AN INVESTIGATION OF GENETIC AND EPIGENETIC FACTORS IN THE REGULATION OF GENE EXPRESSION IN SCHIZOPHRENIA: INSIGHTS INTO PATHWAYS INVOLVED IN PATHOGENESIS.

Thesis submitted to Imperial College London for the degree of Doctor of Philosophy (PhD)

By

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-2011-

Declaration

I declare that this thesis submitted for the degree of Doctor of Philosophy is my own composition and the data presented herein is my own original work.

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ABSTRACT

Schizophrenia is a common psychiatric disorder with a complex aetiology that has a strong genetic component as well as a major contribution from non-genetic factors. Whilst GWAS studies have not yielded substantial evidence of DNA variants linked to schizophrenia, microarray expression studies, which reflect both genetic and environmental factors, have provided evidence of candidate genes whose expression changes significantly in schizophrenia. The present thesis is based on a prospective microarray study carried out on two brain regions implicated in schizophrenia: frontal cortex and superior temporal cortex (Maycox et al., 2009; Barnes et al., 2011). SNP variants in six of these genes were genotyped and allele, genotype and haplotype analysis carried out initially to investigate associations with levels of expression found in these microarray studies. Subsequently SNP associations with disease were investigated in an extended case-control cohort from the East UK region. The selected candidates genes, highlighted from microarray studies, reflected two main functional categories: synaptic plasticity and the Wnt signalling pathway. Initial ANOVA revealed a significant interaction of disease status with genotype/haplotype in ZnT3 with respect to levels of expression. Significant effects of specific genotypes and haplotypes in CACNA1E and FRZB on expression were also observed. Furthermore, ZnT3 was the gene for which the strongest evidence of association with schizophrenia was obtained in the East UK cohort, with four SNPs associated at the allelic and genotypic level. Significant associations of a number of haplotypes of ZnT3 with disease status were also identified. In addition, modest evidence of statistical epistasis between genes within both functional groups was identified. Finally, a method for analysis of CpG island methylation was developed based on

bisulphite sequencing and methylation specific PCR (MSP) using previously validated primers for DKK3 (Yue et al., 2008). Although DKK3 showed a low density of methylation, the application of this method may prove useful for other candidate genes.

Acknowledgments

I thank Professor Jackie de Belleroche for her constant support and guidance throughout my Studentship. Thanks to Dr. Alex Morris for sharing with me his extensive experience in genetic and statistical analysis and for all his help and interest in the project. To the rest of the Neurogenetics group for their assistance and their disposition, particularly to Dr. Praveen Paul and Ms. Han-Jou Chen for the help with the experiments but above all for their friendship.

Many thanks to Dr. Magdalena Sastre and Callum Parr for kindly providing constructs for the Wnt Signalling Pathway analysis. I am indebted to all my colleagues for always taking the time to assist me and teach me, it has been an honour to be able to work with such talented scientist but most importantly such wonderful people. Special thanks to Ms. Sofia Abrahamsson for being friend and companion, for the conversations and her understanding and for being truly one of the most remarkable persons I have known.

I owe my deepest gratitude to my parents for their unwavering support and to my sister for being my inspiration and my guide. I would not be who I am and where I am without you. To the rest of my family and friend for being there and making this process much easier. Finally, to Paul for always holding my hand and for helping me keep my head where it should be. I look forward to the following years.

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CHAPTER 1 General Introduction

Schizophrenia is a common psychotic disorder characterized by a diversity of symptoms that usually vary from patient to patient and some of which may be shared by other disorders. However, consensus has been reached regarding those symptoms that appear to be characteristic of schizophrenia under the criteria that if they appeared in isolation they would lead almost certainly to the diagnosis of this particular disease (McKenna, 1994).

Schizophrenia has a lifetime risk of 1% in the general population and the onset occurs in most cases in late adolescence or early adulthood. It has been observed in a number of studies that male patients tend to have an earlier age of onset, however these findings are not consistent across different populations (Wong et al., 2003). The age of onset has been also related to the severity of disability since a number of studies have reported a differences in cognitive impairment of late-onset schizophrenia patients compared to youth-onset individuals (Rajji et al., 2009; Kao and Liu, 2010).

Although numerous family, twin and adoption studies have revealed a strong genetic component to the pathogenesis of schizophrenia, the fact that there is only 50% of co-transmission in monozygotic twins suggests a high contribution of non genetic factors, such as environmental factors, to the development of the disorder. However, there is still limited information on how these factors interact and to which extent are they contributing to the outcome.

1.1 Diagnosis and characterisation of Schizophrenia

Schizophrenia is characterised by a variety of symptoms that include psychosis but that also extend to impairment of cognitive function and mood disorder. Moreover, there are a number of other disorders that, like schizophrenia, have a strong psychotic component making it harder to provide an accurate diagnosis for each case. Therefore the correct diagnosis of schizophrenia requires the identification of a combination of signs and symptoms that not only account for the alterations of perception but also the impaired cognitive and social function and ultimately the incapacitating character of the disease. The procedure followed currently for the identification of schizophrenia is fully described in the Diagnostic and Statistical Manual of Mental Disorders on its fourth edition (DSM-IV-TR; American Psychiatric A., 2000). Although the criteria contained in this manual are used as a standard method of diagnosis for research around the world another commonly used guide, particularly in Europe is the Chapter V: Mental and behavioural disorders from the International Classification of Diseases on its tenth edition (ICD-10; World Health Organization).

The method for diagnosis described in the DSM-IV-TR consists of six diagnosis criteria: Criterion A defines the characteristic symptoms of the disease while criterion B describes the signs involved in social and occupational dysfunction. Criterion C specifies the duration of the disturbance necessary for the diagnosis of schizophrenia over other psychotic disorders. The three first criteria are complemented by criterion D designed to rule out schizoaffective and mood disorders. Finally, criterion E is aimed to assess the possible effect of substances or general medication while

criterion F explores the additional diagnosis of schizophrenia in individuals suffering from, or with a history of, pervasive developmental disorders such as autism.

1.1.1 Characteristic symptoms of Schizophrenia

Symptoms regarded as characteristic of Schizophrenia are generally described as contained into one of two broad categories: Positive and negative symptoms. The symptoms comprised in these two groups constitute what is known as active-phase symptoms. The presence of two or more of the aforementioned symptoms for a period of at least 1 month when they are accompanied by other signs leading to impaired function.

Symptoms referred to as positive are commonly presented in intermittent and acute episodes and represent 'an excess or distortion of normal function'. Positive symptoms are broadly a reflection of distorted or intensified functions such as perception (hallucinations) or inferential thinking (delusions) and therefore, represent the psychotic component of the disease. However, other symptoms representing the disorganized component of the disease are also listed as positive.

Delusions

Delusions are defined as misinterpretations of perceptions or experiences. They usually manifest as a strong belief that cannot be substantiated and that is held in spite of evidence to the contrary. While these can be related to a variety of themes the most common in schizophrenia cases are those delusions where the individual holds the strong conviction of being tormented, followed or spied on even against clear contradictory evidence. Especially characteristic of the disease are delusions that for the lack of correlation with ordinary life experiences are considered to be

clearly implausible. These delusions have been identified as *bizarre* and include loss of control over mind and body (e.g. belief of thoughts being taken away from the mind of the patient by an external force). Although these are difficult to assess and depend generally on the context, the presence of bizarre delusions is considered to be sufficient to satisfy criterion A in the diagnosis of schizophrenia.

It is important to note that for the purpose of diagnosis, delusion must be recognised from dogmatic ideas or illusions. There is also a distinction between delusion and a belief that is based on incomplete information or on information that due to its nature may not be easily deemed as false (e.g. religious and spiritual beliefs). Finally, the cultural context plays an important role in the diagnosis of delusion. Indeed, this cultural component to the identification of delusion is subjective and it has become a source of debate on whether it is necessary to define more precise models for diagnosis (Mujica-Parody and Sackeim, 2001).

Hallucinations

Hallucinations are considered distortions in perception and it is possible to find them in any sensory modality. Auditory hallucinations however have been reported to be the most common in schizophrenia and are usually perceived as voices distinguishable from the patient's own thoughts. In most cases the content the hallucinations tends to be pejorative or threatening and in particular cases they consists of two or more voices conversing with one another or holding a permanent commentary over the affected person. When this specific type is present it is considered to be the only requirement needed to satisfy criterion A of the diagnosis.

Disorganisation Syndrome

The second component of the positive symptoms includes disorganised speech, often been referred to as *formal thought disorder.* Regarding this component the criteria indicates that the impediment must be severe enough, at least during the active phase of schizophrenia, to account for largely impaired communication. The final component to the disorganisation dimension of the positive symptoms is disorganised behaviour and is commonly manifested as clearly inappropriate behaviour or unpredictable and untriggered agitation among others. In addition, catatonic motor behaviour could also be present and in some cases to the extreme degree denoted by complete unawareness to external inputs, adoption of rigid postures and resistance to be moved or purposeless and unstimulated excessive motor activity.

The negative symptoms of schizophrenia are regarded as 'diminution or loss of normal function' they usually occur early before the onset of the disease and are more enduring and difficult to treat which makes them a reflection of the chronic disability of the disorder. Symptoms referred as negative often include affective flattening, decreased fluency of speech (alogia), lack of motivation to perform goal-directed activities (abolition) and diminished capacity to experience pleasant emotions (anhedonia) among others. The presence of sever negative symptoms may lead to cognitive dysfunction represented by confusion, disorientation and memory and attention impairment. It is particularly important to consider the possibility of some of these negative symptoms manifesting as a response to medication or the experience of positive symptoms.

1.1.2 Schizophrenia Subtypes

Because of the broad spectrum of symptoms associated with schizophrenia the definition of subcategories has been a helpful tool for the diagnosis and treatment of the disorder. Moreover it has been suggested that definition of schizophrenia subtypes might be key in the identification of underlying mechanisms that could be particular to the pathophysiology of defined groups of cases. From the clinical point of view there are five recognised subtypes of schizophrenia described in the DSM-IV and defined on the basis of the predominant symptoms present in each case.

Paranoid

Paranoid schizophrenia is diagnosed when there is prevalence of prominent delusions and hallucinations but relatively normal cognitive function and affection. Delusions in paranoid schizophrenia can be multiple but generally share a common theme to which hallucinations are generally related as well. When compared to other subtypes, the onset for paranoid schizophrenia is presented at a late stage and the characteristic features tend to be more stable with time. Although this subtype is considered as one of the most severe and the chances of prognosis tend to be low, due to an inclination to suicidal and/or violent behaviour, the fact that there is little or no impairment in cognitive function suggests that the possibility of independent living for individuals that respond positively to treatment more likely than for other subtypes.

Disorganised

Disorganised or hebephrenic schizophrenia is characterised by disorganised speech and behaviour and signs of emotional flattening over other symptoms. In individuals

suffering from this type hallucinations and delusions, if present, are generally not associated with one another and do not share a common theme and in most cases there is no evidence for neuropsychological or cognitive impairment. Along with paranoid, disorganised type is deemed to be highly incapacitating due to the inability of most of the affected patients to perform daily-life activities such as preparing meals or dressing as a result of disorganised behaviour. The onset of this subtype is usually an early one with significant remissions being rare.

Catatonic

Diagnosis of catatonic type of schizophrenia only takes when the patient presents a distinctive psychomotor disturbance that cannot be attributed to medication or any other general medical condition. In addition, the individual must meet the full criteria for schizophrenia in order to rule out major manic-depressive disorder. Motor disturbance may be manifested in a number of ways ranging from excessive activity to immobility. The former is usually untriggered and it tends to lack purpose while the latter can be present itself as muscle rigidity (catalepsy) or stupor. Other common symptoms present in this type include voluntary assumption of inappropriate postures, continuous repetition of words or phrases (echolalia) and repetitive imitation of others (echopraxia). In severe cases of catatonic schizophrenia there is an increased risk of patients harming themselves or others. Other potential risks associated with this type include hyperpyrexia, exhaustion and malnutrition.

Undifferentiated and Residual

The two final subtypes of schizophrenia refer to cases that present enough symptoms to meet the criteria for diagnosis of the disease but that do not fall into

any of the three main schizophrenia types previously described. Thus in the undifferentiated type is used to describe cases where the individual presents enough symptoms to comply with criterion A while residual type describes cases where, with the exception of an initial episode, there are virtually no positive symptoms. However, for the latter to be applied continued presence of the disturbance must be observed and might be enunciated by the presence of negative symptoms and/or two ore more attenuated positive symptoms. It is not uncommon for the occurrence of residual type to represent a transition between a "full-blown" episode and remission.

1.2 Neuropathology of Schizophrenia

The once accepted notion of the lack of defined neuropathology to schizophrenia has proved to be inaccurate by the collective evidence from imaging, histological and neurochemical studies. However, some obstacles remain mainly due to the fact that defined and quantifiable morphological abnormalities have been difficult to identify and replicate. However, when considered in the context of other genetic and molecular findings, data collected from imaging and histology studies have provided important insights into the pathogenesis of the disorder.

1.2.1 Macroscopic findings

The possibility conferred by magnetic resonance imaging (MRI) and other imaging techniques to analyse live tissue has proven to be a great advantage in the study of changes in the brain morphology in schizophrenia compared to healthy individuals. Among the most replicated features reported from these studies are: ventricular enlargement, more specifically the third and lateral ventricles, and small reductions

in whole brain volume and weight (Weinberger et al., 1983; Lawrie and Abukmeil, 1998; Wright et al., 2000; Hirayasu et al., 2000; Shenton et al., 2001; Harrison et al., 2003; Steen et al, 2006; Velakoulis et al., 2006; Arnone et al., 2009; Ellison-Wright and Bullmore 2009). These volume differences have been reported to be present in first-episode and drug-naïve patients (Steen et al., 2006) and in unaffected relatives (Lawrie et al., 1999), a fact that seems to link the described macroscopic changes with a genetic predisposition to the illness.

Other notable findings from imaging studies are reductions in volume of hippocampus, amygdala, parahippocampal gyrus and superior temporal gyrus as well as the temporal horn portion of the lateral ventricles, which results in an increase in cerebrospinal fluid (CSF) in the region surrounding these structures (Bogerts et al., 1990; Nelson et al., 1998; Lawrie et al., 1998; Niemann et al., 2000; Matsumoto et al., 2001; Sim et al., 2006) prefrontal and superior temporal cortices (Pearlson et al., 1999; Davidson et al., 2003) and thalamus (Andreasen et al., 1994; Konick and Friedman, 2001). However, these findings are not entirely specific for schizophrenia and some of them may not be present in all cases (Wright et al., 2000; Shenton et al., 2001; Harrison and Weinberger, 2005; Wong and Van Tol, 2003), which makes it difficult to define a neuropathological profile that could be used in diagnosis.

In spite of the differences between studies, advances in brain imaging have and will undoubtedly continue to contribute in the identification of the important structural abnormalities that are key in the understanding of the neurobiology of schizophrenia. Perhaps the most evident conclusion that can be drawn from the number of brain regions affected is that the disturbance of connectivity as a pathological feature of the disorder. As an attempt to identify the origin of this defect a number of theories

have been formulated, many of them proposing the idea that schizophrenia could be regarded as a neurodevelopmental disorder. Some of the data collected from imaging studies seems to support this notion. For instance, many of the structural abnormalities that have been reported from the limbic and temporal region seem to be present particularly in the left side of the brain, which is consistent with the language-related symptoms that characterise the disorder (Crow, 1998; Sommer et al., 2001). This idea has been further extended to the investigation of disrupted connectivity within a neuronal network not only relevant for language but also for memory formation (Nestor et al., 1993; McCarley et al., 1999).

1.2.2 Histological findings

In addition to the macroscopic findings collected from imaging studies on the structural abnormalities present in schizophrenia, post mortem brain studies have provided insight into more detailed neuropathological characteristics of the disease. Although, positive findings have been limited, important information has been gathered from negative studies regarding the nature of the disorder. Such is the case of the consistent lack of evidence for the association of schizophrenia with neurodegenerative processes or gliosis, even in schizophrenia cases presenting dementia (Arnold et al., 1996; Baldessarini et al., 1997; Arnold et al., 1998; Jellinger et al., 1999; Bernstein et al., 2009). These data provide further support to the idea of schizophrenia as a neurodevelopmental disorder rather than a progressive one.

Consistent with this idea of an important developmental component to schizophrenia are histological findings of aberrant location of clusters of neurons in entorhinal cortex (Arnold et al., 1991a; Akil and Lewis, 1997; Falkai et al., 2000) and other cortical regions (Akbarian et al., 1993a; 1993b; 1996; Kirkpatrick et al., 1999;

Eastwood et al., 2003) suggesting dysfunctional cell migration affecting connectivity. However, attempts to reproduce these data have not always been successful, which has led to questions about the origin of these aberrations when present (Beasley et al., 2002a).

Other well replicated data collected from histological studies in post-mortem brain of schizophrenia cases are observations of reduced cell body size of pyramidal neurons in hippocampus and neocortex of cases compared to controls (Benes et al., 1991a; 1991b; 1992; Arnold et al., 1995; Zaidel et al., 1997; Rajkowska et al., 1998; Pierri et al., 2001; Sweet et al., 2003). In addition, these neurons have been reported to have fewer dendritic arborisations and spines (Glantz and Lewis, 2000; Rosoklija et al., 2000; Black et al., 2004). Furthermore, these findings have been supported by a number of studies reporting dysfunctional regulation of expression of two dendritic molecular markers, microtubule-associated protein 2 (MAP2) and spinophilin in hippocampus of schizophrenia subjects (Arnold et al., 1991b; Law et al., 2004). Downregulation of genes coding for presynaptic markers in these regions has also been observed (Young et al., 1998; Thompson et al., 1998; 2003a; 2003b; Halim et al., 2003). Moreover, reductions in density of interneurons and their synaptic projections have also been reported in schizophrenia cases as well as alterations in GABAergic and glutamatergic synaptic populations in dorsolateral prefrontal cortex (DPFC) of affected individuals (Beasley et al., 2002b; Reynolds et al., 2002; Bitanihirwe et al., 2009). Finally, changes in dopaminergic signalling have also been observed (Akil et al., 1999) and the importance of interactions between glutamate and dopamine in the mechanisms underlying neurotransmitter dysfunction in schizophrenia have become apparent (Carlsson and Carlsson, 1990)

Although there are some inconsistencies across studies, overall these findings have provided evidence for deficient synaptic connectivity in schizophrenia. This approach has consistently been supported by following studies pointing to the conclusion that this abnormality of synaptic terminals seems to be mainly molecular and only in part morphological. This is idea is particularly interesting in the context of gene expression and association studies and it suggests an important role of synaptic plasticity processes in the pathophysiology of the disorder.

1.2.3 Neurochemical studies

The analysis of the mechanisms of action of antipsychotic and psychotomimetic drugs has been a useful tool in the attempt to unveil the molecular basis of schizophrenia. These studies provided support for two main theories involving neurotransmitter dysfunction in the aetiology of the disorder: The glutamate hypo-function hypothesis and the dopamine hyperactivity hypothesis.

The first theory was mainly supported by observations of the effects of phencyclidine (PCP) and ketamine, two non competitive antagonists of N-methyl-D-aspartate (NMDA)/glutamate receptors known for inducing not only schizophrenia-like psychosis but also cognitive dysfunction in healthy individuals (Rainey and Crowder, 1975; Snyder, 1980; Javitt and Zukin, 1991) and for exacerbating positive and negative symptoms in schizophrenic patients (Krystal et al., 1994; Lahti et al., 1995). These and other similar drugs have been used subsequently in the development of animal models of schizophrenia (Mouri et al., 2007; Javitt, 2007; Dyck et al., 2007; Broberg et al., 2008; Pedersen et al., 2009; Amann et al., 2010).

In addition to support from imaging and genetic studies, the dopamine hyperactivity hypothesis partly originated from observations of blockade of dopamine D2 receptors by neuroleptic agents (Seeman et al., 1975; Seeman and Lee, 1975; Creese et al., 1976). It was therefore proposed that overstimulation of the dopamine system was likely to be associated with the pathophysiology of psychiatric disorders. This premise has been the basis of extensive research in the search for better and more efficient antipsychotics and to the realization of a correlation between the D2 receptor affinity and the potency of the drug (Seeman and Tallerico, 1999; Kapur et al., 1999; Tauscher et al., 2004). The nature of the dopamine hyperactivity in schizophrenia remains to be fully elucidated. A number of studies have suggested the involvement of dopamine receptor-interacting proteins (Koh et al., 2003; Bai et al., 2004) while other researchers have examined whether the over activity is presynaptic or post-synaptic and what are the implications of each of these possibilities to the aetiology of schizophrenia (Abi-Dargham et al., 2000; Narendran et al., 2006; Graff-Guerrero et al., 2009; Kegeles et al., 2010).

In recent years, attention has turned to the possible intersection of these two hypotheses. The well documented role of dopamine in regulation of cognitive function (Gao et al., 2001; Seamans and Yang, 2004) and the close resemblance of the dysfunction induced by NMDA-receptor antagonists to the negative syndrome of schizophrenia have led to the conclusion of a possible dopaminergic dysregulation in striatal and prefrontal regions of the brain resulting from NMDA hypofunction (Balla et al., 2009; Li et al, 2010).

Serotonergic dysfunction in schizophrenia has also been proposed from neurochemical studies initially from the observation that lysergic acid diethylamide

(LSD), an antagonist of serotonin-2A receptor (5-HT2A), had hallucinogenic properties although, unlike PCP however, LSD-induced psychosis is not accompanied by the negative symptoms characteristic of the disorder (Woolley and Shaw, 1954). However, the basis of more recent studies concerning the role of serotonin in psychiatric disorders is the fact that many neuroleptic drugs seem to be strong 5HT2A antagonist but have relatively reduced affinity for D2 receptors (Meltzer and McGurk, 1999).

1.3 Identification of genetic risk factors for Schizophrenia

In spite of extensive investigation the exact mechanisms underlying the pathophysiology of schizophrenia have remain elusive. Heritability of the disorder calculated from family and twin studies has been reported to be between ~80% which highlights a strong genetic component to its aetiology (McGuffin et al., 1984; Tsujita et al., 1998; Cannon et al., 1998; Franzek and Beckmann, 1998; Cardno et al., 1999; Kläning et al., 2002). However, another important observation from these studies is that in pairs of discordant monozygotic twins the risk of psychosis for offspring of both siblings is similar. Moreover, concordance for schizophrenia in MZ pairs has been calculated as ~50%. Overall, these data suggest an important contribution of non-hereditary factors contributing to the expression of the schizophrenic phenotype.

Although the genetic epidemiology of schizophrenia is yet to be fully understood, it has become clear that the transmission of susceptibility genes occurs in a complex way and that it is likely to involve multiple loci conferring risk for the disorder (Gottesman and Shields, 1967; McGue and Gottesman, 1989). Perhaps one of the

main challenges that the polygenic nature of schizophrenia is the elucidation of the number of susceptibility loci involved and the extent of the risk associated with each of them. In an attempt to answer these questions, two main models have been proposed. The first one considers that there are multiple genetic variants with a small additive or multiplicative effect contributing to the risk of developing the disorder. This common-disease/common-variant hypothesis has proved to be valid for various common complex disorders including Crohn's disease, type II diabetes and obesity (Zeggini et al., 2007; Hampe et al., 2007; Rioux et al., 2007; Wellcome Trust Case Control Consortium et al., 2010). Unlike these examples in the case of schizophrenia, in spite of a number of studies supporting the implication of common polymorphisms with a small effect in the pathophysiology of the disease, there is still some debate as to whether this theory is the more suitable for the investigation of genetic factors conferring risk for the development of the disorder.

A second hypothesis has been proposed based on a growing body of evidence pointing to a significant association of rare large variants with the disease (Basset et al., 2008; Walsh et al., 2008; Xu et al., 2008; ISC, 2008; Stefansson et al., 2008). This common-disease/rare variant hypothesis attributes genetic predisposition to schizophrenia to rare alleles with highly penetrant effects. There is still no consensus regarding which of these models is the most appropriate for the study of schizophrenia. However, evidence seems to suggest that these theories might not be mutually exclusive and that the combination of both in a more general model is more fitting for the study of schizophrenia and other psychiatric disorders.

1.3.1 Candidate genes from Linkage and Association studies

Traditionally, linkage analyses have been regarded as the starting point in the search for genes associated to common complex disorders. This analysis is based on the assumption that two alleles that are close to each other in a specific region of a chromosome are likely to be inherited together. Therefore, by genotyping of polymorphic markers in related individuals it is possible to identify loci associated with risk of a particular disease phenotype. Linkage studies have been applied successfully to Mendelian inherited disorders, but this method has some limitations when applied to complex disorders where it is expected that each of the loci implicated will account for only a small increment in risk. Moreover, epistatic and additive effects among genes are expected to have an implication in the accurate identification of associations (Bureau et al., 2009).

A number of susceptibility loci for schizophrenic phenotype have resulted from linkage analysis including 6p24-22 (Straub et al., 1995) 8p22-21, 13q14-q33 (Blouin et al., 1998; Gurling et al., 2001) 1q21-22 (Brzustowicz et al., 2000), 1q32.2-41 (Hovatta et al., 1999; Ekelund et al., 2001) 10q25.3-26.3, 2p12-22.1 (Lewis CM et al., 2003) and 22q (Williams et al., 2003), although in many cases attempts to reproduce these results in other genomewide studies have not been successful. Possible reasons for this lack of replication are varied, from differences in diagnosis to heterogeneity of the samples analysed (Owen et al., 2005). In spite the difficulties, three meta-analyses have been able to identify some interesting patterns (Badner and Gershon, 2002; Lewis CM et al., 2003; Ng et al., 2009). Amongst the most strongly supported regions are 8p, 22q, 1q, 6p, 2q, 3p, 5q, 14p and 20p.

Although undoubtedly valuable the limited capacity of linkage studies to detect weak linkage (Risch, 1990a; 1990b) has led to suggestions that the positive findings

reported might be representing segregation of rare alleles with a large effect. Alternatively, it is possible that the loci identified might contain several genes conferring small increments in risk.

The availability of information about the human genome has provided the possibility of determining the risk to schizophrenia conferred by specific candidate genes, both within previously identified linkage regions and in other chromosomal regions, thus complementing linkage studies. Association studies are usually performed by comparing frequencies of common allelic variants in affected and unaffected individuals. Common polymorphisms are variations in the DNA sequence that appear with a frequency of at least 1% in a certain population. Single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) are examples of these variations that have been of great relevance to the investigation of genes implicated in complex diseases. Copy number variants are large-scale polymorphisms, this is large fragments of DNA (one kilo base or greater) whose copy-number have been found to contribute to the phenotypic variation in humans and it has been proposed that they also have an important role in susceptibility to disease (Iafrate et al., 2004; Sebat et al., 2009).

The basic premise of these association studies is that when significant differences in frequencies of these polymorphisms are observed in cases compared to controls it is considered that the variant is associated to disease status or is in linkage disequilibrium with another risk variant. Evidence for SNPs associated with schizophrenia has been reported for a number of genes, although further validation has not been successful for all cases and functional relevance is still not clear even for some of the most robust findings.

Amongst the most interesting and most extensively studied candidates genes from linkage and association studies are:

- Dysbindin (DTNBP1) on chromosome 6p22.3 (Straub et al., 2002), also relevant for its importance for presynaptic and glutamate function (Numakawa et al., 2004; Talbot et al., 2004)

- Neuregulin (NRG1) on 8p21-22 (Stefansson et al., 2002; 2003). NRG1 includes cell signalling, axon guidance, synaptogenesis, glial differentiation, myelination and neurotransmission (Corfas et al., 2004; Roy et al., 2007; Buxbaum et al., 2008).

- D-amino-acid oxidase activator (DAOA, G72) in the linkage region of chromosome 13q22-34 (Chumakov et al., 2002). Implication of this gene with the aetiology of schizophrenia has also been supported by previous association studies (Schumacher et al., 2004)

- Regulator of G-protein signalling 4 (RGS4) on 1q22 (Chowdari et al., 2002). RGS4 is an interesting candidate gene for schizophrenia from a functional perspective for a number of reasons: Expression of the gene appears to be regulated by dopaminergic transmission (Taymans et al., 2004). Moreover, evidence of regulation of serotonergic and metabotropic glutamatergic receptors by RGS4 has been reported (De Blasi et al., 2001; Beyer et al., 2004) as well as evidence of interaction of RGS4 and ERBB3 in the brain (Thaminy et al., 2003).

- Catechol-O-methyl transferase (COMT) on chromosome 22q11.2 (Kunigi et al., 1997; Ohmori et al., 1998; Kotler et al., 1999; Murphy et al., 1999; Li et al., 2000; Egan et al., 2001; Murphy, 2002; Wonodi et al., 2003; Chen et al., 2004). Most of the association of COMT with schizophrenia has been related to a particular SNP in exon 4 of the gene, a G→A nucleotide change that results in an amino-acid substitution from valine (val) to methionine (met) at codon 158 of the transcript coding for MB-COMT. This change of amino-acids results in lower stability of the met-COMT enzyme, which implies lower activity compared to the val-COMT allele (Lotta et al., 1995). The importance of COMT in the catabolism of dopamine, especially in the prefrontal cortex (PFC) has further highlighted the possibility of the gene as a risk factor for the disease (Tenhunen et al., 1994; Lotta et al., 1995).

- Disrupted in schizophrenia (DISC1) in chromosome 1q42 (Ekelund et al., 2001; 2004; Hennah et al., 2003; Hodgkinson et al., 2004; Hashimoto et al., 2006; Qu et al., 2007; Chubb et al., 2008). The first suggestion of the implication of DISC1 in schizophrenia came from the observation that the gene was disrupted a balanced chromosomal translocation (1;11)(q42;q14.3) initially identified a Scottish family and strongly associated with the risk of schizophrenia, bipolar disorder and recurrent depression (St Clair et al., 1990; Millar et al., 2001; Blackwood et al., 2001). The implication of DISC1 in regulation of neurodevelopment (Miyoshi et al., 2003; Johnston et al., 2011) and neuronal progenitor proliferation in the adult brain (Kim et al., 2009) as well as the evidence for implications of the genetic variability of this gene in cognitive function in normal and schizophrenic individuals, has made the investigation of this gene more interesting (Hennah et al., 2005; 2009;

Callicott et al., 2005; Burdick et al., 2005; Cannon et al., 2005; Thomson et al., 2005; Liu et al., 2006).

- Brain-derived neurotrophic factor (BDNF). This gene has been considered as a candidate gene for schizophrenia from a number of association and expression studies (Weickert et al., 2003; 2005; Knable et al., 2004; Angelucci et al., 2005; Torrey et al., 2005). The hypothesis that disruption of BDNF might be implicated in the pathophysiology of the disorder is a compelling one due to the importance of the gene in cognition, learning and memory (Linnarsson et al., 1997; Hall et al., 2000) amongst other processes essential for normal neural function.

In addition to the candidate genes identified from point mutation analyses and chromosomal abnormalities, a number of other genes have been considered for association studies on the basis of their functional relevance. The most notable examples are metabotropic glutamate receptor-3 (GRM3) and dopamine receptors D2 (DRD2) and D3 (DRD3). Positive reports for GRM3 include evidence for associations a SNP in intron 3, as well as a number of two and three marker haplotypes, with schizophrenia (Fujii et al., 2003). Report of association of a second SNP in intron 2 with disease also came later (Egan et al., 2004). The author also demonstrated association of this SNP with impaired cognitive function in hippocampus and prefrontal cortex of both affected and unaffected individuals. In addition, they found a strong correlation of the presence of the high-risk allele of the SNP with downregulation of the glial glutamate transporter EAAT2.

In the case of dopamine receptors, reports suggests have implication of a functional SNP in the DRD3 gene responsible for an amino-acid substitution in residue 9 of the protein (Ser9Gly). Although the variation seems to confer small risk to disease, various studies have confirmed an association of the Ser/Ser homozygote with poor response to antipsychotic treatment (Williams et al., 1998; Jonsson et al., 2003; 2004; Hwang et al., 2010). Moreover, one study reported synergistic effect of the Ser9Gly variant and other three polymorphisms, all of which were found to be in strong linkage disequilibrium (Sivagnanasundaram et al., 2000). Finally, other studies have reported significant association of the Ser311Cys polymorphism in the DRD2 gene with schizophrenia (Glatt et al., 2003; Glatt and Jonsson, 2006)

1.3.2 Microarray technology applied to the analysis of Schizophrenia risk factors

Within the last three decades, the development of new and improved techniques as well as the construction of large genomic databases has enabled researchers to perform studies on a larger scale and in a hypothesis-free manner. This has provided a new approach to the investigation of the aetiology of schizophrenia through the use of microarray technology to carry out genome-wide association and expression studies in larger samples in order to increase the power of the analysis. Genome-wide analyses have been a valuable tool not only for the identification of candidate genes for schizophrenia but also for the elucidation of the disruption of certain functional pathways. Notably the data collected so far using microarray technology has confirmed findings that are consistent with previous hypotheses on the pathophysiology of schizophrenia, formulated from other studies.

Microarray technology works on the basis of the chemical composition of DNA and RNA molecules and their properties. Both molecules have the same basic structure, which consists in a chain of sugar molecules linked together by phosphate groups conforming what is called the backbone to which nucleotide bases are attached. Each nucleic acid molecule is constituted by a combination of four different nucleotide bases that are capable of forming hydrogen bonds with only one other base. Thus, thymine (or uracil in RNA) will bind to adenine and cytosine to guanine. This principle creates the possibility of using complimentary sequences for a variety of applications such as genotyping of single nucleotide polymorphisms (SNPs) or quantifying the amount of a given mRNA transcript being expressed in a given tissue or cell sample. In most of the platforms currently available, a number of known probes are attached to a solid substrate (commonly glass) in order to be immobilized. These probes can either be the complementary DNA sequence (cDNA for a specific mRNA transcript) or a synthetic oligonucleotide. The dimensions of the analysis depend on the design of the arrays and range from a few hundred probes to 10^6 samples in a high-density microarray. It is therefore possible to apply microarray technology to a number of applications that include resequencing, detection and genotyping of single nucleotide polymorphisms, identification of specific protein binding sites and analysis of epigenetic mechanisms such as DNA methylation or small RNA-DNA interactions (Brent and Ausubel, 2005).

Microarray technology has been extensively used for the identification of genetic factors contributing to risk of schizophrenia in genome-wide association studies (GWAS). In addition, microarrays have been used for the quantification of mRNA transcript levels in large numbers of tissue or blood samples. This approach has

provided the possibility of identifying differentially expressed genes when comparing groups of samples from controls and affected individuals.

1.3.3 Schizophrenia risk factors identified from genome-wide association studies

Linkage and association have been extremely valuable in the identification of genomic regions associated to schizophrenia. They have also provided a basis for more detailed analysis of the role of candidate genes in the disease through functional analyses. However, this approach has proven to have considerable limitations in the study of common complex traits. As an example of these limitations we can mention that the selection of candidate genes has depended either on their location in previously reported linkage regions or on their participation in functional pathways that are considered relevant to the disorder. This introduces the need for numerous hypothesis-based candidate gene association and expression studies in order to single out particular genes within a broad region. One other important drawback is the reduced power of linkage analysis in the identification of modest effects of common genetic variants. Thus, considering these and other previously discussed factors, the need for improved complementary techniques became evident.

Within recent years the development of faster and more accessible genotyping techniques along with the possibility of using a selected set of polymorphisms as markers as well as the completion of the International HapMap project (International HapMap Consortium, 2005; 2007) have made it possible to perform these studies in a genome-wide manner and without the need of previously established hypotheses.

This genome wide association studies (GWAS) have the advantage over linkage studies that they provide information not only on specific loci associated to schizophrenia but also on groups of genes potentially related through specific pathways. An additional advantage is that GWAS are able to detect small effects with greater power than other previously used techniques when the appropriate considerations are made (Barret and Cardon, 2006). However it is important to note that there are also some limitations associated with this type of studies that need to be considered for the interpretation of data (Wang et al., 2005).

The first important difficulty to be address when performing GWAS is to determine how to limit the occurrence of false positive associations and therefore increasing the possibility of replication. The starting point in approaching this dilemma is the calculation of the threshold for genome-wide significance, in order to address the issue of multiple testing when a large number of markers (typically around 500,000 SNPs) are compared (Thomas et al., 2005). However, the parameters considered in each case to perform these calculations tend to vary and this has to be taken into account when interpreting the data. Recently Dudbridge and Gusnato (2008) performed an estimation of a genome-wide significance threshold for the UK Caucasian population using genotypes from the Wellcome Trust Case-Control Consortium (WTCCC). They proposed a threshold value of $p < 7.2 \times 10^{-8}$ and an additive odds ratio (OR) of 1.5. According to this calculation, at least 12000 samples of each group, controls and cases, is necessary in order to achieve a power of 0.8. Furthermore, following this model it has been calculated that a GWAS that has been carried out in approximately 1000 cases and controls still could identify a risk allele, even at a lower threshold ($p < 10^{-5}$) if there is a relatively large number (> 100). However, replication of these results, even at a nominal level of 0.05, is still only

expected when using samples larger than 2500 cases and controls of these alleles to be detected (O'Donovan et al., 2009). While these calculations are to be considered merely as guidelines they point out the importance of large cohorts in order to minimise false positives and improve the chances of identifying small effects.

SNP-based GWAS

There are to date eight genome wide association studies in schizophrenia (Table 1.1). An initial study was performed using 320 schizophrenia cases and 325 controls of European ancestry. Over 25000 SNPs were screened in this study using a combination of pooled DNA approach and individual genotyping but with no significant genome-wide association reported (Mah et al., 2006). A second GWAS in two small groups of Caucasian samples of 178 cases and 144 controls reported association ($p=3.7 \times 10^{-7}$) of a SNP located in the pseudoautosomal region of chromosome Y to disease (Lencz et al., 2007).

A following larger study of a heterogeneous group of 738 cases and 733 controls failed to identify any significant association to the disorder (Sullivan et al., 2008). Similar results were obtained from two additional studies performed using pooled DNA samples. The first one of these was performed using Bulgarian samples consisting on 574 schizophrenia trios and 605 controls (Kirov et al., 2008a) while the second one used 600 cases and 2,777 controls, all Ashkenazi Jews (Shifman, et al., 2008). It is noteworthy that although no evidence for association with schizophrenia was reported from either of these studies Shifman et al. observed a genre-specific association of an intronic SNP in the reelin gene (RELN) with an increased risk of schizophrenia in women. They were further able to support this finding in a meta-

analysis of 2,000 cases and 4,000 controls. Furthermore a recent study has been able to replicate this sex-specific association in an independent group of 721 schizophrenia and schizoaffective disorder cases and 1455 controls of Ashkenazi Jewish background (Liu et al., 2010). The association of this SNP to schizophrenia is an interesting one, particularly considering previous reports of down-regulation of the RELN gene in schizophrenia (Knable et al., 2004) as well as evidence suggesting that this might be a product of epigenetic mechanisms (Grayson et al., 2005; Tamura et al., 2007)

In a more recent GWAS O'Donovan and colleagues (2008) compared an initial group of 479 cases to 2,937 controls from the Wellcome Trust Case Control Consortium (WTCCC). The most significant loci observed from this initial study ($p < 10^{-5}$) were subsequently validated in two additional cohorts for a total of samples of up to 6,829 cases and 9,897 controls. The best-supported association with schizophrenia was that of an intronic SNP (rs1344706) in the zinc finger protein 804A gene (ZNF804A). Although this association did not reach genome-wide significance ($p= 1.6 \times 10^{-7}$) in the schizophrenia samples, this was achieved ($p=1.0 \times 10^{-8}$) when a bipolar disorder cohort from the WTCCC was included leading to the conclusion that the variations is more likely to be associated with a general psychotic phenotype. Because the precise function of the ZNF804A protein in the brain is still not clear nor are the mechanisms through which this variant contributes to psychosis predisposition. However, an insight was obtained from recent imaging study that reported association of the same SNP with the degree of correlation of hippocampus and dorsolateral prefrontal cortex activities suggesting a role in the disturbance of connectivity across these brain regions (Esslinger, et al., 2009).

Consistent with O'Donovan et al., replication of the association of rs1344706 was observed (p=0.029) from a later study performed by the International Schizophrenia Consortium (ISC, 2009) where 3,322 cases and 3,587 controls were genotyped for a large number of SNPs (> 1 million). However, the most significant finding from this study was the genome wide association ($p= 3.4 \times 10^{-7}$) of a SNP in the myosin 18B gene (MYO18B). The second most significant association observed from this study was that of a group of 450 SNPs spanning a region in the major histone compatibility complex (MHC) on chromosome 6. Interestingly this association has been further supported by two more GWAS. The first one of these was performed using the Molecular Genetics of Schizophrenia (MGS) samples of 2,681 cases and 2,653 controls or European ancestry in addition to 1,286 cases and 973 controls of African-American ancestry with no genome-wide significant associations reported. However, a meta-analysis performed using the MGS European samples, the ISC and the SGENE datasets (for a total of 8,008 cases and 19,077 controls) yielded a significant association (p= 9.54 x 10-9) of a locus within the 6p22.1 region, which includes the MHC and other immunity related genes (Shi et al., 2009). The second GWAS was performed using the SGENE dataset (2,663 schizophrenia cases and 13,498 controls from eight different European locations) for genotyping a total of 314,868 SNPs. Although no significant genome-wide association was observed from this study, further inclusion of the genotyping information obtained from the ISC study resulted in association of several SNPs in the MHC region (p values ranging from 1.1×10^{-9} to 1.4×10^{-12}). In addition, significant association was observed for a polymorphism located upstream of the neurogranin gene (NRGN) on the 11q24.2 region ($p= 2.4 \times 10^{-9}$) as well as for the SNP in intron four of the T cell transcription factor 4 (TCF4) on 11q24.2 ($p=4.1 \times 10^{-9}$) (Stefansson et al., 2009).

Interestingly both these genes are involved in neurodevelopmental and cognitive processes (Huang FL et al., 2007; Flora et al., 2007) with NRGN encoding for a postsynaptic protein kinase substrate abundantly expressed in brain regions relevant to cognitive functions and especially enriched in CA1 pyramidal neurons in the hippocampus (Huang FL et al., 2007). NRGN is known to bind to calmodulin (CaM) and it has been proposed as its reservoir in the postsynaptic compartment for the regulation of NMDA-receptor signalling through calcium/calmodulin-dependent protein kinase II (CAMKII) action in (long-term potentiation in the hippocampus) synaptic plasticity and formation of associative memories in the brain (Bliss and Collingridge, 1993; Lee SJ-R et al, 2009; Rose et al., 2009). TCF4 is in turn involved in a number or important processes including differentiation of neural progenitors through Wnt signalling pathway activation (Hirabayashi et al., 2004)

Finally, a SNP-based GWAS (Need et al., 2009) was performed comparing an initial group of 871 unrelated schizophrenia cases with 863 controls from European background. Further validation of the top 100 SNPs was then carried out using four independent cohorts of similar ancestry for a total of 1,460 cases and 12,995 controls collected from Munich. The authors report a strong association of a SNP (rs2135551) in the 3' UTR of the ADAMTSL3 gene. Although this association did not reach genome-wide significance, it is noteworthy that the small size of the replication sample has a very limited power for the detection of small effects.

