enige voordele waartoe u andersins geregtig is. Sommige lede van die span navorsers wat hierdie projek uitvoer, kan moontlik verantwoordelik wees vir u kliniese versorging. Weiering om deel te neem aan hierdie studie sal nie u kliniese versorging verander nie.

VRAE OOR DIE NAVORSING EN KONTAKBESONDERHEDE:

Indien u wel in genetiese berading geïnteresseerd is, sal u inligting oor waar sodanige berading beskikbaar is, ontvang en 'n nuwe bloedmonster kan op daardie stadium vereis word. DNA-inligting van 'n familielid sal slegs beskikbaar gestel word indien die genetiese berader bevestig dat die familielid oorlede is of nie opgespoor kan word nie en dat die inligting noodsaaklik is vir kliniese berading.

Die navorsers sal enige vrae wat u mag hê oor bogenoemde prosedures of oor die resultate van die projek, beantwoord. Indien u enige navrae het, kan u Christine Lochner by 021 - 938 9179 skakel.

Die Navorsingsubkomitee C van die Universiteit van Stellenbosch het die werwing en deelname van individue aan hierdie projek goedgekeur.

U het 'n afskrif van hierdie toestemmingsvorm ontvang om te bewaar.

INGELIGTE TOESTEMMING:

Ek het die bostaande pasiëntinligting gelees, my vrae is beantwoord, en ek stem vrywillig in om aan hierdie projek deel te neem.

Naam: _____ Handtekening: _____

Datum:_____

Ek het die voorgestelde projek met die deelnemer bespreek en, na my mening, verstaan die deelnemer die voordele, risiko's, en alternatiewe (inlsuitend nie-deelname) en is in staat om toestemming te gee vir vrywillige deelname.

Naam: _____ Handtekening: _____

Navorser of Gemagtigde

Datum:_____

Naam: _____ Handtekening: _____

Getuie (indien van toepassing)

Datum:_____

APPENDIX IX

Articles submitted to peer reviewed journals using data generated in the present study

Identification and assessment of novel obsessive-compulsive disorder candidate genes residing in schizophrenia susceptibility loci (Under review in World Journal of Biological Psychiatry)

Regular Research Article

Craig J. Kinnear (1,2), Lize van der Merwe (3) Sîan M. J. Hemmings (1,2), Christine Lochner (2), Robin A Emsley (4), Dan J. Stein (2,5) Valerie A. Corfield (1), Johanna C. Moolman-Smook (1)

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Running title: OCD candidate genes in schizophrenia loci

Word Count: Abstract: 200

Main Body: 2724

References: 61

Tables: 3

Figures: 1

ABSTRACT

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric disorder that is caused by a complex interplay of uncertain environmental and genetic factors. The majority of genetic OCD studies have focussed on a limited set of genes; hence a need to identify novel OCD candidate genes. One way to achieve this is by utilising knowledge of diseases more amenable to genetic dissection through linkage analysis, and with phenomenological overlap with OCD, such as schizophrenia. Schizophrenia linkage and chromosomal aberration studies have implicated numerous chromosomal regions that may contain genes involved in both schizophrenia and OCD pathogenesis. As proof -of-principle, we analysed genes residing in schizophrenia susceptibility loci using bioinformatic techniques to assess their candidature for OCD susceptibility. Nine credible OCD candidate genes were assessed for their potential role in the aetiology of OCD by case-control association studies in a cohort of Afrikaner OCD and control subjects. The *C*-allele of the *SYN3 -631C>G* polymorphism increased susceptibility to OCD in both an original and extended sample. The heterozygote of the *DLX6 IVS1C>T* polymorphism decreased susceptibility to OCD in both an original and an extended sample, while interaction between these two polymorphisms significantly influenced susceptibility. These associations may point to novel mechanisms involved in OCD development.

Keywords: Obsessive-compulsive disorder, schizophrenia, genetic loci, *DLX6*, *SYN3*

Introduction

Obsessive-compulsive disorder (OCD) is a common, disabling psychiatric condition (Murray and Lopez., 1996) for which the underlying molecular pathophysiology remains unclear. Currently, it is proposed that this disorder is caused by the complex interplay of various environmental and genetic factors, the identity of which remain uncertain.

A number of segregation analyses of OCD have provided evidence for the existence of a major gene locus (Alsobrook *et al*., 1999; Cavallini *et al*., 1999; Nestadt *et al*., 2000) although, to date, no major susceptibility gene has been identified. Nonetheless, a number of plausible candidates have been investigated in several population- and family-based association studies, although divergent results have generally confounded interpretation of such studies.

A possible explanation for inconsistencies is that genes found to be associated with OCD in some populations may only contribute very minor effects to disease susceptibility within those populations, while having no effect in others. This could have serious implications for the identification of genetic factors involved in OCD, given that the current focus of many investigations is to replicate previously reported associations in different populations. A consequence of such replication attempts is that the same genes are habitually analysed, with less effort expended in identifying novel candidate genes that may play larger contributory roles to the pathogenesis of OCD phenomenology in more general populations.

One possible approach to identifying novel candidate genes is to utilise existing knowledge of diseases that possess phenomenological overlap with OCD, but which are more amenable to genetic dissection. Genetic loci for such disorders, identified through linkage analysis, could potentially harbour novel OCD candidate genes.

One such disorder is schizophrenia, where case-control association study data, linkage data and studies of chromosomal aberrations have led to the identification of many chromosomal regions that may contain genes involved in the aetiology of the disorder (reviewed by Owen *et al.,* 2004).

The notion of pathophysiological overlap between OCD and schizophrenia stems from observations of increased comorbidity between these two disorders (Fenton and McGlashan, 1986; Nechmad *et al*., 2003;Poyurovsky *et al*.,1998; Dominquez *et al*., 1999; Tibbo *et al*., 2000; Lysaker *et al*., 2000; Bermanzohn *et al*., 2000; Poyurovsky *et al*., 1999; Fabisch *et al*.,2001; Craig *et al*., 2002; Ohta *et al*., 2003; Eisen *et al*.,1997). It has been suggested that the co-expression of these symptoms may reflect an overlap of the structural and functional brain abnormalities associated with schizophrenia and

OCD. Indeed, based on structural and functional neuroimaging studies, abnormalities in the frontal striatal circuits (Cummings *et al.,*1993), thalamus, basal ganglia and the amygdala complex have been identified in both disorders (Kwon *et al.,*2003), although the noted abnormalities are often at opposite ends of the functional spectrum. For example, in OCD, hyper-functionality of the frontal-striatal system has been implicated (Baxter *et al.,*1988), whereas in schizophrenia, hypo-functionality of this system has been implicated (Kim *et al.,*2000). In addition, studies have shown that, in OCD, the thalamus is enlarged (Kim *et al.,*2001), while in schizophrenia, a decrease in thalamic volume has been reported (Andreasen *et al.,*1994).

Further evidence for a pathological overlap between schizophrenia and OCD stems from retrospective pharmacological studies and case reports. On the one hand, antipsychotic pharmacotherapy has been reported to induce obsessive-compulsive symptoms (OCS) or exacerbate existing OCS in schizophrenic patients (Eales and Layeni, 1994; Morrison *et al.,*1998; de Haan *et al.,*1999; Mottard and De la Sablonniere, 1999; Tibbo and Warneke, 1999). On the other hand, antipsychotics are effective augmentatory agents in treating refractory OCD (Bloch *et a*l. 2006).