There is no doubt that GWAS have provided a valuable insight into genetic risk factors for schizophrenia and possible functional pathways whose disruption might be underlying the pathophysiology of the disease. However, it is also clear from the lack of replication in most cases, even with the use of large cohorts, that there are still

obstacles to overcome before it is possible to determine the extent to which small effects from common variants are able to explain the genetic variation of the disease.

CNVs in Schizophrenia

Chromosomal mutations contributing to susceptibility to schizophrenia have been identified in affected individuals and families from studies previous to GWAS and include disruptions in genes such as DISC1 (Blackwood et al., 2001) and the deletion in the 22q11.2 region of chromosome 6 (Murphy et al., 1999; Liu et al., 2002; Basset et al., 2008). In later years, the use of microarrays provided the means to identify smaller events. An important point on this subject was the limited capacity of the arrays used to perform GWAS in detecting the effects of rare SNPs but their ability to identify large rare copy number variants (CNVs) of moderate effect, even if they are only present in one or very few individuals. The result has been the increasing interest in CNVs associated with schizophrenia with a number of genome wide association studies assessing their incidence in affected individuals (Table 1.2).

One such study performed by Kirov et al. (2008b) using 93 schizophrenia trios and 372 matched controls reported two variations likely to be pathogenic. The first one of these was a deletion in the 2p16.3 region that results in a disruption of the neurexin 1 (NRXN1) gene. The second one is a *de novo* duplication affecting amyloid precursor-binding protein A2 (APBA2) gene at 15q13.1. Both of these genes encode for proteins involved in synaptic development (Sudhof, 2008). Further support for the disruption of neurodevelopmental pathways by CNVs was provided by a later study performed in a cohort of 150 schizophrenia cases and 268 matched controls (Walsh

et al., 2008). From this study, the authors identified mutations affecting type I transmembrane tyrosinekinase receptor for neuregulins (ERBB4) and solute carrier 1 A3 (SLC1A3), a glutamate transporter.

Other studies have provided evidence for the increased frequency of rare variant in individuals with schizophrenia. In their study in Xu et al. (2008) observed a number of *de novo* CNVs with an increased frequency in sporadic cases of schizophrenia as compared to familial. They analysed 359 schizophrenia trios and 154 control trios collected from the Afrikaner population in South Africa. Amongst the most notable polymorphisms reported by this study are microdeletions in the 22q11.2 region disrupting DGCR8, a gene with an important function in miRNA processing (Beveridge et al., 2010). Another member of this pathway, DICER1, was found to be affected in one individual by a duplication in the chromosomal region 14q32.13-32.2.

Two more recent GWAS show the effect of large rare variants. Kirov et al. (2009) used a group of 471 UK recruited cases and 2792 controls from the Wellcome Trust Case Control Consortium (WTCCC) to survey large CNVs (> 100 Kb). From this study, the authors observed that only CNVs larger than 1Mb, particularly deletions, were significantly increased in cases. Two of these deletions were observed in more than one case and include the previously identified 22q11.2 region as well as a deletion in the 17p12 region. In addition they report significant association with schizophrenia of a duplication on 16p13.1 previously reported as a susceptibility region for autism (Straub et al., 1995). Finally, in the previously discussed study of SNPs and CNVs in schizophrenia Need and colleagues (2009) observed 8 large deletions (>2 Mb) in cases but not in controls in a subset of 1,013 cases and 1,084 controls. These deletions also included the previously observed 1q21.1 and 22q11.2 regions and two

more newly reported on 8p22 and 16p13.11-12.4. However overrepresentation of large duplications in this study failed to reach significance. Therefore this data does not provide strong support for the hypothesis proposing a greater frequency of large rare CNVs in schizophrenia.

Although some of these findings are supported from previous studies it is clear that very few of them are consistent across GWAS, perhaps due to the already discussed need for large samples in order to increase the power of the analysis. With this in mind, findings from two larger studies become particularly notable with two CNVs significantly associated with schizophrenia reported by both. The first one initially identified 66 *de novo* CNVs from a study performed in a population-based sample of 9,878 transmissions (2,160 trios and 5,558 parent-offspring pairs) with no history of schizophrenia (Stefansson et al., 2008). They later used an initial sample of 1,433 schizophrenia cases and 33,250 controls of European ancestry to test for associations of the identified CNVs with disease. The results from this first stage were subsequently followed up using a second cohort of 3,285 cases and 7,951 controls. From this study, three CNVs resulted significantly associated with schizophrenia in the 1q21.1, 15q11.2 and 15q13.3 regions. Two of these variations (1q21.1 and 15q13.3) were also reported as conferring risk for schizophrenia by an independent study of 3,391 patients and 3,181 matched controls (ISC, 2008).

From the analysis of these studies it becomes clear that, even with the use of large samples and with careful control of confounding factors, there are only a few polymorphisms whose implication with schizophrenia has been possible to replicate. However when considered collectively this data seems to point to the conclusion that both, common and rare variants, are important and they might need to be analysed

together in order to obtain a complete view of the genetic factors conferring risk to the disorder. Moreover, recent studies have taken this approach to interrogate regions previously reported as associated with schizophrenia in more detail. An example of this is the study of the region 17q23-17q24 (Carroll et al., 2010) where significant linkage to schizophrenia had been previously identified. They screened 1028 SNPs within that region in 1,755 unrelated schizophrenia cases and 3,580 controls of European ancestry. Their most notable finding was the significant association ($p=0.02$) of a SNP in the protein kinase C, alpha (PRKCA) gene, although comparison with independent groups of samples did not yield the same result.

Reference	No. Samples		Ancestry	Genotyping	Notable Findings
	C	S			
Mah et al., 2006	325	320	European	Pooled DNA and individual genotyping	No significant GW association
Lencz et al., 2007	144	178	Caucasian (U.S.)	Individual genotyping	rs4129148 in **CSF2RA** in pseudoautosomal region ($p=3.7 \times 10^{-7}$)
Shifman et al., 2008	600	2771	Ashkenazi Jews.	Pooled DNA	No significant GW association. Genre-specific association of a SNP in **RELN** with risk of schizophrenia
Liu et al., 2010	1455 (834 female)	721 (259 female)	Ashkenazi Jews.	-	Genre-specific association of a SNP in **RELN** with risk of schizophrenia
Kirov et al., 2008a	605	574	Bulgarian (Schizophrenia trios)	Pooled DNA samples. Schizophrenia trios	No significant GW association
Sullivan et al., 2008	733	738	African American, European American and Other	Individual genotyping	No significant GW association
O'Donovan et al., 2008	479	2937	Caucasian (UK) from WTCCC	Individual genotyping	Intronic SNP (rs1344706) in **ZNF804A** associated with psychosis phenotype ($p= 1x10^{-8}$)
	3541	1664	Caucasian (Irish, German and Bulgarian)		
	7897	6666	German, European Ancestry (Australia/USA), Han Chinese and Ashkenazi Jewish		
ISC, 2009	3587	3322	European	Individual genotyping	• SNP in **MYO18B** ($p=3.4 \times 10^{-7}$) • 450 SNPs spanning the region of the **MHC** in chr. 6 • Replication of association of SNP in **ZNF804A**
Shi et al., 2009	2,653	2,681	European	-	No significant genomewide association
	973	1,286	African-American		
Meta-analysis	19,077	8,008	European (MGS, ISC, SGENE)		Locus in **6p22.1** including the **MHC**
Stefansson et al., 2009	13,498	2,663	European (SGENE)		No significant genomewide association
Meta-analysis			ISC	-	• Several SNPs in **MHC** • SNP in **NRGN** and one SNP in **TCF4** genes
Need et al., 2009	863	871	European	Individual genotyping	No significant GW association
	713	298			
	12,995	1,460	Caucasian ancestry deCODE U.S.A.		

Table 1.1 Summary of most recent findings from SNP based GWAS in schizophrenia cases and controls.

Reference	No. Samples		Ancestry	Locus Identified
	C	S		
Kirov et al., 2008b	372	93	Bulgarian proband-parent trios	**2p16.3 and 15q13.1**. Disrupting **NRXN1** and **APBA2**
Walsh et al, 2008	268	150		Disrupting genes in neurodevelopmental pathways (e.g **ERBB4** and **SLC1A3**)
Xu et al., 2008	154	359	Afrikaner population, South Africa Schizophrenia and control trios	Rare *de novo* CNVs with high penetrance associated to schizophrenia including **22q11.2** and **14q32.13-32.2**
Stefansson et al., 2008	33,250 7,951	1,433 3,285	European ancestry	Three significant CNVs: In **1q21.1, 15q11.2** and **15q13.3**
ISC, 2008	3,181	3,391	European ancestry	Support for association of CNVs in: **1q21.1** and **15q13.3**
Kirov et al., 2009	2,792	471	Cases recruited in UK, controls from WTCCC	Only large CNVs (> 1Mb) significantly increased in cases including deletions in **22q11.2** and **17p12** and a duplication in **16p13.1**
Need et al., 2009	1,084	1,013	Caucasian	No significant CNVs 8 large (> 2Mb) deletions observed in cases but not in controls in regions including **1q21.1, 22q11.2, 8p22** and **16p13.11-12.4**

Table 1.2 Summary of copy number variants (CNVs) and large rare polymorphisms associated with schizophrenia.

Most notable associations identified from genomewide microarray studies in schizophrenia cases and controls**.**

1.3.4 Use of microarrays for the identification of differential gene expression in schizophrenia

One of the major applications for microarrays in the study of complex disorders has been the analysis of differences in gene expression in affected individuals as compared to controls. The use of microarray technology for gene expression studies relies on the availability of data about the human transcriptome. Probes complementary to known sequences can then be used to measure the levels of specific mRNA transcripts in a particular tissue sample. Prior to hybridization to the probes, the target mRNA is labelled with a fluorescent dye in order to enable subsequent quantification of levels of gene expression based on the assumption that the level of fluorescence is proportional to the amount of mRNA collected by each site on the array.

While microarray expression profiles have proved to be of great value in the elucidation of molecular pathways possibly disrupted in schizophrenia through the identification of groups of genes differentially expressed in cases, there are certain limitations that need to be considered in the analysis and interpretation of data (Mirnics et al., 2006). Many of these limitations come from the fact that in the study of psychiatric disorders the comparison of transcript levels in affected individuals and healthy controls is commonly performed using post-mortem brain tissue, although peripheral blood cells have also been used for a number of studies. The importance of carefully controlling these and other factors is highlighted by the fact that differences in gene expression in the brain of affected individuals are likely to be relatively small and are therefore more likely to be affected by assay noise.

The first critical issue to be considered with the use of clinical samples is the integrity of the mRNA that could be affected by a number of factors including sample storage and freezing. It is commonly assumed that in samples with a post mortem interval (PMI) of up to 30 hours most of the tissue contains high-integrity mRNA. However, PMI in itself is considered to be a poor predictor of mRNA quality and is often complemented with other methods such as the measurement of brain pH and RNA degradation. The pH of brain samples is an indirect indicator of mRNA integrity because it greatly depends on the terminal state of the patients with low levels commonly observed in tissue samples from individuals with prolonged agonal periods or death after a coma (Bahn et al., 2001; Li et al., 2004; Mexal et al., 2006). It has been recently reported that the pH of post-mortem brain samples seems to have a great effect in gene expression (Li et al., 2004; Mexal et al., 2006). Furthermore, it was possible from these and other studies to identify a "critical" pH value a below which these transcriptional changes occur (Li et al., 2004; Iwamoto et al., 2005). It is therefore important to identify this value in each particular sample empirically.

Moreover, because the samples available for study are in most cases obtained at a much later stage following the initial onset of the disease it is possible that the changes observed are in fact a product of a normal aging process or a reflection of the brain adapting to changes caused by the disease throughout time rather than a direct consequence of disease-causing factors.

One other major limitation that has been encountered when performing microarray expression studies in brain tissue is the fact that these samples are composed of a mixture of different cell types including subtypes of neurons and glial cells. This heterogeneity makes it very difficult to identify certain potentially significant gene

expression changes that might only be taking place in a particular group of cells. In addition to this, it is possible that the left and right hemispheres of the brain have different levels of expression of certain transcripts and this might introduce another source of variation.

Additionally, genetic background, gender and lifestyle as well as neuroleptic treatment are likely to have an effect on the transcript levels and it is important to be able to differentiate these effects from the ones that could potentially be associated with disease. However, in the case of medication, an additional difficulty is presented because post-mortem tissue of untreated patients is hardly ever available. Although animal models and tissue culture provide valuable information about the effects of medication on gene expression in the brain, the question remains on to what extent this information translates into the effects of neuroleptic treatment in the diseased human brain.

Finally, in order to minimise the variation it is ideal for controls and cases to be correctly matched prior to the analysis with respect to age, ethnicity, medication history, post-mortem interval, sex, cause of death and lifestyle amongst other factors. However, the reduced availability of post-mortem introduces the need for finding a balance and resulting in the need for compromises. One of such compromises often implies that the studies are performed with a very limited number of samples and therefore with a reduced power for the identification of significant expression changes.

There are a variety of different microarray platforms available that have been used so far for expression studies in complex disorders. As is the case with other

applications the probes used for hybridization of mRNA transcripts of interest can be cDNA or synthetic oligonucleotides. In order to quantify transcript levels target mRNA needs to be labelled prior to hybridization, a process that is usually performed by reverse transcription of RNA to cDNA and subsequently labelled and transcribed back into cRNA. The samples can be labelled with a single tag or double labelled. Single labelling is a popular technique when using human samples because it allows the comparison of expression data across various experimental groups. Double-labeling is used commonly for comparison of matched samples such as case-control samples.

From the variety of commercially available microarrays perhaps the most commonly used is the Affimetrix GeneChip, an oligonucleotide array. This platform consists on a series of probe-set representing approximately 39,000 transcripts. Probe-sets are conformed by 11 probe-pairs designed for each gene and distributed across the array. Each of these pairs consists of one perfect match oligonucleotide and a mismatched used to quantify and correct for non-specific hybridization and background signals. The chip contains control probes to determine hybridization efficiency. In addition to this and other commercially available microarrays, it is possible that the use of a custom microarray results more adequate when the objective is to study a specific group of genes (e.g. genes involved in a specific pathway). An example of this case is the NIA-neuroarray comprised of 1,128 clones selected representing families of genes that include: transcription factors, structural genes, synaptic, neuronal, glial, cell adhesion molecules, kinases, phosphatases, proteases and oncogenes (Vawter et al., 2001).

As discussed previously, by carefully controlling factors such as quality of the mRNA/cDNA, proper matching of samples and clinical history of the subjects it is

possible to minimise experimental variability and improve reliability of the data. However, even with a rigorous quality control and the application of the appropriate algorithms to interpret the signals, validation of the data is crucial. This is regularly performed through qPCR analysis and verification in an additional cohort might be considered if possible. Moreover, there are still some technical drawbacks associated with the use of microarray technology. One of these limitations is the fact that it is still not possible for microarray platforms to distinguish between splice variants of a gene and therefore it is not possible to identify posttranslational or posttranscriptional changes. In addition to this is the limited ability of microarrays to uncover differences in expression of genes with low abundance. Thus, it becomes important to consider the use of complementary techniques not only for the verification of microarray data but also for further investigation of mechanisms regulating the observed differences in expression.

In spite of the fact that replication of results has not been possible for all studies, presumably due to the differences in study groups, microarray platforms used and methods of analysis, expression microarray studies have provided important information about specific pathways potentially disrupted in schizophrenia. Some of the most replicated results include genes implicated in synaptic function, mitochondria-energy metabolism and ubiquitin degradation and oligodendrocyte function (Table 1.3).

Synaptic function

Perhaps the first microarray study to report differential expression of genes involved in synaptic processes was the one performed by Mirnics et al. (2000) and which

consisted of the expression profiling of over 250 genes in post-mortem tissue from the prefrontal cortex (Brodmann Area 9) of 11 pairs of matched pairs of schizophrenia and control samples. Results from this study revealed a decrease in the expression of a group of transcripts encoding for proteins involved in presynaptic function in schizophrenia. Amongst the changes most consistently replicated across pairs were N-ethylmaleimide sensitive factor (NSF) and synapsin II. In addition genes involved in glutamate (GLU) and GABA neurotransmission were also found to be decreased in schizophrenia compared to controls. These findings were then confirmed by in situ hybridization in a different cohort of schizophrenia samples. Consistent with these results, the same group of genes reported by Mirnics et al. (2000) were found downregulated in a later microarray study of 1127 genes in the same brain region (Vawter et al., 2002). Relative gene expression of 15 pooled schizophrenia samples and 15 matched controls was assessed for this study in order to identify genes whose expression is consistently changed across schizophrenia cases. In addition to reduced expression of genes in the PSYN, GLU and GABA groups, histidine triad nucleotide-binding protein (HINT), ubiquitin conjugating enzyme E2N (UBE2N) and glutamate receptor, ionotropic, AMPA 2 (GRIA2) were found to have reduced expression in schizophrenia.

Interestingly, in a follow-up study using the same dataset Mirnics et al. (2001) observed a number of disease specific changes in the postsynaptic cell. Most notably they identified reduced expression of regulator of G-protein signalling 4 (RGS4) as the most significant change in schizophrenia, which is consistent with previous reports (Bowden et al., 2007). Moreover, in their microarray analysis, Mirnics et al. (2000) reported that expression of ten other RGS family members and over 200 genes involved in G-protein signalling remained unchanged as a group. The

relevance of RGS4 in relation to schizophrenia has been considered not only because RGS-modulated G-protein-coupled receptors (GPCRs) are targets for antipsychotic drugs (Lieberman et al., 1998) but also because it has been identified as a candidate gene for schizophrenia from association and linkage studies (Brzustowicz et al., 2000; Chowdari et al., 2002).

Two more studies have provided evidence for the dysfunction of synaptic pathways in schizophrenia. In the study conducted by Hemby et al. (2002) single-cell microdissection technique was used in satellite neurons from the entorhinal cortex of 8 cases and 9 matched controls (Hemby et al., 2002). A screening of more than 18,000 transcripts using cDNA custom arrays identified, amongst the most significant changes, reduced levels of mRNA encoding for glutamate receptor 3 (GRM3), N-methyl-D-aspartate receptor 1 (GRIN1), synaptic vesicle amine transporter, synaptotagmin I and IV and synaptic plasma membrane proteins SNAP 23 and SNAP 25. Finally Maycox et al. (2009) reported the result of a comparison between to independent microarray expression analyses. In the first study levels of expression of 30,000 mRNA transcripts were assessed in Brodmann Area 10 of the PFC of post-mortem tissue from 28 schizophrenia cases and 23 controls. This data was then compared with that obtained from a second study in BA9 of post-mortem brain samples of 16 schizophrenia cases and 27 controls carefully selected to match criteria used in the first group. The authors identified a total of 49 transcripts commonly regulated in both cohorts including genes implicated in synaptic vesicles recycling/secretion (e.g. ZnT3, VAMP2, SYT5), signal transduction (e.g. CACNB3, CACNG3, CAMKK2) and neurodevelopment (PHYHIP). It is noteworthy from this study that although no association was observed with genes previously proposed as

candidate genes for schizophrenia from genetic studies, some of the changes observed are consistent with disruption of DISC1 and dysbindin networks.

Metabolic function

Additional expression studies have provided evidence pointing to metabolic dysfunction as a possible pathological factor associated to schizophrenia. In an expression study carried out in samples from BA9 in the prefrontal cortex of 10 schizophrenia cases and 10 controls (Middleton et al., 2002) five metabolic pathways were found to be downregulated across schizophrenia samples including the mitochondrial malate shuttle system and ubiquitin metabolism. In a comprehensive analysis Prabakaran et al. (2004) carried out microarray analysis combined with proteomics and metabolome (low-molecular weight intermediates) analyses in post-mortem tissue also from BA9 of 10 schizophrenic samples and 10 matched controls from the Neuropathology Consortium of the Stanley brain collection. Significant downregulation of several genes involved in glycolysis, including pyruvate dehydrogenase (PDH) were detected. Additionally, a group of genes related to response to stress, signal transduction and transcriptional regulation were found to be upregulated in schizophrenia. Similarly, Iwamoto et al. (2005) have reported down-regulation of mitochondria-related genes in schizophrenia and bipolar disorder. Samples from the Stanley Array Collection were used for their study for a total of 35 samples that included post-mortem tissue from PFC (BA46) of individuals diagnosed with schizophrenia and bipolar disorder and healthy controls. The observed reduced expression of mitochondria-related genes was maintained even after considering the effect of pH of the tissue samples.

Interestingly, in a cell-specific study performed by Altar et al. (2005) in hippocampal granule neurons from the dentate gyrus of schizophrenia, bipolar disorder and depression cases the authors reported a group of mitochondrial energy metabolism related genes whose expression was decreased exclusively in schizophrenia. The study included 9 bipolar, 10 depression and 14 schizophrenic cases as well as 15 control individuals, from two different groups. Among the genes whose expression was reduced in both schizophrenia groups were nicotinamide adenine dinucleotide (NADH) and mitochondrial ATPase. In addition, other genes representing mitochondrial function, such as mitochondrial ribosomal protein S28 (MRPS28), solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11 (SLC25A11) and stannin (SNN), were reported by Maycox et al. (2009) as consistently downregulated in schizophrenia.

Oligodendrocytes and myelination genes

Although not consistently replicated, a number of microarray studies have identified genes implicated in oligodendrocyte function as differentially expressed in schizophrenia as compared to controls. An initial study in the BA46 of the PFC of 12 schizophrenia samples and 12 controls observed decreased expression in schizophrenia of a group of 6 genes predominantly enriched in myelin-forming oligodendrocytes (Hakak et al., 2001). Among these genes were the neuregulin receptor tyrosine kinase (ERBB3) and myelin associated glycoprotein (MAG) and lymphocyte protein. In a later microarray study of prefrontal cortex from 10 schizophrenia cases and 19 matched controls downregulation of the proteolipid protein 1 (PLP1) across the majority of the affected individuals was identified (Pongrac et al., 2002). To discount the change as a possible result of neuroleptic

treatment, data from this study was compared to one carried out in a pair of haloperidol and saline treated monkeys (Mirnics et al., 2001) with no replication of downregulation of PLP1.

Consistent with both of these studies were similar findings from microarray expression profiling of BA9 of 15 schizophrenia cases and 15 matched controls, together with 15 bipolar disorder cases from the Stanley Medical Research Institute collection (Tkachev et al., 2003). This data was further confirmed by differential display PCR and showed significant downregulation of PLP1 in schizophrenia in addition to other oligodendrocyte myelin-related genes, including myelin-associated glycoprotein (MAG), oligodendrocyte-specific protein (CLDN11) and myelin oligodendrocyte glycoprotein (MOG) which resides within the MHC locus in chromosome 6p21.3 (discussed in section 1.4.1). Furthermore, a third study of 1373 genes in dorsolateral prefrontal cortex (DLPFC) of schizophrenia cases and controls reported that the highest frequency of significant transcript expression changes were observed in oligodendrocyte and astrocyte-related genes (Sugai et al., 2004). The authors also used tissue samples from non-human primates treated and untreated (n=3 each) with haloperidol for three weeks prior to the study in an attempt to detect effects due to neuroleptic treatment. Amongst the most significant changes were myelin basic protein (MBP), myelin associated oligodendrocytic basic protein (MOBP), T-cell differentiation protein (MAL) and phosphoprotein enriched in astrocytes 15 (PEA15).

In addition to the changes observed in PFC two more studies reported changes in the expression of oligodendrocyte and astrocyte genes in other brain regions. The first one was a study in post mortem tissue samples from middle temporal gyrus to

BA21 of 12 schizophrenia cases and 14 matched controls (Aston et al., 2004). Significantly reduced expression of myelin-related genes in schizophrenia was identified, including plasma membrane proteolipid (PLLP) and the previously reported MAG and ERBB3. Other notable findings from this study were the significant downregulation in schizophrenia of TNF receptor-associated factor 4 (TRAF4), a protein involved in neurogenesis, and histone deacetylase 3 (HDAC3), as well as an upregulation of a cicardian pacemaker protein (PER1). Finally, a study performed using tissue from 15 cortical regions as well as from hippocampus, caudate nucleus and putamen of schizophrenia individuals and controls Katsel et al. (2005) identified significant changes in a number of genes categorised by function. Amongst the top scoring categories, especially in temporal and cingulated gyri and hippocampus, were oligodendrocyte and myelin-related genes. Their findings included significant downregulation of MAG, PLLP, MAL, ERBB3 and MOG.

Expression studies have without question proved to be a valuable tool in the elucidation of molecular pathways implicated in the pathophysiology of schizophrenia. However the question remains as to whether these changes are part of the primary aetiology of the disorder or they are part of regulatory mechanisms more likely to be a reflection of secondary pathology. It is difficult to conclude that expression profiling by itself would be able to give us the answers. It is therefore evident that it is necessary to combine this technique with other studies in order to improve our understanding of this disorder and to determine how the possible combinations of numerous risk factors contribute to its aetiology.

Reference	No. Samples C	No. Samples S	Brain Region	Microarray platform	Notable Findings
Synaptic Function					
Mirnics et al., 2000; Mirnics et al., 2001	Two groups of 6 and 5 S-C pairs.		BA9	UniGEM-V (high density cDNA)	Decreased expression of genes involved in presynaptic function **(PSYN)**, glutamate **(GLUT)** and GABA transmission. Significant downregulation of **RGS4** in schizophrenia
Vawter et al., 2002	15	15	BA9	NIA-Neuroarray	Decreased expression of the same group of genes reported by Mirnics et al. (2000) Significant downregulation of **HINT, UBE2N** and **GRIA2**
Hemby et al., 2002	9	8	EC	Custom microarray	Reduced levels of mRNA encoding for **GRM3, GRIN1, synaptic vesicle amine transporter, synaptotagmin I and IV and SNAP 23 and SNAP 25**
Maycox et al., 2009	23	28	BA10	Affymetrix GeneChip (CX cohort)	49 transcripts commonly regulated in both cohorts: Genes implicated in synaptic vesicles recycling/secretion (**ZnT3, VAMP2, SYT5**), signal transduction (**CACNB3, CACNG3, CAMKK2**) and neurodevelopment (**PHYHIP**)
	27	16	BA9	MAS 5.0 (Harvard Cohort)	
Metabolic function					
Middleton et al., 2002	10	10	BA9	UniGEMV	Five metabolic pathways downregulated in schizophrenia including mitochondrial **malate shuttle system and ubiquitin metabolism**
Prabakaran et al., 2004	10	10	BA9	HG-U133A GeneChip Affymetrix	Downregulation of a group of genes implicated in **glycolysis** and upregulation of genes with a role in **oxidative stress**.
Iwamoto et al., 2005	35		BA46	HI-U133A GeneChip Affymetrix	Reduced expression of **mitochondria-related genes**.
Altar et al., 2005	15	14	Hippocampus (DG)	Agilent Human 1 (cDNA based)	Downregulation in schizophrenia of genes including nicotinamide adenine dinucleotide (**NADH**) and **mitochondrial ATPase**
Maycox et al., 2009	23	28	BA10	Affymetrix GeneChip (CX cohort)	**MRPS28, SLC25A11** and **SNN** downregulated in schizophrenia
	27	16	BA9	MAS 5.0 (Harvard Cohort)	

Table 1.3 Summary of notable Findings from microarray expression studies in schizophrenia.

Myelin and oligodendrocyte-related genes					
Reference	**No. Samples**		**Brain Region**	**Microarray platform**	**Notable Findings**
	C	**S**			
Hakak et al., 2001	12	12	BA46	HuGene F1 Affymetrix GeneChip	Decreased expression in schizophrenia genes enriched in myelin-forming oligodendrocytes, including **ErbB3, MAG** and **lymphocyte protein.**
Pongrac et al., 2002	10	10	PFC	UniGEM-V	Downregulation of **PLP1**
Tkachev et al., 2003	15	15	BA9	Affymetrix GeneChip	Downregulation of **PLP1, MAG, CLDN11** and **MOG** (in the MHC chr. 6p21.3)
Sugai et al., 2004	6	6	DLPFC	Clontech Atlas Human Neurobiology Clontech Atlas Human Cancer	Downregulation of glia-related protein genes including: **MBP, MOBP, MAL** and **PEA15**
Aston et al., 2004	14	12	Temporal Cortex	Affymetrix HgU95A GeneChip	Downregulation of myelin-related genes: **MAG, PLLP, ERBB3** Downregulation of **TRAF4** and **HDAC3** Upregulation of **PER1**
Katsel et al., 2005	13	13	BA 8, 10, 44, 46, 23/31, 24/32, 20, 21, 22, 36/28, 7 and 17. Hippocampus, caudate nucleus, and putamen	Affymetrix HG-U133 GeneChip	Downregulation of myelin-related genes including: **MAG, PLLP, MAL, ERBB3** and **MOG**

Table 1.3 (*Continued*)

1.4 Epigenetic mechanisms in Schizophrenia

The term epigenetics refers to a series of mechanisms that regulate gene expression through processes that do not involve the modification of DNA sequences. It has been proposed that epigenetic regulation may hold the key to the understanding of the interaction of genetic and environmental factors in the development of schizophrenia and other psychiatric disorders. Epigenetic mechanisms are not only dynamic partly in response to external input, they also are transmitted mitotically and in some cases epigenetic changes are able to remain even after gametogenesis and be transmitted across generations (Weaver et al., 2004; McLeod et al., 2007; Roth et al., 2009). Moreover, it has become increasingly evident that in the CNS epigenetic modifications can also occur as part of processes that play an important role in activity-dependent gene expression regulation in fully differentiated cells as a response to environmental factors in cognition and behaviour as well as learning and memory processes (Guan et al., 2002; Korzus et al., 2004; Tsankova et al., 2004; 2006; Vecsey et al., 2007; Miller et al., 2007).

Although there are number of epigenetic mechanisms that have been identified, the term has been most commonly used when referring to the modification of chromatin structure. Chromatin is the complex of DNA and proteins (histones and non-histones) that constitute the structure of chromosomes in the cell nucleus. The basic unit of chromatin is called a nucleosome and consists of ~147 DNA base pairs wrapping a histone octamer core (two copies of each of the four histones H1, H2, H3 and H4). There are two main conformations of chromatin that are relevant to gene expression, a condensed state known as heterochromatin and an extended state or euchromatin which in turn block or allow gene transcription respectively. In the cell nucleus

chromatin exists in a combination of these two states and remodelling of the conformation is possible through various processes such as covalent histone modifications and DNA methylation, which remain the two most extensively studied and best characterised in the brain (Jiang et al., 2008). Other epigenetic mechanisms include post-translational modification of mRNA transcripts by micro RNAs and other non-coding RNA molecules. The regulation of gene transcription through micro RNA-mRNA interactions has become a topic of great interest as a possible regulator of functional pathways in the CNS.

1.4.1 DNA methylation

One of the most important and most extensively studied epigenetic processes is the covalent bonding of a methyl group to DNA in the side chain residues of cytosines that are immediately followed by a guanine known as CpGs. These dinucleotide sequences are much less frequent in the genome that would be expected by chance and the majority are methylated (Cooper et al., 1989). However, the promoter regions of genes usually contain areas with a high density of CpGs that are normally methylated to a lesser extend (Gardiner-Garden et al., 1987; Bird, 1986). It is in these CpG islands where regulation of gene expression by DNA methylation has been observed.

The methylation reaction is carried out by transfer of a methyl group from S-adenosine methyonine (SAM) and catalysed by a group of enzymes known as DNA methyltransferases (DNMTs), which are divided in two categories. The DNMT1 isoform is a "maintenance" enzyme in charge of placing methyl groups in the complementary strands of already methylated cytosines after cell division. The

isoforms 3a and 3b are *de novo* DNMTs and they are responsible for new epigenetic changes (Okano et al., 1998).

A basic model for repression of gene expression has been proposed whereby the methylation of CpGs is followed by the recruitment of methyl-DNA binding proteins. In addition to the methyl-DNA binding domain (MBD), these proteins also possess a transcription regulatory domain (TRD) that binds to scaffolding proteins and ultimately to histone deacetylases (HDACs). These in turn remove acetyl groups from the histone proteins, thus altering chromatin structure and inhibiting transcription (Thomson et al., 2010). Although DNA methylation is generally associated with inhibition of gene expression there are some reports of methylation resulting in increased expression, but the mechanism for this have not been determined (Chahrour et al., 2008; Cohen et al., 2008).

There is some evidence that shows a correlation of the extent of methylation with gene inactivation, which is the basis for the "methylation density" model (Boyes and Bird, 1991; Cameron, et al., 1999). However, there have been reports of cases where there is only the need for methylation of one or few a cytosines located in trascription factor binding sites (Robertson et al., 1995; Pogribny et al., 2000). These studies later gave rise to the "critical site" model.

The implication of epigenetic mechanisms in the aetiology of schizophrenia has been supported by studies that have reported high levels of enzymatic activity of DNMTs in mature CNS neurons (Brooks et al., 1996; Ravindran and Ticku, 2005) as well as a number of studies that have provided evidence for the involvement of DNA methylation in synaptic plasticity and memory formation (Levenson and Sweatt,

2005; Levenson et al., 2006; Miller et al., 2007; Lubin et al., 2008; Nelson et al., 2008).

Moreover, there have been reports of aberrant methylation patterns in genes identified as associated with risk for schizophrenia such as the human Reelin gene (RLN) and GAD1 (Impagnatiello et al., 1998; Guidotti et al., 2000; Knable et al., 2004; Fatemi et al., 2005; Straub et al., 2007; Shifman et al., 2008; Liu et al, 2010). RLN encodes a glycoprotein that is secreted preferentially by GABA interneurons and is critical for neuronal migration during development and in supporting long-term potentiation in the adult CNS as well as regulating glutamate receptor homeostasis (Fatemi et al., 2005; Campo et al., 2009). The GAD1 gene in turn encodes the enzyme GAD_{67}, which is responsible for the synthesis of GABA from glutamate. The important role of these genes in the GABAergic system makes them interesting candidates for the elucidation of the molecular basis of schizophrenia.

From the initial discovery of DNA hypermethylation implicated in decreased expression of RELN and GAD_{67} in cortical and basal ganglia GABAergic interneurons of schizophrenic individuals (Chen et al., 2002; Costa et al., 2002) and the correlation of these findings with abnormal expression of DNA methyltransferase 1 (DNMT1) (Veldic et al., 2004; 2005; Levenson et al., 2006; 2007; Ruzicka et al., 2007), evidence has continued to accumulate in recent years pointing to the implication of aberrant epigenetic patterns in the promoter regions of these genes in the disorder. The majority of the studies performed thus far have attributed the decrease in protein levels to abnormal methylation patterns of CpG islands in the promoter regions of these genes (Abdolmaleky et al., 2005; Grayson et al., 2005; Benes et al., 2007; Huang HS et al., 2007).

Animal models have also been used in a number of studies in an attempt to elucidate whether these epigenetic modifications in RLN and GAD1 contribute to the schizophrenia phenotype. The heterozygote reeler mouse (HRM; Liu et al., 2001), characterised by reduced expression of *reelin* due to a single allele disruption, presents neuroanatomical and behavioural features similar to those observed in humans suffering from schizophrenia such as hippocampal-dependent learning deficit and impairment of hippocampal plasticity amongst others. The HRM has been used as a model for this and other disorders associated with cognitive impairment (Qiu et al., 2006). Moreover, treatment of mice with L-methionine (MET), necessary for DNMT catalytic activity, resulted in schizophrenic like behaviour in mice as well as replication of increased methylation of the RLN promoter and downregulation of both RLN and GAD1 genes in cortical interneurons (Dong et al., 2005). Additionally, the use of DNMT and HDAC inhibitors in neuronal progenitor cells has been shown to activate RLN and GAD1 expression (Kundakovic et al., 2009).

Other schizophrenia candidate genes for which abnormal patterns of CpG island methylation have been reported include Brain-derived neurotrophic factor (BDNF) and catecholamine-O-methyltransferase (COMT). Two studies have reported changes in methylation status of COMT in schizophrenia. One such study showed methylation of the MB-COMT isoform of the gene to be reduced in schizophrenia and bipolar disorder cases, which yielded higher levels of expression of MB-COMT when compared to controls. Moreover, inverse correlation was observed between the MB-COMT and dopamine D2 receptor gene (DRD2) expression. Finally, the authors reported an increase in the frequency of the 158-val allele of COMT in individuals presenting higher levels of methylation in MB-COMT (Abdolmaleky et al., 2006). The second report came from a microarray study (Mill et al., 2008) and although no

association of level of methylation with disease was reported, high levels of exonic methylation were consistently observed in individuals homozygous for the 158-val allele.

In the case of BDNF there is evidence pointing to a specific temporal and spatial regulation of specific promoter regions in the gene in an activity-dependant manner (Lauterborn et al., 1996), in spite of the complex structure of the gene (Liu QR et al., 2006; Aid et al., 2007; Pruunslid et al., 2007). In recent years a number of studies have made it evident that DNA methylation plays an important role in chromatin remodelling for learning-induced regulation of expression of BDNF (Martinowich et al., 2003; Lubin et al., 2008; Roth et al., 2009). However, only one study provided evidence for association of methylation status with genotype of SNP rs6265 (val66met) located in the vicinity of the gene in tissue from frontal cortex of schizophrenia and bipolar disorder individuals (Mill et al., 2008).

Genomewide epigenetic studies

There is only one study to date that has taken a genomewide approach to the analysis of epigenetic mechanisms possibly implicated in psychosis. In their study, Mill et al. (2008) analysed post-mortem brain tissue from the frontal cortex of 35 schizophrenia cases, 35 bipolar disorder cases and 35 matched controls from the Stanley Medical Research Institute. Using CpG island microarrays they identified a number of loci whose methylation status was associated with psychosis. Notably, they report disruption of loci relevant for glutamatergic and GABAergic neurotransmission, neurodevelopment and mitochondrial function and stress response. Amongst the most interesting findings from the study are the observed

downregulation of the vesicular glutamate transporter 1 (VGLU1) in schizophrenia cases, which correlated with hypermethylation of the promoter region of the gene in female schizophrenic individuals. The authors also reported increased expression of VGLU2 in the brains of schizophrenia cases, consistent with the reduced levels of methylation in the promoter region of this gene in female schizophrenia patients compared to controls. Other glutamate and GABA related genes highlighted in this study include Secretogranin II (SCG2) and MARLIN-1. The latter of these two is a RNA-binding protein known to have a role in regulation of the production of GABA receptors and was consistently hypermethylated in the three analysed groups (schizophrenia, bipolar disorder and major-psychosis). Finally, a significant correlation was observed in the methylation status of the gene encoding mitogen-activated protein kinase kinase I (MEK1) with lifetime antipsychotic use in schizophrenia male subjects.

1.4.2 Histone modifications

Another major epigenetic mechanisms for chromatin structure modulation is the post-translational tagging of histone proteins. Covalent modifications to histones are various and they occur in specific sites of their N-terminal tail. To date, there are five post-translational modifications that have been thoroughly characterised: Acetylation and ubiquitination at lysine residues, methylation at lysine or arginine residues, phosphorylation at serine or threonine residues and ADP-ribosylation at glutamate residues.

Acetylation of histones is perhaps the best characterised of these modifications in the CNS. The transfer of an acetyl group from acetyl-CoA to the $-NH^+$ group of the lysine residue of the histones is catalysed by a group of enzymes known as histone

acetyltransferases (HATs) (Tanner et al., 1999, 2000; Lau et al., 2000). Acetylation is associated with increased gene activation with hyperacetylation generally thought to promote gene activity through chromatin decondensation. However acetylation is a reversible process and there is evidence that suggests that it is actually the dynamic combination of acetylation and deacetylation that regulates the activation of gene expression. The process of deacetylation is catalysed by a group of lysine deacetylases generally known as histone deacetylases (HDACs). These enzymes perform their function mainly through Zn^{2+}-dependant charge-relay system (Finnin et al., 1999) or through nicotinamide adenine dinucleotide (NAD^{+})-dependent mechanism (Buck et al., 2004). Although the intracellular pathways that regulate histone acetylation in the CNS have not been well established, there are two signalling cascades that have been implicated thus far. The first one involves the mitogen-activated protein kinase (MAPK) super family. In this pathway the transcription factor cyclic AMP (cAMP)-response element binding protein (CREB) is phosphorylated through a series of signalling pathways that include cAMP, Ca^{+2} and extracellular-signal regulated kinase (ERK). Phosporylation in turn leads to activation of CREB and the recruitment of CREB binding protein (CBP), a transcriptional co-activator with intrinsic HAT activity. The second identified pathway involved in the regulation of histone acetylation in the CNS is the nuclear factor κ□ (NFκB) signalling pathway. While it has been established that this regulation of NFκB depends on the action of the inhibitor of κB kinase (IKK), however the precise mechanism are yet to be defined (Yeh et al., 2004; Lubin and Sweatt, 2007).

Following reports that HDACs inhibitors resulted in increased expression of REELIN in vitro (Chen et al., 2002), the first study to provide evidence of histone modification in schizophrenia was the one performed by Akbarian et al. (2005) who observed high

levels of histone methylation in prefrontal cortex (PC) of a subset of schizophrenia patients. Furthermore, they observed a correlation of this increased level of methylation with downregulation of four genes implicated in metabolic pathways. Following studies have found increased histone methylation correlating with decreased expression of GAD1 (Huang et al., 2007). Other findings include increased expression of histone deacetylase 1 (HDAC1) in prefrontal cortex of schizophrenic individuals (Sharma et al., 2008) but decreased HDAC3 in temporal cortex (Aston et al., 2004).

Overall, while it is clear that epigenetic mechanisms have an important contribution to the schizophrenia phenotype, further analysis is still required in order to elucidate to which extent DNA and histone modifications might aroused as a response to stochastic events, environmental stimuli during development, maturation or aging or as a secondary effect of psychosis. Thus, perhaps one of the major challenges will be determining not only why these changes occur but also at which stage are they occurring in order to have a better understanding of their role in the development of the disease.

1.4.3 Micro RNAs

The once common assumption that proteins are the primary regulator of cellular interactions in eukaryotic organisms has been challenged in recent years. In particular the revelation that the number of protein-coding genes was found to be much lower than expected in complex organisms and that the ratio of non-coding to protein-coding DNA increases proportionally to the complexity of each species (Mattick, 2001; Mattick and Makunin, 2005; Ambros, 2004; Zeng, 2009). Moreover, a growing body of evidence has led to the conclusion that RNA molecules generated

from non-coding DNA are implicated in regulatory processes much more complex than just cis-regulation of gene expression on the translational or transcriptional level, that include synapse regulation during development (Bicker and Schratt, 2008). A very particular group of these non-coding RNA molecules are micro RNAs (miRNAs). These are small endogenous RNA molecules that bind to mRNA in order to act as post-transcriptional negative regulators of gene expression (Lee, et al., 1993; Reinhart et al., 2000; Lee and Ambros, 2001; Lagos-Quintana et al., 2001).