Thus, the OCD/schizophrenia co-morbidity data, the overlap of implicated brain regions from structural and functional studies and the possible role that antipsychotic medication may play in mediating OCS, suggests that these two disorders may share neurophysiological aspects, driven at least in part by genetics, of a common pathological pathway (Insel and Asikal, 1986; Gross-Isseroff *et al*., 2003). It may be further hypothesised that, although some susceptibility genes may be shared, the functional characteristics of the actual susceptibility variants may be opposed. Since several linkage studies have identified numerous schizophrenia susceptibility loci, we hypothesised that these loci may also harbour genes increasing susceptibility to OCD. Hence, to test this hypothesis, we bioinformatically searched selected schizophrenia susceptibility loci for credible OCD candidate genes and explored their involvement in OCD susceptibility in a case-control association study.

Materials and Methods

Study subjects

The protocol was approved by the Ethics Committee of the University of Stellenbosch, (protocol number 99013) and all subjects provided written informed consent, after being presented with a complete description of the study. All case and control subjects participating in the present study were of Afrikaner descent. The South African Afrikaner population has frequently been used, as a homogeneous population, in case-control association studies: the Afrikaner population is of Dutch, German and French origin and their history and population dynamics over the past 350 years have

led to a relatively small gene pool with an above-average frequency of rare genetic illnesses, due to founder effects (Starfield et al., 1997; Moolman-Smook et al., 1999; de Jager et al., 1999). For the purpose of this investigation, subjects were classified as Afrikaners if at least three of their four grandparents were of Afrikaner descent.

Unrelated control subjects were recruited throughout South Africa by trained clinical psychologists and via media advertisements. All controls were required to complete a questionnaire pertaining to his/her personal demographic data and present state of physical health. Unrelated OCD patients, between nine and 65 years of age, were recruited through physician referral, media advertisements, the Mental Health Information Centre (MHIC) and the OCD Association of South Africa (OCDSA). To be eligible for inclusion in the study, patients had to meet the DSM-IV criteria (APA, 1994) for a primary diagnosis of OCD on the Structured Clinical Interview for Axis I disorders – Patient Version (SCID-I/P) (First et al., 1998). All diagnoses were made by trained clinicians at the MRC Unit on Anxiety and Stress Disorders.

Identification of schizophrenia susceptibility loci

Literature searches were conducted using the publicly available PUBMED database (http://www.ncbi.nlm.nih.gov/PubMed), in order to identify previously reported schizophrenia susceptibility loci. The PUBMED database was searched by the terms “schizophrenia linkage”, “schizophrenia genetic linkage” and “schizophrenia genetics”.

Prioritising genes within each locus as OCD candidate genes.

The Ensembl (http://www.ensembl.org), the University of California Santa Cruz (http://www.genome.ucsc.edu) and the NCBI databases were scanned to derive *in silico* catalogues of all annotated and predicted genes within each of the identified loci. Once all the genes within the selected loci were catalogued, they were prioritised as plausible OCD candidate genes based on function (http://harvester.embl.de/), expression profile and known pathogenic role (http://www.ncbi.nlm.nih.gov/OMIM). Brain tissue expression was a prerequisite in the selection of plausible candidate genes; the expression profiles for all genes within the loci were determined using the Unigene EST database (http://www.ncbi.nlm.nih.gov/unigene).

Genes that encode proteins with unknown function were analysed by homology searches to predict a possible function based on their protein domain composition using the pfam (http://www.sanger.ac.uk/Software/Pfam) and prosite (http://www.expasy.org/prosite/) algorithms.

Previously reported polymorphisms within randomly selected candidate genes that had been targeted in past psychiatric case-control association studies were selected for investigation in the current analysis. Where no investigations of a candidate gene had been reported in the literature, target polymorphisms were selected from a publicly available single nucleotide polymorphism databases (http://www.ncbi.nlm.nih.gov/SNP; http://www.hapmap.org) based on a minor allele frequency of greater than 0.3.

Candidate gene association analyses

The target genomic fragment containing a selected polymorphism was amplified by means of the polymerase chain reaction (PCR) and genotyped using allele-specific restriction enzyme analysis (ASREA) according to previously published data for the following polymorphisms: synaptosomal-associated protein of 25 kDa (*SNAP-25*) *Mnl*I (Barr et al., 2000); synaptosomal-associated protein of 29 kDa (*SNAP-29*) *C56T* (Saito et al., 2001); AMPA receptor subunit GluR4 (*GRIA4*) rs609239 (Makino et al., 2003); N-methyl-D-aspartate receptor NR1 subunit (*GRIN1*) rs11146020 (Martucci et al., 2003); distal-less like homeobox 6 (*DLX6*) *IVS1C>T* (Nabi et al., 2003); peripheral benzodiazepine receptor (*BZRP*) *Ala147Thr* (Kurumaji et al., 2001); dopamine beta hydroxylase (*DBH*) *I/D* (Yamamoto et al., 2003) and synapsin III (*SYN3) -631C>G* (Tsai et al., 2002). Primers amplifying the rs10887523 polymorphism in the glutamate receptor, ionotropic delta 1 (*GRID1*) gene were created using Primer 3 (Rozen and Skaletsky, 2000) (primer sequences available on request from first author).

Statistical analysis

Logistic regression was used to model case-control status as a function each genotype. Each genotype factor was modelled as two variables, one counting the number of *SNAP25/Mnl*I *G, SNAP29 C56T T, GRIA4 rs630567 T, GRIN1/1 G, DLX6 IVS1C>T T*, *BZRP Thr, DBH I* and *SYN3 -631C>G G* alleles (0, 1 or 2) (the additive effect) and another taking the value 0 for any homozygote and 1 for the heterozygotes (the heterozygous effect) as described by Cordell and Clayton, 2005. The resulting models are described and summarised in the results.

Functions from base R and R packages (R Development Core Team, 2007) were used for all statistical analyses.

Results

Bioinformatic searches of schizophrenia susceptibility loci for plausible OCD candidate genes.

Twenty-one schizophrenia susceptibility loci were searched for plausible OCD candidate genes, based on function and expression. For the purpose of this proof-of-principle investigation, only one

polymorphism in nine candidate genes from nine randomly selected schizophrenia loci were assessed for their potential role in OCD pathogenesis.

Association analysis of selected candidate genes

All control groups were in Hardy-Weinberg equilibrium for each of the polymorphisms tested. Genotype and allele frequencies for each of the analysed polymorphisms are shown in Table 1. Table 2 summarises the logistic regression models for case-control status for the polymorphisms showing significant effects and Table 3 summarises the logistic regression models for the remaining polymorphisms

.

The *G* allele of the *SYN3 -631C>G* polymorphism was found to be protective, with the presence of each *G*-allele effectively halving the odds of presenting with OCD (OR = 0.51 [95% CI: 0.30-0.83]) (Table 2). The *TC* heterozygote of the *DLX6 IVS1C>T* polymorphism was also found to be protective (OR = 0.38 [95% CI: 0.18-0.81])(Table 2). No significant associations were observed for any of the other polymorphisms investigated (Table 3).

In order to improve the power of the two significant findings, the sample size of the case and control groups was increased, and a joint analysis of the original and new subjects performed (Table 3). Once again, the additive, protective effect of the *G* allele of the *SYN3 -631C>G* polymorphism (OR = 0.62 [95% CI: 0.42-0.89]) and the protective effect of the *TC* heterozygote of *DLX6 IVS1C>T* polymorphism (OR = 0.54 [95% CI: 0.30-0.96]) was observed.

We also tested for, and observed, a significant interactive effect between the *SYN3 -631C>G* and *DLX6 IVS1C/T* polymorphisms and OCD (p = 0.039), where individuals who were heterozygous *CG* for the *SYN3 -631C>G* polymorphism, as well as homozygous *CC* for the *DLX6 IVS1C/T* polymorphism, had increased susceptibility to developing OCD, while individuals who were heterozygous at both these loci were protected against OCD (Figure 1).

Discussion

In the present investigation, one sequence variant in each of nine candidate genes from as many schizophrenia-implicated chromosomal regions was assessed as OCD candidate genes in a population-based case-control association study. Logistic regression analysis implicated *DLX6 IVS1C/T* and *SYN3 -631C>G* variants in the development of OCD (Table 2).

The *CT* heterozygote of *DLX6 IVS1C/T* polymorphism was found to confer protection against the development of OCD. A protective effect of the *T* allele was also observed, but this was not

statistically significant. Here, the odds of developing OCD was significantly reduced in heterozygous individuals (Table 2). Whether the *DLX6 IVS1C/T* intronic polymorphism has any functional significance is yet to be determined; hence explanations for the described association are currently speculative.

One possible explanation is that the *DLX6 IVSC/T* polymorphism is in linkage disequilibrium (LD) with a functional polymorphism, either within *DLX6* itself or an adjacent gene that is associated with OCD pathogenesis. In fact, *DLX6* forms a bigene cluster with another *DLX* gene, *DLX5*, which is a gene that, like *DLX6*, has been shown to be involved in basal ganglia development (Liu *et al*., 1997; Zerucha et al., 2000).

SYN3, encoding synapsin III, a protein involved in synaptogenesis of hippocampal neurons (Ferreira *et al*., 2000) and regulation of neurotransmitter release (Kao *et al*., 1998), has been the focus of many association studies in schizophrenia (Ohmori *et al*., 2000; Ohtsuki *et al*., 2000; Imai *et al* 2001; Tsai *et al*., 2002; Porton *et al*.,2004; Lachman *et al*., 2005; 2006). Three of these studies (Ohmori *et al*., 2000; Ohtsuki *et al*., 2000; Tsai *et al*., 2002) specifically investigated the role of the *SYN3 -631C>G* polymorphism in this disorder, but failed to show any statistically significant association.

In the present study, the *G*-allele of the *SYN3 -631C>G* polymorphism was found to confer protection against OCD, under an additive model (Table 2). The functional significance of the *SYN3 -631C>G* polymorphism remains undetermined. Since this polymorphism resides in the promoter region of the gene, it is tempting to speculate that it may disrupt binding of transcription factors, thereby altering *SYN3* gene expression. Ohmori *et al.* (2000) previously scanned the *SYN3* promoter sequence for known transcription factor recognition sites using the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html; Fogel *et al*., 2005), but found that *SYN3 -631G>C* was not located in any known transcription factor binding site, thereby concluding that the variant has no effect on gene expression. However, care should be taken when interpreting data generated by transcription binding site recognition programs, since such programs rely on pre-existing knowledge of experimentally determined recognition sequences for known transcription factors. Therefore, one cannot exclude the possibility that *SYN3 -631C>G* may disrupt a recognition sequence for an unidentified transcription factor.

Another possible explanation for the observed association between *SYN3* and OCD in the present study is that the *SYN3 -631C>G* polymorphism is in LD with a functional polymorphism, either within *SYN3* or an adjacent gene. Interestingly, the *SYN3 -631C>G* polymorphism was found to be in almost complete linkage disequilibrium with another polymorphism, *-196G>A*, in the promoter

region of *SYN3* in a Japanese schizophrenia cohort (Ohmori *et al*., 2000) and in an Italian multiple sclerosis cohort (Liguori *et al*., 2004). The *A* allele of the latter polymorphism results in a 6bp base match to the core recognition octamer sequence of the Oct-1 transcription factor (Lachman *et al*., 2006). The level of LD between these two polymorphisms in the Afrikaner population has not been determined; however, based on the aforementioned observations, one could speculate that the association of *SYN3 -631C>G* polymorphism with OCD observed in the present study may reflect an association with the *SYN3 -196G>A* polymorphism.

Another interesting observation was the interaction between the *DLX6 IVS1C>T* and *SYN3 -631C>G* polymorphisms (Figure 1). One could speculate that, since *DLX6* is a transcription factor, the interactive effect reported here may be a consequence of *DLX6* regulating the transcription of *SYN3*. However, *in silico* analysis of the *SYN3* promoter region using the Gene2Promoter program (http://www.genomatix.de/products/Gene2Promoter/index.html) did not reveal any *DLX6* binding sites. Therefore, explanations for the interactive effect between *SYN3* and *DLX6* remain unclear and warrant further investigation.

The identification of genetic susceptibility factors to psychiatric disorders such as OCD and schizophrenia remains a daunting task. Nonetheless, recent advances in technologies and statistical methodologies have provided researchers with valuable tools and novel approaches to identify, and assess, potential candidate genes for these disorders. With the availability of resources such as gene and protein micro-arrays, the increasing amount of information on the human genome sequence and whole genome association analysis, much of the "guess-work" in identifying potential candidate genes for the psychiatric disorders may be a thing of the past. These technologies, however, are costly and researchers therefore need to continue to develop innovative approaches to identify candidate genes for psychiatric disorders.

The preliminary data presented in the current study warrants further investigation in larger sample sets, and ideally, Hapmap tagSNPs and haplotypes should be examined across all genes in order to increase the power of case-control association studies (Akey *et al.,* 2001, Daly *et al.,* 2001). Furthermore, in this study multiple-testing correction has not been used as there is currently no international consensus on what constitutes generally applicable and appropriate multiple testing correction. The Bonferroni correction is known to lead to over-conservative p-values, risking the rejection of important findings, while Bayesian methods rely on knowledge of prior probability of involvement, which is currently unknown for most variants (Campbell and Rudan, 2002). Nonetheless, this proof-of-principle study illustrates how unconventional approaches can be used to identify plausible candidate genes for a complex psychiatric disorder such as OCD.

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STATEMENTS OF INTEREST

None.

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Table 1: Genotype and allele counts of polymorphisms investigated.