It is now known that miRNAs, along with other types of small RNAs, are implicated in the initiation of a complex and highly specific phenomenon known as RNA interference (RNAi) or gene silencing. This process involves the formation of an RNA-protein complex known as miRISC that recognizes the mRNA target to start the gene silencing process by means of mRNA translation (Doench et a., 2003; 2004; Zeng et al., 2003) or destabilization that results in target mRNA degradation (Bagga et al., 2005). In plants miRNAs commonly bind to target mRNAs with almost exact complementarity inducing mRNA cleavage (Rhoades et al., 2002). In contrast, perfect matching of metazoan miRNAs to their mRNA targets rarely occurs. The result of this type of interaction is not cleavage but repression of translation through mechanisms that are still a subject of intense investigation. Because of the imperfect pairing of miRISCs and mRNA, prediction target sequences has not been an easy task, however the efforts for the development of reliable genome-wide computational algorithms for the identification of these targets have been greatly aided by the use of microarray technology (Lewis et al., 2005; Nielsen et al., 2007).

To date researches have agreed a series of important requirements for miRNAs target recognition and the successful repression of mRNA translation (Lewis et al.,

2003; Bartel, 2009). Binding sites are in most cases located in the 3' untranslated region (UTR) of the mRNA target. One of the most important factors identified so far for mRNA target recognition is the presence of a region complementary to the nucleotides 2-7 of the 5' end of the miRNA, known as "seed region" and that the specificity of the pairing requires a perfect-matching sequence of at least 4 to 5 nucleotides (Lewis et al., 2003; 2005). This finding was further supported by a study reporting that the ability of miRNA to repress translation of mRNA largely depends on the binding free energy between the target mRNA and the first 8 nucleotides at the 5' end of the miRNA (Doench and Sharp, 2004). This data suggests that each mRNA might target several mRNAs, an assumption consistent with recent microarray studies that have shown that miRNAs are able to downregulate many target mRNA containing sequences complementary to their "seed" regions (Lim et al., 2005). Recently it has been shown that miRNA can also exert their regulatory function when binding sites are artificially introduced to 5' UTR or coding regions of mRNA targets although the extent of this effect has not been conclusively established yet (Lytle et al., 2007; Easow et al., 2007)

Microarray analysis has also been used for identification and expression profiling of miRNAs in mammalian tissue samples (including human). Data collected from these studies shows that a significant proportion of the miRNAs identified so far are expressed in a tissue specific manner (Babak et al., 2004; Thomson et al., 2004; Sempere et al., 2004). Furthermore, these and other studies provided evidence to conclude that approximately 70% of the miRNAs detected so far are present in the brain and that they seem to be implicated in brain development (Krichevsky et al., 2003; Miska et al., 2004; Thomson et al., 2004; Liu et al., 2004; Babak et al., 2004; Barad et al., 2004; Schratt et al., 2006; Kye et al., 2007; Bak et al., 2008). It is

therefore not surprising that miRNAs have become the focus of extensive investigation, in particular concerning their possible role in neurodevelopmental and psychiatric disorders.

In a study using a mouse model of the human 22q11.2 microdeletions Stark et al. (2008) observed abnormal biogenesis of a number of miRNAs expressed in the brain due to disruption of the DiGeorge syndrome critical region gene-8 (DGCR8) resulting in the presence of the schizophrenia-like symptoms similar to those observed in individuals containing this chromosomal defect. Notably, an miRNA expression profiling study performed by Beveridge et al. (2010) in superior temporal gyrus and dorsolateral prefrontal cortex revealed a significant increase in global miRNA expression in schizophrenia that corresponded to an increase in DGCR8 and primary miRNA processing. This data supports previous findings of the expression of a number of miRNAs decreased in prefrontal cortex of schizophrenia patients (Perkins et al., 2007). An additional study in post-mortem samples from temporal cortex reported that miR-18 was significantly dysregulated in schizophrenia (Beveridge et al., 2008). Although evidence for the involvement of miRNAs in the aetiology of schizophrenia is still in an early stage, microarray analysis and other techniques have already proved to be a valuable resource in the identification and prediction of the effects of these molecules. Such is the case of miR-18 whose predicted mRNA targets include calcium sensor gene visinin-like1 (VSNL1) and the AMPA glutamate receptor subunit (GRIA2). These interactions were further confirmed by in vitro assays using HEK293 and SH-SY5Y cells.

The identification miRNAs expressed in the brain and the constant improvement in methods of mRNA binding site prediction has also provided new possible approaches

in the investigation of miRNA implication in schizophrenia and other psychiatric disorders. One example of this is a single nucleotide polymorphism (SNPs) association study in schizophrenia cases and controls in which SNPs were selected, and all of them were located within +/- 100 bp from mature miRNA transcripts previously documented as exclusively expressed in the brain (Hansen et al., 2007). The study revealed that two SNPs (rs17578796 and rs17001) were significantly associated to disease status. Interestingly, it was observed that the two miRNAs harbouring these polymorphisms (miR-206 and miR-198) shared 15 common targets. A second example is the study performed by Zhu et al. (2009) in which they observed that a particular miRNA (miR-346) targeted susceptibility genes for schizophrenia (from Schizophrenia Gene database) twice as frequently as expected. They then identified that the gene encoding miR-346 was located within intron 2 of the glutamate receptor ionotropic delta 1 (GRID1) gene. Finally they went on to demonstrate that expression of both miR-346 and GRID1 was significantly reduced in dorsolateral prefrontal cortex of individuals with schizophrenia. It is noteworthy that GRID1 has been proposed from previous studies as a candidate gene for schizophrenia (Treutlein et al., 2009).

Other studies have identified miRNAs implicated in processes that have long been regarded as associated to the pathology of schizophrenia. One such study showed that miR-219 was involved in NMDA receptor hypofunction in the prefrontal cortex of mice (Kocerha et al; 2009). These findings have encouraged further investigation to elucidate the precise role of miRNAs in the development of schizophrenia.

1.5 Rationale for this project

The pathophysiology of schizophrenia is not well understood, but it is thought to have a strong genetic component with some environmental influences on aetiology. Despite the potential of GWAS studies to reveal genetic association of common alleles with schizophrenia, including the major histocompatibility complex region alleles (Stefansson et al 2009), the variants detected to date may account for as little as 4% of the risk of schizophrenia in a population (ISC et al., 2009). The fact remains that no marker or loci has reached genome-wide significance in any single study. There are a number of reasons that could account for these limitations, such as population stratification within a sample (SNPs varying in frequency across geographical regions) or inadequate coverage of common alleles within genes. In addition, in the majority of GWAS where 500,000 SNPs are routinely screened and p values of the order of 10^{-7} are necessary for an association to be deemed significant. Under these circumstances, larger case-control cohorts (from 2,000-4,000 individuals), are being used in order to have the power to detect the small genetic effects expected from specific loci (Thomas et al., 2005). This has often led to the argument that "there are no genes of large effect in schizophrenia". However, the GWAS approach for analysis of complex traits is based on single SNP associations and does not consider the underlying linkage disequilibrium (LD) patterns between loci. Data available from HapMap project has made evident that most of the human genome can be organized in haplotype blocks of SNPs in strong LD with each other. Indeed tag-SNPs used in GWAS do not represent all haplotype blocks and hence do not represent the true genetic variation present in the population (Morris, 2005).

To circumvent the limitations of GWAS, a number of alternative approaches have been proposed. One of them is to investigate associations with schizophrenia of specific candidate genes, selected on the basis of their strong biological plausibility. This approach has been used successfully by a number of studies, including three recent haplotype-block based analyses (Voisey et al 2010a; 2010b; Burdick et al., 2010) where SNP association was assessed using smaller cohorts of controls and cases (total sample size from 310 to 410 individuals).

The present study uses the above described candidate gene approach as the basis of its experimental design. Instead of making no assumptions about which genes are involved, candidate genes have been selected from those showing robust changes in expression in schizophrenia in brain regions implicated in functional abnormalities occurring in this condition from two previous microarray expression studies. Expression of over 30,000 mRNA transcripts was determined in post-mortem tissue from two brain regions associated with the pathophysiology of the disease (Brodmann Area 10: anterior prefrontal cortex and Broadmann Area 22: superior temporal cortex). Transcripts were screened in 28 schizophrenic and 23 control patients (Charing Cross Hospital Prospective Collection: CCHPC) The first publication reported the results from the prefrontal cortex, along with a direct comparison of microarray data between two large independent studies, the first comparison of this sort that has been made in schizophrenia. Furthermore, this is also the first microarray report comparing two demographically distinct cohorts - the Harvard Brain Bank (United States) and the CCHPP (United Kingdom) cohorts (Maycox et al., 2009). This data has been validated and common candidate genes show the same direction and magnitude of effect in the Charing Cross Hospital prospective study and the Harvard Brain Bank.

To date, there has only been limited consistency between studies of small size but the data discussed above show that it is possible to detect significant and common changes in gene expression between independent cohorts. Moreover, the overlapping set comprises genes of relatively restricted function with significant enrichment in genes associated with synaptic function/ plasticity. Overall, 49 genes were identified that are differentially regulated in two independent schizophrenia cohorts. Interestingly, these genes cluster into a relatively small number of functional groups that are associated with synaptic function via synaptic vesicle regulation, signal transduction or cytoskeletal dynamics (Figure 1.1).

The second publication concerns expression changes observed in BA22 region of schizophrenic subjects compared to controls and is being reviewed for publication (Barnes et al., 2011). Similarly to what was observed from candidate genes changed in BA10, specific pathways seem to be highlighted, particularly genes whose function seems to be crucial during neurodevelopmental stages.

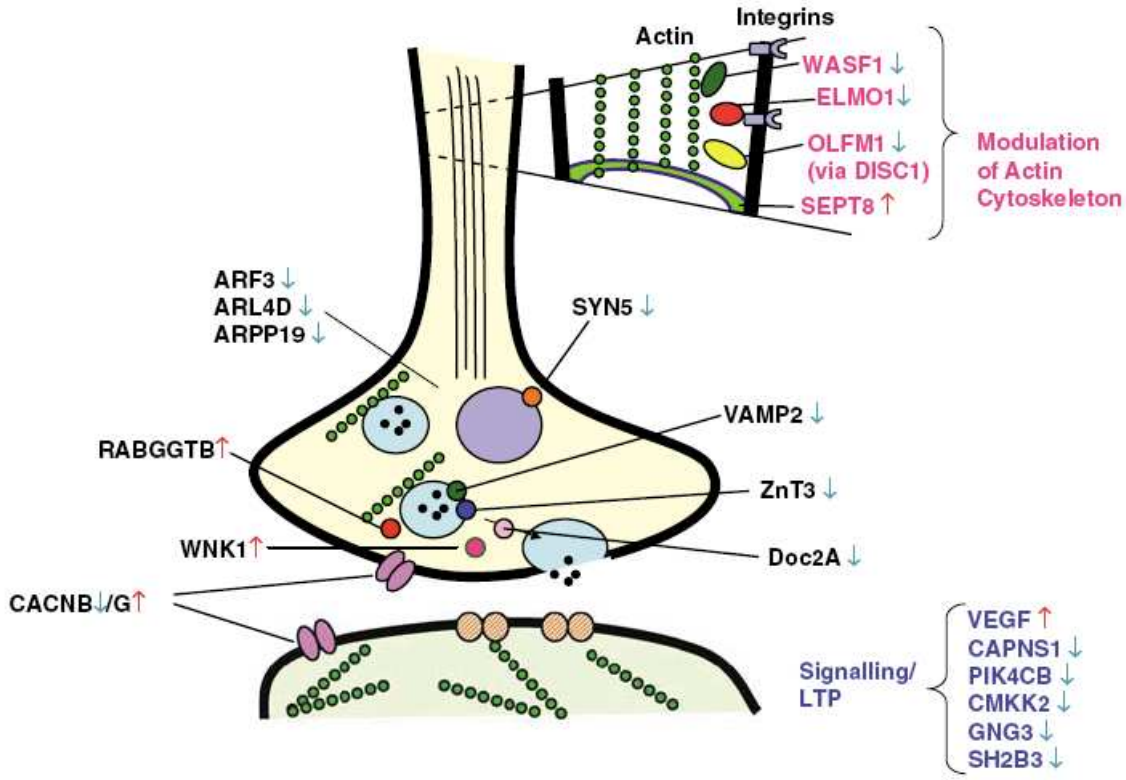


Figure 1.1 Schematic representation of functional localization of schizophrenia candidate genes. (Maycox, et al., 2009).

The study identified a number of genes implicated in synaptic function, signal transduction and cytoskeletal regulation that were differentially expressed in schizophrenia when compared to controls. In this figure, blue circles represent small synaptic vesicles, purple circles represent large dense cored vesicles while red hatching represent postsynaptic receptors and actin polymers are depicted as green small circles.

The aim of the present project is to investigate whether the candidates identified in this gene expression study can provide insight into the aetiology of schizophrenia and potential susceptibility factors. A candidate gene based approach was therefore proposed for analysis of correlation of genetic variance with gene expression as well as analysis of possible association of selected candidate genes with disease. Candidate genes were selected not only on the basis of their differential expression in schizophrenia but also because they are implicated in one of two major functional categories: Genes implicated in synaptic processes and cellular communication and genes participating in the Wnt signalling pathway. A similar pathway-based approach

has been successfully used in a recent analysis of GWAS data that revealed association of genes related to glutamate metabolism, apoptosis and inflammation (Jia et al., 2010). These associations were not detected by the original single SNP GWAS analysis.

Three main hypotheses were formulated for the present study:

1. SNP variants/ haplotypes in genes whose expression is robustly altered in schizophrenia are associated with levels of gene expression and disease status.

In this study, we will genotype SNPs in 6 candidate genes in 3 matched cohorts from the UK, including samples from the Charing Cross Hospital Prospective Collection, here designated Imperial College Prospective Study (ICPH) cohort. It is proposed to genotype up to 6 SNPS per candidate genes. SNP genotype data from the ICPS cohort will be used for associations with levels of expression. Factorial analysis of variance (ANOVA) will be used to identify significant correlations of expression with disease status, genotype/haplotype and interactions between variables. Bonferroni's *post-hoc* tests will be used to correct for multiple testing.

A subsequent association study will be carried out in a case-control cohort from East UK (East Anglia and East Yorkshire) with a total sample size of 321 individuals, a similar cohort size to the one used successfully by other association studies (Burdick et al., 2010). Golden Helix© software will be used for determination of allele and genotype frequencies between controls and schizophrenia subjects to determine their association with the disease. A logistic regression model and the χ^2 test will be used for identification of allelic associations and genotypic associations. In addition,

logistic regression will be used to test three other genetic models to evaluate the increase in risk for disease conferred by genotypes containing the associated allele (‘*D*’) to the genotype with only the wild-type allele (‘*d*’).

A moving-window analysis of association of haplotype with disease will be performed using Golden Helix©, while Haploview (Barrett, 2005; 2009) software will be used to determine LD patterns across SNP. PHASE program (Stephens et al., 2001; Stephens and Scheet, 2005) will be used for estimation of diplotypes for each individual. SNP and haplotype frequencies will also be correlated with age at onset as a clinical marker. To address the problem of multiple testing, p values were corrected using false discovery rate (FDR) values, following the Benjamini-Hochberg method (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). This is a similar analytical approach used by Voisey et al. (2010a: 2010b) in their studies of similar cohort size (160 schizophrenia cases and 250 controls).

In addition, correlation of SNP genotypes with other endophenotypes (such as age at onset) was considered for the present study. However, this information was not available for all individuals across the two main cohorts used: East UK and ICPS.

2. Epistatic interactions may be detected between genes involved in common pathways.

In recent years attention has been turned to gene-gene interactions, also known as epistasis, and their contribution to complex disorders (Carlborg and Haley, 2004; Daly and Altshuler, 2005; Marchini et al., 2005; Moore, 2005). In a simplified manner, if we consider an interaction between two loci, epistasis refers to a situation where the effect of the first gene on a particular phenotype depends on the

genotype of the second one. This could also be the case for interactions between several genes. It has been suggested that interactions between loci as well as environmental factors account for the non-linear correlation between genotype and phenotype observed in complex disorders, such as schizophrenia, and that these interactions have a more substantial effect in the risk for disease.

Evidence of epistasis between candidate genes in schizophrenia has been reported from a number of studies, although very few of them involving interactions between genes within specific pathways (Straub et al., 2007; Nicodemus et al., 2007; 2010). In order to determine the possible association of defined functional pathways with disease, gene-gene interactions will be analysed in both groups of genes using an online application, GAIA (Macgregor and Khan, 2006), that allows testing for additive and dominant interaction gene-gene effects using case-control data for SNP genotypes.

3. Epigenetic factors may be responsible for changes in expression of candidate genes.

In order to explore this possibility, the promoter region of the DKK3 gene will be analysed in controls and cases under the assumption that, if present, CpG island methylation of this region would be associated with lower levels of expression and or disease status.

CHAPTER 2 Materials and Methods

2.1 Population samples description

DNA samples from three independent cohorts of European ancestry were used for this study. Tissue samples collected as part of prospective programme at Imperial College (ICPS), used in previous expression studies, were included in the study for correlation of genetic variants with expression. In addition, blood samples from unrelated schizophrenia patients and controls were collected from East Anglia (EA) and East Yorkshire (EY) regions and constitute our two larger cohorts. Informed written consent was obtained from all subjects, with agreement of nearest relative or authorised representative, for use of tissue obtained post-mortem for research.

2.1.1 Tissue samples from the Imperial College prospective collection

The Imperial College Prospective Study (ICPS) cohort was comprised of 38 schizophrenia cases and 37 controls collected from Charing Cross Hospital, London. These included 28 post-mortem tissue samples from BA10 of schizophrenic individuals (19 male and 9 female) and 23 controls (13 male and 10 female) included in the expression study performed by Maycox et al. (2009; Table 2.1). Schizophrenia samples consisted of a group of elderly patients whose condition in many cases had started before the introduction of antipsychotic medication and who had remained in psychiatric nursing facilities over long periods. Diagnosis of these patients was performed prospectively based on DSM-III criteria, using the available clinical data and through scrutiny of medical notes. All cases were diagnosed with residual schizophrenia and presented pronounced negative symptoms and intellectual dysfunction. Mean age of onset for patients in this cohort was 26 years old and the mean period between onset and death was 48 years. Thirteen patients were never

discharged after admission while only five had more than four periods of discharge between first admission and death in hospital. Eleven patients had been treated by electroconvulsive therapy and most of them were treated with neuroleptic drugs when they became available. The mean duration of treatment amongst patients was 33 years although one patient was drug naïve at the time of death. Histological screening was performed in tissue from cases and those with evidence of Alzheimer's disease, Parkinson's disease or multiple sclerosis were excluded.

Controls were recruited from Charing Cross Hospital and local nursing homes. All of these samples were collected from mentally normal tissue donors. Causes of death were similar in controls and cases with bronchopneumonia being the most common cause, followed by carcinoma (lung, bowel, prostate, bladder, oesophagus) and to a lesser extent ischemic heart disease and coronary artery occlusion. Autopsy revealed no major atrophy of brains. Controls and cases were collected over the same period and stored under the identical conditions.

	Schizophrenic	*Control*
BA10 region		
Number of samples	28	23
Gender (male/female)	19/9	13/10
Age		
Mean	73.3	69.0
s.d.	15.2	21.6
Median	78.0	71.0
Range	28–97	25–94
Post-mortem delay (hours)		
Mean	8.1	9.4
s.d.	6.5	4.4
Median	4.8	9.5
Range	3–30	4–17
Brain pH		
Mean	6.2	6.5
s.d.	0.2	0.3
Median	6.2	6.5
Range	5.7–6.6	5.7–6.9

Table 2.1 Summary of demographics of samples from ICPS cohort used in microarray study (Maycox et al., 2009)

2.1.2 Sample composition of the East UK cohort

The East UK cohort used for analysis of genetic association of the proposed candidate genes with disease, consisted of blood samples from schizophrenia patients and matched controls, collected from the East Anglia and East Yorkshire regions in England. All controls and cases were of European ancestry. Eighty-five schizophrenia patients (46 males and 39 females) were recruited from the rehabilitation and acute services at Fulbourn Hospital in Cambridge, East Anglia. Forty-nine patients were taking clozapine at the time that the blood samples were collected and were categorised as responding or non-responding to this antipsychotic. Patients were diagnosed according to the DSM-III-R criteria. From the group of schizophrenics (SEA) one female of Afro-Caribbean descent and one male of Arabic descent were excluded. Seventy-nine controls (39 males and 40 females) of matched age and gender distribution were recruited from the oral surgery and ophthalmology clinics in Addenbrooke's Hospital, Cambridge and were included in this cohort. From controls (CEA), one male of Asian descent and another male with a family history of schizophrenia were excluded. None of the remaining controls had any family history of major depression, bipolar disorder, schizophrenia or schizoaffective disorder (Table 2.2).

The population sample collected from the East Yorkshire region contains a large proportion of individuals whose families may have been settled in this area for many generations with limited immigration. Seventy-two blood samples form schizophrenic patients (40 males and 29 females and 3 for whom information regarding sex was not provided) were collected from the psychiatric outpatient clinic based at De La Pole Hospital in Hull, EY. Six patients were treated with clozapine from which three

responded positively and one showed moderate improvement. The patients were diagnosed using the DSM-IV criteria, through which 38 patients were diagnosed with paranoid schizophrenia, 15 with disorganised type, 1 with catatonic type, 5 with undifferentiated type. Although no endophenotype data was available for this cohort, the mean age of onset was known and registered as 19.75 with the earliest onset observed at 9 years old and the latest at 47 years. Eighty-five control subjects (38 males and 41 females plus 6 for whom information regarding sex was not provided) were recruited from the Hull Royal Infirmary. None presented with a family history of major depression, bipolar disorder, schizophrenia or schizoaffective disorder. Mean age of controls was matched to that of cases, as well as gender distribution (Table 2.2).

Disease Status	Geographic Region-UK	n	Age-at - Ascertainment		Gender	
			Mean	S.D.	Male	Female
Controls	East Anglia	79	38.7	18.9	39 (49.4%)	40 (50.6%)
	East Yorkshire	79 (85)	36.9	10.2	38 (48.1%)	41 (51.9%)
Schizophrenics	East Anglia	85	44.6	15.1	46 (54.1%)	39 (45.9%)
	East Yorkshire	69 (72)	48.2	17.5	40 (58.0%)	29 (42.0%)
Total		312 (321)				

Table 2.2 Summary of demographics of samples from East UK cohort.

Mean age at ascertainment and gender distribution for the East UK cohort are presented in the table. The 9 samples for which no information regarding sex was available (collected from the EY region) were not used in the gender proportion calculation but are included in the total number of samples (321).

2.2 Analysis of DNA

2.2.1 DNA extractions and purification

Extractions of DNA from blood and tissue samples were performed using the BACC Nucleon genomic extraction kit (Tepnel Life Sciences) according to the supplier instructions. After ethanol precipitation and washing (with 70% ethanol) DNA was resuspended in 0.1 ml of deionised water (SIGMA-ALDRICH) and subsequently diluted to a final concentration of 20 μg/μl for use as template for PCR amplification. Stock DNA samples were placed at -20º C for long-term storage while dilutions were kept at 4º C while genotyping of SNPs.

DNA purification was performed using SureClean (Bioline) or QIAquick PCR Purification Kit (QIAGEN) following the procedure recommended by the manufacturers. Purity of DNA was assessed by determination of the UV light absorbance ratio at 260 nm and 280 nm using a NanoDrop Analyser (Thermo Scientific), an absorbance ratio ($A_{260/280}$) of 1.6-1.8 being obtained for pure DNA. Concentration of DNA was also determined based on the optical density (OD) values measured at a wavelength of 260 nm based on the assumption that an OD value of 1 corresponds to a concentration of 50 μg/ml for double-stranded DNA (Sambrook and Russell, 2001).

2.2.2 DNA amplification with polymerase chain reaction (PCR)

Standard PCR reactions were carried out using a mixture containing 60 to 100 ng of DNA template, 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTP mix, forward and reverse primers to a final concentration of 0.5μM and one unit of Platinum® Taq polymerase (Invitrogen) or GoTaq® polymerase (Promega) using pure deionised water to make a

total reaction volume of 30 μl. PCR amplification was performed using a Px2 Thermohybaid® thermocycler as follows: 95 ºC for 3.5 minutes, 1 cycle followed by 38 cycles of 30s at 95 ºC, 30 s at the optimized annealing temperature (generally 50-64 ºC) and 1 min at 72° C and a final cycle of 6 min at 72 ºC.

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used in order separate DNA fragments according to their size, after amplification or digestion. In some cases, it was also used as an intermediate step for purification of DNA for cloning or sequencing. Gels were prepared using agarose 'Electran' (BDH) in a 0.5x TBE Buffer (43.75 mM of Tris base, 44.48 mM of boric acid and 1mM of EDTA pH8.0). Agarose gel concentrations varied from 0.7 to 1.5% depending on the resolution required and the size differences between the DNA fragments that were being separated. A 0.5 μg/ml solution of ethidium bromide (SIGMA) was used for detection of DNA under UV light. Two markers were used: ΦX174 DNA/BsuRI (Hae III) marker 9 (Fermentas) and HypperLadder II™ (Bioline) for different fragment size identification.

2.2.4 DNA sequencing

For each sample, a sequencing mixture was prepared containing either 600 ng of purified DNA plasmid or 100 ng/Kb of PCR product, along with 3.2 pmole of sequencing primer adding distilled water (SIGMA) to give a total volume of 10 μl. DNA sequencing was then performed by the MRC CSC Genomics Core Laboratory, Imperial College London.

2.3 Molecular biology techniques

2.3.1 Preparation of DNA constructs

Plasmid DNA constructs were prepared using PCR for cloning of desired sequence with further agarose gel electrophoresis for identification and separation of bands. DNA fragments were later excised from the gel and purified using QIAquick gel extraction kit (QIAGEN). Ligations were performed using one unit of Taq DNA ligase (NEB) with 1X reaction buffer and an insert to vector ratio of 1:3. 25 ng of vector plasmid DNA were used for each reaction and volume was adjusted to 10 μl using molecular biology rate water (SIGMA). The reaction mix was maintained at 16ºC overnight in order to ensure successful ligation. 5 μl of the ligation product were subsequently transformed into 50 μl of One Shot TOP10 chemically competent *E. Coli* cells (Invitrogen) by incubating at 4 ºC for 30 minutes followed by a 30 seconds heat shock at 42 ºC and finally recovery at 4 ºC for 2 minutes. After transformation bacteria were left to grow in SOC medium at 37 ºC and shaking at 200 rpm for one hour before spreading onto agar plates containing 100 μg/ml of ampicillin or 50 μg/ml of kanamycin.

2.3.2 Selection and screening of positive colonies

For constructs containing a Lac Z site, positive colonies were selected using blue white screening method (Sambrook and Russell, 2001). After transformation bacterial solution was spread across agar plates containing 50 μg/ml of ampicillin (Sigma-Aldrich) and 40 μl of a 2% X-gal (Calbiochem) solution combined with 7 μl of a 20% isopropyl-β-D-thio-galactoside (IPTG; Calbiochem). The presence of the insert was later confirmed by PCR. For constructs without the Lac Z site, at least 10 to 20 colonies were harvested and screened for the insert using PCR. For expression

constructs orientation of the insert was confirmed by enzyme digestion. Positive colonies were then cultured in order to extract DNA plasmid using QIAprep mini prep kits (QIAGEN) for sequencing.

2.3.3 Cell culture and transfection

N2a and COS7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing10% FBS (Dubelco) at 37 °C and 5% CO_2. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturers directions. Cells were allowed to achieve 90% confluence before transfection. For activation of Wnt signalling pathway, cells were treated with a 5μM solution of the GSK-3β VIII (EMD Calbiochem) inhibitor overnight before fixing.

2.3.4 Immunocytofluorescence

Cells used for analysis of protein subcellular localization and immunofluorescent staining were seeded in 24-well plates on round cover slips coated with 0.01% poly-lysine slips. In the case of transfections with fluorescent constructs and Wnt3A construct were fixed 48 hours after transfection using a 4% paraformaldehyde (PFA) solution in phosphate buffer saline (pH 7.3), for 20 minutes at room temperature. Permeabilisation of cells was performed using a 0.5% solution of Triton-X (Sigma-Aldrich) in PBS, for 15 minutes, followed by at least three PBS washes (of 5 minutes each) before counterstaining by incubation with a 0.5 μg/ml DAPI solution in PBS buffer for 10 minutes. Cover slips were finally mounted onto slides using Fluorosave reagent (Calbiochem)

Immunostaining of endogenous β-catenin was performed by plating cells as described above and allowing them to grow for 24 hours before fixing them with 4%

PFA solution. After permeabilisation of the cells with 0.5% Triton, and three PBS washes, cover slips were incubated with a 10% FBS/PBS solution for an hour at room temperature for blocking. Cells were then incubated with rabbit anti-β-catenin (1:2000) at 4ºC overnight followed by secondary anti-rabbit Alexa Fluor 594 antibody (1:1,000;Molecular Probes) and DAPI counterstaining and mounting.

2.3.5 Western Blot

For Western Blot analysis cells were seeded in 6-well plates and transfected as previously described. Cells were lysed, 48 hours after transfection, in 20mM Tris·HCl (pH 8), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2mMEDTAcontaining 1mMPMSF, 1mMsodium orthovanadate, 5mMsodiumfluoride, and a mixture of protease inhibitors (Roche). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad). Samples were separated by 10% SDS/PAGE gel electrophoresis using a Mini-PROTEAN Tetra Cell (Bio-Rad) and a Precision Plus Protein All Blue Standards (Bio-Rad) as protein size marker. Bands were then transferred to a nitrocellulose membrane (Amersham Hybond™ ECL™, GE Healthcare) using a, Semi-Phor semi-dry transfer system (Hoefer Scientific Instruments). Membranes were blocked in 5% nonfat milk/PBS-Tween (0.1%) and incubated with primary antibody at 4ºC overnight.

For analysis of β-catenin we used polyclonal rabbit anti-β-catenin (1:4000; Sigma-Aldrich) while analysis of DKK3 was performed by incubation with rabbit polyclonal anti-GFP antibody (1:10000; Abcam). Primary antibody incubation were followed by three washes using PBS-Tween buffer before a second incubation with an anti-rabbit HRP (1:3000; Vector) for 1 hour at room temperature and then developed using the

Immun-Star HRP chemiluminescent kit (Bio-Rad) and Amersham Hyperfilm ECL (GE Healthcare).

Western blotting of secreted DKK3 was carried out by replacing medium of GFP-DKK3 transfected cells with serum free DMEM, 24 hours after transfection. Cells were harvested and lysed as specified above after additional 24 hours and the medium collected for protein precipitation. Precipitation was carried out by adding an equal volume of a 20% trichloroacetic acid (TCA) solution to medium samples (for a final TCA concentration of 10%) and incubating them at 4º C for 45 minutes. Precipitated was recovered by centrifugation at 13000 rpm at 4º C for 30 minutes. The pellet obtained was washed with a 90% cold acetone solution followed by a second wash with cold acetone (100%). Protein was re-suspended in 2X SDS buffer for Western blot analysis.

2.3.6 Luciferase Assays

N2a cells were seeded in 96-well plates and allowed to achieve 90% confluence before being transfected with the luciferase reporter TOPFlash or FOPFlash (A gift from Dr. Magdalena Sastre from Imperial College London), renilla luciferase pRL-CMV vector (PROMEGA) as a transfection control, RFP-β-catenin and GFP-DKK3 or GFP empty vector. In addition, cells transfected with either TOPF or FOPFlash, renilla pRL-CMV vector, and GFP and RFP empty vectors were used as controls. Three wells containing N2a cells were then transfected with each of the six combinations of DNA constructs for luciferase activity measurement. A Dual-Luciferase Reporter assay system from Promega was used for this purpose. Cells were harvested 48 hours after transfection and lysed by adding 70 μl of 1X passive lysis buffer (PLB) provided with the kit to each well and incubating at room temperature for 15 minutes on a rocking

platform. Luciferase activity was measured immediately after completion of lysis using a FluoroSkan Ascent microplate reader (Thermo Scientific). Firefly luciferase activity of TOPFlash and FOPFlash was measured initially by adding 50 μl of luciferase assay reagent II (LARII) to 50 μl of cell lysates. Once this reaction was complete, renilla luciferase activity of the transfection control vector was measured by adding 50 μl of 1X Stop & Glo reagent to the lysate and LARII mix.

The procedure described above was repeated at least three times for N2a and COS7 cells for optimisation of transfection conditions and to minimise variation in measurement of luciferase activities. For experiments performed to determine the effect of DKK3 on Wnt signalling activation, optimised DNA ratios of reporter constructs with respect to the RFP-β-catenin construct were defined as 1:1. Similarly, DNA ratio of GFP-DKK3, or GFP empty vector, with respect to RFP-β-catenin was maintained as 1:1. Finally, 1.4 ng of pRL-CMV vector were used for each transfection. The total amount of DNA was constant throughout all experiments and was set as 200 ng per well. For the case of 'reporter only' transfections GFP and RFP empty vectors were used as controls.

CHAPTER 3 Correlation of gene expression with genotype and genotype with schizophrenia of genes involved in synaptic processes and intracellular signalling.

3.1 Introduction

The involvement of synaptic dysfunction and deficient connectivity in the aetiology of schizophrenia has been supported by evidence from a variety of studies. Findings from structural and neurochemical studies point to a contribution of deficient neurotransmitter function underlying the pathophysiology of the disorder (e.g. Carlsson and Carlsson, 1990; Akil et al., 1999; Beasley et al., 2002b; Reynolds et al., 2002; Bitanihirwe et al., 2009). In addition, linkage, association and expression studies have also provided a number of candidate genes with important roles in synaptic and memory formation processes (e.g. Numakawa et al., 2004; Talbot et al., 2004; Corfas et al., 2004; Roy et al., 2007; Buxbaum et al., 2008).

The present study has its basis in data collected from microarray mRNA expression studies in post-mortem brain samples from Brodmann areas 10 and 22 (BA 10 and BA22) of schizophrenia cases and controls (Maycox et al., 2009; Barnes et al., 2011). In this section we present results from the analysis of three candidate genes selected for their involvement in synaptic processes, neurotransmitter release and cell-cell communication pathways. The analysis consists of two parts; the first one involves the correlation of genotype with levels of expression while the second part of the study was the analysis of association of genotype with disease.

3.1.1 Overview

3.1.1.1 Correlation of genotype with levels of expression

The aim for the first part of the analysis was to identify significant correlations between genetic variation and mRNA levels of the candidate genes. In order to do so, a number of SNPs were selected in regions (within, proximal and distal) containing each of our candidate genes. Genotypes from these SNPs in controls and cases from the ICPS cohort (Section 2.1.1) were then used to explore correlations with expression levels. Using the data-set reported by previous microarray studies (Maycox et al., 2009; Barnes et al., 2011) SNP genotypes of all candidate genes were then correlated with expression data of controls anda cases. Three-way analysis of variance (ANOVA) was used to evaluate the effect of all variables on levels of expression of each candidate genes, as well as the interactions between these variables. For analysis of single SNPs, disease status and genotype were considered as independent variables. Multilocus associations were assessed by grouping haplotypes according to their likely evolution, based on regions of strong LD across SNPs. The software Haploview (Barrett, 2009) was used to estimate LD values. PHASE v. 2.1 (Stephens et al., 2001; Marchini et al., 2006) was used to predict both haplotype pairs for each individual (diplotype). Correlation of diplotype with expression levels was then performed using a two-way ANOVA. Following these analyses, *post-hoc* Bonferroni-corrected individual comparisons were performed to correct for multiple testing.

3.1.1.2 Analysis of association of genotype with schizophrenia

For the second part of this analysis we tested the hypothesis that these variants might also be associated with schizophrenia. For this purpose, SNP genotype data was collected for an additional larger group of schizophrenia cases and unrelated matched controls, the East UK cohort (Sections 2.1.2). This cohort comprised controls and cases of European ancestry from East Anglia and East Yorkshire regions in England. These cohorts were combined in view of the expected similarities between them and the low levels of population stratification in England compared to those across Europe (Wilson et al., 2001; Capelli et al., 2003; O'Dushlaine et al., 2010). The software G*power (Faul et al., 2007; 2009) was used to calculate size effects (w) for particular changes in minor allele frequencies (MAF) between controls and cases. These 'w' values were subsequently used to estimate the power of a case-control study to detect significant SNP effects (α= 0.05), using the East UK cohort (n=321). From this calculation, it was determined that the analysis had sufficient power (1-β= 0.8 to 1, where β represents type II error) to detect significant effects of SNPs with a difference in MAF of 8% or greater in cases compared to controls. This was true for all the MAF values of controls used for the power calculation (Table 3.1).

	Increase of MAF in Cases		
Control MAF	7%	8%	10%
50%	0.14	0.16	0.2
40%	0.142887	0.163299	0.204124
30%	0.152753	0.174574	0.218218
20%	0.175	0.2	0.25
10%	0.233333	0.266667	0.333333

Power 70-78%
Power ≥ 80%
Power ≥ 88%
Power ≥ 95%

Table 3.1 Power calculation for the East UK cohort.

Power heat map calculated using the software G*Power. Effect size (w-values) are represented in the table, for different Case/Control MAFs at α=0.05, considering the East UK cohort, with a total sample size of 321 individuals.

The genotype data collected from the East UK cohort was then used to perform a candidate gene based genetic association study. Deviations from Hardy-Weinberg equilibrium and LD values were calculated using the software Haploview in controls and cases. Golden Helix© SNP and Variation Suite 7 (SVS7) software version 7.3.0 (Golden Helix, Inc., Bozeman, Montana) was used for application of a logistic regression to assess allele associations while χ^2 test was applied to analyse genotypic associations. In addition, three models of disease penetrance were tested in order to compare the effect of genotypes containing the disease associated allele '*D*' to those containing the wild-type allele '*d*'. All p-values were corrected for multiple testing using the in-built feature of the software, which calculates false discovery rate (FDR) values based on the Benjamini-Hochberg method (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001; Storey and Tibshirani, 2003). This method has been used in a number of candidate gene association studies with similar sample sizes to

the East UK cohort (Voisey et al., 2010a; 2010b; Jia et al., 2010) and it is considered more appropriate for SNP association studies compared to Bonferroni's for a number of reasons. It has been the subject of debate that Bonferroni-type corrections can be extremely conservative and might decrease power when statistical tests are not independent, which is the case for SNPs in strong LD with each other (Macregor and Khan, 2006; Straub et al., 2007; Evans, 2008).

Analysis of the effect of haplotypes on risk of schizophrenia was performed using two methods. The first one of these was a moving window type analysis performed using Golden Helix©, where χ^2 test was applied to compare haplotype frequencies between controls and cases from the East UK cohort. For this analysis, p values were corrected by calculating FDR values. The second method for analysis of haplotype association with disease consisted in defining groups of similar haplotypes and comparing their frequencies in controls and cases using Fisher's exact test. This approach has the advantage that it reduces the degrees of freedom for the analysis and avoids grouping together rare haplotypes of large and small effect (Morris, 2005; 2008).

Haplotype analysis is commonly regarded to be a more powerful tool to detect genetic association with disease when compared to the single SNP approach. This is due to the fact that a haplotype approach considers LD patterns between loci, which provides an advantage since it has been observed that much of common variation can be structured in haplotype blocks of SNPs in strong LD with one another (International HapMap consortium, 2005). Another important consideration supporting haplotype analysis is that protein function is determined by the linear

DNA sequence corresponding to each copy of the coding gene and therefore will be affected by variation in the maternal or paternal haplotypes (Clark, 2004).

Finally, a third hypothesis was considered that there might be gene-gene interactions potentially contributing to an increased risk of disease. In order to explore this idea, analysis of epistatic interaction was also performed between all pairs of genes using genotype data from controls and cases of the ICPS and the East UK cohorts. For this analysis, the online based application GAIA was used to calculate p values for interaction between loci as well as an overall p value for significance of association over and above SNP association (Macgregor et al., 2006; http://gump.qimr.edu.au/GAIA/gaia.html). For this analysis 1000 permutations were used to calculate empirical p values.

3.1.2 Selected candidate genes

Three candidate genes were selected for this part of the study. The first candidate, the solute carrier family 30 member A3 (SLC30A3) is also known as ZnT3 and is the third member of the family of proteins known as zinc transporters. ZnT3 has been identified as the only source of chelatable zinc (Zn^{+2}) in synaptic vesicles of zinc secreting neurons (Palmiter et al., 1996; Wenzel et al., 1997; Cole et al., 1999; Salazar et al., 2004; Suh et al., 2005; Linkous et al., 2008; Chi et al., 2008). Release of Zn^{+2} from presynaptic buttons and its translocation to postsynaptic terminals results in important effects on the modulation of excitatory synapses through regulation of glutamate and γ aminobutiric acid (GABA) receptors (Xie et al., 1994; Palmiter et al., 1996; Jacob et al., 1998; Marin et al., 2000; Sheline et al., 2002). Along with these findings, the reported downregulation of ZnT3 in post-mortem cortical brain tissue from the BA10 region of schizophrenia cases (Maycox et al.,

2009; Figure 3.1) provided strong support for selection of this gene as candidate for the present study. A significant decrease in gene expression was observed from microarray data in schizophrenia cases compared to controls (p=0.003). Validation of this data by qPCR later confirmed this result (p=0.009).

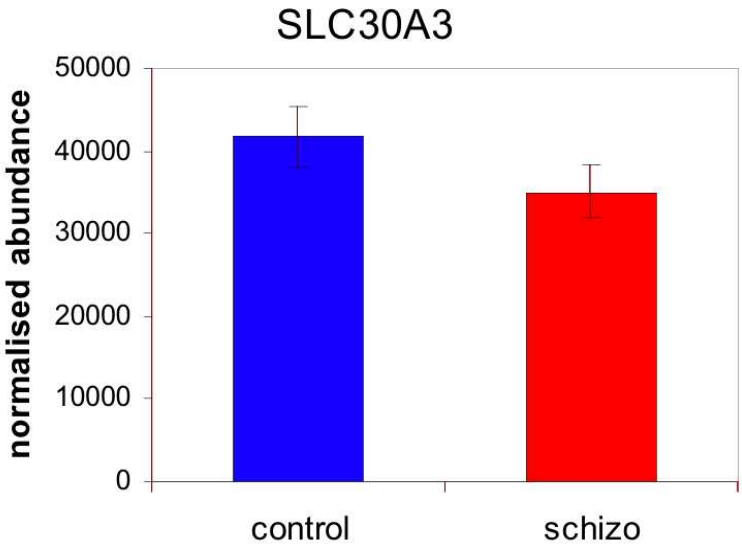


Figure 3.1 Levels of expression of SLC30A3 (ZnT3) in BA10 post-mortem tissue from schizophrenia cases and controls (Maycox et al, 2009)

Validation analysis of microarray study was carried out by QPCR and confirms a significant decrease in expression of SLC30A3 in schizophrenia (p=0.009). Data is obtained from Maycox et al (2009).