SNAP25/MnlI	***TT***	***TG***	***GG***	**Total**	***T***	***G***	**Total**
OCD	31 (32.2)	42 (51.9)	8 (9.9)	**81**	104 (64.2)	58 (35.8)	**162**
Control	30 (46.9)	29 (45.3)	5 (7.8)	**64**	87 (69.5)	39 (30.5)	**128**
SNAP29 C56T	***CC***	***CT***	***TT***		***C***	***T***	
OCD	14 (24.1)	29 (50.0)	15 (25.9)	**58**	57 (49.1)	59 (50.9)	**116**
Control	9 (14.1)	40 (62.5)	15 (23.4)	**64**	58 (45.3)	70 (54.7)	**128**
***GRIA4* rs630567**	***AA***	***AT***	***TT***		***A***	***T***	
OCD	23 (33.8)	41 (60.3)	4 (5.9)	**68**	87 (63.9)	49 (36.1)	**136**
Control	31 (34.8)	48 (53.9)	10 (11.3)	**89**	110 (618)	68 (38.2)	**178**
***GRIN1* rs11146020**	***CC***	***CG***	***GG***		***C***	***G***	
OCD	12 (15.4)	21 (26.9)	45 (57.7)	**78**	45 (28.8)	111 (71.2)	**156**
Control	10 (16.4)	21 (34.4)	30 (49.2)	**61**	41 (33.6)	81 (66.4)	**122**
***BZRP* Ala147Thr**	***Ala/Ala***	***Ala/Thr***	***Thr/Thr***		***Ala***	***Thr***	
OCD	10 (14.9)	25 (37.3)	32 (47.8)	**67**	45 (33.6)	89 (66.4)	**134**
Control	9 (9.9)	36 (39.6)	46 (50.5)	**91**	54 (29.7)	128 (70.3)	**182**
DBH I/D	***II***	***ID***	***DD***		***I***	***D***	
OCD	20 (26.6)	40 (53.4)	15 (20)	**75**	80 (53.3)	70 (46.6)	**150**
Control	17 (28.4)	34 (56.6)	9 (15)	**60**	68 (56.6)	52 (43.3)	**120**
***GRID1* rs10887523**	***CC***	***CA***	***AA***		***C***	***A***	
OCD	21 (42)	23 (46)	6 (12)	**50**	65 (65.0)	35 (35.0)	**100**
Control	20 (31.3)	33 (55.1)	11 (17.2)	**64**	73 (57.0)	55 (43.0)	**128**

DLX6 IVS1C>T	***CC***	***CT***	***TT***		***C***	***T***	
OCD	14 (33.3)	16 (38.1)	12 (28.6)	**42**	44 (52.4)	40 (47.6)	**84**
Control	10 (13.3)	47 (58.1)	24 (34.6)	**81**	67 (41.4)	95 (58.6)	**162**
OCD*	15 (16.9)	39 (43.8)	35 (39.3)	**89**	69 (38.8)	109 (61.2)	**178**
Control*	13 (9.7)	76 (56.7)	45 (33.6)	**135**	102 (31.1)	166 (61.9)	**268**
SYN3* -631 *C>G	***CC***	***CG***	***GG***		***C***	***G***	
OCD	37 (43.1)	42 (48.8)	7 (8.1)	**86**	116 (67.4)	88 (54.3)	**172**
Control	24 (29.6)	40 (49.3)	17 (21.1)	**81**	56 (32.6)	74 (45.7)	**240**
OCD*	47 (37.1)	65 (50)	17 (12.9)	**127**	164 (62.1)	100 (37.9)	**264**
Controls*	33 (23.2)	77 (54.2)	32 (22.6)	**142**	143 (50.4)	141 (49.6)	**284**

Genotype and allele percentages in brackets.

*numbers of OCD and control subjects after additional samples genotyped.

Abbreviations: *BZRP*, peripheral benzodiazepine receptor; *DBH*, dopamine beta hydroxylase; *DLX6,* distal-less like homeobox 6; *GRID1*, glutamate receptor, ionotropic, delta 1; *GRIA4,* AMPA receptor subunit GluR4; *GRIN1,* N-methyl-D-aspartate receptor NR1 subunit; *SNAP25* Synaptosomal-associated protein of 25kDa: *SNAP29*, Synaptosomal-associated protein of 29kDa; *SYN3*, synapsin III

Table 2: Summary of logistic regression models for case-control status for the polymorphisms showing significant effects. Odds ratios (ORs), 95% confidence intervals (CIs) for the ORs and p-values are given for the original as well as the combined cohort.

	Combined cohort			Original cohort		
DLX6 IVS1 C>T	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
CC homozygote	1					
Each *T* allele	0.82	0.53-1.27	0.371	0.69	0.41-1.15	0.158
Heterozygote	0.54	0.30-0.96	0.038	0.38	0.18-0.81	0.013
SYN3 -631C>G	**Combined cohort**			**Original cohort**		
CC homozygote	1					
Each *G* allele	0.62	0.42-0.89	0.011	0.51	0.30-0.83	0.009
Heterozygote	0.97	0.59-1.59	0.891	1.36	0.71-2.69	0.362

Abbreviations: *DLX6,* distal-less like homeobox 6; *SYN3*, synapsin III; OR, odds ratio; CI, confidence interval

Table 3: Summary of logistic regression models for case-control status for each of the other polymorphisms investigated.

SNAP2 5MnI	**OR**	**95% CI**	**p-value**
TT homozygote	1		
For each *G* allele	1.24	0.68-2.37	0.484
Heterozygote	1.13	0.51-2.43	0.763
SNAP29 C56T	**OR**	**95% CI**	**p-value**
CC homozygote	1		
For each *T* allele	0.802	0.457-1.39	0.432
Heterozygote.	0.581	0.278-1.2	0.145
GRIA4.1	**OR**	**95% CI**	**p-value**
AA homozygote	1		
For each *T* allele	0.73	0.73-1.39	0.344
Heterozygote	1.57	1.57-1.48	0.248
GRIN 1	**OR**	**95% CI**	**p-value**
CC homozygote	1		
For each *G* allele	1.118	0.687-1.81	0.648
Heterozygote	0.745	0.343-1.61	0.455
BZRP	**OR**	**95% CI**	**p-value**
Ala/Ala homozygote	1		
For each *Thr* allele	0.79	0.47-1.31	0.362
Heterozygote	0.81	0.39-1.66	0.571
***GRID1* rs1088532**	**OR**	**95% CI**	**p-value**
CC homozygote	1		
For each *A* allele	0.721	0.392-1.28	0.272
Heterozygote	0.921	0.419-2.05	0.838
DBH (I/D)	**OR**	**95% CI**	**p-value**
DD homozygote	1		
For each *I* allele	1.26	0.74-2.19	0.395
Heterozygote	0.79	0.39-1.60	0.517

Abbreviations: *BZRP*, peripheral benzodiazepine receptor; *DBH*, dopamine beta hydroxylase; *GRID1*, glutamate receptor, ionotropic, delta 1; *GRIA4,* AMPA receptor subunit GluR4; *GRIN1,* N-methyl-D-aspartate receptor NR1 subunit; *SNAP25* synaptosomal-associated protein of 25kDa: *SNAP29*, synaptosomal-associated protein of 29kDa

OR: odds ratio; **CI**: confidence interval for odds ratio.

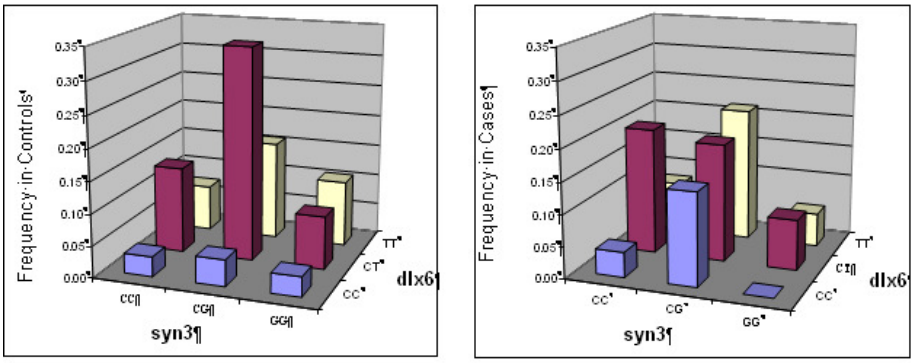

Fig 1. Bar graphs representing joint *DLX6IVS1C>T* and *SYN3-631C>G* genotype frequencies **A Genotype frequencies for the control panel, B Genotype frequencies for the OCD patients.**

Investigating the role of the brain-derived neurotrophic factor (BDNF) *val66met* variant in obsessive-compulsive disorder (OCD)

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Regular Research Article

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ABSTRACT

Although evidence from family studies suggest that genetic factors play an important role in mediating obsessive-compulsive disorder (OCD), results from genetic case-control association analyses have been inconsistent. Discrepant findings may be attributed to the lack of phenotypic resolution, and population stratification. The aim of the present study was to investigate the role that the *val66met* variant within the gene encoding brain-derived neurotrophic factor (*BDNF*) may play in mediating the development of selected OCD subtypes accounting for the aforementioned confounding factors. One hundred and twelve OCD subjects and 140 controls were selected from the South African Afrikaner population. A significant association was observed in the male subgroup, with the *met66* allele implicated as the risk allele in the development of OCD. This allele was also found to be associated with an earlier age at onset of OCD in males. On the other hand, the *val66val* genotype was associated with more severe OCD in the female population. No evidence of population stratification was observed in Afrikaner control subjects. These preliminary results point towards genetically distinct characteristics of OCD mediated by dysfunctions in BDNF. The present investigation forms part of ongoing research to elucidate the genetic components involved in the aetiology of OCD and OCD-related characteristics.

Keywords: Obsessive-compulsive disorder; Brain-derived neurotrophic factor; BDNF; population structure

INTRODUCTION

Obsessive-compulsive disorder (OCD), a disorder which affects a sizeable portion of the general population, has a complex aetiology. Although a large number of association studies have examined genetic components possibly involved in OCD (see review by Hemmings and Stein, 2006), very few have yielded consistent results, due to potential confounders. One such confounder may be insufficient resolution of research subjects, which increases heterogeneic noise, to identify genetic risk factors; this resolution can pertain to two levels within a sample set: clinical phenotypic resolution and population stratification.

Firstly, present data indicate that OCD is a clinically and genetically heterogeneous disorder - the disease phenotype characterised as OCD may represent a final common pathway of multiple aetiologies. Although genes encoding serotonergic (5-HT) and dopaminergic components are most commonly investigated, it is likely that the behavioural manifestations of OCD are mediated by a large network of interconnected neurotransmitter and signalling pathways, of which different combinations of functional genetic variants underlie particular traits. Thus, classifying OCD according to clinically-defined characteristics should be more informative and statistically powerful (Silverman and Palmer, 2000), since such traits are probably controlled by fewer loci and environmental factors, while targeting candidate genes which pertain temporally or functionally more strongly to the particular phenotypic subtype may yield more meaningful results.

Secondly, population stratification arises when the genetic background of the source populations differ between cases and controls (Cardon and Palmer, 2003). The South African Afrikaner population has often been used in case-control association studies: the Afrikaner population is of Dutch, German and French origin, and their history and population dynamics over the past 350 years have led to a relatively small gene pool, and above-average frequency of rare genetic illnesses, due to founder effects (Starfield et al., 1997; Moolman-Smook et al., 1999; de Jager et al., 1999). However, no formal analysis has been conducted into whether the Afrikaner population represents a unified, genetically homogeneous population, or whether certain “sub-populations” exist, which may confound genetic association studies. As recent investigations in the Icelandic populations demonstrated, substructure may exist in populations originally thought to be genetically homogeneous (Helgason et al., 2005).

One intriguing OCD candidate gene from both a developmental and neurotransmitter etiological perspective is the brain-derived neurotrophic factor (BDNF) gene. It encodes an activity-dependent endogenous neurotrophin involved in neurodevelopment, neuronal survival, morphology and differentiation (Hoglinger et al., 1998), which may promote the function and growth of 5-HT neurons

in the brain (Mamounas et al., 1995; 2000), and modulate the synaptic plasticity of DRD3-secreting neurons in the striatum (Guillin et al., 2003). Recently, a common SNP occurring at nucleotide 196 (*196G/A*; rs6265) in the terminal exon of the proBDNF sequence, resulting in an amino acid substitution (*val66met*), was found to be associated with early-onset (EO) OCD in a family-based case-control association study (Hall et al., 2003). On the other hand, in a more recent family-based association study on a group of EO OCD patients, no significant association was noted between this variant and EO OCD (Mossner et al., 2005). The *met66*-allele has previously been found to affect the intracellular processing of the pro-BDNF polypeptide, thereby inhibiting the release of BDNF from activated neurons (Egan et al., 2003). This allele has also been implicated as the risk allele in a restrictive subtype of anorexia nervosa (Ribases et al., 2003; 2004; 2005), which has features in common with OCD. Moreover, it has also been found that the *met66*-allele may act as a risk factor in the development of anxiety disorders (Jiang et al., 2005).

In the present, preliminary study, the role that the *BDNF val66met* polymorphism may play in the development of particular subtypes of OCD was investigated in the Afrikaner population. Furthermore, to determine whether any population stratification exists within the Afrikaner population, the sample set used in the present study was investigated using a Bayesian model-based algorithm, *Structure* (Pritchard et al., 2000).

METHODS AND MATERIALS

The protocol was approved by the Ethics Committee of the University of Stellenbosch, and all subjects provided written informed consent, after being presented with a complete description of the study. All case and control subjects participating in the present study were of Afrikaner descent. For the purpose of this investigation, subjects were classified as Afrikaners if at least three of their four grandparents were of Afrikaner descent.

Unrelated control subjects were recruited throughout South Africa by trained clinical psychologists and via media advertisements. All controls were required to complete a questionnaire pertaining to his/her personal demographic data and present state of physical health. Unrelated OCD patients, between 9 and 65 years of age, were recruited through physician referral, media advertisements, the Mental Health Information Centre (MHIC) and the OCD Association of South Africa (OCDSA). To be eligible for inclusion in the study, patients had to meet the DSM-IV criteria (APA, 1994) for a primary diagnosis of OCD on the Structured Clinical Interview for Axis I disorders – Patient Version (SCID-I/P) (First et al., 1998). All diagnoses were made by trained clinicians at the MRC Unit on Anxiety and Stress Disorders.

The Yale-Brown Obsessive-Compulsive Symptom Checklist (YBOCS-CL) and severity scale (YBOCS-SS) (Goodman et al., 1989) were used for the assessment of the typology and severity of obsessive-compulsive symptoms, respectively. The dimensional YBOCS (DY-BOCS) interview (Rosario-Campos et al., 2006) was conducted to allow for assessment of the typology and severity of OCD symptoms. In the genetic analyses, both the categorical phenotype of OCD diagnosis and the quantitative phenotype of total YBOCS score were considered. The presence/absence of tics (current and past) and the nature thereof (e.g. motor and/or vocal) was assessed with the SCID-OCSD and the Yale Global Tic Severity Scale (YGTSS), respectively (Leckman et al., 1989).

Questions pertaining to head injury, current medication, medical screening, developmental history, the presence of tics (current or past) and family history was also administered. OCD patients with a significant history of neurological disease, schizophrenia, schizo-affective disorder, other psychotic conditions or a history of substance dependence, as determined from the interviews or previous medical records, were excluded from the study.