The second candidate gene considered was the voltage dependent, R-type, α1E subunit or calcium channel $Ca_V2.3$ (CACNA1E). In the central nervous system (CNS) calcium channels play and important role in neurotransmitter release, neuronal excitability and other calcium dependent processes. In the brain at least five genes coding for alpha 1 subunits of these channels have been identified, namely alpha 1A to 1E. The expression of each one of these subunits regulates the properties of specific calcium channels. The 1E subunit, particularly, has been associated with high voltage R-type currents in rat and human brain (Ellinor et al., 1993; Williams et al.,

1994). R-type voltage-gated calcium channels (α_{1E}) are expressed primarily in neurons, particularly in dentate granule, entorhinal cortex and hippocampal CA3 pyramidal neurons (Day et al., 1996). In presynaptic terminals the main function of these channels is to trigger fast neurotransmitter release whilst those located in cell bodies and dendrites, they elicit depolarization. In hippocampus, R-type channels are particularly important in long-term potentiation (LTP) and therefore have a major role in synaptic plasticity, learning and memory processes (Breustedt et al., 2003). The biology of R-type calcium channels has not been clearly elucidated. However, inhibition of R-type currents by activation of glutamate and GABA receptors in a G-protein dependent manner has been reported (Wu et al., 1998; Yu and Shinnick-Gallagher, 1998; Toro-Castillo et al., 2007; Meza et al., 2007).

The functional relevance of voltage-gated calcium channels in neurological processes has guaranteed their extensive investigation in relation to a variety of neurological disorders including association of R-type channels with neuropathic pain (Greenberg, 1997; Matthews et al., 2007; Benarroch, 2010). In addition, evidence for association of a SNP in the CACNA1C ($Ca_V1.2$) gene with bipolar disorder has been identified (Ferreira et al., 2008) and more recent reports provide evidence of association of this polymorphism with major depression and schizophrenia (Green et al., 2010; Nyegaard et al., 2010). CACNA1E was initially selected for the present study because it was one of the transcripts most significantly changed in BA22 in schizophrenia, from a previous microarray expression study using samples from the ICPS cohort (Appendix A: Barnes et al., 2011). After validation, a significant increase (1.24 fold, $p=0.003$) in mRNA levels of CACNA1E was confirmed in affected individuals when compared to controls.

The third candidate gene selected was the S100 calcium binding protein A8 (S100A8), also known as myeloid-related protein 8 (MRP8) and calgranulin A. This gene is part of a family of EF-hand Ca^{2+} binding proteins involved in regulation of a variety of cell processes including differentiation, survival and proliferation (Fritz et al., 2010). Together with the S100A9 protein (MRP14, calgranulinB), S100A8 has been found to be particularly abundant in neurotrophils in the form of a heterodimer known as calprotectin (Hessian et al., 1993; Stroncek et al., 2005). Calprotectin has a high affinity for arachidonic acid and binding of this lipid has been observed to have an effect on activation of the NADPH oxidase complex in phagocytes (Bouzidi et al., 2004). The role of calprotectin in chronic inflammation has been well documented (Lügering et al., 1995; Foell et al., 2004; McCormick et al., 2005). In addition, this heterocomplex is known to have chemotactic, antimicrobial, apoptotic and growth-regulating properties (Rammes et al., 1997; Murao et al., 1999; Ghavami et al., 2010). However, little is known about the function of S100A8 in the CNS.

The relevance of S100A8 as a candidate gene for schizophrenia is strengthened when considering that it is located in chromosome 1q25-31, a region associated with schizophrenia from previous linkage studies (Hovatta et al., 1999; Brzustowicz et al., 2000; Ekelund et al., 2001), and also contains the CACNA1E (Figure 3.2). Like CACNA1E, S100A8 was also found to be significantly upregulated in BA22 (Appendix A, Barnes et al., 2011) and BA10 (Maycox et al, 2009) of schizophrenia cases compared to controls from the ICPS cohort (Appendix B).

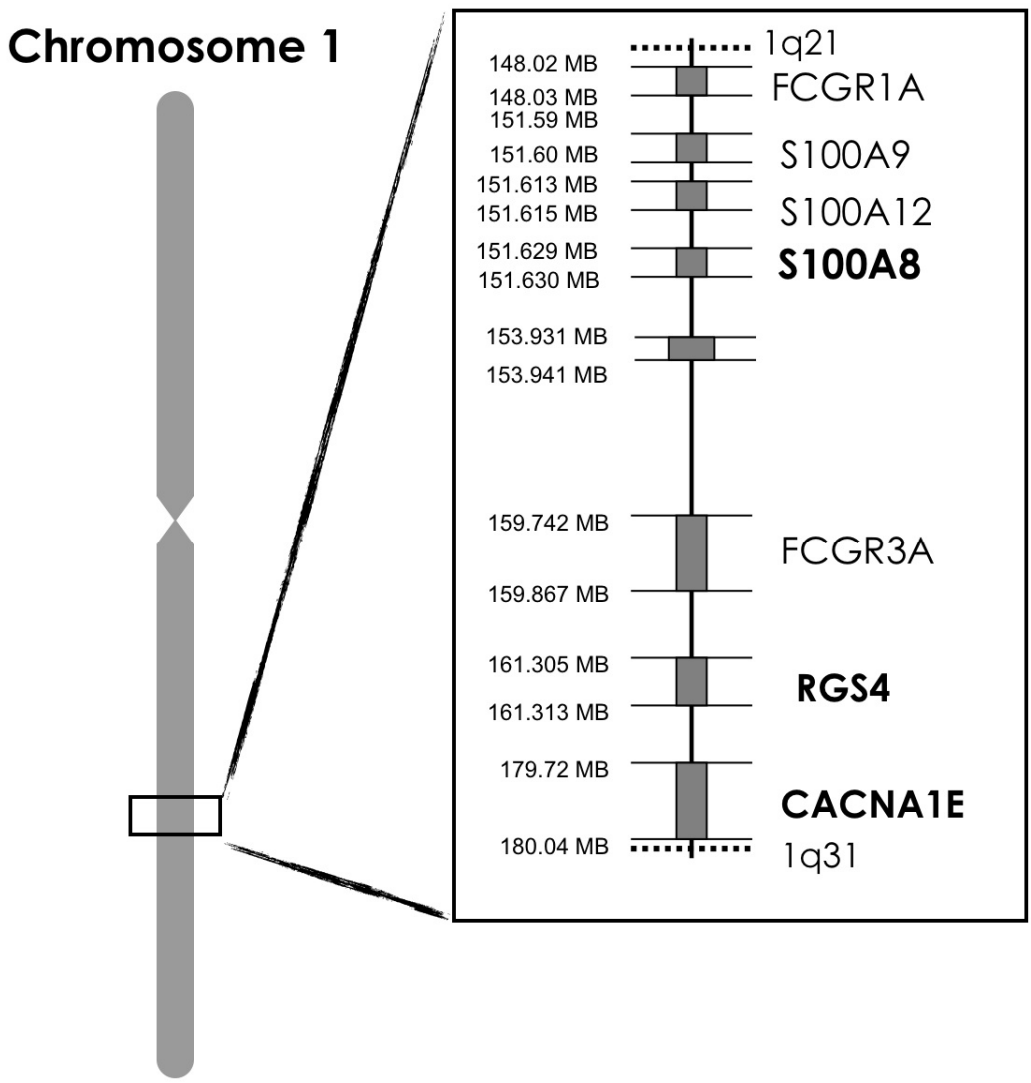


Figure 3.2 Schematic representation of the localization of schizophrenia candidate genes in chromosome 1q21-q31.

With the exception of RGS4, expression mRNA levels of all these genes were identified from the ICPS as significantly increased in post-mortem tissue from PFC of schizophrenia compared to controls (Appendix B, Barnes et al., 2011). RGS4 has been identified as a candidate gene for schizophrenia from a number of studies (Mirnics et al., 2000; 2001; Chowdari et al., 2002).

3.2 Methodology

3.2.1 Selection of single nucleotide polymorphisms (SNPs)

The selection of SNPs of interest was performed by using data from the open access HapMap database (http://hapmap.ncbi.nlm.nih.gov), following a series of well established guidelines that enhance the power of the study (Pettersson et al., 2009). Initially, known polymorphisms within the gene region for which genotype data was available were selected. SNPs within coding regions, preferably non-synonymous or located in regulatory regions of the gene were given priority for selection. Proximity of the selected SNPs to regions identified in GWAS as being associated with schizophrenia was also considered as high priority. Additionally, in order to avoid the difficulties associated with the interpretation of frequency differences from SNPs with low minor allele frequencies (MAFs), polymorphisms with a MAF of between 20%-30% in samples of European ancestry were preferred (Table 3.2). Finally, two more important criteria and, in some cases, the deciding factor for SNP selection were considered: the characteristics of the sequence that might make the SNP difficult to genotype and the availability of restriction enzymes that could be used to distinguish between the two alleles.

For the analysis of association of genetic variants in ZnT3 with schizophrenia, four SNPs were selected within a 14.1 Mb region in chromosome 2. SNP 1 (rs6547521) is located in the 5' end of the gene at 4.7 Kb upstream from the ATG start codon. The second SNP (rs11126936) is located in intron 1, while SNPs 3 (rs2083363) and 4 (rs11126931) are both located in the 3' flanking region at a distance of 1.9 Kb and 2.9 Kb, respectively, from the end of the gene. Although none of the selected SNPs

are located in coding regions of ZnT3, they are located in, or close to, regions with potential for regulation of gene expression (Chen et al., 2006).

For analysis of the CACNA1E gene, five genes were selected. SNP1 (rs17743547) was selected for its location in a splice site, while SNPs 3 (rs35737760) and 5 (rs704326) were selected as they are non-synonymous variants and cause a change in the amino-acid residue of the protein. Two more polymorphisms, SNP 2 (rs2280865) in intron 22 and SNP 4 (rs4652678) in exon 32 were also included in the analysis. Although the latter is located in a coding region of the gene, the base change does not result in a change of amino acid residue as is therefore deemed as a synonymous change.

Finally, although genotype information for SNPs in the S100A8 gene region was limited, two polymorphisms in this gene were used in this study, one located in an intronic region (rs3795391; intron 1) and the other one (rs3006488) located in the untranslated region (UTR) at the 3' end of the gene.

ZnT3			Frequency (%)			
	SNP Location	n	G/G	G/C	C/C	C
rs6547521	5'	65	52.3	40.0	7.7	27.7
			G/G	G/T	T/T	T
rs11126936	Intron 1	113	61.1	34.5	4.4	21.7
			G/G	G/C	C/C	C
rs2083363	3'	61	54.1	41.0	4.9	25.4
			C/C	C/T	T/T	T
rs11126931	3'	65	56.9	38.5	4.6	23.8
CACNA1E						
			G/G	G/A	A/A	A
rs17743547*	Intron 10 (splice site)	23	78.3	21.7	0.0	10.9
			T/T	T/C	C/C	C
rs2280865	Intron 22	120	28.3	38.3	33.3	47.5
			T/T	T/A	A/A	A
rs35737760	Exon 23 [D⇒E]	37	N.A	N.A	N.A	5.6
			T/T	T/C	C/C	C
rs4652678	Exon 32 (syn)	112	53.1	38.1	8.8	27.9
			G/G	G/A	A/A	A
rs704326	Exon 47 [A⇒T]	112	28.6	45.5	25.6	48.7
S100A8						
			A/A	A/G	G/G	G
rs3795391	Intron 1	113	83.2	15.9	9.0	8.8
			T/T	T/C	C/C	C
rs3006488	3' UTR	113	83.2	15.9	9.0	8.8

Table 3.2 HapMap database frequency data of SNPs selected in ZnT3, CACNA1E and S100A8

Frequency data available from the HapMap database for all SNPs in genes involved in synaptic processes and cell communication. The data shown in this table above corresponds to the HapMap CEU cohort, composed of unrelated individuals of European ancestry from the CEPH collection. Genotype data for SNP rs17743547 (*) corresponds to the AFD-EUR-PANEL, which comprises 23 unrelated individuals of European ancestry from the CEPH collection.

3.2.2 Genotyping

Genotyping was performed by polymerase chain reaction (PCR) amplification of the gene fragment containing the SNP of interest, followed by restriction enzyme digestion for determination of genotype frequencies (Figure 3.3).

Primer pairs for SNP genotyping were designed with the aid of Primer 3 v. 0.4.0 (http://frodo.wi.mit.edu) so that the CG content was not higher than 55% (Supplementary Table 2; Appendix B). Theoretical Tm values for the primer pairs were calculated and used as a guideline for optimization of PCR conditions. Annealing temperature gradients, modification of template concentrations, increase of Taq polymerase units and/or addition of an enhancing solution (Invitrogen) were necessary for optimization of amplification conditions of some SNPs.

Restriction enzymes suitable for each polymorphism were selected using a web-based tool (http://tools.neb.com/NEBcutter2/index.php) from New England Biolabs. This application provides a list of all the commercially available enzymes that have recognition sites matching one or several points of a given sequence. In order to ensure a complete digestion, 1 unit of enzyme was used for each digestion and the reactions were carried out for 16 h under mineral oil at the recommended temperature for each enzyme (normally 37º C). Once the digestion was completed digestion products were analysed by agarose gel electrophoresis. Gels were prepared using 1.7 to 2.0 % (by weight) of agarose (depending on the size of the bands analysed) in TBE buffer (ph= 8) stained with 1% ethidium bromide. Band sizes were determined by comparison ϕX174 DNA/Bsru (HaeIII) marker 9 (Fermentas).

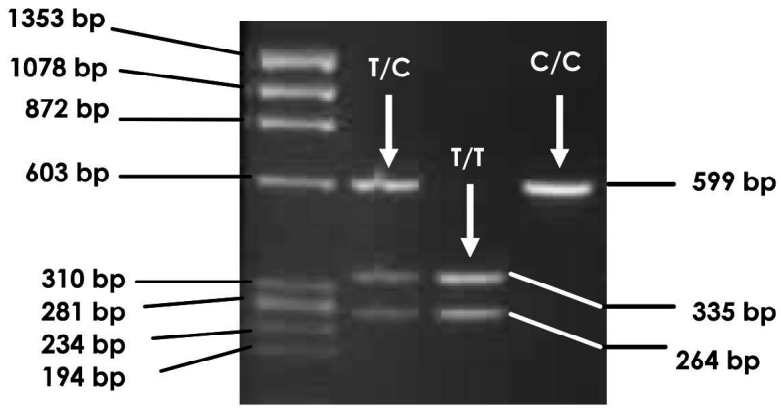


Figure 3.3 Example of genotyping of one of the SNPs in the CACNA1E gene.

An example of the method used for determination of genotypes by restriction enzyme digestion and agarose gel electrophoresis analysis. For this SNP, one restriction site is present for the BspHI (NEB) restriction enzyme when the 'T' allele is present but not for the 'C' allele. This means that two bands can be observed after digestion only when the 'T' allele is present while the intact 599 pb fragment is obtained for 'C'. A ϕX174 DNA/Bsru (HaeIII) marker 9 (Fermentas) DNA ladder was used to determine the size of the bands.

3.2.3 Analysis of the correlation of genetic variation with gene expression

Correlation of genetic variation with gene expression was performed using genotype data from cases and controls from the ICPS cohort. Expression data for the same cohort was obtained from previous microarray studies (Maycox et al., 2009; Barnes et al., 2011). Genotypic associations with mRNA levels of single SNPs for all candidate genes were tested by analysis of variance (ANOVA). Deviation from normality of the data was assessed by in-built Prism tests such as the D'Agostino and Pearson and Kolmogorov-Smirnov normality tests. For each gene an initial three-way ANOVA was performed using the application Rweb module (http://www.biostatisticien.eu/cgi-bin/Rweb/buildModules.cgi; ©J. Banfield; Montana State University). For this analysis three independent variables were defined; disease status, genotype and SNP ID with expression as the outcome variable. Further two-

way ANOVA was then performed using Prism v. 5 software (Graph Pad), with only disease status and genotype as independent variables and expression as the outcome. Due to the low numbers of rare allele homozygotes for most SNPs, two genotype groups were considered, one containing individuals carrying both copies of the common allele (1/1) and a second one containing individuals carrying one or two copies of the rare allele (1/2+2/2). *Post-hoc* comparisons between controls and cases were performed for each genotype group and Bonferroni corrected p values were obtained.

In addition to single SNP analysis, analysis of haplotype effects on gene expression was performed. Because both gene copies represent the genetic make up of each individual, correlations with levels of expression were performed considering diplotypes using a similar approach to the one used for correlations with genotypes of single SNPs. Due to the presence of rare alleles of very low frequency and in order to reduce the dimensionality of the analysis, haplotypes were grouped together according to their similarity and LD across haplotypes. Similarity was based on the abundance of alleles shared by haplotypes within a group. LD values were calculated using the Haploview software from the Daly Lab at Broad Institute, MIT (Barrett, 2009;http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). For analysis of the effect of haplotypes on the levels of expression of candidate genes, PHASE software v2.1 (http://stephenslab.uchicago.edu/software.html) was used to calculate haplotype frequencies and to predict diplotypes for each individual in the ICPS cohort. PHASE has been demonstrated to infer haplotype phase with a 95% accuracy in previous studies based on data from unrelated individuals (Stephens et al., 2001; Marchini et al., 2006). For all three candidate genes two groups of similar haplotypes were

defined: A and B. Individuals containing diplotypes composed of two haplotypes from group A were subsequently compared to diplotypes comprising one or two copies of haplotypes in group B.

3.2.4 Analysis of SNP association with disease status.

Analysis of association of candidate genes with schizophrenia was performed using schizophrenia samples and matched controls within the East UK cohort (Section 2.1.2). Previous to SNP association analysis, deviations from Hardy-Weinberg equilibrium in all cohorts were assessed for all SNPs using the χ^2 goodness-of-fit test determined with Prism v.5, comparing observed with expected values. These results were later confirmed by analysis using the Haploview software.

Allelic and genotypic associations with disease were assessed using the Golden Helix© software. Initially, a logistic regression model was applied in order to estimate odds ratio and p value for allele associations, while genotypic associations were evaluated using a χ^2 test. In addition, a logistic regression model was also applied to the analysis in order to incorporate models for disease penetrance. Three basic models were used, comparing the effect of genotypes carrying the disease associated allele *'D'* to those containing the wild type allele *'d'*. The first model considered was the additive model where risk for disease is increased by γ (γ>1) for genotype *d/D* and of 2γ for genotype *D/D* relative to genotype *d/d*. The parameter γ refers to a measurement of genetic penetrance and is often calculated as odds ratio (OR). The second model tested was the common recessive, in which risk of disease only increases when both copies of the *'D'* allele are present. Finally, the common dominant model was tested where it is assumed that only one copy of the associated allele is sufficient to produce an increase in risk of disease of γ. Additionally, the

multiplicative model of disease is commonly used, although it has not been considered in the present study. According to this model the risk for disease increases by γ with each copy of the *A* allele present (Morris, 2008; Clarke et al., 2011; Table 3.3).

	Penetrance			Relative Risk	
Disease model	***d/d***	***d/D***	***D/D***	***d/D***	***D/D***
Additive	f_0	$f_0\gamma$	$2f_0\gamma$	γ	2γ
Common recessive	f_0	f_0	$f_0\gamma$	1	γ
Common dominant	f_0	$f_0\gamma$	$f_0\gamma$	γ	γ
Multiplicative	f_0	$f_0\gamma$	$f_0\gamma^2$	γ	γ^2

Table 3.3 Disease penetrance models from Clarke et al., 2011.
Shown in the table (modified from Clarke et al., 2011) are disease penetrance functions of genotypes *d/d, d/D and D/D* with baseline disease penetrance f_0 for the non-associated genotype *d/d*. Relative risk of disease are represented as well for genotypes *d/D* and *D/D* compared to wild type genotype *d/d,* where the genetic penetrance parameter is $\gamma>1$.

To address the problem of multiple testing, false discovery rate (FDR) values were calculated according to the Benjamini-Hochberg method of correction for multiple comparisons (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001; Storey and Tibshirani, 2003). This method has increasingly been used for other association studies as an alternative to Bonferroni-type corrections, often regarded as overly conservative since they do not take into account LD patterns between markers (Voisey et al., 2010a; 2010b; Jia et al., 2010). FDR values are calculated as part of the analysis performed by the Golden Helix© software. The Benjamini-Hochberg correction is also included as an in-build option in PLINK (Purcell et al., 2007; http://pngu.mgh.harvard.edu/~purcell/plink/), and other software packages widely used for whole-genome genetic association studies. An additional method for

multiple testing corrections used in the present study is the calculation of empirical p values by permutation testing (Evans, 2008). Although this approach is computationally demanding, most of the software packages available for analysis of SNP association are able to perform it.

Following assessment of single locus association with disease, haplotype analysis was performed. Haplotype analysis is commonly regarded to be a more powerful tool to detect genetic association with disease when compared to the single SNP approach. This is due to the fact that a haplotype approach considers LD patterns between loci. This is advantageous since it has been observed that much of common variation can be structured in haplotype blocks of SNPs in strong LD with one another (International HapMap consortium, 2005). Another important consideration supporting haplotype analysis is that protein function is determined by the sequence contained in each copy of the gene and therefore will be affected by variation in either maternal or paternal haplotypes (Clark, 2004).

The Haploview software was used to estimate LD, represented as values of D' where $0.7<D'\leq 1$ is considered as strong LD. The expectation maximisation (EM) was implemented within the Golden Helix software to calculate haplotype frequencies (Excoffier and Slatkin, 1995). Test for association was performed using a moving window approach in the Golden Helix© software. For this analysis the software generates haplotype blocks according to the settings specified by the user. For example, in an analysis comprising four SNPs, one might opt for a case/control comparison of two, three or four SNP haplotype frequencies. The number of markers selected defines the size of the 'window' that slides across the LD spine to generate

the haplotype blocks. A χ^2 test is then used to identify significant differences in haplotype block frequencies between controls and cases.

A second approach to the haplotype analysis was taken by comparing four SNP haplotype frequencies in controls and cases. In order to reduce the degrees of freedom and increase the power of the analysis, two haplotype groups were generated. These groups were defined by the likely evolution of the haplotypes and considering LD patterns across SNPs. Analysis was then carried out using a Fisher's and χ^2 test to compare haplotype and diplotype frequencies in controls and cases (analogous to the determination of allelic and genotypic associations). Once more, PHASE version 2.1, was used to predict haplotype pairs (diplotypes) for each individual.

3.2.5 Analysis of epistatic interactions between candidate genes

In accordance with the pathway approach of the present study, it was important to determine whether statistical epistasis between pairs of genes functionally related could be contributing to an increase in risk of disease. An exploratory analysis of statistical epistasis was performed using genotype data for the three candidate genes involved in synaptic and cellular communication processes from controls and cases of the East UK cohort, used for the association study. The selected tool for this analysis was, the web-based application GAIA (http://gump.qimr.edu.au/GAIA/gaia.html). This application allows the calculation of p values for interaction over and above any main effect ("interaction only" term). It also includes an "overall" p-value, which tests for significance of the joint main effects for both SNPs and the interaction effect over the mean effect. This results in a test with 3 degrees of freedom for an additive model. For the present study, the "interaction only" test was used because it has

been reported to be the most suitable of the two for candidate gene based analysis (Macgregor and Khan, 2006). Calculation of an empirical p value for the most significant interaction was carried out using 1000 permutations in each case. In addition, the Benjamini-Hochberg method was also applied in order to calculate corrected p-values for individual interaction values.

3.3 Results

In this section the results from the analysis of SNP genotype and haplotype effect on mRNA levels and disease status of three genes implicated in synaptic function and cell-cell communication processes are described. The analysis of gene expression confirmed our previous reports of significant differences in regulation of these candidate genes in schizophrenia subjects compared to healthy controls. Although no significant effect of any individual SNP variant on levels of expression was observed for any of the polymorphisms in ZnT3 or S100A8, associations were detected for three SNPs in CACNA1E. Furthermore, an overall association was detected for the four SNPs in ZnT3 contributing to a significant interaction of disease status and genotype with respect to gene expression.

In the second stage, carried out using the East UK cohort, four polymorphisms in the ZnT3 gene were found to be significantly associated with risk of schizophrenia. Allelic and genotypic associations obtained through the application of a logistic-regression model, remained significant for all four SNPs after multiple comparisons correction. Moreover, a moving-window analysis revealed a number of haplotypes significantly associated with disease status. A different approach for haplotype analysis later confirmed the significant association with disease of two groups of similar haplotypes.

Finally, evidence for statistical epistasis was observed between SNP rs11126931 in the 3' flanking region of ZnT3 and rs2280865 in intron 22 of CACNA1E. Additionally, SNP rs4652678, a synonymous polymorphism located in exon 23 of CACNA1E, was also found to significantly interact with both SNPs analysed in S100A8: rs3795391

and rs3006488. Although these interactions were not maintained after correction for multiple testing, the indication of epistatic interaction between genes that are so closely related in function makes it an interesting subject for further exploration.

3.3.1 ZnT3: Analysis of SNP genotype effect on expression levels and study of association with disease

3.3.1.1 Correlation of SNP genotypes and haplotypes with mRNA levels in the ICPS cohort

Genotype data for four SNPs in ZnT3 in controls and cases of the ICPS cohort was used for correlation with validated gene expression data of this gene in BA10 (Supplementary Table 4; Appendix C). As it has been discussed previously, ZnT3 was previously reported as significantly downregulated in the BA10 region in schizophrenia (Maycox et al., 2009). Initial three-way ANOVA was performed considering disease status, genotype and SNP ID as independent variables with mRNA levels as the outcome variable. This analysis resulted in a significant association of disease status with levels of expression ($p= 5.33 \times 10^{-4}$). Although no significant effect of either genotype or SNP ID was identified, the analysis yielded a significant p value for interaction between disease status and genotype ($p= 0.029756$). In order to further explore this interaction a two-way ANOVA analysis was carried out using only disease status and genotype as independent variables. For this analysis genotype groups (1/1 or 1/2+1/2) for each SNP were considered as levels of the variable 'genotype'. This analysis confirmed the significant association of disease status with expression levels ($p=3.0 \times 10^{-4}$), although no significant interaction of disease status with genotype was observed. Finally, post-hoc Bonferroni comparisons made between controls and cases across all genotypes did

not result in significant p-values. However, it was interesting to observe that the most substantial differences in ZnT3 mRNA levels between controls and cases were observed in individuals with the 1/1 genotype across all four SNPs (Figure 3.4).

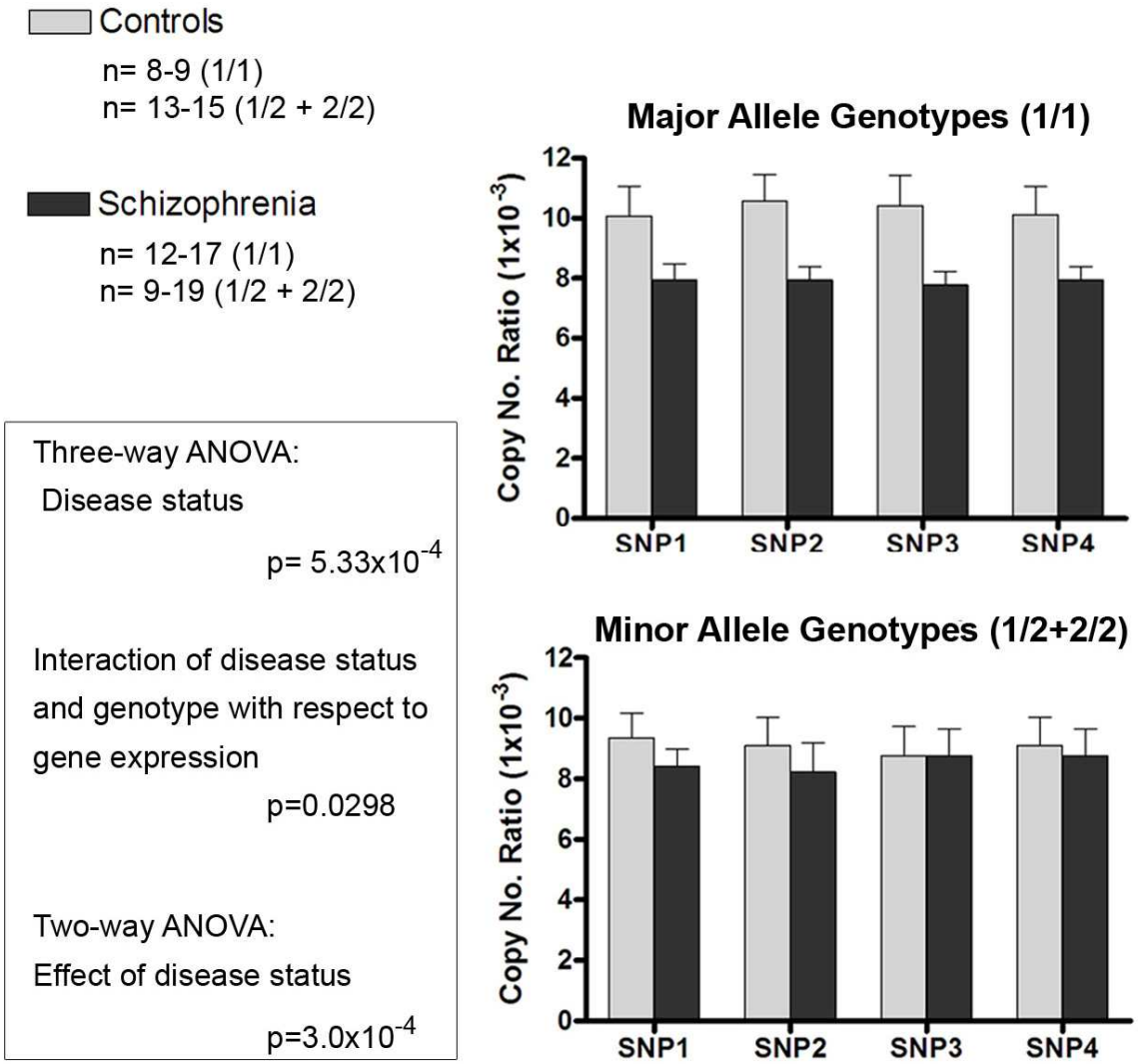


Figure 3.4 Analysis of SNP and disease status effect on ZnT3 mRNA levels.

Levels of expression of ZnT3 in BA10 are presented in this figure for controls and schizophrenia cases. For comparison, mRNA levels of ZnT3 for individuals homozygous of the major allele (1/1), of the four SNPs analysed, are presented in a separate histogram to those corresponding to subjects carrying at least one copy of the minor allele (1/2 + 2/2). Three and two-way ANOVA were performed by comparing all genotypes in controls and cases (1/1: controls n=8-9; cases n=12-17 with 1/2+2/2: controls n=13-15; cases n=9-10) across all SNPs.

In order to further explore possible SNP pair interactions, analysis of two SNP haplotypes was performed using phased data predicted from PHASE v. 2.1. For each SNP pair, expression data for individuals carrying both common haplotypes (Common diplotype: 1-1/1-1) was compared to expression data of those carrying at least one copy of the rare haplotypes (Rare diplotypes: any diplotype formed by at least one copy of haplotypes 1-2, 2-1 and 2-2). Using a similar analysis of the effect of individual SNPs on expression of ZnT3, a factorial ANOVA was performed initially considering three independent variables: disease status, diplotype and SNP pair. This approach revealed a significant effect of disease status ($p=3.1 \times 10^{-5}$) but no effect of either diplotype or SNP pair. However, a significant interaction was observed between disease status and diplotype with respect to gene expression ($p=7.1x\ 10^{-4}$), which is even more striking than for the individual SNPs. In order to evaluate these effects in more detail, a two-way ANOVA was then performed using only disease status and diplotypes as independent variables, where diplotype groups for each SNP pair were considered levels of the variable 'diplotype' and the outcome variable was gene expression. From this analysis, a significant effect of disease status on mRNA levels was confirmed ($p< 1.0 \times 10^{-4}$) but no significant direct effect between disease status and diplotype was observed. Bonferroni post-tests were used once more for comparison of ZnT3 levels of expression between controls and cases for all diplotypes. Although these comparisons did not yield any significant p values, once again it was interesting to observe that consistent with the results from analysis of single SNP genotypes, the differences in levels of expression between controls and cases were greater for individuals carrying both copies of the most common haplotype (1-1), across all SNP pairs. (Figure 3.5).

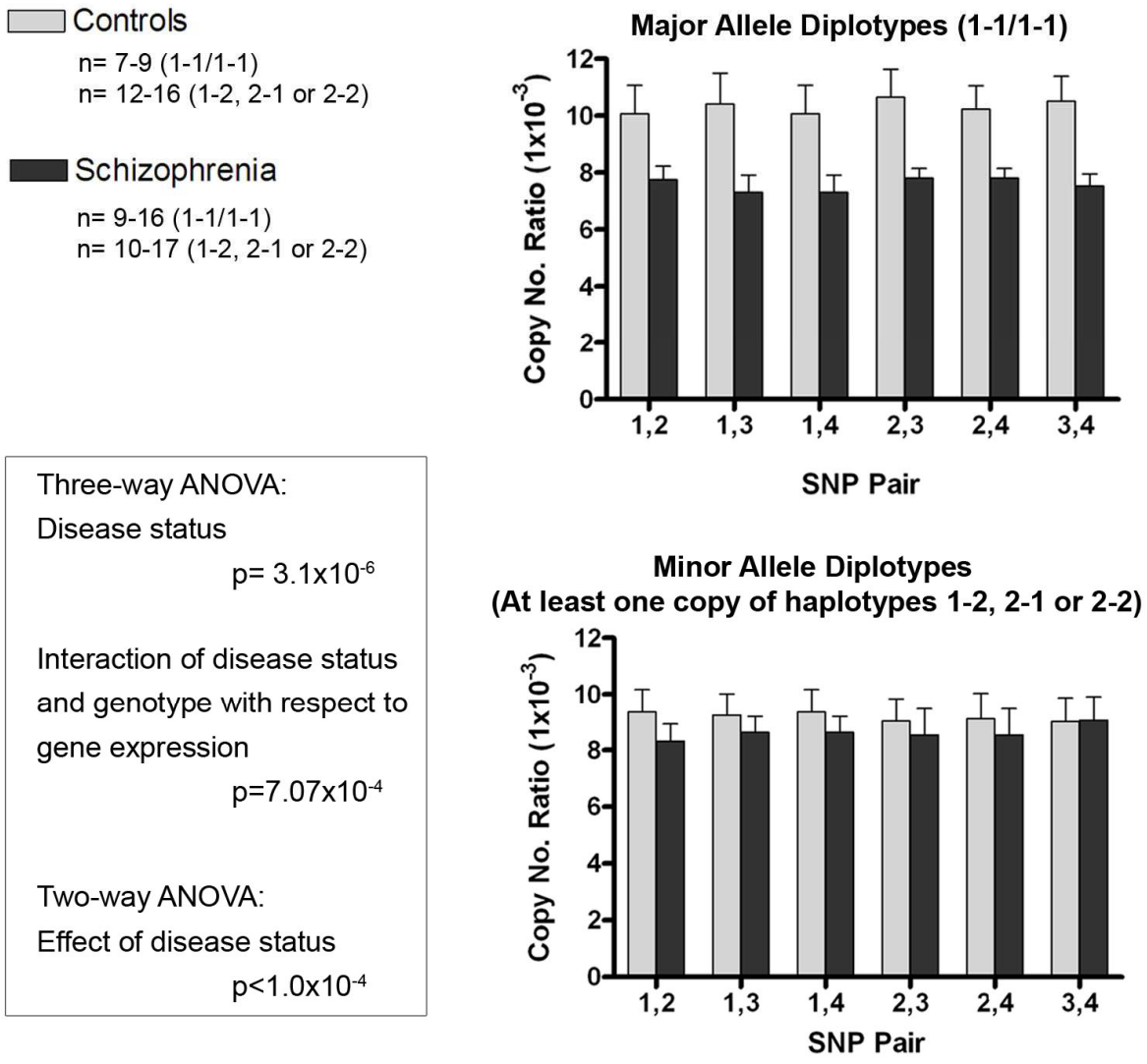


Figure 3.5 Analysis of the effect of two SNP diplotypes of ZnT3 on mRNA levels in controls and cases of the ICPS cohort.

Levels of expression of ZnT3 in BA10 are presented in this figure for controls and schizophrenia cases across all SNP pairs. mRNA levels of ZnT3 are presented in two separate histograms for purposes of clarity: One for individuals carrying both copies of the most common haplotype (1-1/1-1) and a second histogram for those carrying at least one copy of haplotypes 1-2, 2-1 or 2-2. Three and two-way ANOVA was performed by comparing both diplotype groups (1-1/1-1: controls n=7-9; cases n=9-16 or diplotypes containing one copy of 1-2, 2-1 or 2-2: controls n=12-16; cases n=10-17) across all SNP pairs.

Although no significant effects of any individual SNP genotype or 2 SNP diplotype on expression levels was observed thus far, the fact that the largest differences in ZnT3 mRNA levels are observed between controls and cases carrying two copies of the common alleles (for either one or two SNPs) prompted the analysis of the effect of four SNP haplotypes. In order to address the problem posed by the presence of very rare haplotypes and in order to increase the power of the analysis by reducing the degrees of freedom, two groups of haplotypes were defined using a method described by A. Morris (2005; 2008). Instead of grouping haplotypes according to their frequency, which may lead to a lack of power for identifying the effect of rare haplotypes, categories were defined according to likely evolution (Table 3.4). For this study, similarity between haplotypes was determined by the frequency of alleles they shared and considering the strong LD patterns observed in ICPS cohort across all four SNPs but particularly for SNPs 2, 3 and 4. A two-way ANOVA was performed in order to compare mRNA levels of ZnT3 between controls and cases carrying two copies of any combination of haplotypes in group A (A,A) with those carrying at least one haplotype from group B (A,B; B,B). No significant effect of disease status or diplotype on the expression of ZnT3 was observed, nor any significant interaction between these factors was identified (Figure 3.6).

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1-1	58.81	56.18
	1-1-2-1	2.89	0.11
	2-1-1-1	5.77	18.68
	1-2-1-1	0.01	1.43
Group B	1-2-2-2	1.08	10.93
	2-2-1-1	0.04	0.61
	2-2-1-2	1.36	0.59
	2-2-2-2	29.76	11.25

Table 3.4 Haplotype blocks defined for correlation with ZnT3 expression.

The table presents two haplotype groups defined according to frequency of alleles shared and considering strong LD patterns of all four SNPs in the ICPS cohort.

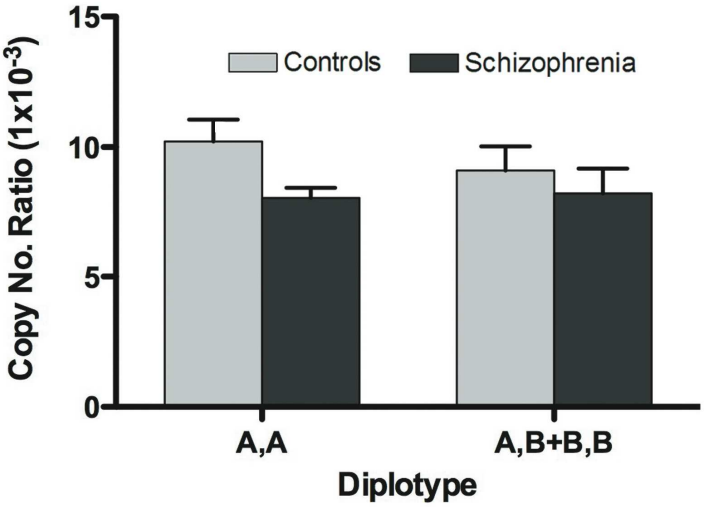


Figure 3.6 Analysis of the effect of four SNP diplotypes of ZnT3 on mRNA levels in controls and cases of the ICPS cohort.

For this analysis haplotypes were grouped according to their likely evolution in two groups (described in section 3.2.3). Both haplotype copies were considered for each individual, analogous to the analysis of individual SNPs genotypes. Two-way ANOVA was performed to compare frequencies of controls and cases carrying both haplotypes belonging to group A (controls n=10; cases n=16) with those carrying at least one of the haplotypes from group B (controls n=10; cases n=13). No significant effect of disease status or diplotype was observed nor a significant interaction between variables.

3.3.1.2 Analysis of association of SNPs and haplotypes in ZnT3 with schizophrenia in the East UK cohort

Analysis of SNP association with disease status was performed by genotyping the four selected SNPs in the ZnT3 gene (Figure 3.7) in the East UK cohort of controls (n=164) and cases (n=157) collected from the East Anglia (EA) and East Yorkshire (EY) regions in England (Section 2.1.2). Prior to the association study, deviations from Hardy-Weinberg equilibrium were assessed for all SNPs in controls and cases using Haploview software. No significant deviations were observed for any of the SNPs. Application of the χ^2 test to the analysis, using Golden Helix© resulted in significant allelic association of all four SNPs with schizophrenia. These associations remained significant after correction using FDR values (Table 3.5). In addition, the χ^2 test for genotypic association also revealed significant associations of genotype with disease for SNP 3 as well as SNP 4, and these associations remained significant after correction (Table 3.6). Further examination of the genotype data suggested that disease penetrance for all four variants could be consistent with a dominant model. This was evident when comparing odds ratio (OR) values of genotypes containing the minor allele *'D'* (*d/D* and *D/D*) with the major allele genotype (*d/d*). Indeed, application of a dominant model to a logistic regression analysis (with a χ^2 distribution and one degree of freedom) resulted in an increased power for detecting genotypic associations with disease for all four SNPs. These values remained significant after correction using FDR values (Table 3.6).

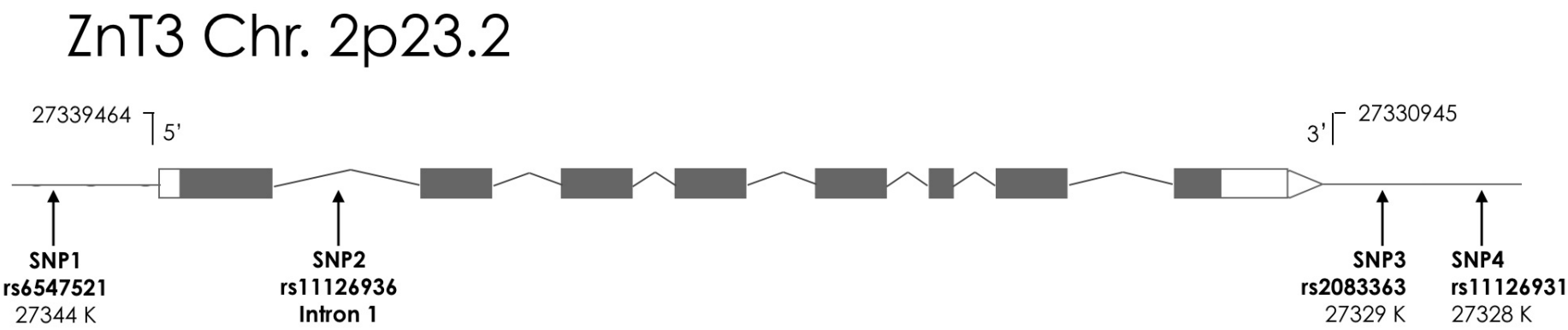


Figure 3.7 Schematic representation of four SNPs in the ZnT3 gene.