Candidate gene association analyses

The target genomic fragment in *BDNF* (*val66met*, or rs6265) containing the selected polymorphism, was amplified by means of the polymerase chain reaction (PCR) and genotyped using published primer sequences (Sen et al., 2003). Allele specific restriction endonuclease analysis (ASREA) was employed to genotype the *BDNF val66met* polymorphism. The 274bp amplimer was digested with 0.5U *Nla*III (New England Biolabs, Beverly, MA., USA) for 3 hours. Two *Nla*III restriction enzyme sites were present, one constitutive site producing fragment sizes of 57bp and 217bp, and the other polymorphic. If the *val66*-allele was present, the *Nla*III enzyme did not recognise the second polymorphic site. If, however, the *met66*-allele was present, the restriction enzyme recognised the polymorphic site within the PCR product, generating fragments of 140bp, 77bp and 57bp in size.

Statistical analyses

Initial demographic analyses were conducted to determine whether between-group differences existed in gender (Fisher test for equality of proportions) and age (Wilcoxon test for equality between medians). Categorical variables were represented as counts, with their associated frequencies. Confidence intervals (CI) for medians of groups stratified by gender and *BDNF* genotype were set at 95% CIs simultaneous for all groups.

Logistic regression was used to model case-control status, and linear regression was used to model YBOCS score (severity) and age at onset. (There was no need to analyse age at onset with survival analysis techniques, because there were no censored ages at onset.) Each of the three response

variables: case-control status, YBOCS and age at onset, was initially modelled as a function of gender and *BDNF* genotype (the main effects), including the interaction between gender and *BDNF* genotype. The *BDNF* genotype factor was modelled as two variables, one counting the number of *met66*-alleles (0, 1 or 2: the additive effect [add]) and the other taking the value 0 for any homozygote and 1 for the heterozygotes (the dominance [dom] effect) as described by Cordell and Clayton (2005). For each of the three models, a stepwise procedure was employed to discard those terms that did not contribute significantly to the model. The resulting optimal models are described and summarised in the results.

Functions from base R and R packages (R Development Core Team, 2006) were used for all statistical analyses.

Structure

The possibility of population substructure was investigated by employing a Bayesian clustering method, *Structure* (version 2.0) (Pritchard, 2000; http://pritch.bsd.uchicago.edu/). *Structure* assigns individuals probabilistically to one or more sub-populations based on allelic frequencies at each locus. The procedure places individuals into 'K' number of clusters, where 'K' is chosen in advance, but can be varied across independent runs of the *Structure* algorithm. The value of K that maximised the posterior probability of the data was selected as representing the true number of clusters (genetically determined sub-populations) within the Afrikaner population. K was varied between one and five, and each analysis was repeated 10 times to assess convergence. Default values were used for all other parameter settings. Thirty-one polymorphisms were included in *Structure* analysis (Table 6; primer sequences and PCR protocols available upon request).

RESULTS

Analysis of clinical data

One hundred and twelve Afrikaner OCD subjects (57 [50.9%] male; 55 [49.1%] female) and 140 Afrikaner control subjects (33 [23.6%] male; 107 [76.4%] female) were investigated in the study (Table 1). The proportion of each gender was found to differ significantly between the OCD and control subgroups ($p < 0.001$). The median age for males in the control group (43 years [95% CI: 39-47]) was significantly higher than for any of the other three groups ($p < 0.001$). The median ages of the females in the OCD and control groups differed by 3.5 years, and were not significantly different.

Total YBOCS scores were recorded in 110 (98%) of the OCD patients; the median YBOCS score was 21.5 (95% CI: 20.0-23.0). No statistically significant differences in YBOCS scores were

observed between male and female subjects (p = 0.46) (Table 2). Age at onset of OCD was recorded for 102 (91.7%) of the OCD subjects, with the median age at onset of OCD 14.5 years (95% CI: 12.8-16.2). Although the median age at onset of OCD was lower in males compared to females (14 [95% CI: 12.3-15.7] and 16.0 [95% CI: 12.8-19.2] years, respectively), the difference was not significant (p = 0.55) (Table 2).

Analysis of genetic data

The final logistic regression model for case-control status is summarised in Table 3a. In it, both dominance terms (main effect and interaction) were discarded, indicating that the variant has a different additive effect for each gender, but no dominance effect. This final model fitted the data well, with a deviance difference of χ^2 = 346.2-320.6 = 25.6 at 3 df, p-value = 0.001. The additive term for males was independently statistically significant (p-value = 0.047), but not for females. This means that for males, the odds of having OCD increases significantly with each additional *met66*-allele. The modelled odds ratio of a female OCD subject versus a male OCD subject was 0.447 (95% CI: 0.234-0.845). The modelled odds ratios of OCD with the given genotype versus the *val66val* genotype are presented separately for each gender in Table 3b. The significant increase in the odds of OCD with increasing number of *met66*-alleles in males is apparent, as is the absence of a significant effect in females.

For analysis of the impact of gender and genotype on YBOCS score, there were only six individuals (one female) who possessed the *met66met* genotype (Table 2). The YBOCS scores of these six individuals were similar to those of the heterozygote individuals; thus these individuals were grouped together, creating a dichotomous genotype: *val66val* and "not *val66val*"; no dominance effect could therefore be estimated. No terms were removed from the linear model during stepwise selection. The optimal model for YBOCS analysis is summarised in Table 4a. Modelled YBOCS scores are shown in Table 4b. From these tables, it can be seen that, for males, those homozygous for the *val66*-allele do not possess significantly different YBOCS scores from those who are heterozygous or homozygous for the *met66*-allele. However, females homozygous for the *val66*-allele had significantly higher YBOCS scores compared to those who possess at least one *met66*-allele.

For analysis of the impact of gender and genotype on age at onset, only six individuals (five males) were found to possess the *met66met* genotype (Table 2). The single *met66met* female was grouped with heterozygous females for the modelling. All terms, including the dominance terms, were retained in the model. The model indicates that the genotype effect differed between genders, and also included a dominance effect. The final model fitted the data well ($F_{5,96}$ = 3.029; p = 0.0139) and

is summarised in Table 5a. The modelled age at onset for males and females for each genotype is provided in Table 5b. The significant heterozygote effects in Table 5a denotes that males and females have similar age at onset for heterozygotes and those homozygous for the *val66*-allele, whereas males possessing the *met66met* genotype present with significantly earlier age at onset of OCD. The fact that only a single female carrying the *met66met* genotype was included in the group means that any association between this genotype and age at onset in females could not be resolved.

Analysis of Afrikaner control population structure

A total of 40 Afrikaner controls were selected for genotyping for inclusion in the determination of population substructure in the present study. These individuals were genotyped for 31 unlinked autosomal polymorphisms, selected on the basis of location. The polymorphisms, their exact HWE values and heterozygosities are portrayed in Table 6. The mean heterozygosity for all 31 polymorphisms was 0.514. All of the polymorphisms were found to obey HWE.

The posterior probability values of K, assuming a uniform prior on K between 1 and 5, are provided in Table 7. The posterior probabilities favoured a K of 1. This is indicative of an absence of population structure within the Afrikaner population utilised in the present study. This result was corroborated by the examining the membership coefficients (Q) for each individual for each value of K: the proportion of the population assigned to each cluster was roughly symmetric for K = 2 to K = 5.