Four SNPs were selected in ZnT3 using data from the HapMap project for analysis of association of the gene with schizophrenia. SNP 1 (rs6547521), located in the 5' flanking region of the gene is 4.7 Kb from the ATG codon. SNPs 3 (rs2083363) and 4 (rs11126931), both located at the 3' end are at a distance of 1.9 Kb and 2.9 Kb from the end of the gene, respectively.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%	False Discovery Rate p value
SNP 1 rs6547521: 5'		C				
Schizophrenia	258	36.1	0.023*	1.59	1.07-2.37	0.045*
Controls	210	26.2				
SNP 2 rs11126936: Intron 1		T				
Schizophrenia	274	33.2	0.028*	1.57	1.05-2.35	0.037*
Controls	212	24.1				
SNP3 rs2083363: 3'		C				
Schizophrenia	266	31.2	0.002**	2.00	1.29-3.11	0.008**
Controls	200	19.0				
SNP4 rs11126931: 3'		T				
Schizophrenia	266	29.3	0.033*	1.58	1.04-2.42	0.033*
Controls	212	20.8				

Table 3.5 Summary of statistical analysis for allelic association of SNPs of ZnT3 in the East UK cohort.

Minor alleles of all four SNPs in the ZnT3 gene were found to significantly contribute to the risk for disease. All p values and FDR values were calculated using Golden Helix© software. Significant p values are indicated by * when $p<0.05$ and by ** when $p<0.01$.

East UK Samples	n	Genotype Frequency (%)			χ^2 Test p value (FDR)	Dominant Model Logistic regression p value (FDR)	Dominant Model Logistic regression Odds Ratio (CI95%)
SNP 1 rs6547521: 5'		**G/G**	**G/C**	**C/C**			
Schizophrenia	129	41.9	44.2	14.0	0.079	0.028*	1.78
Controls	105	56.2	35.2	8.6	(0.11)	(0.038)*	(1.06-2.99)*
SNP 2 rs11126936: Intron 1		**G/G**	**G/T**	**T/T**			
Schizophrenia	137	47.4	38.7	13.9	0.096	0.031*	1.75
Controls	106	61.3	29.2	9.4	(0.096)	(0.031)*	(1.05-2.94)*
SNP3 rs2083363: 3'		**G/G**	**G/C**	**C/C**			
Schizophrenia	133	48.1	41.4	11.0	0.008**	0.002**	2.29
Controls	100	67.0	28.0	5.0	(0.035)*	(0.009)**	(1.33-3.93)**
SNP4 rs11126931: 3'		**C/C**	**C/T**	**T/T**			
Schizophrenia	133	50.4	40.6	9.0	0.046*	0.015*	1.91
Controls	106	66.0	26.4	7.5	(0.093)	(0.029)*	(1.13-3.24)*

Table 3.6 Summary of statistical analysis of genotypic associations of SNPs in ZnT3 in the East UK cohort.

The P-values and FDRs presented in the table were calculated using Golden Helix© from the application of a χ^2 to identify genotype associations with disease. In addition, a logistic regression model was applied as a trend test in which three genetic models were tested, comparing the effect of genotypes containing the disease allele *'D'* to those with the wildtype allele *'d'*: (1) Additive, where *DD* > *Dd* > *dd*. (2) Dominant, where (*DD*, *Dd*) > *dd*. (3) Recessive, where *DD* > (*dd*,*Dd*). Significant p-values are indicated by * when $p<0.05$ and ** when $p<0.01$. The association of schizophrenia with the SNP alleles, when adjusted for the FDR, retained significance for all SNPs and this corresponded to the dominant model of genotype association (See Appendix D; **Supplementary Table 5** for best fit of data with disease penetrance models).

In order to assess a possible contribution of one or more haplotypes to the risk of schizophrenia, a moving-window analysis was performed using Golden Helix©. Linkage disequilibrium (LD) for all SNPs in controls and cases was also estimated using the HaploView platform and represented as percentage values of D' (Figure 3.8). Overall, higher LD values were seen in controls compared to cases from the East UK cohort across all polymorphisms. These differences in LD structure observed between controls and cases are interesting because they might indicate significant differences in haplotype distribution. Lower LD values in schizophrenia indicate a greater degree of chromosomal crossover between these SNPs, leading to an increase in haplotype classes that are found in schizophrenia (Figure 3.9).

This suggestion was later confirmed by the results from the haplotype analysis performed using Golden Helix©. Significant associations with disease were obtained for a number of haplotypes. In particular, it was interesting that two and four SNP haplotypes containing the common alleles of all SNPs had a protective effect while those formed entirely from minor alleles significantly increased the risk of disease (Table 3.7). Notably all common allele haplotype associations remained significant after multiple testing corrections. In addition, association of the two SNP haplotype constructed from the minor alleles of SNPs 3 and 4 also remained significant after correction.

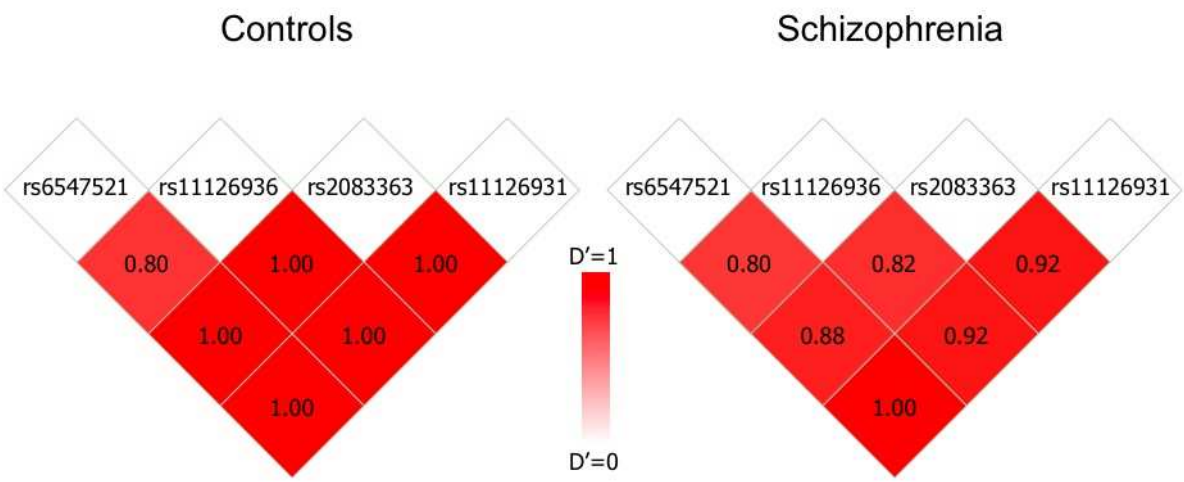


Figure 3.8 Linkage disequilibrium values for four SNPs of ZnT3 in the East UK cohort.

Linkage disequilibrium (LD) diagram obtained from analysis using Haploview for the East UK cohort. LD values are represented as D'. Although strong LD was observed across SNPs in both controls and cases (denoted by D' values rank from 0.8 to 1), LD appeared stronger in controls with a very well defined haplotype block of SNPs rs11126936, rs2083363 and rs11126931.

Sliding Haplotype Windows	2 SNP Haplotypes	Association with Schizophrenia							
SNP#	1 2 3 4	EM Cases Freq	EM Controls Freq	χ^2 p value	χ^2 FDR	Odds Ratio	Odds Ratio Lower 95% CI	Odds Ratio Upper 95% CI	At Risk or Protective Associations
Haplotype Sequences	G G	0.600	0.711	0.0147*	0.0440*	0.609	0.409	0.908	Protective
	G G	0.643	0.772	0.0032**	0.0191*	0.530	0.347	0.810	Protective
	G C	0.681	0.808	0.0024**	0.0283*	0.507	0.326	0.789	Protective
	C T	0.293	0.206	0.0355*	0.0609	1.598	1.030	2.478	Risk
	T C	0.275	0.190	0.0365*	0.0547	1.619	1.029	2.549	Risk
	C T	0.2754	0.1817	0.0196*	0.0391*	1.712	1.087	2.697	Risk
	4 SNP Haplotypes								
	G G G C	0.589	0.729	0.007**	0.048*	0.561	0.368	0.854	Protective
	C T C T	0.267	0.186	0.037*	0.074	1.649	1.028	2.646	Risk

Table 3.7 Analysis of association for haplotypes of ZnT3 with schizophrenia in East UK cohort.

Haplotype frequencies, p-values for association and odds ratio (OR) values were calculated using Golden Helix©. Corrected p-values were also calculated using FDR values. OR<1 for haplotypes composed of all major alleles suggest a protective effect of these haplotypes, while haplotypes composed of minor alleles significantly increased risk for disease. Significant p-values are indicated by * when $p<0.05$ and ** when $p<0.01$.

In order to obtain an overall p-value for association of four SNP haplotypes, data collected from the analysis from Golden Helix analysis was used to calculate an empirical p-value through a Monte Carlo method using the CLUMP software (Sham and Curtis, 1995). A significant overall p-value was obtained from this analysis (p=0.036). Moreover, the differences in frequencies of haplotypes GGGC (1111) and CTCT (2222) between controls and cases confirmed the significant effects observed from the previous analysis (Figure 3.9).

Finally, an additional haplotype analysis was performed using the approach described by Morris (2008). In an attempt to increase the power of the analysis by reducing degrees of freedom, haplotypes were grouped into two categories. Instead of defining these categories according to haplotype frequency, they were defined by haplotype similarity. This approach takes into account LD patterns across SNPs and it was preferred because it avoids pooling together rare haplotypes with large and small effect, which may result in false negative association with disease (Morris, 2006; 2008). For the analysis two groups of similar haplotypes were defined and Fisher's exact test was used to assess association with disease (Table 3.8). A significant difference was observed when comparing the frequencies of these haplotype groups between controls and cases (p=0.009), with an increase in the frequency of haplotypes of group B from 21% in cases to 31% in schizophrenia. Additionally, diplotypes were estimated using PHASE v. 2.1 and a χ^2 test was used for the analysis, analogous to the one carried out to detect genotypic associations. A significant association of diplotypes with disease status was also observed (p=0.0145; Figure 3.10).

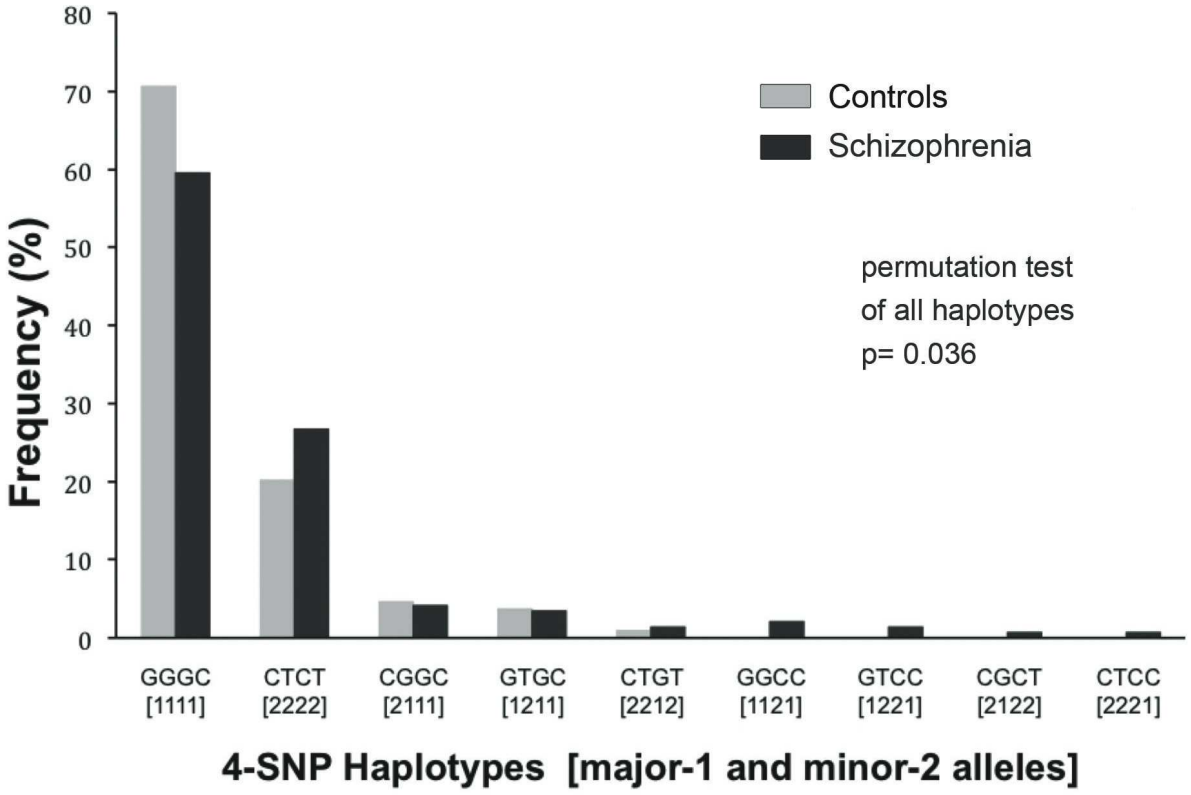


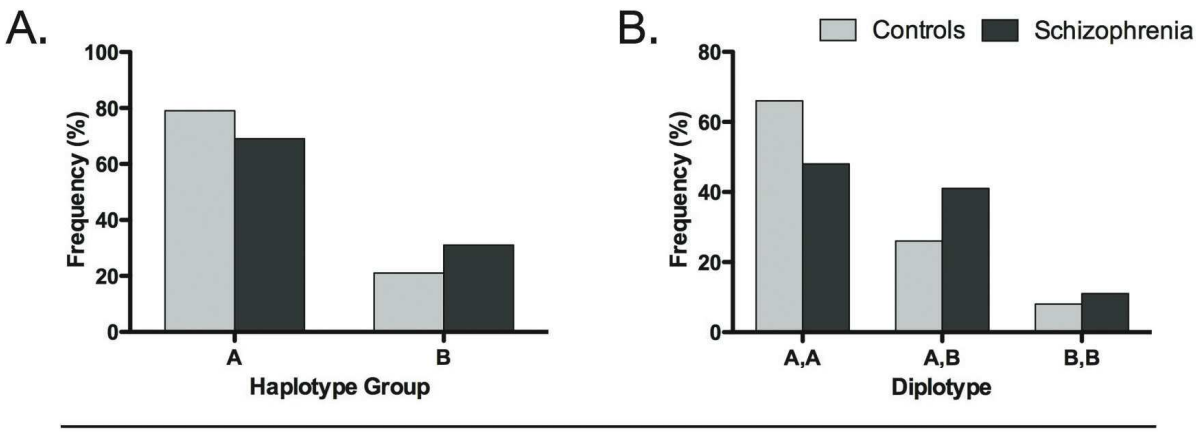
Figure 3.9 Four SNP haplotypes of ZnT3 in controls and cases from the East UK cohort.

Frequencies for four SNP haplotypes in controls and cases are presented. A significant overall p-value (p=0.036) was obtained from permutation test using the Monte Carlo based CLUMP software. The increased representation of the haplotype conformed by all common alleles (1111), as well as the decrease in frequency of the one formed by the rare alleles (2222), in controls compared to cases is consistent with results from the moving window analysis.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1-1	70.06	59.94
	1-1-2-1	0.00	0.71
	1-2-1-1	4.07	3.81
	2-1-1-1	4.84	4.34
Group B	1-2-2-2	0.01	3.50
	2-1-2-2	0.08	1.53
	2-2-1-2	1.36	0.59
	2-2-2-1	0.87	0.83
	2-2-2-2	18.72	26.77

Table 3.8 Haplotype groups defined for ZnT3 in the East UK cohort.

Considering LD patterns observed in controls and cases of the East UK cohort, two haplotype groups were defined based on haplotype similarity for association with disease status. For this purpose, data obtained from PHASE was used in order to predict both haplotypes in each individual and to calculate haplotype frequencies in controls and cases.



		Frequency (%)				
		Haplotypes		Diplotypes		
	n	Group A	Group B	A,A	A,B	B,B
East UK Schizophrenia	147	68.7	31.3	48.3	40.8	10.9
East UK Controls	110	79.1	20.9	66.4	25.4	8.2

Figure 3.10 Frequencies of two haplotype groups of SNPs in ZnT3 in East UK cohort.

Two groups of similar haplotypes were defined. Frequencies of haplotypes in Group A were compared to those in Group B in controls and cases using Fisher's exact test (**A**). A significant association was obtained for haplotype with disease due to an increase in frequencies of haplotypes in group B in schizophrenia compared to controls (p=0.009). In addition, phased data was used to perform a comparison of diplotype frequencies using the χ^2 test (**B**). This also resulted in significant association with disease status (p=0.0145).

3.3.2 CACNA1E: Analysis of SNP association with expression levels and disease status

3.3.2.1 Correlation of SNP genotypes and haplotypes with mRNA levels in the ICPS cohort

Five SNPs in the CACNA1E gene were genotyped in controls and cases from the ICPS cohort for correlation with expression data for this gene in BA22 (Supplementary Table 4; Appendix C). CACNA1E was found to be upregulated in BA22 of schizophrenia subjects when compared to healthy controls from the ICPS cohort (Appendix A: Barnes et al., 2011). Initial factorial ANOVA performed using three variables, resulted in a significant association of disease status with levels of expression ($p=0.0017$). No significant association of either genotype or SNP ID on expression was observed and no significant interactions between variables were found. A subsequent two-way ANOVA performed by considering only disease status and genotype as independent variables confirmed the significant effect of disease status ($p<1.0x10^{-3}$). Subsequent Bonferroni *post-hoc* comparisons between controls and cases across all genotypes revealed significant differences for major allele homozygotes of SNP1 and 3 as well as minor allele carriers of SNP 2 with respect to gene expression (Figure 3.11).

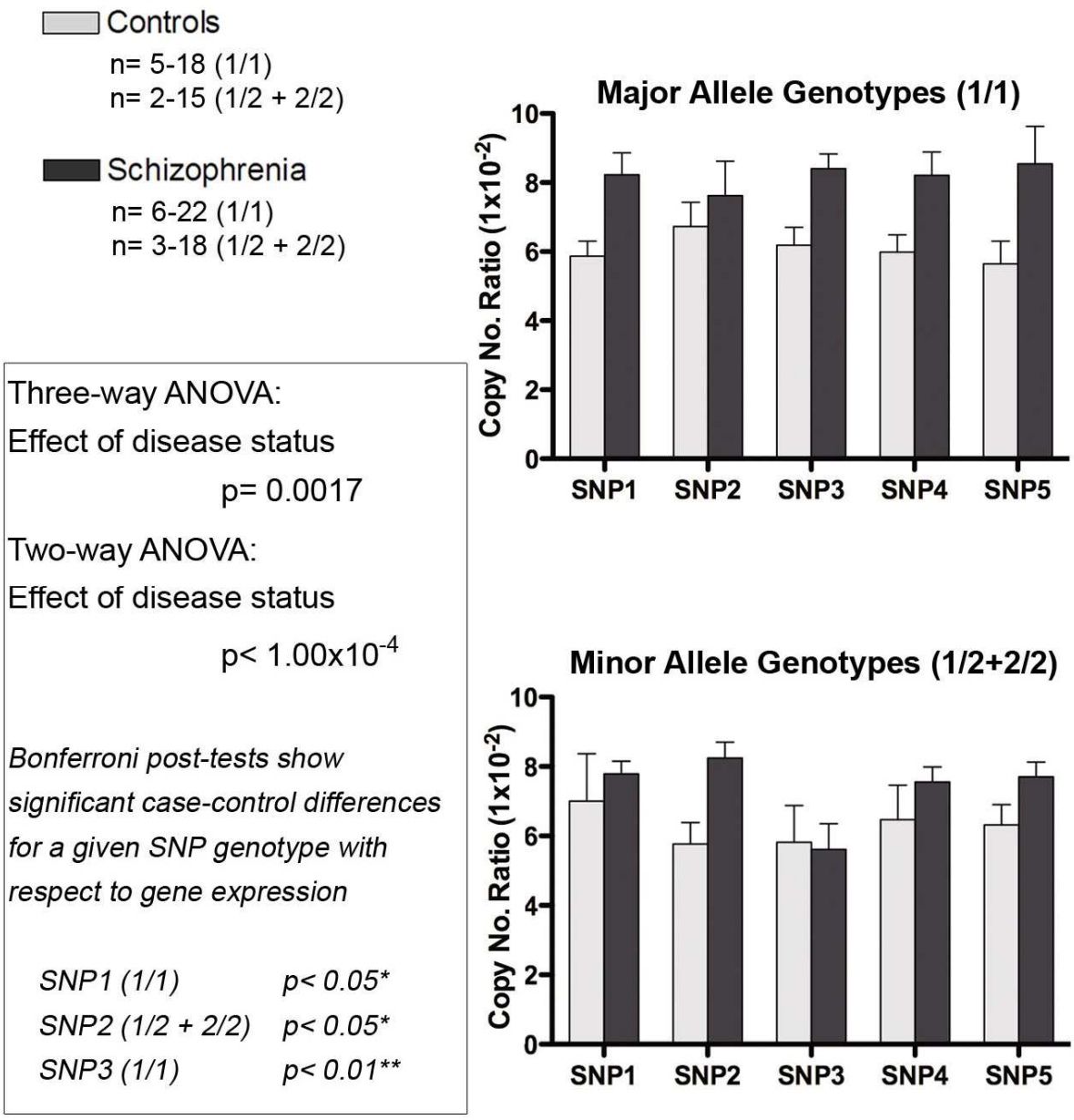


Figure 3.11 Analysis of SNP and disease status effect on CACNA1E mRNA levels.

Levels of expression for five SNPs of CACNA1E in BA22 are presented in this figure for controls and schizophrenia cases of the ICPS cohort. For clarity purposes, mRNA levels for individuals homozygous for the major allele (1/1), of the five SNPs analysed, are presented in a separate histogram to those corresponding to subjects carrying at least one copy of the minor allele (1/2 + 2/2). However, three and two-way ANOVA was performed by comparing both genotype classes in controls and cases (1/1: controls n=5-18; cases n=6-20 with 1/2+2/2: controls n=2-16; cases n=4-19) across all SNPs.

In order to determine haplotype effects on levels of expression a two SNP haplotype analysis analogous to the one performed for ZnT3 was carried out. Expression levels of CACNA1E were compared between controls and cases carrying either two copies of the common haplotype (1-1/1-1) or at least one copy of a rare haplotype (1-2, 2-1 or 2-2), using a two-way ANOVA. Two SNP pairs, SNP 2 and 5 and SNPs 4 and 5 could not be included due to insufficient number of samples in one of the diplotype groups (Figure 3.12). The analysis confirmed a significant effect of disease status ($p=3x10^{-4}$) but no effect of diplotype and no significant interactions between these variables. Bonferroni *post-hoc* comparisons between controls and cases across diplotype groups resulted in significant p-values for diplotypes of the major alleles (1-1/1-1) of SNP pairs 1,3, 1,4 and 3,4. In additon, significant differences in mRNA levels of CACNA1E between controls and cases carrying at least one copy of the less common haplotypes (1-2, 2-1 or 2-2) was observed for SNP pairs 1 and 2 and 2 and 4.

Analysis of the five-SNP haplotype effect on mRNA levels of CACNA1E was performed next using the approach described previously for ZnT3. Haplotypes were group into two categories according to their of similarity, defined by the alleles they shared (Table 3.9). Diplotypes for each individual were estimated using PHASE v 2.1 in order to compare mRNA levels of CACNA1E between controls and cases carrying two copies of any combination of haplotypes in group A (A,A) with those carrying at least one haplotype from group B (A,B and B,B). A two-way ANOVA revealed a border-line but not significant effect (p=0.0561) of disease status on levels of expression but no effect of diplotype and no significant interactions between variables either (Figure 3.13).

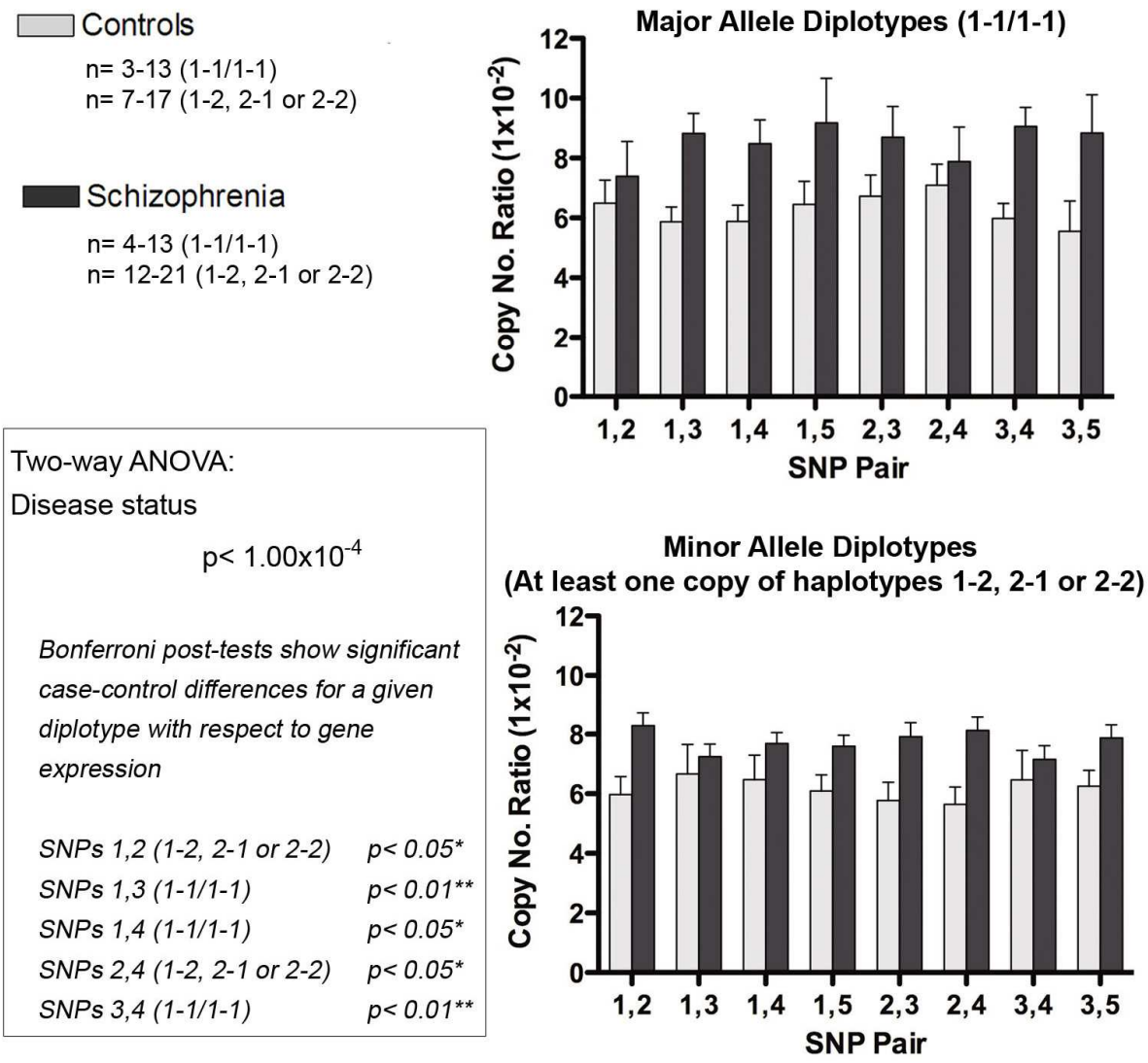


Figure 3.12 Analysis of the effect of two SNP diplotypes of CACNA1E on mRNA levels in controls and cases of the ICPS cohort.

Levels of expression of CACNA1E in BA22 are presented in this figure for controls and schizophrenia cases across SNP pairs. Haplotypes of SNPs 2 and 5 and SNPs 4 and 5 were not included due to limited number of samples in one of the diplotype groups. Two-way ANOVA was performed in order to compare expression levels in controls and cases for both diplotype groups (1-1/1-1: controls n=7-9; cases n=9-16 or diplotypes containing one copy of 1-2, 2-1 or 2-2: controls n=12-16; cases n=10-17) across all SNP pairs. In this figure mRNA levels of CACNA1E are presented in two separate histograms for clarity purposes.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1-1-1	12.44	11.82
	1-1-1-1-2	42.96	40.51
	1-1-1-2-1	2.02	3.40
	1-1-2-1-2	0.14	2.85
	1-2-1-1-1	10.37	16.02
	1-2-1-1-2	2.04	1.82
	1-2-1-2-1	4.85	3.87
	2-1-1-1-2	1.66	3.67
Group B	1-2-2-2-1	10.14	2.17
	1-2-2-2-2	1.36	0.58
	2-2-1-1-2	1.10	0.33
	2-2-1-2-1	3.40	0.62

Table 3.9 Haplotype blocks defined for correlation with CACNA1E expression.

Haplotypes were grouped according to their similarity into two blocks. Grouping was performed considering the shared alleles and LD values, particularly across SNPs 1 to 4.

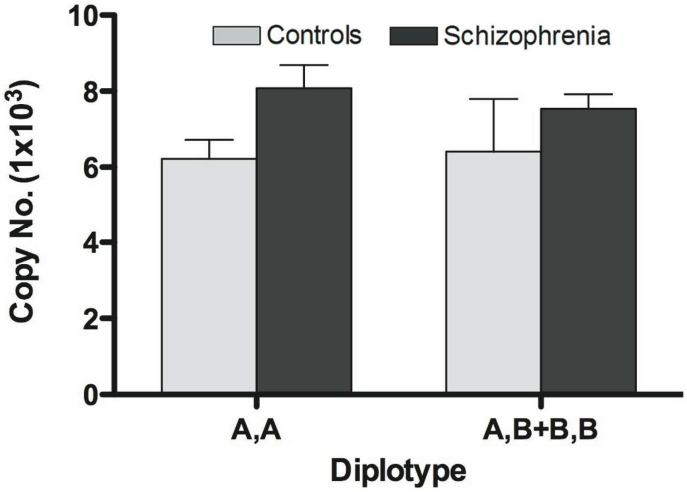


Figure 3.13 Analysis of the effect of five SNP diplotypes of CACNA1E on mRNA levels in controls and cases of the ICPS cohort.

For this analysis haplotypes were grouped according to their likely evolution in two groups (described in section 3.2.3). Both haplotype copies were considered for each individual, analogous to the analysis of individual SNPs genotypes. Two-way ANOVA revealed no significant effect of disease status, genotype or interactions. *Post-hoc* Bonferroni's corrected t-test comparisons were made between controls and cases carrying either two copies of haplotypes in Group A (controls n=14; cases n=17) or those carrying at least one of the haplotypes from Group B (controls n=6; cases n=8). No significant differences were observed in CACNA1E expression levels between controls and cases for any of the diplotype groups.

3.3.2.2 Analysis of association of SNPs and haplotypes in CACNA1E with disease status in the East UK cohort

Considering SNP frequencies in the ICPS cohort (Supplementary Table 4; Appendix C) and taking into account the power calculation previously performed (Section 3.2.4) only three SNPs in CACNA1E were considered for further study. Analysis of SNP 2 (rs2280865), SNP 4 (rs4652678) and SNP 5 (rs704326) in CACNA1E was performed using controls and cases from the East UK cohort. Even though the two alleles in SNP5 have very similar frequencies, the fact that this variant is non-synonymous was a decisive factor for the inclusion of the polymorphism in this analysis (Figure 3.14).

Previous to analysis of genetic association with disease, Hardy-Weinberg equilibrium was assessed for each group with no significant deviations observed. From analysis using a χ^2 test no allelic association with disease were observed (Table 3.10). Genotype contribution to risk of disease was also assessed using Golden Helix© to perform a χ^2 test, with no significant associations observed. In addition logistic regression analysis was performed applying the three models for disease penetrance (additive, dominant and recessive) with no significant associations detected. (Table 3.11).

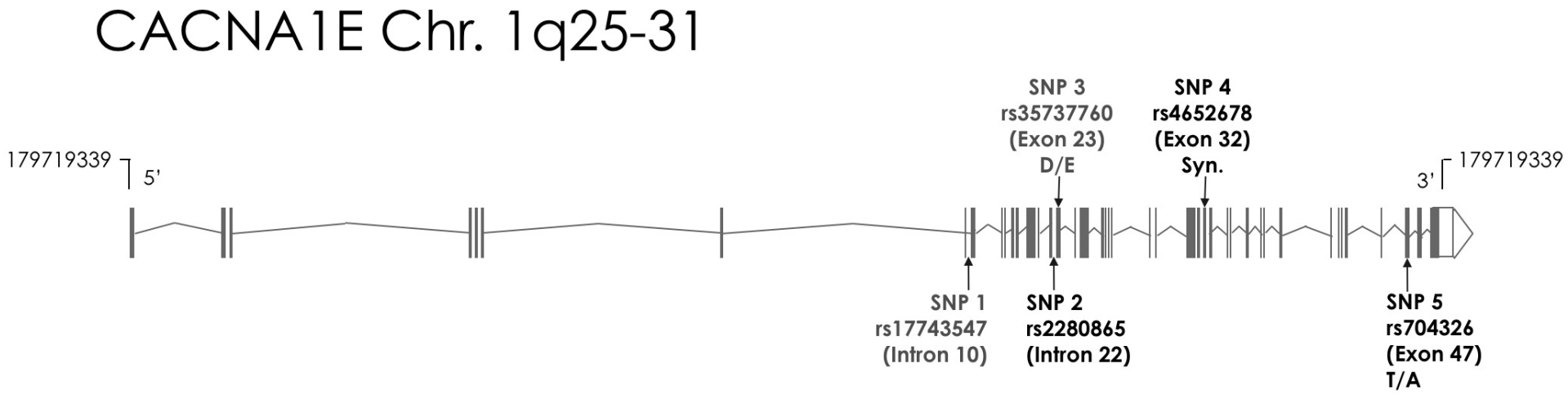


Figure 3.14 Schematic representation of the five selected SNPs in the CACNA1E gene.

From the five SNPs selected in CACNA1E using data from the HapMap project, only three were used for analysis of association of the gene with schizophrenia. SNP 1 (rs17743547), located in a splicing site and SNP 3 (rs35737760) in exon 23 (in grey) were not included in the analysis. SNP 5 (rs704326) was included in spite of its high MAF because is located in a coding area of the gene and represents a change in the amino acid residue from threonine to alanine.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%
SNP 2					
rs2280865: Intron 22					
		C			
Schizophrenia	214	42.1	0.381	0.83	0.58-1.25
Controls	172	46.5			
SNP 4					
rs4652678: Exon 32					
		C			
Schizophrenia	260	27.7	0.858	1.04	0.68-1.59
Controls	182	26.9			
SNP5					
rs704326: Exon 47 [T/A]					
		A			
Schizophrenia	270	46.3	0.831	0.96	0.66-1.40
Controls	186	47.3			

Table 3.10 Summary of statistics for allelelic association of SNPs in CACNA1E in the East UK cohort.

Results form the analysis of allelic associations of three SNPs in CACNA1E with schizophrenia. Summarised in the table are p-values and odds ratio (OR) obtained from application of the χ^2 test using Golden Helix©.

East UK Samples	n	Genotype Frequency (%)			Genotype χ^2 Test p value
SNP 2 rs2280865: Intron 22					
		T/T	**T/C**	**C/C**	
Schizophrenia	107	29.9	56.1	14.0	0.325
Controls	86	29.1	48.8	22.1	
SNP 4 rs4652678: Exon 32					
		T/T	**T/C**	**C/C**	
Schizophrenia	130	50.8	43.1	6.1	0.308
Controls	91	56.0	34.1	9.9	
SNP5 rs704326: Exon 47 [T/A]					
		G/G	**G/A**	**A/A**	
Schizophrenia	135	22.2	48.2	29.6	0.720
Controls	93	25.8	43.0	31.2	

Table 3.11 Summary of statistics for analysis of genotypic association of SNPs in CACNA1E in the East UK cohort.

Analysis of genotype effect on risk for schizophrenia of three SNPs in CACNA1E was performed using the χ^2 test. No significant associations were observed.

In order to identify possible haplotype associations with disease, the same two methods as those used for ZnT3 were applied for CACNA1E. The first approach was a moving-window analysis using Golden Helix© while the second one involved grouping haplotypes according to their similarity in order to decrease the degrees of freedom for the analysis. Linkage disequilibrium across the three SNPs in CACNA1E was estimated using Haploview with genotype data from controls and cases from the East UK cohort and represented as D'. Values of D' were similar between controls and cases with strong LD observed particularly between SNPs 1 and 2 in both groups (Figure 3.15).

Moving-window analysis did not reveal significant association of haplotypes with schizophrenia. Haplotype frequencies were very similar for controls and schizophrenia cases except for a difference observed in the most common 3-SNP haplotypes containing the major alleles for SNPs 2 and 4 and the minor allele of SNP 5 (112; Figure 3.16). A second haplotype analysis was performed by defining two groups of similar haplotypes (Table 3.12) Fisher's exact test was used to compare frequencies of haplotypes in group A and group B between controls and cases. Additionally, diplotypes were estimated for all individuals in the East UK cohort using PHASE v. 2.1. Subsequently, χ^2 test was used to compare haplotype frequencies between controls and cases carrying both haplotypes belonging to either of the two groups (A,A or B,B) or one haplotype from each group (A,B). No significant association of haplotype with disease were identified from this analysis (Figure 3.17).

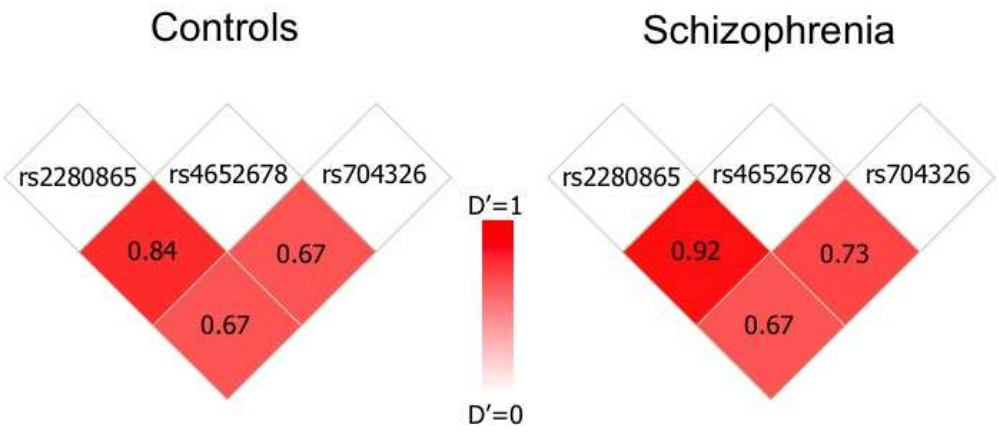


Figure 3.15 Linkage disequilibrium values for three SNPs in CACNA1E in the East UK cohort.

Linkage disequilibrium (LD) values for three SNPs in CACNA1E calculated with Haploview, using genotyping data from controls and cases of the East UK cohort. Strong LD is observed across these three SNPs in both groups with D' values between 0.67 and 0.92.

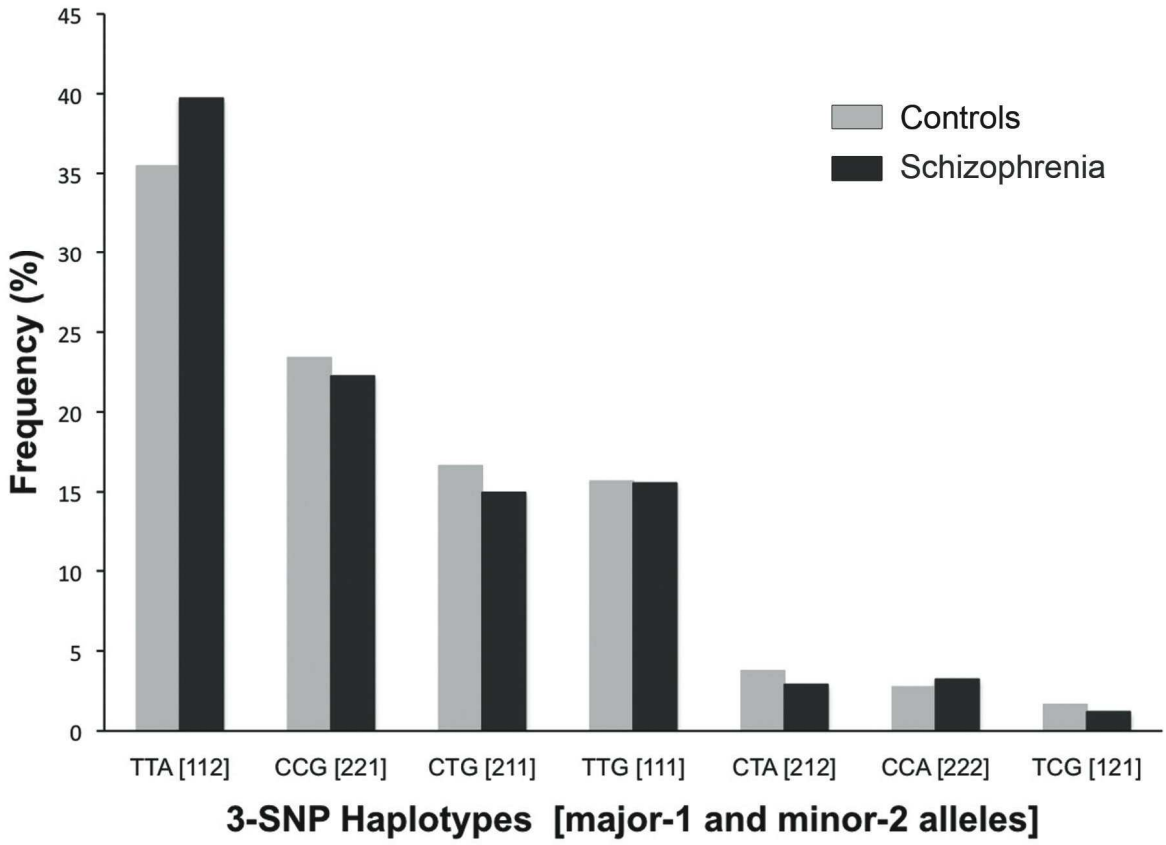


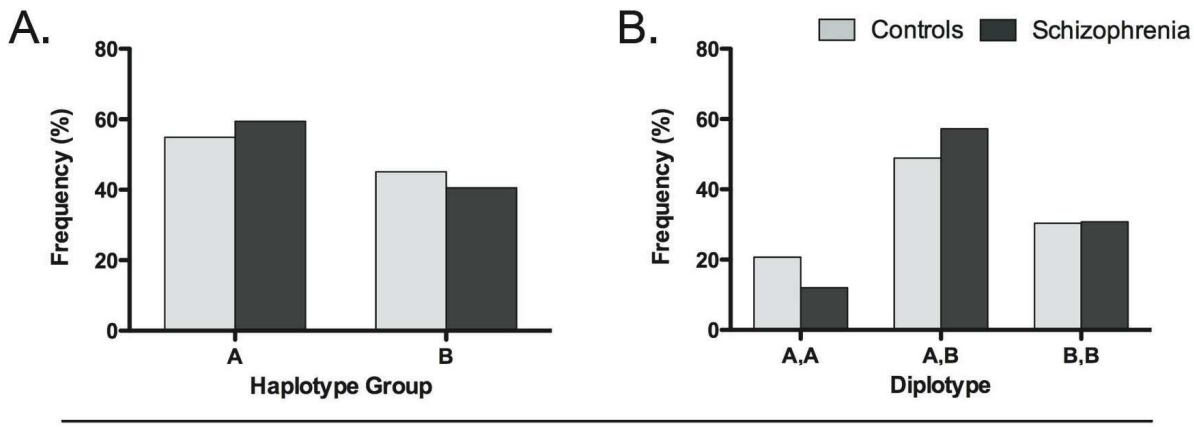
Figure 3.16 Frequencies of CACNA1E haplotypes in controls and cases of the East UK cohort.

Frequencies of 3-SNP haplotypes of SNPs 2 (rs2280865), SNP 4 (rs4652678) and SNP 5 (rs704326) in CACNA1E, in controls and cases from East UK.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1	15.62	15.21
	1-1-2	36.67	40.42
	1-2-1	1.56	1.37
Group B	2-1-1	17.88	13.85
	2-1-2	4.10	3.05
	2-2-1	21.13	22.69
	2-2-2	2.68	3.27

Table 3.12 Haplotype groups defined for CACNA1E in the East UK cohort.

Two haplotype groups for CACNA1E were defined for the analysis of their contribution to risk for schizophrenia in the East UK cohort. Shared alleles and LD across markers was considered, especially between SNPs 2 and 4. No significant association was observed.



		Frequency (%)				
		Haplotypes		Diplotypes		
	n	Group A	Group B	A,A	A,B	B,B
East UK Schizophrenia	133	59.4	40.6	12.0	57.2	30.8
East UK Controls	92	54.9	45.1	20.7	48.9	30.4

Figure 3.17 Frequencies of two haplotype groups of SNPs in CACNA1E in East UK cohort.

Two groups of similar 3-SNP haplotypes were defined for CACNA1E. Frequencies of haplotypes in Group A were compared to those in Group B in controls and cases using Fisher's exact test (**A**). Phased data was also used to compare diplotype frequencies in controls and cases using χ^2 test (**B**). No significant associations of haplotype with disease were identified from this approach.

3.3.3 S100A8: Analysis of SNP genotype effect on gene expression and study of association with disease

3.3.3.1 Correlation of SNP genotypes and haplotypes with mRNA levels in the ICPS cohort

Allelic and genotypic effects of two SNPs in S100A8 with levels of expression of the gene were explored using RT-PCR validated data from BA10 region of controls and cases from the ICPS cohort. Genotype data of these SNPs is available in Supplementary Table 4 (Appendix C). Analysis was performed as described above for the other two genes, an initial factorial ANOVA was performed with three independent variables defined as disease status, genotype and SNP ID. The analysis revealed a significant effect of disease status on mRNA levels ($p= 0.0083$) but no effect of genotype. There was no significant interaction between these variables was found either (Figure 3.18). Bonferroni post-tests were performed in order to compare expression levels of each genoytpe between controls and cases with no significant differences observed.

Finally, haplotype effects on gene expression were analysed. For this analysis, two haplotype groups were defined with the most common haplotype (1-1) in Group A, while the rare haplotypes (1-2, 2-1 and 2-2) were in Group B. Following the same methodology as the one used for the previous two genes, values of gene expression were correlated with genotype information for each individual, taking into account both haplotype pairs (diplotypes). Two-way ANOVA was performed to compare S100A8 mRNA levels in controls and cases either carrying both common haplotypes (A,A) or one or both haplotypes from group B (A,B and B,B). No significant effect of either disease status or diplotype was observed. Also, no significant interaction between these variables was found (Figure 3.19).

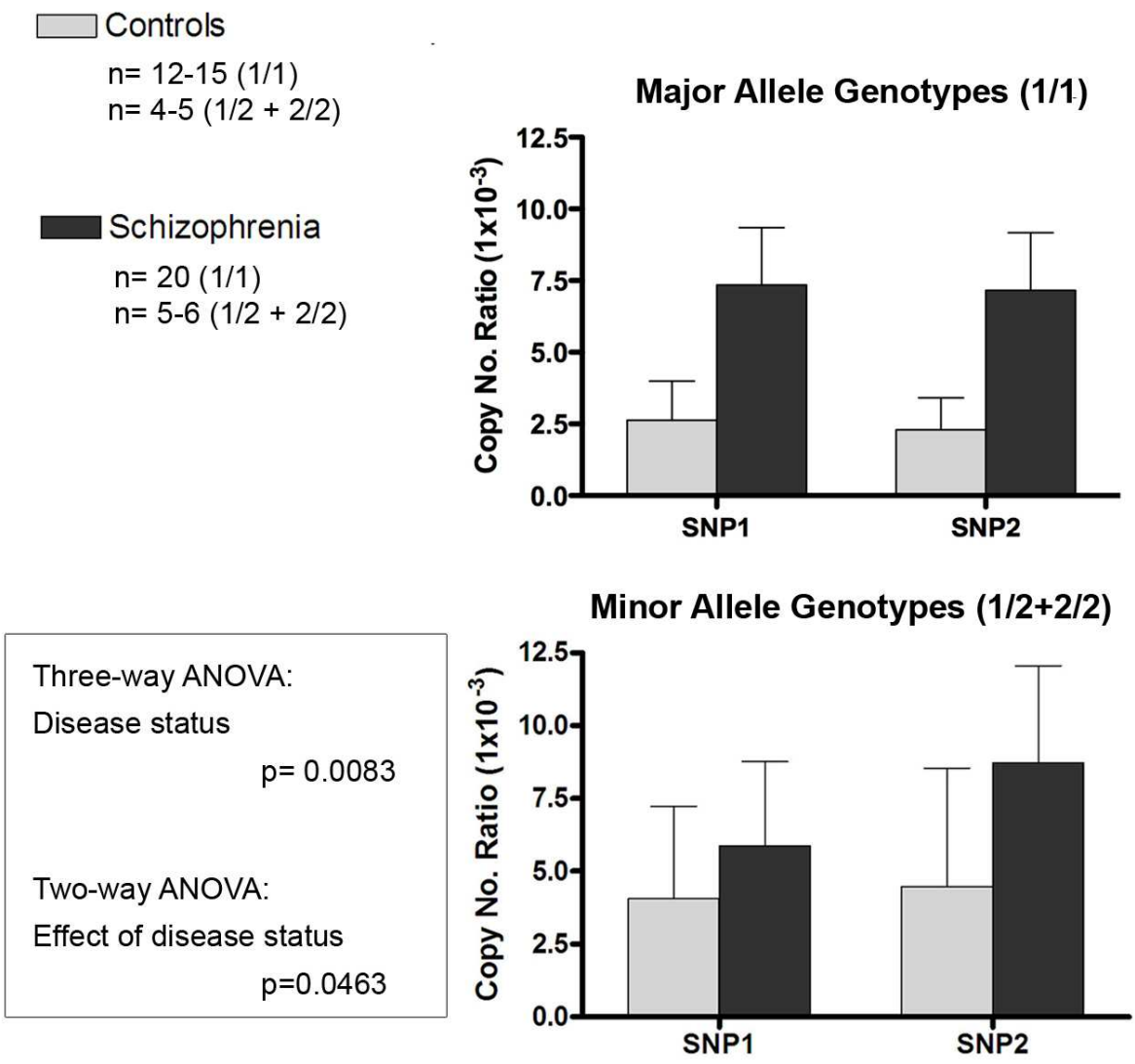


Figure 3.18 Correlation of genotype of two SNPs in S100A8 with expression levels in controls and cases of the ICPS cohort.

The figure presents S100 mRNA levels of controls and cases according to the genotype of two SNPs in this gene. For clarity purposes the figure presents expression levels of individuals carrying two copies of the major allele separate to those with at least one copy of the minor allele. For three and two-way ANOVA values of expression were compared between controls and cases across all genotypes.

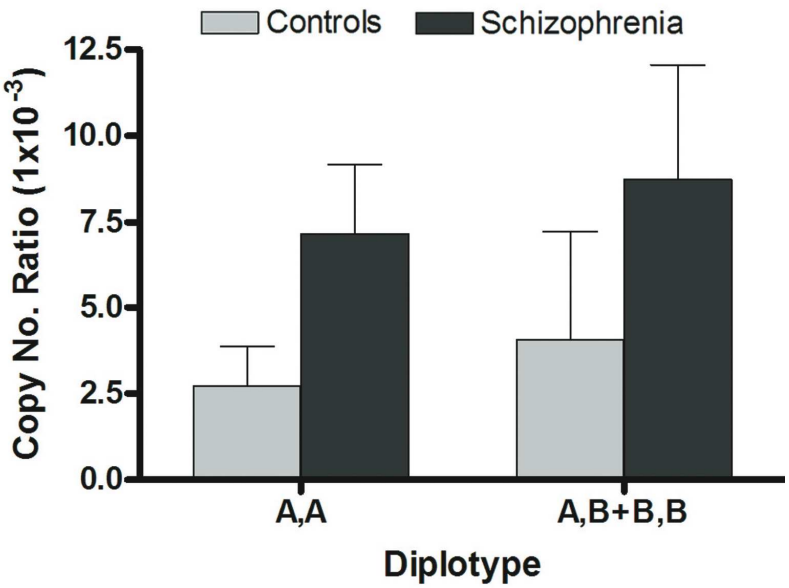


Figure 3.19 Analysis of the effect of two SNP diplotypes of S100A8 on mRNA levels in controls and cases of the ICPS cohort.

For this analysis haplotypes were grouped in two groups (described above). Both haplotype copies (diplotypes) were considered for each individual. Two-way ANOVA was performed in order to compare S100A8 mRNA levels between controls and cases carrying either two copies of haplotypes in group A (controls n=18; cases n=20) or those carrying at least one of the haplotypes from group B (controls n=5; cases n=7). The analysis did not reveal any significant effect of disease status, genotype or interactions between these factors.

3.3.3.2 Analysis of association of SNPs and haplotypes in S100A8 with disease status in the East UK cohort

Analysis of allelic and genotypic association of S100A8 with schizophrenia was carried out using genotype data for the two selected SNPs, in controls and cases from the East UK cohort. Both polymorphisms selected are located in potentially regulatory regions of the gene (Chen et al., 2006; Lomelin et al., 2010; Figure 3.20). Although none of them was identified as significantly correlated with levels of expression of the gene the possibility that they might contribute to risk of disease was interesting to explore. A χ^2 test was applied in order to determine the contribution of alleles to risk of schizophrenia was assessed using Golden Helix©. No significant association resulted from this analysis for any of the two polymorphisms (Table 3.13). Analysis of genotype contribution to risk of disease was carried out using χ^2 test and disease penetrance models (Additive, dominant and recessive) were tested using a logistic regression analysis. No significant associations of genotype with disease were observed by any of these two analyses (Table 3.14).

Finally, in order to identify possible pairwise associations of the two SNPs in S100A8 with schizophrenia, haplotype analysis was also performed using Golden Helix©. Haplotype frequencies between controls and cases were compared using χ^2 test. No significant associations were observed from this analysis.

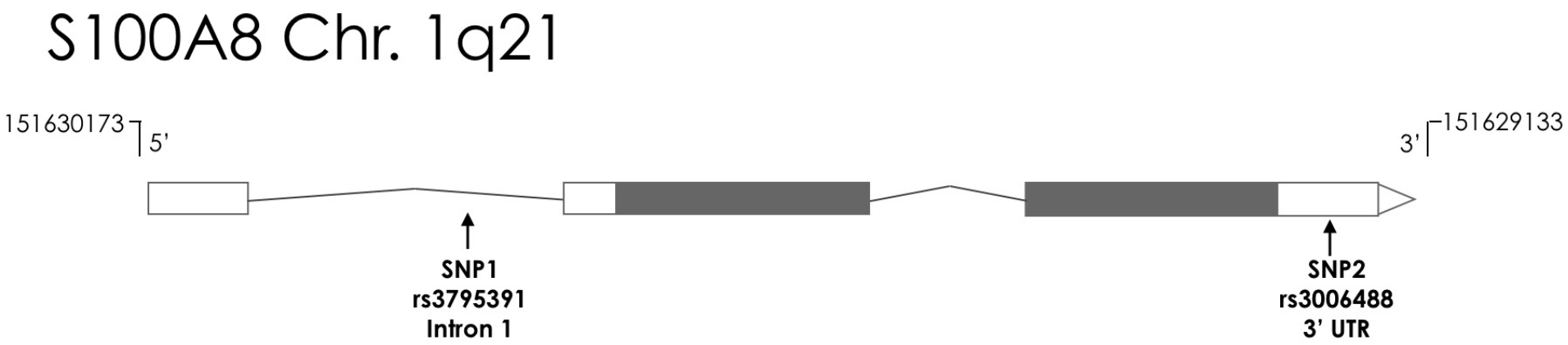


Figure 3.20 Schematic representation of the two selected SNPs in the S100A8 gene.

Although limited SNP data was available for S100A8 from HapMap project database, two polymorphisms were selected and used for analysis of association of the gene with schizophrenia. Both SNPs are located in potentially regulatory regions of the gene.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%
SNP1 rs3795391: Intron 1					
		G			
Schizophrenia	264	12.9	0.274	1.40	0.76-2.56
Controls	188	9.6			
SNP2 rs3006488: 3' UTR					
		C			
Schizophrenia	294	14.6	0.129	1.52	0.88-2.66
Controls	208	10.1			

Table 3.13 Summary of statistical analysis for allelelic association of SNPs in S100A8 in the East UK cohort.

Allele frequencies were calculated from Golden Helix© analysis. OR and p values for association with schizophrenia were obtained using a χ^2 test. No significant association with disease was observed for any of the two SNPs in East UK cohort.

East UK Samples	n	Genotype Frequency (%)			χ^2 Test p value
SNP1 rs3795391: Intron 1					
		A/A	**A/G**	**G/G**	
Schizophrenia	132	76.5	21.2	2.3	0.478
Controls	94	83.0	14.9	2.1	
SNP2 rs3006488: 3' UTR					
		T/T	**T/C**	**C/C**	
Schizophrenia	147	74.1	22.5	3.4	0.305
Controls	104	80.8	18.3	0.9	

Table 3.14 Summary of statistical analysis of genotypic associations of SNPs in S100A8 in controls and cases of the East UK cohort.

Analysis of genotypic association of two SNPs in S100A8 with disease was performed using a χ^2 test.

3.3.4 Analysis of epistatic interaction between genes involved in synaptic processes and intracellular signalling

Analysis of gene-gene interactions was performed using the online application GAIA (http://gump.qimr.edu.au/GAIA/gaia.html). For this analysis, genotype data from controls and cases of the East UK cohort was used. Individual SNP associations observed for ZnT3 were confirmed by GAIA analysis. In addition a significant interaction was observed between SNP 4 (rs2083363) in ZnT3 and SNP 2 (rs2280865) in CACNA1E (p=0.025). Moreover, interaction between SNP 4 (rs4652678) of CACNA1E and both SNPs in S100A8 (p=0.0193: rs3795391 and p=0.0476: rs3006488) were also observed to increase the risk for disease (Figure 3.21).

Multiple testing was addressed by performing a permutation test (in-built in GAIA) in order to calculate an empirical p value. As discussed previously, this method was preferred over the Bonferroni's correction because the latter is considered as too conservative since it does not take into account underlying LD patterns across markers. Although none of these interactions remained significant after correction using 1000 permutations, the interactions suggested by this analysis and the function of the three candidate genes suggest that gene-gene interactions within this group should be explored using a larger sample size and functional studies.

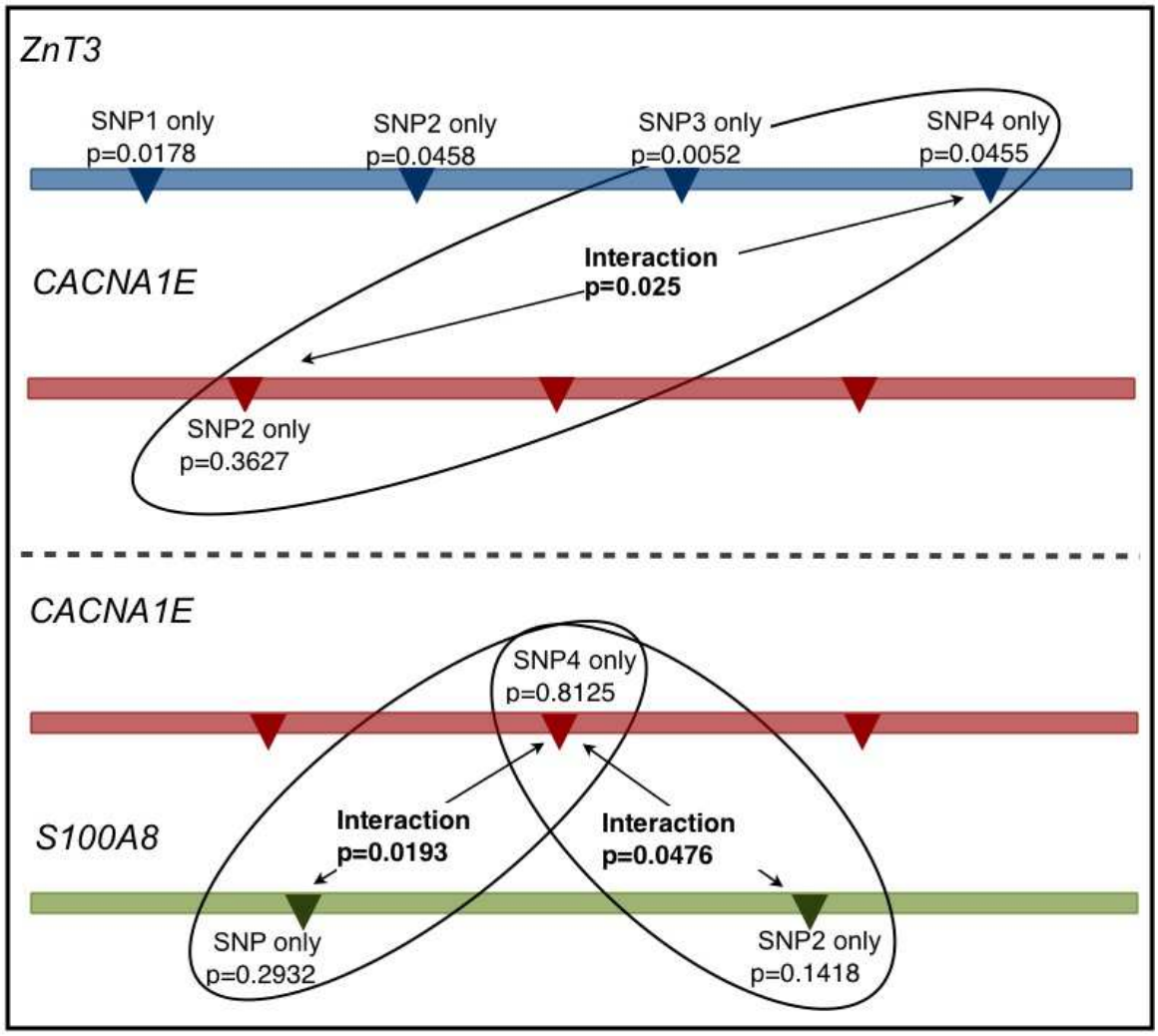


Figure 3.21 Analysis of epistatic interactions between ZnT3, CACNAIE and S100A8.

Gene-Gene interactions observed between SNP4 in ZnT3 and SNP 2 in CACNA1E. Significant interactions were also observed between SNP 4 in CACNA1E and both SNPs in S100A8. However, none of the empirical p values were significant after 1000 permutations.

3.4 Discussion

3.4.1 Analysis of SNP genotype effect on expression levels of ZnT3, CACN1E and S100A8 confirmed significant differences between controls and cases

In the present study four SNPs in ZnT3, five SNPs in CACNA1E and two SNPs in S100A8 were selected in order to explore possible genotype effects on levels of expression of these candidate genes. For this purpose, validated expression data resulting from two previous expression profiles in BA10 and BA22 (Maycox et al., 2002; Barnes et al., 2011) was used. From these studies expression of ZnT3 was reported as significantly decreased, while S100A8 was upregulated in BA10 of schizophrenic individuals when compared to controls from the ICPS cohort. Both CACNA1E and S100A8 were reported as upregulated in BA22 of schizophrenia cases of the ICPS cohort when compared to controls. RT-PCR data was available for expression of ZnT3 and S100A8 in BA10, as well as for expression of CACNA1E in BA22 of controls and cases from the ICPS cohort.

A significant association of disease status with levels of expression of the three candidate genes analysed in this section was confirmed. The differences in mRNA levels between controls and cases of the ICPS cohort observed across all genotypes were consistent with the direction of the changes reported from microarray studies for each one of the three candidate genes. Particularly interesting was the case of ZnT3 where the analysis revealed a significant genotype by disease status interaction. Although Bonferroni *post-hoc* comparisons did not result in significant p values for any of the genotypes, it was remarkable that the differences that could account for the significant effect of disease status on expression of the gene were

observed only between controls and cases with the most common genotype (1/1) for all four SNPs. Furthermore, a significant interaction of disease status and diplotype was also observed from the analysis of the effect of two SNP haplotypes on ZnT3 expression in controls and cases. As seen in single SNP genotype analysis, the greatest differences in expression levels between controls and cases were observed almost exclusively in individuals carrying both copies of the most common haplotype (1-1) for all SNP pairs.

In contrast to what was observed for ZnT3, where the significant interaction of genotype and disease status with respect to gene expression reflected an overall genotype effect, in the case of CACNA1E there was no overall effect however, significant associations of individual genotypes were observed. Bonferroni post-hoc comparisons revealed significant differences in levels of expression of CACNA1E between controls and cases with the common genotype (1/1) of SNP 1 (rs17743547) and SNP 3 (rs35737760) and controls and cases with at least one copy of the rare genotypes (1/2 and 2/2) of SNP 2 (rs2280865). Additionally, significant differences in levels of expression were observed between controls and cases with at least one copy of the rare haplotypes (1-2, 2-1 and 2-2) of SNP pairs 1,2 and 2,4. The fact that no significant association of genotype with levels of expression was observed for SNP 4 (rs4652678) individually, affirms the importance of a haplotype-based approach that might reflect the underlying LD patterns across variants. These results are noteworthy in spite of the absence of an overall effect that could contribute to a significant interaction between disease status and genotype with respect to gene expression.

The results described above suggest that the four SNPs in ZnT3 contributing to an overall association of genotype with expression, and the four SNPs in CACNA1E whose genotype was significantly associated with expression levels, could be in strong LD with polymorphisms with regulatory properties. SNP rs6547521 is located in the 5' region of ZnT3, which is interesting in the particular case of zinc transporter proteins since there is evidence of transcriptional regulation through zinc induced binding of metal-regulatory transcription factor 1 (MTF1) to metal-response elements (MREs) in target genes usually located in the 5' UTR (Zheng et al., 2008). In addition, SNP rs11126936, in ZnT3 and SNPs rs17743547 and rs2280865 in CACNA1E are all located in intronic regions of the genes. It is currently a widely accepted that intronic DNA plays and important role in regulation of gene expression, although the precise mechanisms through which this regulation takes place are not yet clear and are the subject of extensive investigation (Lomelin et al., 2010). Thus it remains a possibility that these variants might be in strong linkage with other variants directly affecting gene expression.

3.4.2 Significant association of single SNPs and haplotypes of ZnT3 with schizophrenia

Analysis of four SNPs in ZnT3 revealed significant allelic associations with schizophrenia for all markers when comparing controls and cases from the East UK. All of these associations remained significant after correction using FDR values. Moreover, genotypic association was also observed for SNP 3 (rs2083363) located in the 3' flanking area of the gene. Interestingly, when a dominant model for disease penetrance was applied, all four polymorphisms were found to be associated with disease and these associations remained significant after correction for multiple

testing. Logistic regression models are a common method used in association studies because they are more flexible than traditional tests (Clarke et al., 2011). This approach has increasingly been used in recent years for GWAS and candidate gene-base studies of association (Le Hellard et al., 2010; Bae et al, 2011).

Finally, a moving-window haplotype analysis revealed significant association with disease of two-SNP haplotypes and four-SNP haplotypes containing either major alleles for all SNPs (1-1 or 1111) or minor alleles of all SNPs (2-2 or 2222). It was seen that those associated haplotypes containing only major alleles had a protective effect, while associated haplotypes with only minor alleles, significantly increased the risk of disease. These results are consistent with associations with schizophrenia observed from analysis of single SNPs and with the strong LD patterns observed across all four polymorphisms in controls and cases of the East UK cohort. In addition, four-SNP haplotype analysis revealed a significant association with disease when common and rare haplotypes were grouped, according to their evolutionary similarity, into two categories and their frequencies were compared between controls and cases.

In summary, these findings suggest that ZnT3 may be an important candidate gene for schizophrenia, further to the previously reported downregulation of ZnT3 in BA10 of patients compared to controls, which was confirmed in two independent cohorts (Maycox et al., 2009). The identification of ZnT3 as sole transporter of the presynaptic chelatable zinc makes it the more functionally relevant in relation to schizophrenia. Although knock out of the gene in mouse and rat models had failed to produce a strong recognisable phenotype except for a relatively increased susceptibility to induced seizure (Cole et al., 1999; 2000; 2001), a recent study has

reported a seemingly age dependent cognitive phenotype in ZnT3 knock out mice at 6 months but not at 3 months of age. These findings were supported by significantly reduced expression of proteins implicated in memory and learning in older mice, including NMDAR2A/R2B subunits and AMPAR (Adlard et al., 2010). These data are consistent with previous findings of the effects of dietary zinc deficiency in age-dependant cognitive impairment in rats (Keller et al., 2001) as well as observations that deficiency of maternal dietary zinc affects expression of NMDAR subunits in pups, which persists until adult life (Chowanadisai et al., 2005).

3.4.3 No significant association of SNP or haplotypes of CACNA1E or S100A8 with schizophrenia

Although no significant differences were observed for single SNPs or haplotypes in either CACNA1E or S100A8 with disease status, these genes remain worthy of consideration not only due to the previously reported upregulation of these genes in BA22 (and of S100A8 in BA10) but also because of their location in chromosome 1q21, a region previously linked with schizophrenia (Brzustowicz et al., 2000; Hovatta et al., 1999; Ekelund et al., 2001). A number of factors could account for the lack of genetic association reported in the present study of CACNA1E or S100A8 with schizophrenia in a case-controls sample of European background. Amongst the drawback encountered in the present study it is perhaps important to consider the limited availability of characterised markers within the S100A8 region, as well as the fact that some of the polymorphisms analysed in CACANA1E turned out to be uninformative.

Other reports provide additional support for the study of these genes in the context of schizophrenia. Knock out mouse models of CACNA1E (-/- $Ca_V2.3$) have been

generated with a variety of phenotypes. Notably, alterations in pain perception (Saegusa et al., 2000; 2002), response and tolerance to morphine and cocaine (Han et al., 2002; Yokoyama et al., 2004; Osanai et al., 2006), impaired spatial memory (Kubota et al., 2001) as well as behavioural changes including increased anxiety and fear (Lee et al., 2002). Other phenotypes observed in the -/- $Ca_V2.3$ models include low seizure susceptibility (Weiergraber et al., 2007; 2008). In addition, there has been a suggestion of an important role of S100A8 in embryogenesis (Passey et al., 1999) and cell differentiation (Siegenthaler et al., 1997).

An interesting possibility to be explored is that the increased expression of CACNA1E and S100A8 in schizophrenia might be indicative of disturbances in the inflammatory response. In recent years there has been an increasing interest in the investigation of inflammatory processes in the context of schizophrenia pathophysiology. One reason for this is the apparent close relation between chronic inflammation and metabolic syndrome (Monteiro and Azevedo, 2010) and the high incidence of antipsychotic-induced lipid abnormalities and disturbed glucose metabolism (Arranz et al. 2004; McEvoy et al., 2005) in schizophrenia patients. These findings are particularly interesting when considering the growing evidence of metabolic disturbance in schizophrenia patients, as well as reports of increased risk of type-2 diabetes in schizophrenia patients across different populations (Sugawara et al., 2010; Lin and Shuldiger, 2010). Decreased glucose tolerance and insulin secretion have also been reported in mouse models of $Ca_V2.3$ deficiency (Matsuda et al., 2001; Pereverzev et al., 2002; Jing et al., 2005), consistent with recent evidence of SNP associations in the CACNA1E gene with type 2 diabetes (Holmkvist et al., 2007). Furthermore, there have been reports of increased expression of another

member of the S100 family of proteins, S100B in schizophrenia, a gene that has been associated with insulin resistance (Steiner et al., 2010).

Moreover, recent studies have reported increased expression of inflammation-related genes such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) in schizophrenia cases compared to controls, even in drug-naïve patients (Saetre et al., 2007; Reale et al., 2011). Interestingly, a number of reports have proposed that one of the mechanisms of regulation of the S100A8 gene occurs through TNF, INF-γ and interleukin-10 (IL-10) in macrophages (Xu and Geczy, 200; Xu et al., 2001). The latter is a type of activation that has been also observed in and colon cancer cells in a process that involves activation of the MAPK and JNK/p38 pathways (Ichikawa et al., 2011).

3.4.4 Evidence of modest epistatic interaction between two SNPs in ZnT3 and CACNA1E and four SNPs in S100A8 and CACNA1E

In a simplified manner, if we consider an interaction between two loci, epistasis refers to a situation where the effect of the first gene on a particular phenotype depends on the genotype of the second one. This phenomenon has increasingly been investigated in relation to diseases susceptibility in relation to complex disorders and particularly to schizophrenia (Carlborg and Haley, 2004; Daly and Altshuler, 2005; Moore, 2005). Indeed, evidence of epistasis between candidate genes in schizophrenia has been reported in a number of studies, particularly involving the COMT, NRG1, the gene coding for AKT1 protein, RGS4, DAOA and the disrupted in schizophrenia 1 (DISC1) genes (Straub et al., 2007; Nicodemus et al., 2007; 2010; Sei et al., 2010). Epistatic interactions of COMT with the aldehyde dehydrogenase 3B1 (ALDH3B1) gene have also been reported (Wang et al., 2009).

The exploratory analysis of gene-gene interactions performed using GAIA with genotype data from the East UK cohorts revealed significant nominal p values for interactions between SNP rs4652678 in ZnT3 and SNP rs2280865 in CACNA1E and between SNP rs4652678 in CACNA1E with both SNPs in S100A8 (rs3795391 and rs3006488). The analysis also confirmed the significant association of the four SNPs analysed in ZnT3 with schizophrenia. Although empirical p values calculated by 1000 permutations in GAIA for these effects did not remain significant, the indication of epistatic interactions among these genes, contributing to risk of schizophrenia is worthy of further exploration. Particularly interesting are gene-gene interactions between CACNA1E and S100A8 for their close functional relation and possible implication to metabolic syndrome in schizophrenia.

3.4.5 Conclusion

The results presented in this section provide evidence for association of four SNPs in ZnT3 with schizophrenia in the East UK cohort of controls and cases of European descent. In addition, significant haplotype associations with disease were also observed when grouping haplotypes by their similarity, in order to avoid comparisons between rare and common haplotypes (Morris, 2008). Considering the LD patterns of the four analysed SNPs in ZnT3, these findings are consistent with previous studies in which the use of a haplotype-based approach resulted in an increased power to detect associations of candidate genes with schizophrenia (Petterson et al., 2010; Vosey et al., 2010a; 2010b; Burdick et al., 2010) Moreover, the results further support the implication of ZnT3 in schizophrenia pathophysiology suggested from a previous microarray study (Maycox et al., 2009) from which the gene was found to be significantly downregulated in BA10 of schizophrenia subjects in two independent

cohorts. Even though analysis of the effect of SNP genotype on levels of expression of ZnT3 did not result in any significant correlations, it is possible that the associated markers might be in strong LD with another SNP with a regulatory effect on transcription.

ZnT3 is a strong candidate gene for schizophrenia due the specific localization of the gene in synaptic vesicles of different neuronal populations (Palmiter et al., 1996; Wenzel et al., 1997; Paoletti et al., 2009). Although the specific function of metallic zinc and of the ZnT3 regulation in synaptic processes has not been clearly established, there are some clues that point to its role in a number of processes, including memory and learning (Frederickson et al., 1990; Whittle et al., 2009). As an example, it has been established that zinc-releasing neurons are also glutamatergic and that their somata are mainly located in cerebral cortex and limbic structures (Sindreu et al., 2003; Frederickson et al., 2005; Paoletti et al., 2009), indicating a role in telencephalic information processing (Slomianka et al., 1990). Furthermore, it has been shown that endogenous zinc has a role in modulation of synaptic plasticity through inhibition of NMDA receptors in different brain regions (Vogt et al., 2000; Low et al., 2000; Coughenour and Barr, 2001; Sindreu et al., 2003; Izumi et al., 2006; Madry et al., 2007; Gielen et al, 2009; Takeda et al., 2010). Finally, association of other zinc-related genes with schizophrenia has been previously reported. Perhaps the most notable case being the SNP rs13447006 in the gene for zinc finger protein 804A (ZNF804A) identified and further replicated from two GWAS (O'Donnovan et al., 2008; ISC, 2009).

Finally, although no association of either CACNA1E or S100A8 with disease status was observed, significant epistatic interactions between these two genes were

suggested by an exploratory analysis. This was also the case for CACNA1E and ZnT3. The evidently complex regulation of the calcium channels and the numerous processes in which they are implicated makes the elucidation of their implication in schizophrenia more of a challenge. In addition it is important to consider the possibility that the observed changes in regulation of the gene are part of either a compensatory mechanism or related to co-morbidity of the disorder. An example of this could be a possible relation between the observed association of CACNA1E with glucose metabolism and insulin release (Matsuda et al., 2001; Jing et al., 2005) and the metabolic syndrome observed in patients under antipsychotic treatment.

CHAPTER 4 Correlation of gene expression with genotype and genotype with disease status of genes involved in Wnt signalling pathway.

4.1 Introduction

Wnt signalling refers to intracellular processes initiated by activation of cysteine-rich secreted ligands known as Wnt proteins. Wnt proteins have been shown to control a variety of mechanisms through different pathways. Perhaps the most extensively studied of these is the canonical Wnt signalling pathway, which is characterised by the activation of target genes through the translocation of the protein β-catenin to the cell nucleus. In addition, two other non-canonical pathways have been recognised: The planar cell polarity pathway and the calcium (Ca^{2+}) pathway (Figure 4.1 from Rao and Kühl, 2010).

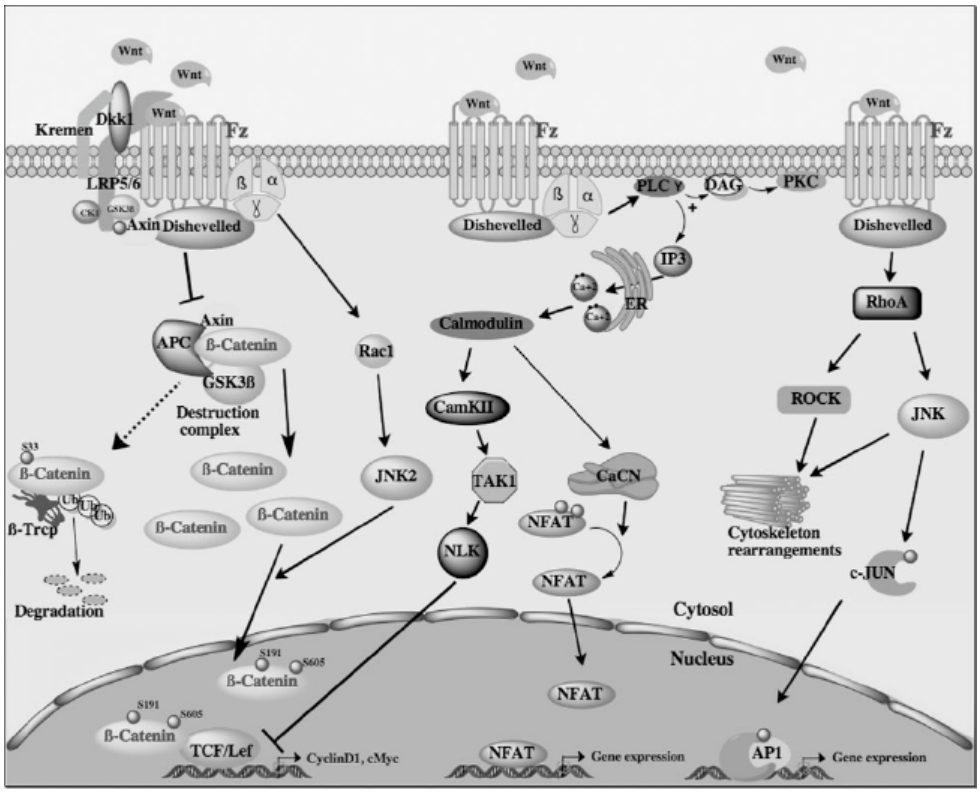


Figure 4.1 Simplified representation of Wnt signalling pathways.

The three types of Wnt signalling are represented from left to right: The canonical Wnt signalling pathway, which involves regulation of β-catenin; the calcium Wnt pathway, characterised by mediation of calcineurin (CaCN) and finally the planar cell polarity pathway, which involves jun N-terminal kinase (JNK) induced cytoskeletal rearrangements (From Rao and Kühl, 2010)

While the important role of Wnt signalling in processes such as cell proliferation, cell fate specification and cell migration during developmental stages has been well established over the past twenty years (Gavin et al., 1990; Moon et al., 1997; 2004; Wodarz and Nusse, 1998; Dale, 1998; Miller, 2002; Logan and Nusse, 2004), it was only recently recognised that Wnt pathways are also involved in the modulation of synaptic function and memory consolidation in adult CNS in mammals (Sokol et al., 1991; Ahmad-Annuar et al., 2006; Lucas and Salinas, 1997; Hirabayashi et al., 2004; Chen et al., 2006; Beaumont et al., 2007; Maguschak and Ressler, 2008; Wexler et al., 2008; Salinas and Zou, 2008; Avila et al., 2010). These findings have suggested that disruptions in Wnt signalling, or related proteins, might be implicated in the pathophysiology of schizophrenia and other neurological disorders. Increased immunoreactivity of Wnt-1 has been observed in pyramidal neurons of CA3 and CA4 regions in brains of schizophrenic subjects when compared to unaffected individuals (Miyaoka et al., 1999). The opposite was observed for β-catenin and γ-catenin whose expression was reduced in hippocampus of schizophrenia cases (Cotter et al., 1998). In addition, downregulation of the glycogen synthase kinase 3 beta (GSK-3β) has been demonstrated in frontal cortex of schizophrenia cases (Kozlovsky et al., 2000; 2004; Beasley et al., 2001). Finally, it was recently suggested that some atypical antipsychotics contribute to activation of canonical Wnt signalling in a dishevelled-3 dependant manner via D_2 dopamine receptors (Sutton et al., 2007).

It has been proposed that interactions between genetic and environmental factors during the early stages of neurodevelopment could account for the abnormalities in morphology and connectivity observed in the schizophrenic brain (Harrison, 1999; Gregorio et al., 2009; Kobayashi et al., 2010). The potential involvement of a disruption in Wnt signalling in the pathophysiology of schizophrenia is consistent with

this neurodevelopmental hypothesis and represents an intriguing subject for investigation.

4.1.1 Overview

4.1.1.1 Correlation of genotype with levels of expression

In the second part of the analysis, three genes involved in the canonical Wnt signalling pathway whose expression was significantly changed in schizophrenia from previous studies (Ftouh et al., 2005; Maycox et al., 2009; Barnes et al., 2011) were selected. Polymorphisms in each gene were genotyped in controls and cases from the ICPS cohort. Expression data provided from the previously described microarray studies (Maycox et al., 2009; Barnes et al., 2011) was then used to determine the effect of genotype/haplotype on levels of expression, as described in section 3.1.1.1. Haplotype associations with gene expression were analysed by grouping haplotypes according to their ancestral similarities, taking into account LD patterns across SNPs. Haploview was used to estimate LD values while diplotypes for each individual were estimated using PHASE v. 2.1. Correlation of diplotype with expression levels was then performed using a two-way ANOVA.

4.1.1.2 Analysis of association of genotype with schizophrenia

Further analysis of association with disease was carried out using the East UK cohort. A logistic regression model was applied for identification of significant allelic effect on risk of schizophrenia. Genotypic associations were tested using χ^2 test and logistic regression was used for testing three other genetic models where the effects of genotypes containing the associated allele '*D*' are compared to those carrying the wild-type allele only '*d*': An additive model where risk of disease is DD>Dd>dd, a dominant model where (DD,Dd>dd) and a recessive model where DD>(Dd,dd) were

tested. All tests were applied using Golden Helix© software. Analysis of association of haplotypes with disease was performed using the same two approaches used for previously described candidate genes. Initially a moving-window haplotype analysis was performed using Golden Helix©. A second analysis was later performed by grouping similar haplotypes and comparing their frequencies in controls and cases using Fisher's χ^2 test. Additionally, PHASE v. 2.1 was used to estimate diplotypes for each individual and compare their frequencies in controls and cases, using a χ^2, as it has been previously described.

Finally, we considered the possibility of an interaction between polymorphisms located in different genes associated with Wnt signalling on the risk of schizophrenia. For this reason, statistical epistasis between pairs of genes was tested as well, in the East UK cohort, using GAIA.

4.1.2 Selected candidate genes

For this part of the study, three candidate genes were selected for their involvement in the Wnt signalling pathway. Polymorphisms in the secreted Frizzled–related protein 3 (SFRP3 or FRZB), the Wnt1 inducible signalling pathway protein 3 (WISP3) and the third member of the Dickkopf family of proteins (DKK3) were genotyped for analysis of association with gene expression and disease status.