DISCUSSION

The *BDNF val66met* variant was found to be associated with EO OCD in the male population, with the *met66*-allele increasing the risk for developing EO OCD. It has been suggested that EO OCD represents a developmental subtype of OCD (Rosenberg and Keshavan, 1998; Geller et al.,2001). EO OCD has been found to exhibit distinct patterns of neuropathology (Busatto et al., 2001) and phenotypic expression compared to LO OCD: it has been associated with male preponderance (Geller et al., 1998; Millet et al., 2004; Fontenelle et al., 2003) and a higher rate of Tourette Syndrome and comorbid tic and disruptive disorders (Geller et al., 1996; Millet et al., 2004; Rosario-Campos et al., 2001).

There is a large body of evidence supporting the involvement of the 5-HT and dopaminergic systems in the aetiology of OCD (Zohar et al., 1987; Greist et al., 1995; Hollander et al., 1992; Marazzitti et al., 1992). Interestingly, both 5-HT and dopamine (specifically, DRD3) [Goggi et al., 2003; Guillin et al., 2003]) have been found to interact closely with BDNF in the brain. Infusions of BDNF have been found to upregulate 5-HT metabolites in the brain (Siuciak et al., 1996; 1998), and BDNF has been

reported to promote and augment the function and growth of 5-HT neurons in the brain (Mamounas et al., 1995; 2000). The interaction between BDNF and dopamine in general may also be particularly relevant, given an association between a polymorphism in the gene encoding the dopamine receptor 4 (DRD4) and EO OCD has previously been observed in the South African Caucasian population (Hemmings et al., 2004). This possible interaction does, however, require further investigation.

In a recent investigation by Hall et al. (2003), it was found that the *val66*-allele, rather than the *met66*-allele, was *over*transmitted to EO OCD probands (although their sample was not stratified according to gender). One reason for the contradictory results may be population-based differences in allele frequencies of polymorphisms: although the alleles associated with age at onset differ between the studies, it may be that the *val66met* variant is not itself the causal variant, but that either of these alleles are in LD with the disease-susceptibility variant depending on the population involved.

Interestingly, in the present study, females carrying the *val66val* genotype were found to be possess higher YBOCS scores than those carrying at least one *met66*-allele. This finding is intriguing, given present observations where the *met66*-allele was found to increase susceptibility to EO OCD in males, but not in females. These findings may indicate differences in the mechanism whereby BDNF contributes to the respective OCD subtypes, and/or female-specific epistatic or epigenetic interaction(s), which increase the risk for a more severe form of OCD in females, but not in males.

No evidence for cryptic population substructure in the Afrikaner control population was observed in the current genetic analyses: classification of the Afrikaner individuals into clusters demonstrated symmetry, with roughly the same proportion of each individual's genome assigned to each cluster. The number of clusters, chosen to vary from K = 1 to K = 5, was based on the reported ancestry of the Afrikaners (the population has been proposed to originate from five Northern European populations: Dutch, German, French, Belgian and British [Botha and Beighton, 1983]). The evidence thus suggested that no cryptic substructure exists within the Afrikaner population, supporting the use of the population in genetic case-control association studies.

A limiting factor to detecting population substructure in the present investigation is that, if substructure within the Afrikaner population does exist, it is likely to be very subtle, given their past geographical and cultural isolation, and derivation from geographically closely-related Northern European groups. Detection of such subtle substructure may require the use of many more markers, increasing the amount and cost of genotyping to a great extent. It should also be noted that the most informative marker to use when investigating whether population stratification exists would be those that possess large frequency differences between the proposed subpopulations (Campbell et al.,

2005). It is thought that the so-called ancestry-informative markers that are currently available will not be sufficient to detect structure in closely related populations; identification of such markers in the Afrikaner population will entail assessing the frequencies of a large number of variants across the proposed contributing sub-populations.

In conclusion, the cumulative, preliminary results suggest that the *BDNF met66*-allele increases the risk for developing EO OCD in males, and that the *val66val* genotype increases the severity of OCD in females. The hypothesis that BDNF may be regulated, at least in part, by estrogen makes for an intriguing result, in that one could thus hypothesise that sex-specific estrogen-mediated effects, perhaps acting at certain stages of neurodevelopment, may underlie the aetiology of some aspects (particularly the age at onset and development of co-morbid tics) of the disorder.

The small sample size remains the most important limitation of the present study and efforts are underway to increase the Afrikaner OCD and control sample size, in order to improve the power of the study. In addition, given the close relationships between BDNF and serotonin, and BDNF and dopamine, it is important to assess the epistatic interactions between these genes, as it is likely that susceptibility alleles will act in unison with one another to bring about the clinical phenotype.

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Table 1. Genotype and allele distributions in OCD and control individuals.

	Genotype (%)			Total	Allele (%)	
	$n_{val66val}$ (%)	$n_{val66met}$ (%)	$n_{met66met}$ (%)		n_{val66} (%)	n_{met66} (%)
OCD	73 (65.2)	33 (29.5)	6 (5.3)	112	179 (79.9)	45 (20.1)
Control	95 (67.9)	43 (30.7)	2 (1.4)	140	233 (83.2)	47 (16.8)
Male OCD	33 (57.9)	19 (33.3)	5 (8.8)	57	85 (74.6)	29 (25.4)
Male control	25 (75.8)	8 (24.2)	0 (0.0)	33	58 (87.9)	8 (12.1)
Female OCD	40 (72.2)	14 (25.5)	1 (1.8)	55	94 (85.5)	16 (14.5)
Female control	70 (65.4)	35 (32.7)	2 (1.9)	107	175 (81.8)	39 (18.2)

Abbreviations: **OCD**: obsessive-compulsive disorder.

Table 2. Summary statistics for YBOCS scores and age at onset of OCD in the total OCD sample, and stratified by gender.

	Total OCD sample[a]			Male			Female		
Genotype	**n**	**median**	**95% CI**	**n**	**median**	**95% CI**	**n**	**median**	**95% CI**
YBOCS	**110**	21.5	20.0-23.0	**56**	21.0	18.7-23.3	**54**	22.0	20.1-23.9
met66met	**6**	18.5	13.3-23.7	**5**	18.0	12.3-23.7	**1**	19.0	-
val66met	**32**	18.0	14.9-21.1	**18**	20.5	16.8-24.2	**14**	16.0	10.9-21.1
val66val	**72**	22.5	20.9-24.1	**33**	21.0	17.7-24.3	**39**	23.0	21.0-25.0
met66met + val66met	**38**	18	15.2-20.8	**23**	18	14.9-21.1	**15**	16	11.7-21.0
AGE AT ONSET	**102**	14.5	12.8-16.2	**53**	14.0	12.3-15.7	**49**	16.0	12.8-19.2
met66met	**6**	9.5	2.7-16.3	**5**	5.0	0-11.7	**1**	45.0	-
val66met	**31**	14	10.9-17.1	**17**	13.0	9.2-16.8	**14**	16.0	8.8-23.2
val66val	**65**	15	13.2-16.8	**31**	15.0	13.2-16.8	**34**	15.5	12.2-18.8
met66met + val66met	**37**	14	11.1-16.9	**22**	13.0	8.3-17.7	**15**	18.0	11.1-24.9

[a]:Total OCD sample for whom total YBOCS or age at onset was recorded**.**

Abbreviations: OCD: Obsessive-compulsive disorder; **CI:** confidence interval; ***BDNF:*** brain-derived neurotrophic factor.