The first candidate gene, SFRP3 or FRZB belongs to a family of Wnt modulators of Wnt signalling. These proteins were initially considered to be Wnt antagonists due to the fact that they possess a cysteine rich domain (CRD) homologous to the Wnt binding domain of the Frizzled proteins. Several studies confirmed this interaction (Wang et al., 1997a; Lin et al., 1997; Bafico et al., 1999) however, data collected

from other studies suggest the existence of alternative mechanisms of interaction of SFRP3 with Wnt and Frizzled proteins that in some cases could also lead to activation of Wnt signalling (Wang et al., 1997b; Yoshino et al., 2001; Bovolenta et al., 2008; Kress et al., 2009; Mii and Taira, 2009; von Marschall and Fischer, 2010).

A number of studies have provided evidence suggesting that FRZB and other SFRPs might have an important role in regulation of cell proliferation (Bi et al., 2009) and as a tumour suppressor in various types of cancer (Zi et al., 2005; Veeck et al., 2006; 2008a; Dahl et al., 2007; Guo et al., 2008; Qu et al., 2008; Berndt et al., 2009; Kongkham et al., 2010). We selected FRZB for analysis of genetic association with schizophrenia based on the highly significant reduction in FRZB mRNA levels in BA10 in schizophrenia patients when compared to controls (Figure 4.2; Maycox et al., 2009). From the microarray data analysis a 1.6 fold decrease in mRNA levels of FRZB was observed in patients when compared to controls ($p=0.003$). Validation of this data using qPCR, later confirmed the significant downregulation of FRZB in schizophrenia ($p=0.008$). To our knowledge, no other reports of the involvement of FRZB in psychiatric disorders have been made previously.

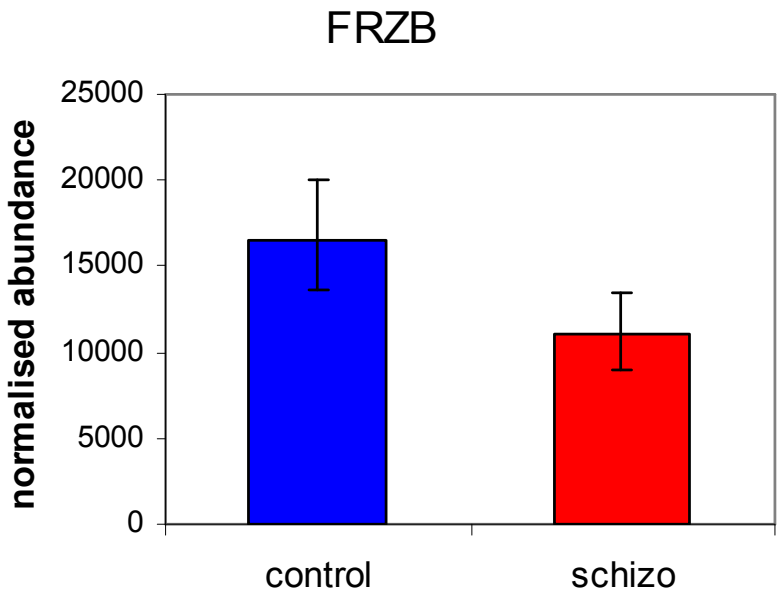


Figure 4.2 Significant difference in levels of expression of FRZB (SFRP3) in BA10 post-mortem tissue between schizophrenia cases and controls (Maycox et al., 2009)

Data from the microarray performed by Maycox et al., (2009) using two independent assays, one of which includes samples from the ICPS cohort. A 1.6 fold decrease in the expression of FRZB was observed in schizophrenia subjects when compared to controls (p=0.003). Further validation by QPCR confirmed this result (p=0.008).

The second candidate gene selected for this section was the Wnt1 inducible signalling pathway protein 3 (WISP3). Along with WISP1 and WISP2, WISP3 is a member of the CCN family of secreted growth factors, which also contains (and owes its name to) connective tissue growth factor (Ctgf), cysteine-rich protein 61 (Cyr61) and nephroblastoma over-expressed (Nov). CCN proteins have been recognised as important extracellular matrix (ECM) modulators of cellular response to environmental input. CCN proteins have been shown to participate in the regulation of embryonic development and in adult organisms they have been found to be important in inflammatory responses and injury repair. Moreover, there is evidence for the involvement of CCN factors in tumour cells proliferation, apoptosis and haematopoietic stem cells self-renewal processes (Chen et al., 2009).

Investigation of WISP3 has mainly focused on mutations that cause progressive pseudorheumathoid dysplasia (PPD), an autosomal recessive disorder characterised by progressive degeneration of the cartilage. The onset of the disease is typically between three and eight years of age and with no extraskeletal symptoms reported to date in PPD patients (Hurvitz et al., 1999, Nakamura Y et al., 2007). These findings led to the conclusion that human WISP3 acts mainly as a modulator of skeletal growth and homeostasis. However, reports of differential expression of WISP3 in colon cancer cells, compared to normal cells, have opened up a second line of research for the elucidation of the functional roles of this gene. From these first reports, several studies have been carried out to explore the mechanisms through which WISP3 contributes to tumorigenesis in colon and other types of cancer. These mechanisms seem to be closely related to the role of WISP3 in Wnt signalling processes and have been extensively explored within this context (Pennica et al., 1998; Kleer et al., 2002; 2004; 2007; Cervello et al., 2004; Zhang et al., 2005; Huang et al., 2010; Frank et al., 2010).

Although up until now, there are no reports of the involvement of WISP3 in schizophrenia aetiology, a significant increase in the mRNA levels of WISP3 in schizophrenia patients compared to controls was found in a microarray study of BA22 described previously (Appendix A; Barnes et al., 2011). Moreover, further validation of these findings in controls and cases of the ICPS cohort using qPCR confirmed the up-regulation of the gene in schizophrenia ($p=0.03$).

The third gene considered for this part of the study was a member of the Dickkopf family of proteins (DKK3). Dickkopf proteins are secreted glycoproteins particularly known for their role in regulation of the Wnt signalling pathway. The family of

human Dickkopf proteins consists of DKK1, 2, 3 and 4 and is characterized by two highly conserved cysteine-rich domains (Cys-1 and Cys-2). An additional fifth member, *soggy,* has been identified and seems to be structurally related to DKK3 but lacks the cysteine domains (Krupnick et al., 1999). From the five members of this family, DKK1 is the most studied as it was identified as a potent inhibitor of the Wnt signalling pathway during head induction in *Xenopus* embryos (Glinka et al., 1998).

DKK3 has been extensively investigated in the context of cancer due to the observed down-regulation of the gene in immortalized cells when compared to normal cells (Tsuji et al., 2000; 2001). For this reason the gene is also known as REIC (reduced expression in immortalised cells). Down-regulation of DKK3 has also been reported in a variety of cancer cell lines and tumour tissue samples, in many cases due to epigenetic mechanisms (Nozaki et al., 2001; Kobayashi et al., 2002; Abarzua et al., 2005; 2007; Edamura et al., 2007; Roman-Gomez et al., 2004; 2007; Untergasser et al., 2008; Lee EJ et al., 2009; Veeck et al., 2008b; Chim et al., 2008; Yue et al., 2008).

Although DKK3 has been mainly regarded as a tumour suppressor there are a few reports that suggest a possible involvement of this gene in the pathophysiology of schizophrenia. The present work extends previous findings from a post-mortem study of tissue samples from BA22 of controls and schizophrenia cases carried out by Ftouh et al. (2005). Using suppression subtractive hybridization, the authors identified a sequence whose expression was reduced in schizophrenia. The sequence was later recognised to be part of the DKK3 gene. Further validation of these results by slot blot hybridization revealed that expression of DKK3 in schizophrenia was reduced by 35% compared to mRNA levels in controls ($p<0.026$). Moreover,

quantitative PCR (RT-PCR) confirmed these findings with a significant reduction of mRNA levels of DKK3 in cases ($p<0.012$). Additional support for the implication of DKK proteins in the aetiology of schizophrenia has been provided by a recent report of an association of a SNP in DKK4 (rs2073665) with the disease from both family and case/control studies (Proitsi et al., 2008).

4.2 Methodology

4.2.1 Selection of polymorphisms

The selection of SNPs of interest was performed as described in section 3.2.1 using data from the open access HapMap database (http://hapmap.ncbi.nlm.nih.gov; Table 4.1). Four SNPs were selected in the FRZB gene: SNP 1 (rs10206992) and SNP 2 (rs7607737) are both located in intron 1, SNP 3 (rs288329) is located in intron 3 and finally SNP 4 (rs16823799) is located in the 3' untranslated region (UTR) of the gene. These four SNPs were of interest due to their location in regions with potential for regulation of gene expression (Chen JM et al., 2006; Lomelin et al., 2010). For analysis of WISP3 only three SNPs were selected: SNP 1 (rs2280153) and SNP 2 (rs1230348) are both located in intron 2 while SNP 3 (rs130345) is a coding variant located in exon 3 resulting in a change in the amino acid residue of the protein from glutamine (Q) to histidine (H). Finally, three more SNPs were selected for analysis of DKK3. SNP 1 (rs2291599) located in intron 5, SNP 2 (rs7480026) and SNP 3 (rs3206824) in exon 8. The latter is also a non-synonymous variant resulting in a change in amino acid from arginine (R) to glycine (G). All three SNPs are present in the two identified isoforms of the DKK3 gene.

FRZB			Frequency (%)			
	SNP Location	n	T/T	T/G	G/G	G
rs10206992	Intron 1	113	54.9	38.1	7.1	26.1
			C/C	C/T	T/T	T
rs7607737	Intron 1	113	75.2	23.0	1.8	13.3
			C/C	C/T	T/T	T
rs288329	Intron 3	62	72.6	21.0	6.5	16.9
			G/G	G/A	A/A	A
rs16823799	3'	113	88.5	8.8	2.7	7.1
WISP3						
			G/G	G/A	A/A	A
rs2280153	Intron 2	113	60.2	36.3	3.5	21.7
			G/G	G/A	A/A	A
rs1230348	Intron 2	113	43.4	44.2	12.4	34.5
			G/G	G/T	T/T	T
rs1230345	Exon 23 [Q⇒H]	113	58.4	37.2	4.4	23.0
DKK3						
			G/G	G/A	A/A	A
rs2291599	Intron 5	62	61.3	35.5	3.2	21.0
			C/C	C/T	T/T	T
rs7480026	Intron 6	113	87.6	12.4	0.0	6.2
			G/G	G/A	A/A	A
rs3206824	Exon 8 [R⇒G]	113	54.9	41.6	3.5	23.3

Table 4.1 HapMap frequency data of SNPs selected in FRZB, WISP and DKK3

Frequency data available from the HapMap database for all SNPs in genes involved in Wnt signalling pathway. The data shown in the table above corresponds to the HapMap CEU cohort, composed of unrelated individuals of European ancestry from the CEPH collection.

4.2.2 Genotyping

Genotyping was performed by polymerase chain reaction (PCR) amplification of the gene fragment containing the SNP of interest, followed by restriction enzyme digestion for determination of genotype frequencies, as described in section 3.2.2. Primer pairs for SNP genotyping were designed so that the CG content was not higher than 55% (Supplementary Table 3; Appendix B).

4.2.3 Analysis of correlation of genetic variation with gene expression

Correlation of genetic variation with gene expression was performed using genotype data from cases and controls from the ICPS cohort using the methodology described in section 3.2.3. . Genotypic associations with mRNA levels of single SNPs for all candidate genes were tested by analysis of variance (ANOVA) using Prism v. 5 software (Graph Pad). Deviation from normality of the data was assessed by in-built Prism tests such as the D'Agostino and Pearson and Kolmogorov-Smirnov normality tests. For each gene an initial three-way ANOVA was performed using disease status, genotype and SNP ID as three independent variables with expression as the outcome. Further two-way ANOVA was then used to compare expression levels between controls and cases and across genotypes.

Analysis of the effect of haplotypes on gene expression was performed using both haplotype copies for each individual (diplotypes) as described in section 3.2.3. For each candidate gene two groups of haplotypes were defined according to their ancestral similarity: A and B. Individuals containing diplotypes composed of two haplotypes from group A were then compared to diplotypes comprising one or two copies of haplotypes in group B.

4.2.4 Analysis of SNP association with disease status

Analysis of association with schizophrenia of the three candidate genes involved in Wnt signalling pathway was also performed using schizophrenia samples and controls from the East UK cohort. The analysis was carried out using the same procedure described in section 3.2.4.

For estimation of allelic association with disease, a logistic regression model was applied in order to estimate odds ratio and p values using Golden Helix©. Genotypic associations were evaluated using a χ^2 test and logistic regression in order to incorporate the three models for disease penetrance previously described. Corrections for multiple testing were performed by calculating false discovery rate (FDR) values using Golden Helix© SVS7.

Haplotype analysis was performed using a moving window approach in the Golden Helix© software as well as a second analysis performed by comparing frequencies in controls and cases of diplotype, as described in section 3.2.4.

4.2.5 Analysis of epistatic interactions between candidate genes

For the participation of these candidate genes in the Wnt signalling pathway, it was considered of great interest to explore the possible statistical epistasis within this group contributing to an increased risk of schizophrenia. This analysis was carried out using genotype data for FRZB, WISP3 and DKK3 in controls and cases from the East UK cohort. For this analysis, the web-based application GAIA was used for calculation of p values for interaction (as it has been discussed in section 3.2.5).

4.3 Results

In this section, the analysis of the correlation of genotype with mRNA levels and disease status of FRZB, WISP3 and DKK3, three genes implicated in Wnt signalling pathway, are described. The effect of genotype on gene expression in the ICPS cohort supported the significant differences in expression of these candidate genes in schizophrenia subjects compared to healthy controls previously reported from the microarray study. Significant association of genotype with gene expression was observed for four SNPs in FRZB. Moreover, significant associations between mRNA levels were detected for individual diplotypes of FRZB using ANOVA to compare gene expression levels of controls and cases with two-SNP haplotypes (1-1/1-1) or at least one copy of the rare haplotypes (1-2, 2-1 and 2-2).

The results of genotype association with schizophrenia using the East London cohort are also described in this chapter. Although not maintained after correction for multiple testing, a nominal significant association with disease was identified for the minor allele of SNP 2 (rs7480026) in DKK3, using a χ^2 test. In addition a significant effect of genotype of the same SNP 2 on disease status was identified when using logistic regression for application of an additive model of disease penetrance. This association however, was not maintained after correction for multiple testing using FDR values.

Analysis of the effect of haplotype on disease status was performed using a moving-window approach with the Golden Helix© software. This analysis resulted in significant association with disease of three haplotypes in WISP3 and four haplotypes in DKK3. In order to correct for multiple testing, FDR values were calculated based

on the Benjamini-Hochberg method. After this procedure, none of the haplotypes identified remained significantly associated with schizophrenia, in spite of the notable differences in LD patterns between controls and cases for all three genes.

Finally, an exploratory analysis of gene-gene interactions between the three candidate genes analysed in this chapter, resulted in modest evidence for statistical epistasis between SNP 1 (rs10206992) in FRZB and SNP 2 (rs1230348) in WISP3. Once more, correction for multiple testing resulted in a loss of significance for this interaction so further investigation of statistical epistasis between these two genes is necessary.

4.3.1 FRZB: Analysis of SNP genotype effect on expression levels and study of association with disease

4.3.1.1 Correlation of SNP genotypes and haplotypes with mRNA levels in the ICPS cohort

Four SNPs in FRZB were genotyped in controls and cases of the ICPS cohort (Supplementary Table 4; Appendix C) for correlation with mRNA data from BA10. FRZB was reported from a previously discussed microarray study as significantly downregulated in schizophrenia compared to controls in BA10 (Maycox et al., 2009). An initial three-way ANOVA revealed a significant association of disease status with expression ($p= 6.49 \times 10^{-11}$). No significant effect of genotype or SNP ID was identified and no interactions between variables were observed. Further two-way ANOVA confirmed the significant effect of disease status on levels of expression ($p<0.0001$). Furthermore significant associations of individual SNPs with expression levels were obtained after Bonferroni *post-hoc* comparisons. Significant differences in mRNA levels of FRZB were seen between controls and cases with the common

genotype (1/1) of SNP 2 ($p<0.05$), SNP 3 ($p< 0.01$) and SNP 4 ($p< 0.05$), as well as those with at least one copy of the minor allele (genotypes: 1/2 and 2/2) of SNP 1 ($p< 0.01$; Figure 4.3).

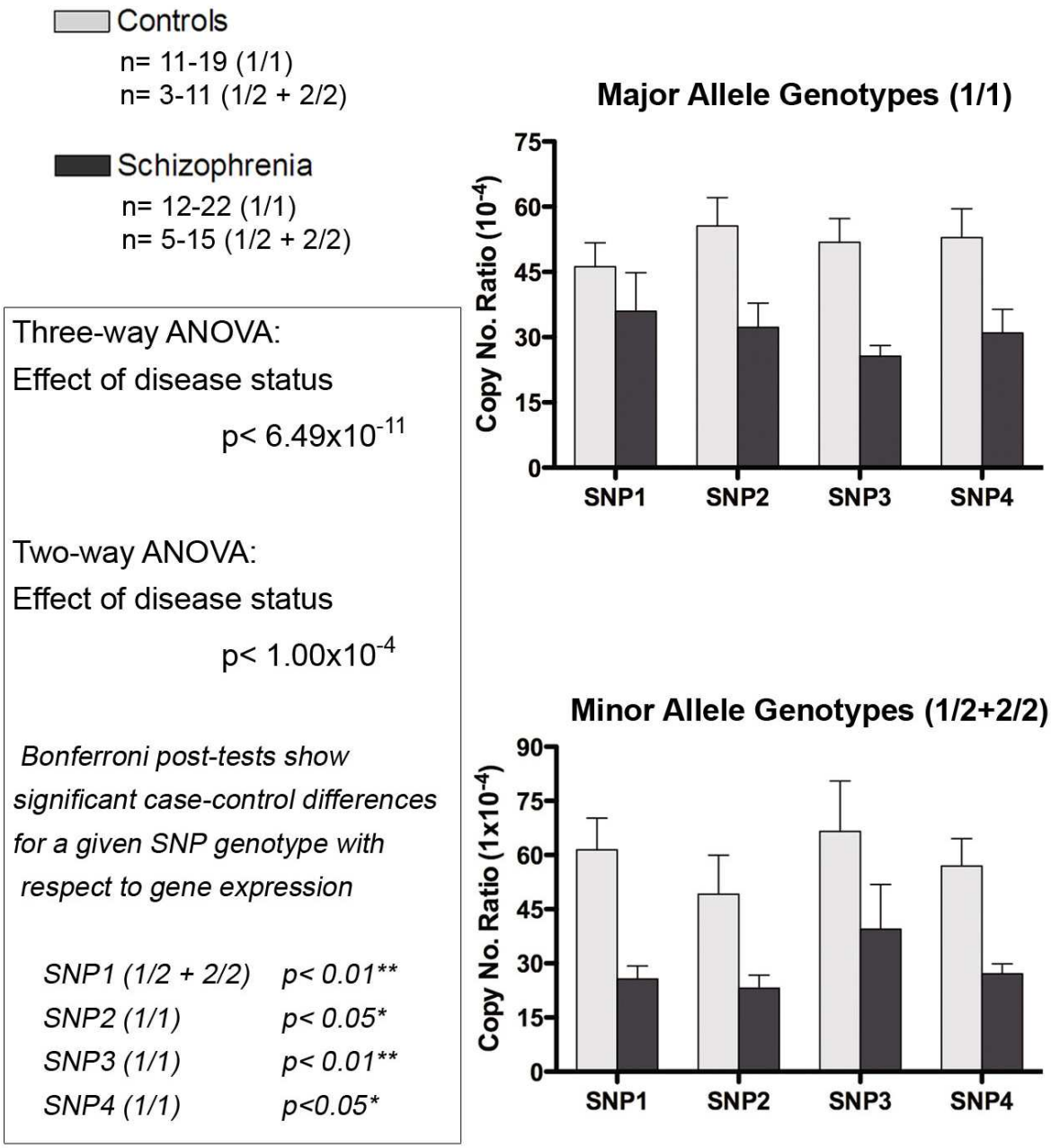


Figure 4.3 Analysis of SNP genotype and disease status effect on mRNA levels of FRZB.

Levels of expression of FRZB in BA10 are presented in this figure for controls and schizophrenia cases of the ICPS cohort. For comparison, two separate histograms are shown, for of individuals homozygous for the major allele (1/1), for the four SNPs analysed, and mRNA and for subjects with at least one copy of the minor allele (1/2 + 2/2). Three and two-way ANOVA was performed by comparing all genotypes in controls and cases across all SNPs.

Following single SNP analysis, the effect of two-SNP haplotypes on levels of expression was assessed. Two-way ANOVA was used to compare mRNA levels of controls and cases for subjects with two copies of the common haplotype (1-1/1-1) and subjects with at least one copy of the minor allele haplotypes (1-2, 2-1 and 2-2). Association of individual diplotypes with gene expression was identified from Bonferroni post-tests, that resulted in significant differences in expression levels for individuals with at least one copy of haplotypes 1-2, 2-1 or 2-2 of SNPs 1 and 2 ($p< 0.0001$), SNPs 1 and 3 ($p< 0.01$), SNPs 1 and 4 ($p< 0.01$) and SNPs 2 and 4 ($p< 0.05$) as well as for individuals with both copies of the most common haplotype of SNPs 2 and 3 ($p< 0.05$; Figure 4.4).

Finally, four-SNP haplotypes were correlated with levels of expression by grouping haplotypes into two groups according to their likely evolution. Phased data was obtained for controls and cases of the ICPS cohort, using PHASE v. 2.1 and Haploview was used to determine LD patterns across the four polymorphisms. Since linkage disequilibrium values for controls and cases were highest between SNP 1 and SNP 2 ($D'=1$), haplotype groups were defined as those containing the major allele for SNP 1 in Group A and those carrying the minor allele for SNP 2 in Group B (Table 4.2). Once these groups were defined, expression levels were compared between controls and cases carrying both copies of haplotypes in Group A (A,A) with those having at least one copy of haplotypes in Group B (A,B and B,B). Two-way ANOVA yielded a significant effect of disease status ($p= 7.0 \times 10^{-4}$) and a significant interaction of disease status with diplotype (0.0143). Furthermore, Bonferroni post-tests revealed a significant difference in mRNA levels for FRZB of controls compared to cases carrying at least one of the haplotypes in Group B (Figure 4.5). These

results further confirmed the significant association of defined diplotypes with levels of expression, which was also evident from the previous analysis of two-SNP haplotypes.

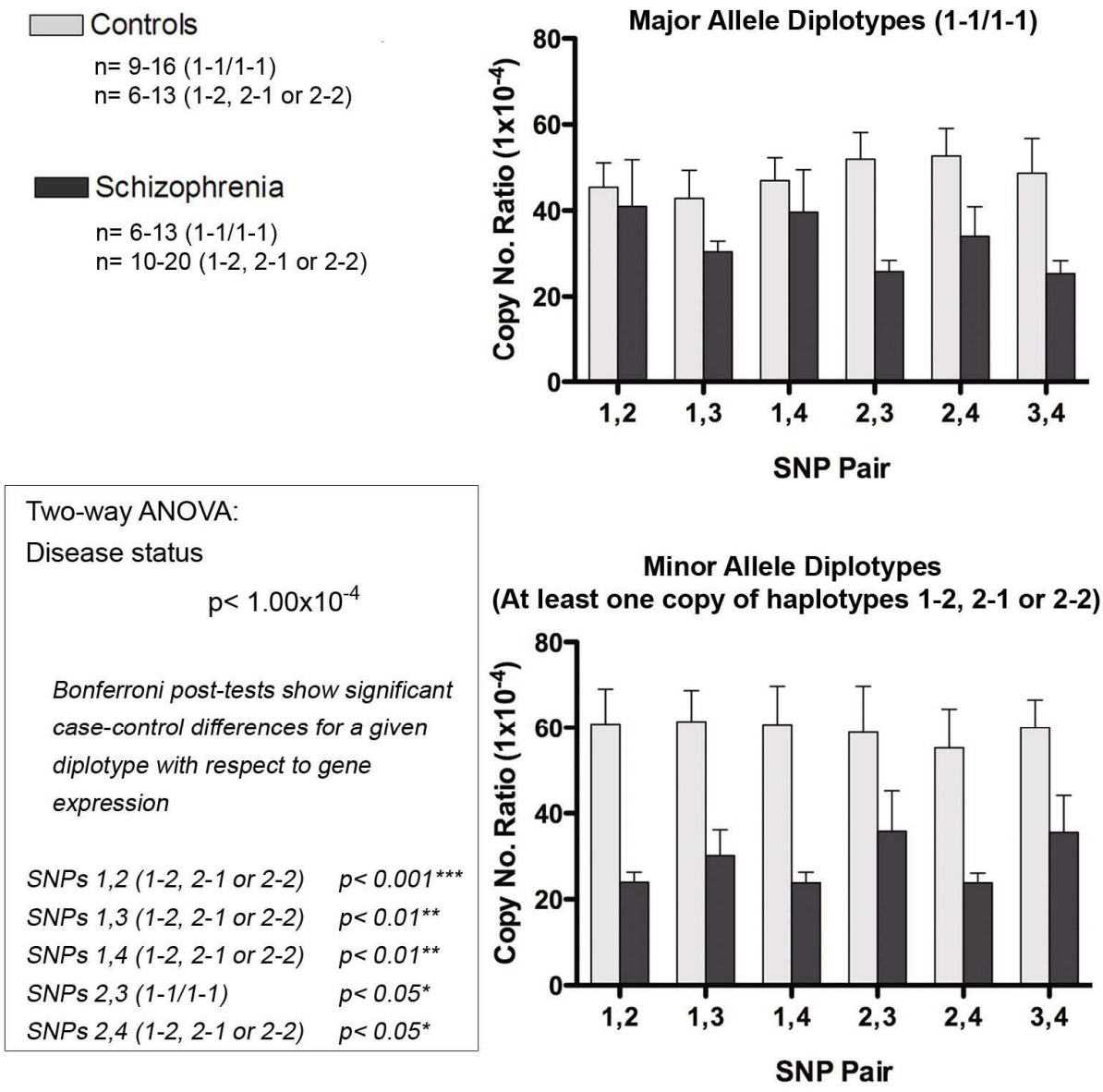


Figure 4.4 Analysis of the effect of two-SNP diplotypes of FRZB on mRNA levels in controls and cases of the ICPS cohort.

Levels of expression of FRZB in BA10 are shown in this figure for controls and schizophrenia cases across all SNP pairs. mRNA levels are presented in two separate histograms for clarity: one for individuals carrying both copies of the most common haplotype (1-1/1-1) and a second histogram for those carrying at least one copy of haplotypes 1-2, 2-1 or 2-2. Three and two-way ANOVAs were performed by comparing both diplotype groups.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1-1	58.93	42.37
	1-1-1-2	1.22	1.29
	1-1-2-1	5.91	11.79
	1-2-1-1	1.27	5,14
	1-2-1-2	2.31	2.81
	1-2-2-1	2.41	9.48
Group B	2-1-1-1	22.97	18.48
	2-1-1-2	4.27	4.05
	2-1-2-1	0.66	2.35

Table 4.2 Haplotype groups defined for FRZB in the ICPS cohort.

This table presents two haplotype groups defined according to LD patterns across SNPs in the ICPS cohort. Major and minor alleles of SNP1 were used to determine haplotypes in each group.

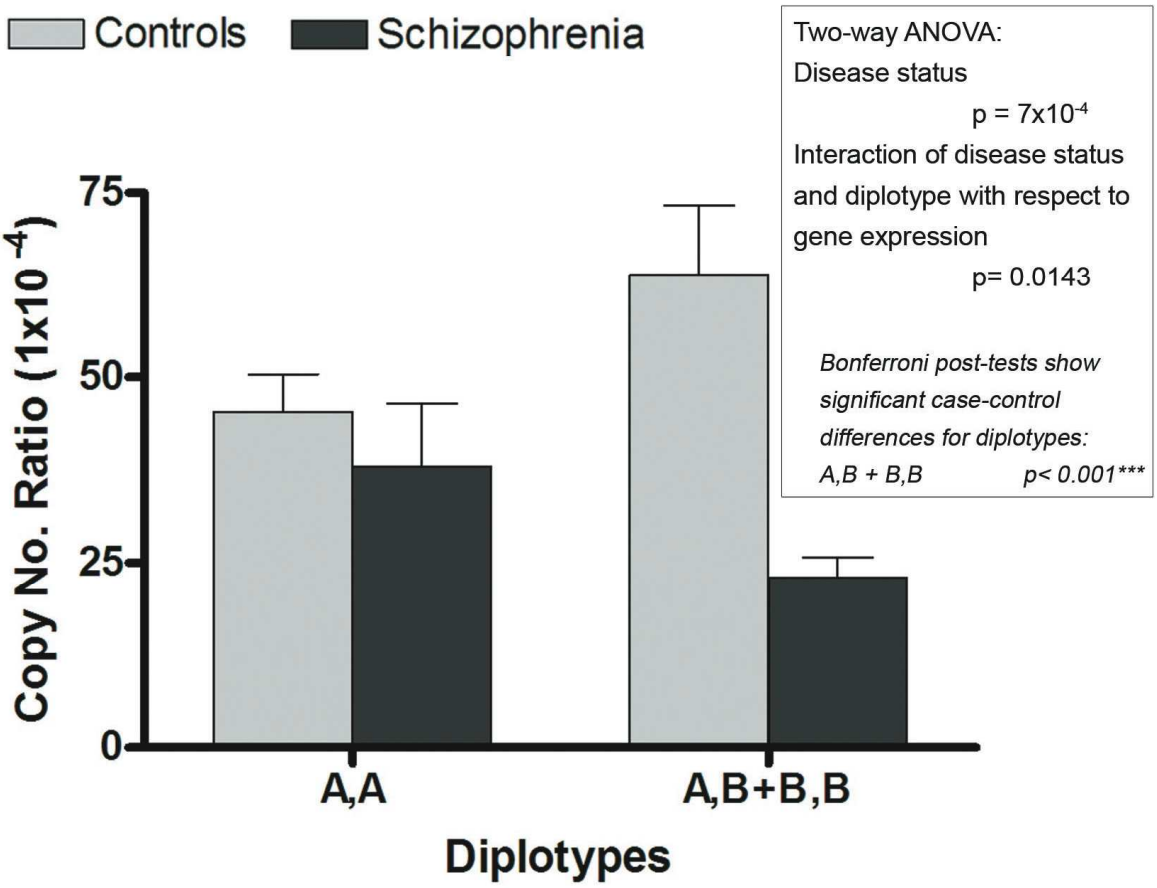


Figure 4.5 Analysis of the effect of four SNP diplotypes of FRZB on mRNA levels in controls and cases of the ICPS cohort.

For this analysis haplotypes were grouped according to their likely evolution in two groups (described above). Both haplotype copies were considered for each individual, analogous to the analysis of individual SNPs genotypes. Two-way ANOVA was performed to compare mRNA levels of controls and cases carrying both haplotypes belonging to Group A (controls n=12; cases n=13) with those carrying at least one of the haplotypes from Group B (controls n=10; cases n=14).

4.3.1.2 Analysis of association of SNPs and haplotypes in FRZB with disease status in the East UK cohort

The four SNPs used above (Figure 4.6) were genotyped in the East UK cohort of controls and cases (total n=321) for analysis of SNP association with disease status. No deviations from Hardy-Weinberg equilibrium were observed for any SNPs using Haploview software. Application of a logistic regression model to the analysis, using Golden Helix© did not result in significant allelic associations (Table 4.3). Also, no significant genotype effect on disease status resulted from the χ^2 test. Finally, application of logistic regression to test disease penetrance models did not result in significant association of genotype with disease (Table 4.4).

Even though no SNP associations with disease were obtained, haplotype associations were explored because analysis of LD patterns across polymorphisms provides a powerful approach in detecting significant association of candidate genes with disease status. Initially, LD values were calculated using Haploview in the East UK cohort. Variations in LD patterns were observed between controls and cases, which indicates possible significant differences in haplotype distribution (Figure 4.7). Moving-window haplotype analysis did not result in significant associations for two, three or four-SNP haplotypes, consistent with the similar frequencies of four-SNP haplotypes in controls and cases of the East UK cohort (Figure 4.8).

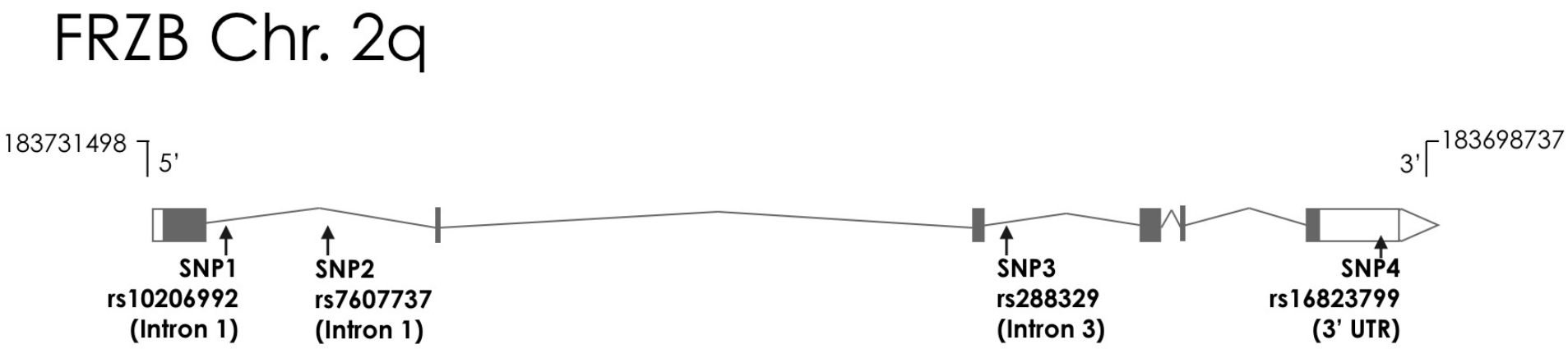


Figure 4.6 Schematic representation of four SNPs in FRZB.

Four SNPs in FRZB were genotyped in the East UK cohort analysis of association with schizophrenia.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%
SNP 1 rs10206992: Intron 1					
		G			
Schizophrenia	288	20.8	03532	0.82	0.54-1.25
Controls	218	24.3			
SNP 2 rs7607737: Intron 1					
		T			
Schizophrenia	266	12.4	0.4554	0.82	0.48-1.39
Controls	210	14.8			
SNP3 rs288329: Intron 3					
		T			
Schizophrenia	266	15.4	0.8666	1.04	0.73-1.74
Controls	202	14.9			
SNP4 rs16823799: 3' UTR					
		A			
Schizophrenia	264	7.2	0.8386	0.93	0.47-1.86
Controls	208	7.7			

Table 4.3 Summary of statistics for allele association of SNPs in FRZB in the East UK cohort.

No significant associations were seen from application of a logistic regression model to calculate p values and odds ratio (OR) using Golden Helix©.

East UK Samples	n	Genotype Frequency (%)			Genotype χ^2 Test p value
SNP 1 rs10206992: Intron 1		**T/T**	**T/G**	**G/G**	
Schizophrenia	144	61.1	36.1	2.8	0.2389
Controls	109	58.7	34.0	7.3	
SNP 2 rs7607737: Intron 1		**G/G**	**G/A**	**A/A**	
Schizophrenia	133	75.9	23.3	0.8	0.2614
Controls	105	61.3	29.2	9.4	
SNP3 rs288329: Intron 3		**G/G**	**G/A**	**A/A**	
Schizophrenia	133	70.7	27.8	1.5	0.9099
Controls	101	72.3	25.7	2.0	
SNP4 rs16823799: 3' UTR		**C/C**	**C/T**	**T/T**	
Schizophrenia	132	85.6	14.4	0.0	0.8316
Controls	104	84.6	15.4	0.0	

Table 4.4 Summary of statistics for analysis of genotypic associations of SNPs of FRZB in the East UK cohort.

P-values are presented in this table following the application of χ^2 to test for genotype associations with disease using Golden Helix©. No significant associations were observed for any one of four polymorphisms analysed.

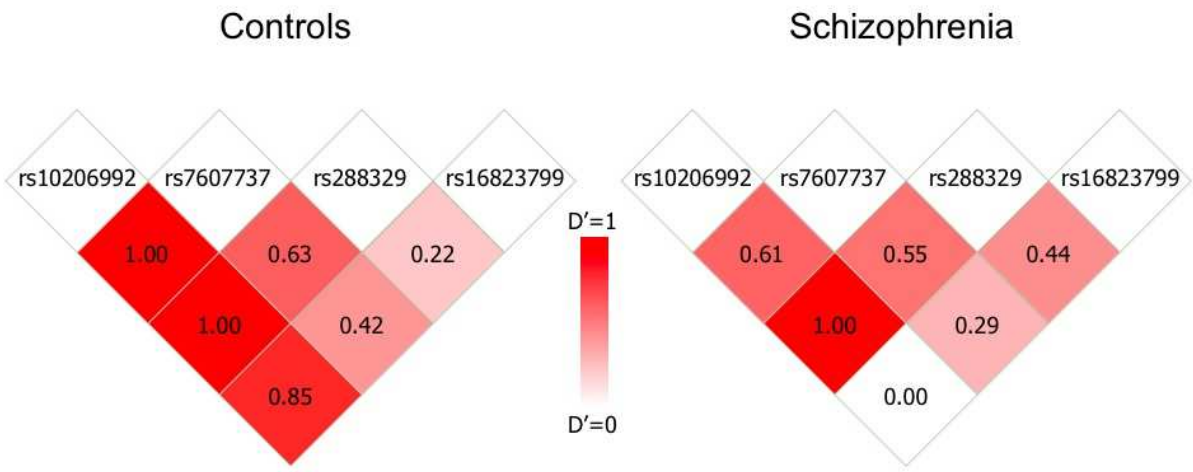


Figure 4.7 LD values for four SNPs of FRZB in the East UK cohort.

Linkage disequilibrium (LD) diagram obtained using Haploview for controls and cases of the East UK cohort. Although strong LD was observed for SNP 1 with all other polymorphisms in controls (D' values rank from 0.85 to 1), clear differences in this pattern were observed in schizophrenia cases.

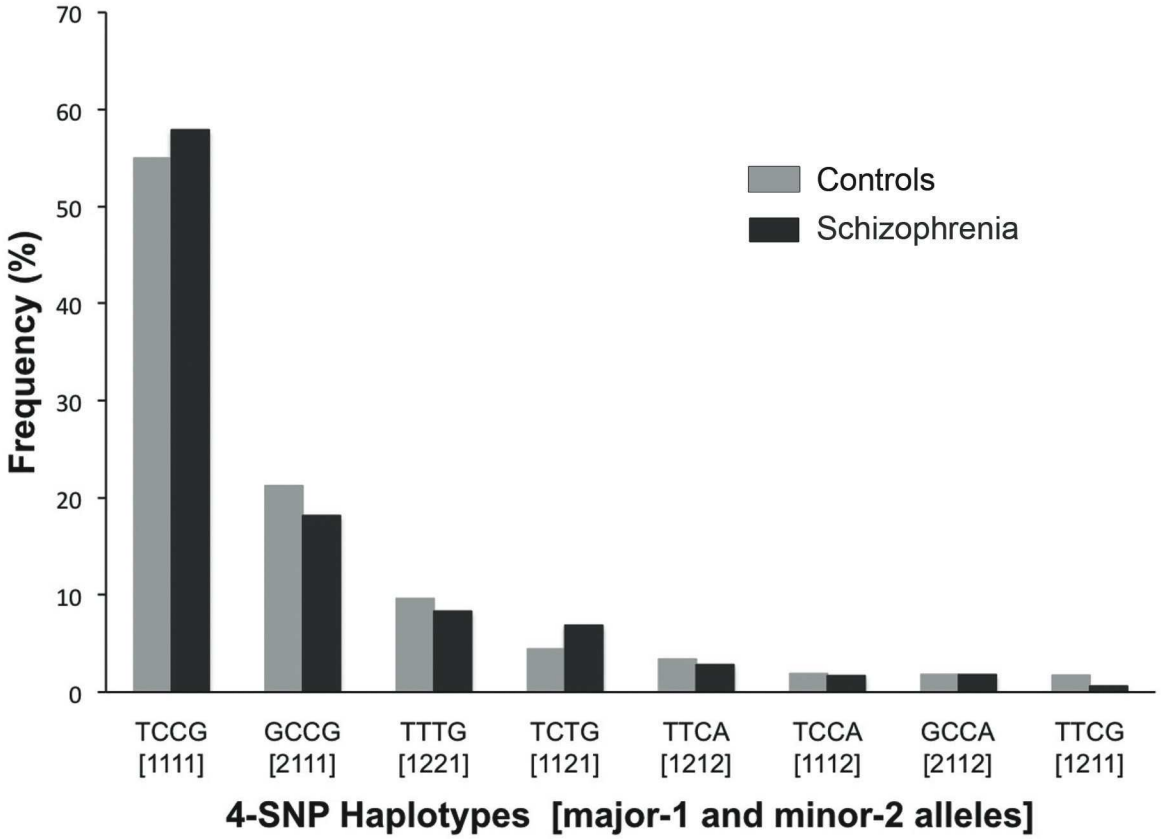


Figure 4.8 Frequencies of four-SNP haplotypes of FRZB in East UK cohort.

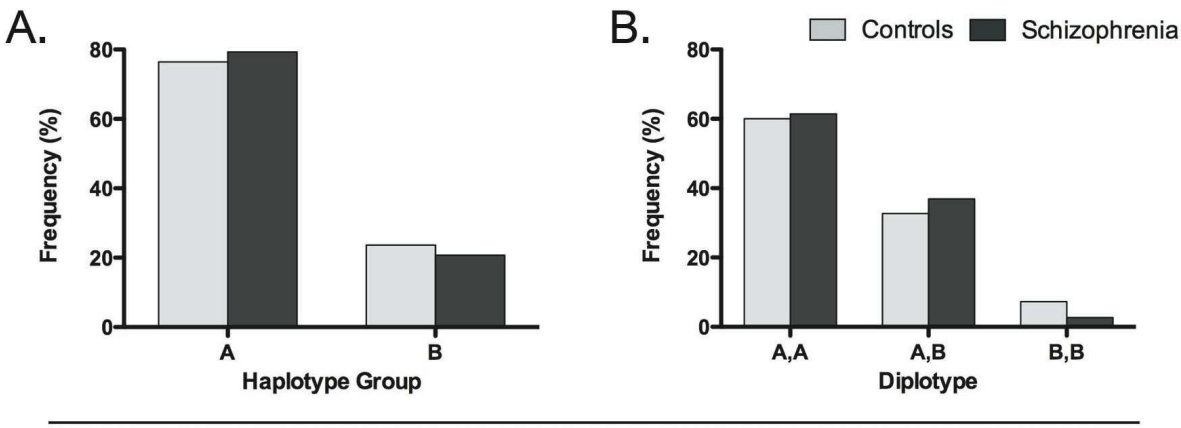
Frequencies of four-SNP haplotypes of FRZB as calculated by Golden Helix©. No haplotype associations with disease were observed from moving-window analysis.