Table 3a. Summary of optimal logistic regression model for case-control status. ($\chi^2_3 = 6 = 25.6$, p = 0.001).

	Effect	SE	z-value	p-value
(Intercept)	1.10	0.37	2.93	0.003
Female	-2.00	0.48	-4.14	<0.001
add	0.86	0.43	1.99	0.047
Female:add	-1.21	0.57	-2.13	0.033

add = number of *met66*- alleles in genotype (0, 1 or 2).

Table 3b. Modelled simultaneous odds ratios (ORs) for males and female subjects separately. Each OR represents the odds of an individual with the given genotype having OCD versus not having it relative to the same odds for someone with the *val66val* genotype.

.Genotype	OR	
	male	female
val66val	1.000	1.000
val66met	2.367	0.779
met66met	5.605	0.606

Table 4a. Summary of optimal YBOCS linear regression model. ($F_{3,106} = 3.746$; p = 0.013).

	Effect	SE	t-value	p-value
(Intercept)	19.83	1.59	12.47	<0.001
Female	-3.76	2.53	-1.49	0.140
val66val	0.66	2.07	0.32	0.751
Female:*val66val*	6.81	3.11	2.19	0.031

Table 4b. Modelled YBOCS scores for male and female OCD subjects.

Genotype	Modelled YBOCS score	
	Male	Female
val66val	20.49	23.54
not *val66val*	19.83	16.07

Table 5a. Summary of optimal linear regression model of age at onset. ($F_{5,96} = 3.029$; p = 0.0139).

	Effect	SE	t-value	p-value
(Intercept)	17.13	1.62	10.58	<0.001
Female	-0.31	2.24	-0.14	0.892
add	-4.57	2.17	-2.11	0.038
dom	3.33	3.08	1.08	0.283
Female:add	32.75	9.40	3.49	0.001
Female:dom	-30.11	9.82	-3.07	0.003

add = number of *met*66-alleles in genotype (0, 1 or 2); **dom** = 1 if *val66met* heterozygote, 0 otherwise.

Table 5b. Modelled ages at onset for male and female OCD subjects.

Genotype	Modelled age at onset of OCD	
	Male	Female
val66val	17.13	16.82
val66met	15.88	18.21
met66met	7.98	-

The single met66met female was grouped with heterozygous females for the modelling, therefore we did not estimate age at onset for the *met66met* female group.

Table 6. Genetic markers used in "*Structure*" analysis, indicating the chromosomal location, major allele frequency and p-value for HWE test and heterozygosity values for each variant

Marker Name	Location	Variant type	Genbank Identifier	HWE p-value	Heterozygosity
FXIIIB	**1q31-32**	*Alu* ins/del	AY69222.3	0.260	0.343
TPOX	**2p23-2pter**	VNTR (AATG)n	M68651	0.815	0.827
D2S441	**2p**	VNTR (ATAG)n	G08191.1	0.402	0.871
DRD3	**3p21**	SNP	rs6280	1.000	0.379
D3S1766	**3p21**	VNTR (GATA)n	G08269	0.337	0.719
DRD1	**5p35.1**	SNP (A-48G)	X58987	0.143	0.333
DAT	**5q15.3**	40bp VNTR	M95167	0.608	0.230
5-HT1Dβ	**6q13**	SNP	rs6296	0.176	0.282
ESRα	**6q25.1**	SNP	rs9340799	1.000	0.454
MOG	**6p21**	VNTR (CA)n	BX120002	0.135	0.731
DLX-6	**7q21-22**	SNP (intron 1 C-T)	AC004774.1	0.316	0.418
D7S820	**7q**	VNTR (GATA)n	AC004848	0.851	0.742
TPA25	**8p12**	*Alu* ins/del	AY291060.1	0.073	0.323
ADRA1C	**8p21**	SNP	cys492arg	0.463	0.419
GRIN1	**9q34**	SNP	rs10870198	1.000	0.515
BDNF	**11p15.5**	SNP	rs6265	0.595	0.275
DRD2	**11p23.2**	SNP	rs1800497	0.590	0.286
GRIN2B	**12p13.3**	SNP	rs1806201	1.000	0.446
5-HT2A	**13q14-21**	SNP	rs6311	0.543	0.550
CHGA	**14q32.12**	SNP	rs735726	0.169	0.264
GABRA5	**15q11**	VNTR (CA)n	AC131310.3	0.160	0.741
FES	**15q25**	VNTR (GATA)n	X06292	0.133	0.742
PV92	**16q24**	*Alu* ins/del	AF302689.1	0.575	0.237
GRIN2A	**16p32**	VNTR (GT)n	AF443855	0.923	0.828
D16S539	**16q22**	VNTR (GATA)n	G07925	0.584	0.839
ACE	**17q11**	*Alu* ins/del	AF118569.1	0.729	0.541
5-HTT	**17q11**	VNTR	AF126506.1	1.000	0.482
D18S51	**18q21.3**	VNTR (GAAA)n	X91254	0.224	0.856
PLCG1	**20q12**	SNP	rs8192707	1.000	0.389
GNAS	**20q13.2**	SNP	rs7121	0.744	0.487
ABCG1	**21q22.3**	SNP	G2457A	0.499	0.425

Abbreviations: **HWE**: Hardy-Weinberg equilibrium**; FXIIIB:** Factor 13B; **TPOX:** thyroid peroxidase**; DRD3**: dopamine receptor 3**; DRD1**: dopamine receptor 1**; DAT:** dopamine transporter**; 5-HT1Dβ:** serotonin receptor type 1D beta**; ESRα:** estrogen receptor alpha**; MOG**: myelin oligodendrocyte**; DLX-6:** Distal-less like homeobox 6; **TPA25:** Tissue plasminogen activator Alu insertion; **ADRA1C**: Adrenergic receptor α1C; **GRIN1**: glutamate receptor type 1; **BDNF**: brain-derived neurotrophic factor; **DRD2**: dopamine receptor type 2; **GRIN2B**: glutamate receptor , ionotropic, N-methyl D-aspartate 2B; **5-HT2A:** serotonin receptor 2A; **CHGA:** chromogranin A (parathyroid secretory protein 1)**; GABRA5:** gamma-aminobutyric acid (GABA) A receptor, alpha 5**; FES:** feline sarcomere oncogene**; PV92:** : predicted variant Alu insertion repeat; **GRIN2A:** Glutamate receptor, ionotropic, N-methyl D-aspartate 2A; **ACE:** angiotensin-converting enzyme**; 5-HTT:** serotonin transporter**; PLCG1:** phospholipase-gamma 1; **GNAS**: guanine nucleotide-binding α subunit of G_{s}; **ABCG1:** ATP-binding cassette, sub-family G (WHITE), member 1; **VNTR**: variable number of tandem repeats; **SNP**: single nucleotide polymorphism.

Table 7. Estimated posterior probabilities of K for the combined control and OCD samples.

K	Total Sample	
	ln P (X|K)[a]	Posterior Probability (P[K|X]) [a]
1	-1972.96	0.90
2	-1977.64	0.01
3	-1976.12	0.04
4	-1981.42	0.00
5	-1975.72	0.06

[a]The probability that an individual will occupy a particular cluster (sub-population), given the observed genotype data
Abbreviation: OCD: Obsessive-compulsive disorder.

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