A second approach was used for haplotype analysis was taken by defining two groups of haplotypes using SNP 1 as reference for definition of haplotype groups following the procedure described above. PHASE v. 2.1 was used to calculate haplotype frequencies and to estimate diplotypes for each individual in the East UK cohort. Haplotypes containing the major allele for this variant were present in Group A and those containing the minor allele were classified as Group B (Table 4.5). Fisher's exact test was used to compare frequencies of haplotype groups in controls and cases. In addition, phased data was used to compare diplotype frequencies in controls and cases using χ^2 test (Figure 4.9). There were no significant associations with schizophrenia resulted from this approach.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1-1	54.66	57.39
	1-1-1-2	2.32	2.50
	1-1-2-1	4.88	7.26
	1-2-1-1	1.90	1.10
	1-2-1-2	2.13	2.64
	1-2-2-1	9.22	7.74
Group B	2-1-1-1	21.47	18.72
	2-1-1-2	2.03	1.88
	2-2-1-1	0.15	0.49

Table 4.5 Haplotype groups defined for FRZB in the East UK cohort.

Two groups of haplotypes were defined for SNPs in FRZB considering LD patterns calculated from Haploview. Haplotype frequencies in controls and cases for this analysis were calculated from PHASE v 2.1.



		Frequency (%)				
		Haplotypes		Diplotypes		
	n	Group A	Group B	A,A	A,B	B,B
East UK Schizophrenia	145	79.3	20.7	61.4	35.9	2.7
East UK Controls	110	76.4	23.6	60.0	32.7	7.3

Figure 4.9 Frequencies of two haplotype groups of SNPs in FRZB in East UK cohort.

Two groups of similar haplotypes were defined. Frequencies of haplotypes in Group A were compared to those in Group B in controls and cases using Fisher's exact test (**A**). Phased data was also used to perform a comparison of diplotype frequencies using χ^2 test (**B**). No significant associations were observed from this analysis.

4.3.2 WISP3: Analysis of SNP association with expression levels and disease status

4.3.2.1 Correlation of SNP genotype with mRNA levels of WISP3 in the ICPS cohort

Correlation of genotypes for three SNPs in WISP3 with levels of expression of the gene was performed using data from the ICPS cohort (Supplementary Table 4; Appendix C). WISP3 mRNA levels in BA22 were significantly upregulated in schizophrenia (Barnes et al., 2011; Appendix A). Three-way ANOVA analysis using three independent variables (disease status, genotype and SNP ID) with gene expression as the outcome, revealed a significant effect of diseases status ($p=0.00033$) but no effect of genotype or SNP ID. There were no significant interactions between variables. Further two-way ANOVA confirmed these findings with a significant effect of disease status on mRNA levels ($p=0.0004$) but no effect of genotype and no interactions between factors (Figure 4.10).

Two-SNP haplotype analysis was not performed because of very low numbers of individuals in either one of the haplotype groups: subjects carrying both copies of the common haplotype (1-1/1-1) or those carrying at least one copy of the less common haplotypes (1-2, 2-1 or 2-2). Three-SNP haplotype analysis was performed by defining two groups of similar haplotypes (Table 4.6). Diplotypes were estimated using Phase v. 2.1 and two-way ANOVA was used to compare mRNA levels between controls and cases with either two copies of haplotypes from group A or at least one copy of any haplotypes from group B (Figure 4.11). This analysis did not result in any significant effect of haplotypes on WISP3 expression, although the effect of disease status showed border-line significance ($p=0.054$).

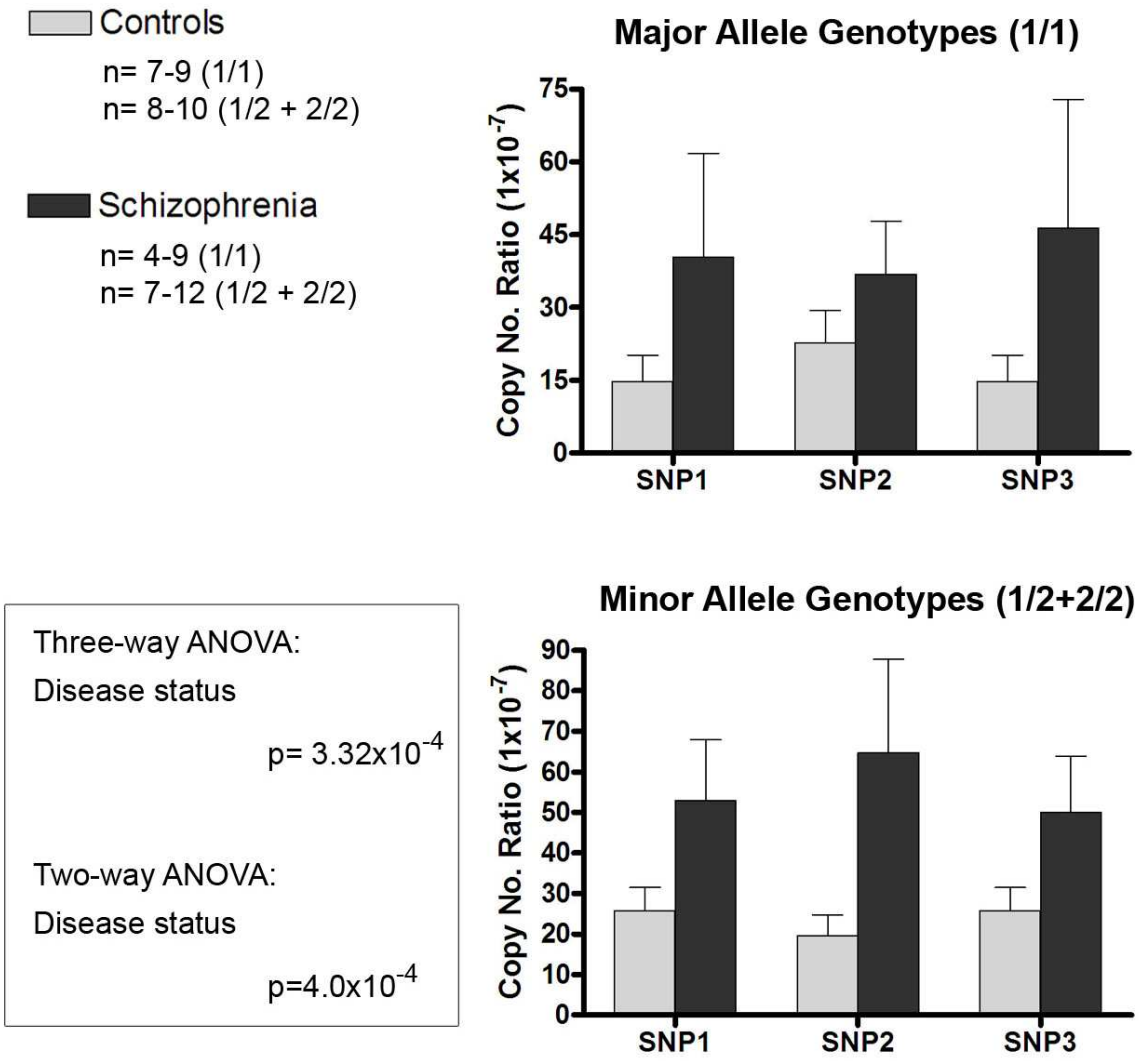


Figure 4.10 Analysis of SNP genotype and disease status effect on mRNA levels of WISP3.

Levels of expression of WISP3 in BA22 for controls and schizophrenia cases of the ICPS cohort are shown for major and minor allele genotypes. Two separate histograms are shown for clarity, one for individuals homozygous for the major allele (1/1) in the three SNPs analysed, and a second one for mRNA values of subjects with at least one copy of the minor allele (1/2 + 2/2). Three and two-way ANOVA was performed by comparing all genotypes in controls and cases across all SNPs.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1	39.86	39.41
	1-1-2	0.06	1.55
	1-2-1	29.77	25.70
Group B	2-1-1	1.02	0.00
	2-1-2	28.76	33.29

Table 4.6 Haplotype groups defined for WISP3 in the ICPS cohort.

The table presents two groups of similar haplotypes defined for analysis of haplotype effect on gene expression levels.

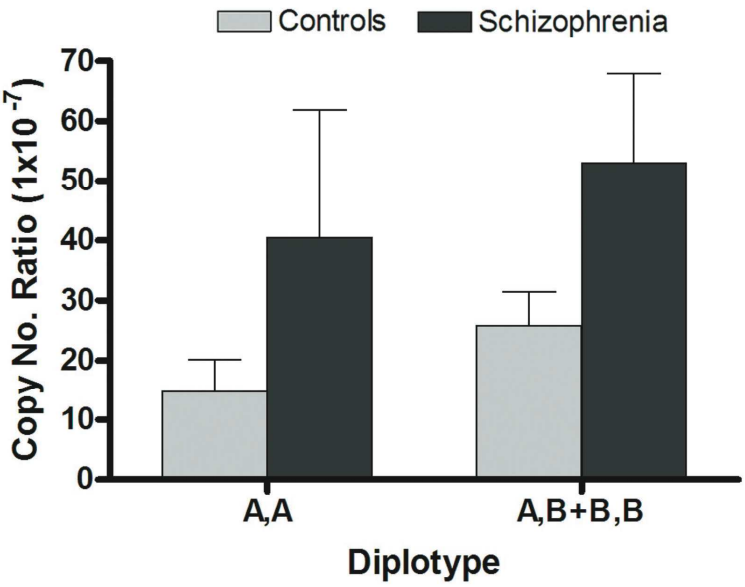


Figure 4.11 Analysis of the effect of three-SNP diplotypes of WISP3 on mRNA levels in ICPS cohort.

Haplotypes were grouped according to their likely evolution into two groups (described above). Both haplotype copies for each individual were analysed using two-way ANOVA, in order to compare frequencies of controls and cases carrying both haplotypes belonging to Group A (controls n=7; cases n=5) with those carrying at least one of the haplotypes from Group B (controls n=10; cases n=11).

4.3.2.2 Analysis of the association of SNPs and haplotypes in WISP3 with disease status in the East UK cohort

The three WISP3 SNPs selected for correlation with tissue expression in the ICPS cohort were used for analysis of association with disease in the East UK cohort (Figure 4.12). No significant allelic associations were obtained from application of a logistic regression model using SVS7 Golden Helix© (Table 4.7). Similarly, no significant association of genotype with disease were seen using the χ^2 test (Table 4.8). In addition, the application of three other genetic models (additive, dominant and recessive) using logistic regression did not result in significant associations.

Although no associations were seen from single SNP analysis, a moving-window haplotype analysis revealed a significant protective effect of two-SNP haplotypes containing both major alleles for SNP 1 and SNP 2 and for SNP 2 and SNP 3. A significant protective effect of the haplotype containing all major alleles for the three SNPs was found (Table 4.9). The difference in frequencies of haplotype 1-1-1 (C-C-G) between controls and cases was the largest observed across all three-SNP haplotypes (Figure 4.13). A second analysis was performed by grouping haplotypes according to their evolutionary similarity and LD patterns. Haploview was used to calculate D' values across all SNPs. Both controls and cases showed strong LD (D' from 0.82-1.0) although some differences in the pattern across SNPs were observed between groups (Figure 4.14). Haplotype groups were defined (Table 4.10) and frequencies were compared between controls and cases using the Fisher's exact test. Phase v.2.1 was also used to predict haplotype pairs for each individual, in order to compare diplotype frequencies between controls and cases using a χ^2 test (Figure 4.15). No significant differences in frequencies in either haplotype or diplotypes were observed.

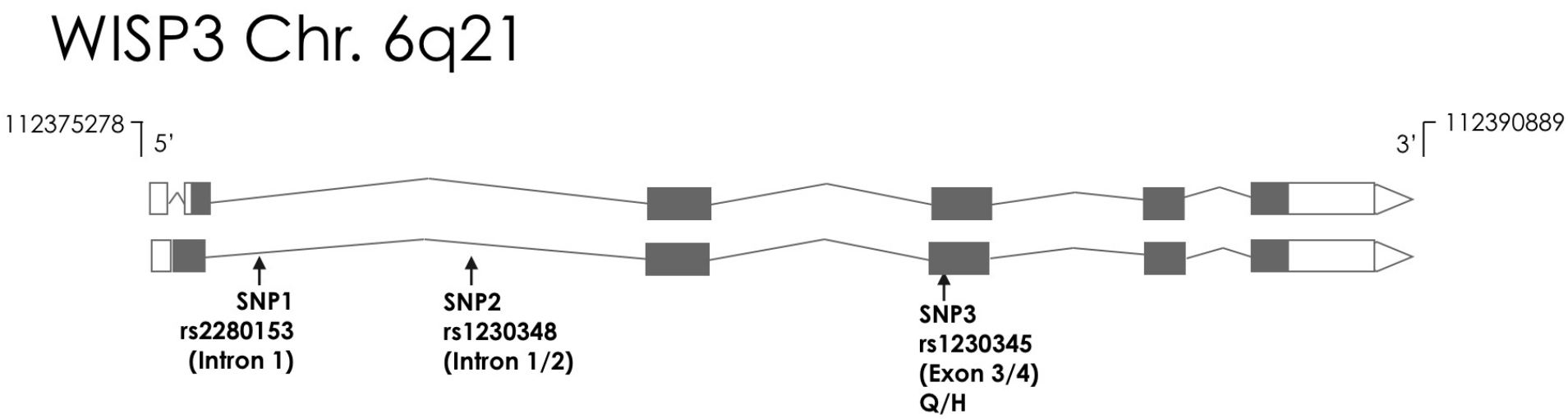


Figure 4.12 Schematic representation of three SNPs in WISP3.

Three SNPs in WISP were genotyped in the East UK cohort for analysis of association with disease status. Two transcripts are presented in the figure with reference number NM_003880.3 (above) and NM_198239.1 (below). The second transcript was used as a reference for SNP position.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%
SNP 1					
rs2280153: Intron 1					
		T			
Schizophrenia	278	28.4	0.668	1.09	0.73-1.63
Controls	210	26.7			
SNP 2					
rs1230348: Intron 1					
		T			
Schizophrenia	258	32.6	0.074	1.43	0.96-2.13
Controls	202	36.0			
SNP3					
rs1230345: Exon 3 [Q/H]					
		T			
Schizophrenia	264	27.6	0.445	1.18	0.77-1.80
Controls	196	24.5			

Table 4.7 Summary of statistics for allele association of SNPs in WISP3 in the East UK cohort.

Summarised in this table are p values and odds ratio (OR) calculated by applying a logistic regression model using Golden Helix©.

East UK Samples	n	Genotype Frequency (%)			Genotype χ^2 Test p value
SNP 1 rs2280153: Intron 1					
		C/C	**C/T**	**T/T**	
Schizophrenia	139	50.4	42.4	7.2	0.0579
Controls	105	59.0	28.6	12.4	
SNP 2 rs1230348: Intron 1					
		C/C	**C/T**	**T/T**	
Schizophrenia	129	41.1	45.7	13.2	0.2037
Controls	101	52.5	38.6	8.9	
SNP3 rs1230345: Exon 3 [Q/H]					
		G/G	**G/T**	**T/T**	
Schizophrenia	132	53.0	38.6	8.4	0.2805
Controls	98	61.2	28.6	10.2	

Table 4.8 Summary of statistics for analysis of genotypic associations of SNPs of WISP3 in the East UK cohort.

The table presents p-values for genotype associations with disease using a χ^2 and Golden Helix©. No significant associations were observed for any one of three polymorphisms analysed.

		Association with Schizophrenia							
Sliding Haplotype Windows	2 SNP Haplotypes	EM Cases Freq	EM Controls Freq	χ^2 P-value	χ^2 FDR	Odds Ratio	Odds Ratio Lower 95% CI	Odds Ratio Upper 95% CI	At Risk or Protective Associations
SNP#	1 2 3								
Haplotype Sequences	C C	0.352	0.465	0.0133*	0.0797	0.620	0.425	0.906	Protective
	C G	0.366	0.464	0.0307*	0.0920	0.655	0.446	0.962	Protective
	3 SNP Haplotypes								
	C C G	0.335	0.452	0.0098**	0.0783	0.601	0.446	0.962	Protective

Table 4.9 Analysis of association for haplotypes of WISP3 with schizophrenia in the East UK cohort.

Haplotype frequencies, p-values for association and odds ratio (OR) values were calculated using Golden Helix©. Corrected p-values were also calculated using FDR values. Significant p-values are indicated by * when $p<0.05$ and ** when $p<0.01$.

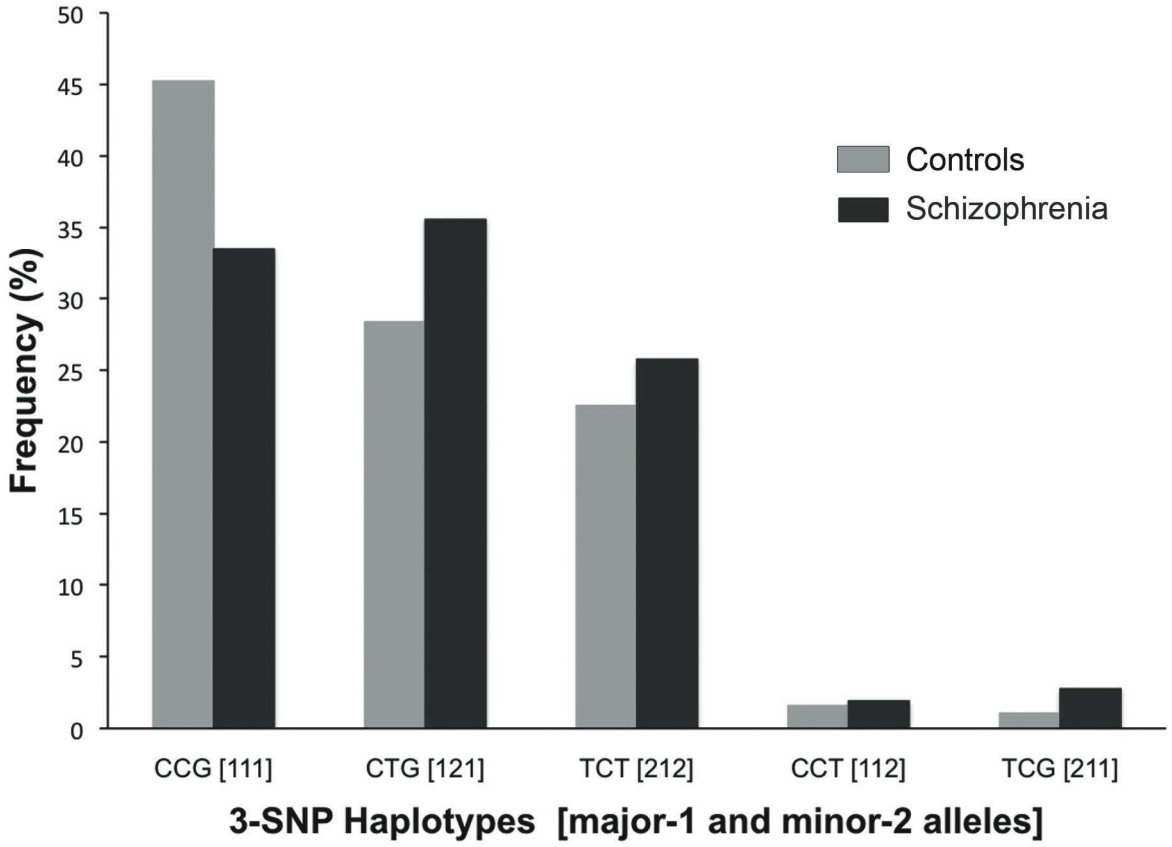


Figure 4.13 Frequencies of three-SNP haplotypes for WISP3 in the East UK cohort.

Frequencies of three-SNP haplotypes of WISP3 as calculated by Golden Helix©. No haplotype associations with disease were observed from moving-window analysis following FDR correction.

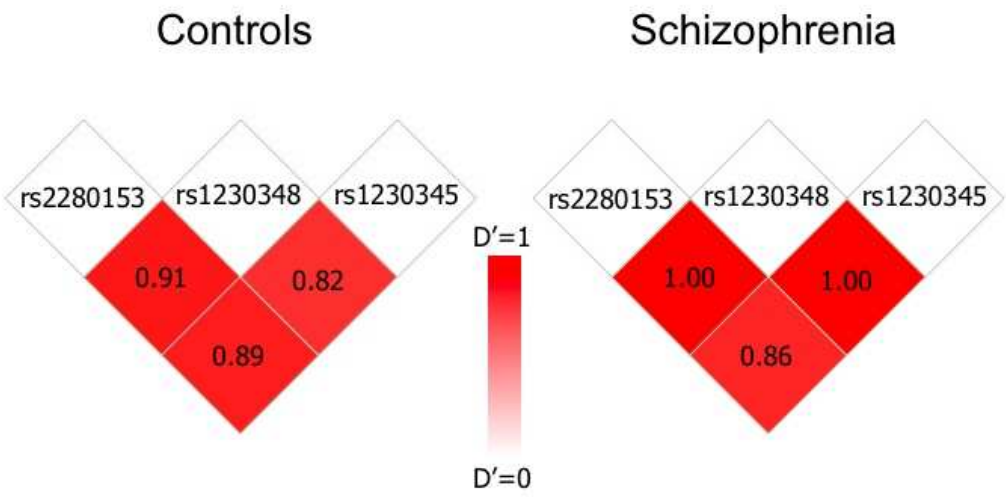


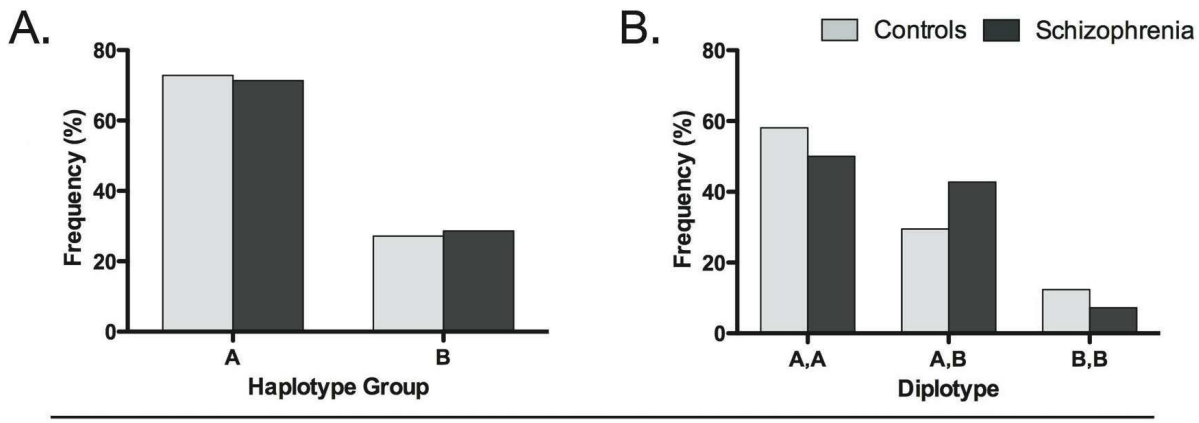
Figure 4.14 LD values for four SNPs of WISP3 in the East UK cohort.

Linkage disequilibrium (LD) maps for three SNPs in WISP3 in controls and cases of the East UK cohort. D' values calculated using Haploview indicated strong LD across all SNPs in both groups (D' values rank from 0.82 to 1).

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1	45.22	33.28
	1-1-2	1.64	2.35
	1-2-1	26.00	35.09
	1-2-2	0.54	0.73
Group B	2-1-2	23.18	24.87
	2-1-1	2.85	3.44
	2-2-2	0.53	0.19

Table 4.10 Haplotype groups defined for WISP3 in the East UK cohort.

Haplotype frequencies in controls and cases calculated using PHASE v 2.1 for two groups of haplotypes of SNPs in WISP3 considering LD patterns calculated from Haploview.



		Frequency (%)				
		Haplotypes		Diplotypes		
	n	Group A	Group B	A,A	A,B	B,B
East UK Schizophrenia	138	71.4	28.6	50.0	42.7	7.3
East UK Controls	105	72.9	27.1	58.1	29.5	12.4

Figure 4.15 Frequencies of two haplotype groups of SNPs in WISP3 in the East UK cohort.

Two groups of similar haplotypes were defined. Frequencies of haplotypes in Group A were compared to those in Group B in controls and cases using Fisher's exact test (**A**). Phased data was also used to perform a comparison of diplotype frequencies using χ^2 test (**B**). No significant associations were observed from this analysis.

4.3.3 DKK3: Analysis of SNP genotype effect on expression levels and study of association with disease

4.3.3.1 Correlation of SNPs and haplotypes of DKK3 with mRNA levels in the ICPS cohort

Correlation of genotypes and haplotypes of DKK3 SNPs with levels of expression was performed with data available from RT-PCR analysis using samples from BA22 of subjects from the ICPS cohort. Expression of this gene was not identified as significantly changed in schizophrenia from previously described microarray studies (Maycox et al., 2009; Barnes et al., 2011). However, a previous study identified expression of DKK3 as being reduced by 35% in BA22 in schizophrenic post-mortem samples from the BA22 by 35% compared to healthy controls, using suppression subtractive hybridization as well as slot blot hybridization and RT-PCR for validation (Ftouh et al., 2005).

Three SNPs were genotyped in the ICPS cohort (Supplementary Table 4; Appendix C). Correlation of genotype with levels of expression for three DKK3 SNPs in controls and cases were performed using ANOVA. Factorial analysis with disease status, genotype and SNP ID as independent variables did not result in any significant effects of any of these factors or interactions between them. A two-way ANOVA confirmed this result. Expression levels of controls and cases were similar for all genotypes and all SNPs (Figure 4.16).

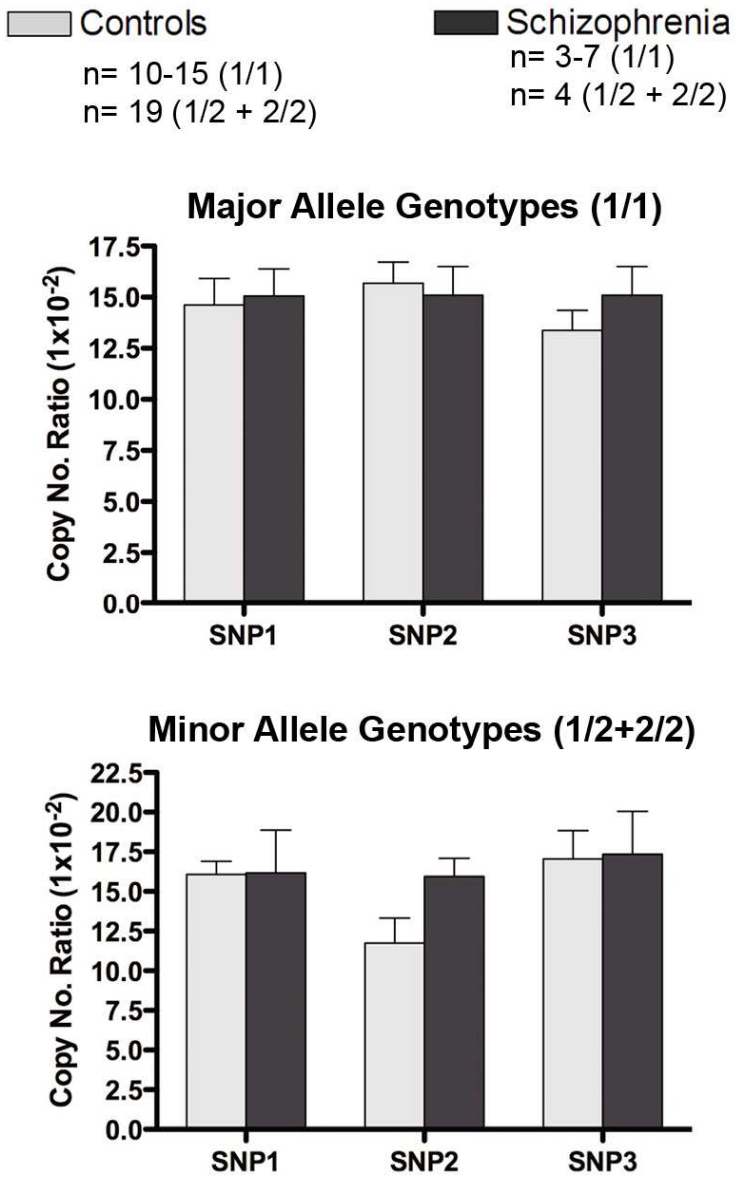


Figure 4.16 Analysis of DKK3 SNP genotype and disease status effect on mRNA levels.

Levels of expression of DKK3 in BA22 for different genotypes are presented in this figure for controls and schizophrenia cases of the ICPS cohort. Two separate histograms are shown for clarity, one for individuals homozygous for the major allele (1/1) in the four SNPs analysed, and another for subjects with at least one copy of the minor allele (1/2 + 2/2). Three and two-way ANOVA was performed by comparing all genotypes in controls and cases across all SNPs. No significant effects of either disease status or genotype observed.

The effect of DKK3 haplotypes on mRNA levels was assessed as previously described. Initially two-SNP haplotypes were analysed using two-way ANOVA with no significant effect of either disease status or diplotype observed and no significant interactions between variables. Once more, levels of expression between controls and cases were very similar for both diplotype groups across all SNP pairs (Figure 4.17). In order to analyse the effect of three-SNP haplotypes on levels of expression, data obtained from PHASE analysis was used to define two groups of similar haplotypes (Table 4.11). Levels of expression of controls and cases from both diplotype groups were compared using a two-way ANOVA with no significant differences resulting from the analysis (Figure 4.18).

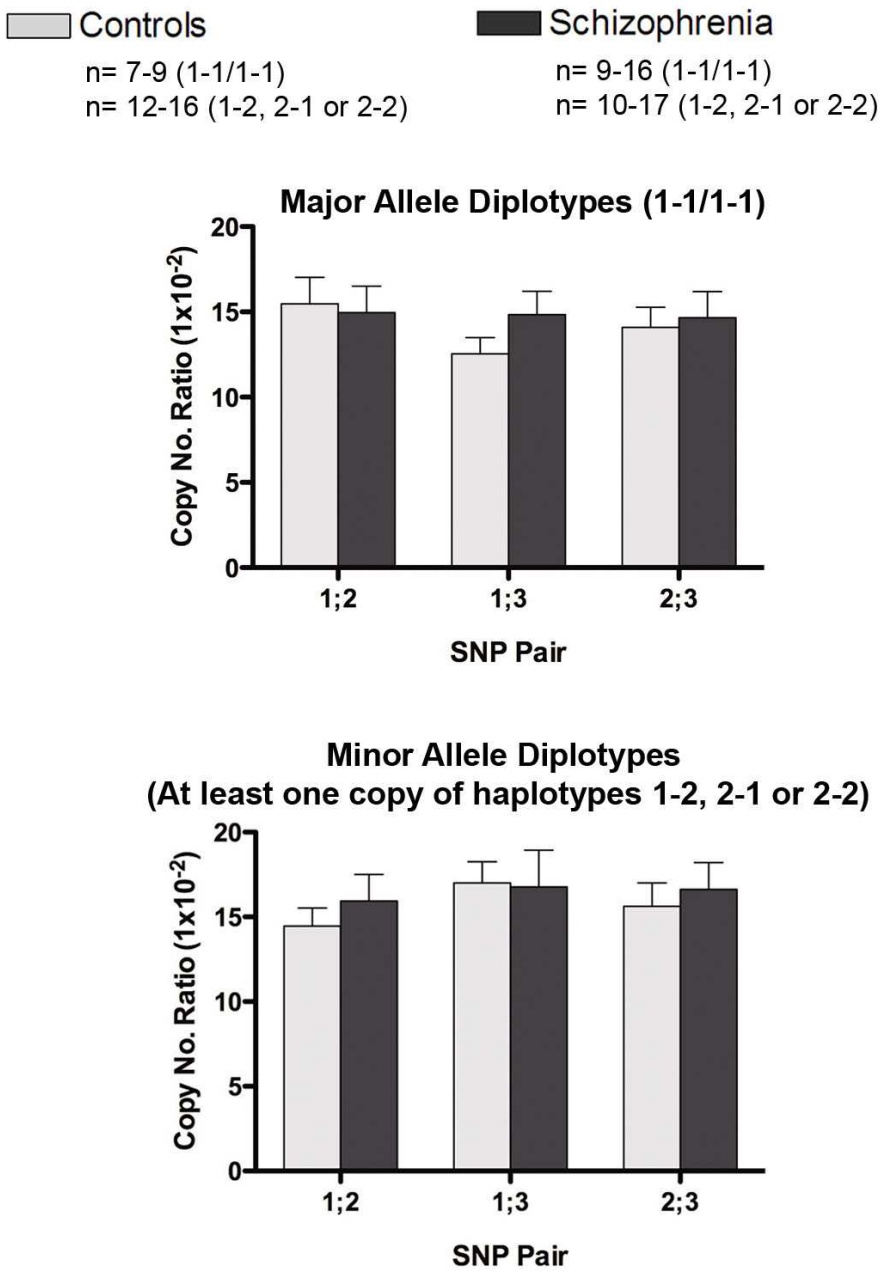


Figure 4.17 Analysis of the effect of two-SNP diplotypes in DKK3 on mRNA levels the ICPS cohort.

Levels of expression of DKK3 in BA22 shown for major and minor allele diplotypes in controls and schizophrenia cases of the ICPS cohort for all SNP pairs. For clarity purposes, mRNA levels are presented in two separate histograms, one for individuals carrying both copies of the most common haplotype (1-1/1-1) and a second histogram for individuals with at least one copy of haplotypes 1-2, 2-1 or 2-2. Three and two-way ANOVA was performed by comparing both diplotype groups. No significant differences in expression levels were observed between controls and cases for any diplotype.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1	62.80	76.35
	1-1-2	12.87	5.69
	1-2-1	5.40	6.71
Group B	2-1-1	3.41	2.92
	2-1-2	15.50	8.28

Table 4.11 Haplotype groups defined for DKK3 in the ICPS cohort.

Two haplotype groups defined according LD patterns across SNPs in the ICPS cohort are shown. Major and minor alleles of SNP1 were used to determine haplotypes in each group.

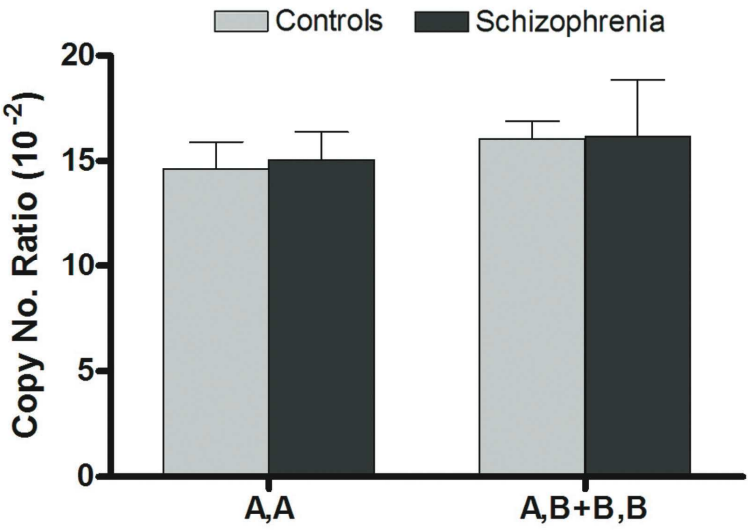


Figure 4.18 Analysis of the effect of three-SNP diplotypes of DKK3 on mRNA levels in controls and cases of the ICPS cohort.

Levels of expression of DKK3 are shown in the figure for controls and cases carrying both haplotypes belonging to Group A (controls n=13; cases n=19) and those carrying at least one of the haplotypes from Group B (controls n=5; cases n=4). Two-way ANOVA was performed to compare mRNA levels between controls and cases in both haplotype groups.

4.3.3.2 Analysis of association of SNPs and haplotypes in DKK3 with disease status in the East UK cohort

For analysis of association of DKK3 with schizophrenia the three selected polymorphisms in this gene were genotyped in controls and cases of the East UK cohort (Figure 4.19). A significant allelic association of SNP 2 with disease was observed. The odds ratio (OR) obtained for this variant indicated a large effect of the polymorphism on risk for disease (OR=2.27). In spite of this, the association did not remain significant after FDR correction (Table 4.12). No genotypic associations resulted from the analysis using a χ^2 test to compare genotype frequencies between controls and cases. However, a significant effect of SNP 2 resulted from the application of logistic regression with an additive model of disease penetrance (where the risk of disease increases as *DD >Dd> dd*), although this association did not remain significant after correction (Table 4.13).

A moving-window analysis revealed significant association with disease for four haplotypes. In all cases the risk for disease was increased in these haplotypes. Although OR values for all four haplotypes indicated a large effect, their frequencies were very low and none of these associations remained significant after FDR correction for multiple testing (Table 4.14). No significant differences in frequencies of more common haplotypes were observed between controls and cases (Figure 4.20). Finally haplotypes were grouped according to their ancestral similarity and LD patterns across SNPs (Figure 4.21). Two haplotype groups were defined (Table 4.15) and frequencies haplotypes in groups A and B were compared between controls and cases using Fisher's exact test. Moreover, PHASE v. 2.1 was also used to predict diplotypes for each individual in order to compare diplotype frequencies between

controls and cases using the χ^2 test. No significant association of haplotypes with disease status resulted from this approach.

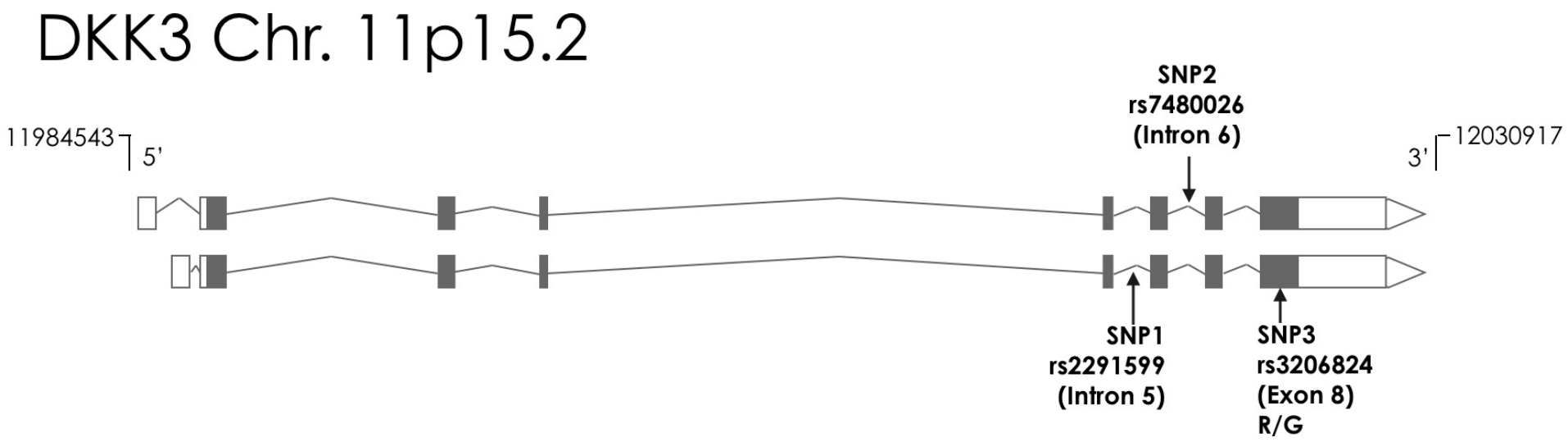


Figure 4.19 Schematic representation of three SNPs in DKK3.

The figure presents two transcripts identified for DKK3; NM_013253.4 (above) and NM_015881.5 (below). The three SNPs selected in DKK3 for analysis of association with disease are represented. SNP position is the same for both transcripts.

East UK Samples	n	Frequency (%) Minor Allele	p value	OR	CI 95%	False Discovery Rate p value
SNP 1 rs2291599: Intron 5						
		A				
Schizophrenia	276	16.67	0.560	0.87	0.54-1.39	0.560
Controls	214	18.69				
SNP 2 rs7480026: Intron 6						
		T				
Schizophrenia	288	8.68	0.031*	2.27	1.04-4.97	0.094
Controls	224	4.02				
SNP3 rs3206824: Exon 8 [R/G]						
		A				
Schizophrenia	280	16.43	0.297	0.79	0.50-1.24	0.446
Controls	230	20.0				

Table 4.12 Summary of statistics for allele association of SNPs of DKK3 in the East UK cohort.

Minor allele frequencies, calculated using SVS7 from Golden Helix©, for three SNPs in DKK3 in the East UK cohort. A logistic regression model was used to calculate p-values and odds ratio (OR) values. False discovery rate was used to correct for multiple testing. Significant p values are indicated by * when $p<0.05$.

East UK Samples	n	Genotype Frequency (%)			χ^2 Test p value (FDR)	Additive Model Logistic regression p value (FDR)	Additive Model Logistic regression Odds Ratio (CI95%)
SNP 1 rs2291599: Intron 5		**G/G**	**G/A**	**A/A**			
Schizophrenia	138	68.8	29.0	2.2	0.745	0.556	0.87
Controls	107	66.4	29.9	3.7	(0.745)	(0.556)	(0.54-1.39)
SNP 2 rs7480026: Intron 6		**C/C**	**C/T**	**T/T**			
Schizophrenia	144	84.0	14.6	1.4	0.116	0.035*	2.20
Controls	112	92.0	8.0	0.0	(0.347)	(0.106)	(1.01-4.78)
SNP3 rs3206824: Exon 8 [R/G]		**G/G**	**G/A**	**A/A**			
Schizophrenia	140	69.3	28.6	2.1	0.393	0.302	0.79
Controls	115	65.2	29.6	5.2	(0.590)	(0.452)	(0.50-1.24)

Table 4.13 Summary of statistics for analysis of genotypic associations of SNPs in DKK3 in the East UK cohort.

The P-values and FDRs presented in the table were calculated using Golden Helix© from the application of a χ^2 to identify genotype associations with disease. In addition, a logistic regression model was applied to test three genetic models to assess the effect of genotypes containing the disease allele *'D'* to those with the wildtype allele *'d'*. Significant p-values are indicated by * when $p<0.05$.

Sliding Haplotype Windows	2 SNP Haplotypes			Association with Schizophrenia							
SNP#	1	2	3	EM Cases Freq	EM Controls Freq	χ^2 P-value	χ^2 FDR	Odds Ratio	Odds Ratio Lower 95% CI	Odds Ratio Upper 95% CI	At Risk or Protective Associations
Haplotype Sequences	G	T		0.093	0.039	0.0209*	0.1253	2.548	1.124	5.775	Risk
		T	G	0.087	0.038	0.0320*	0.0960	2.409	1.055	5.503	Risk
	3 SNP Haplotypes										
	G	T	G	0.092	0.035	0.0169*	0.1355	2.775	1.164	6.619	Risk
	A	C	G	0.044	0.010	0.0329*	0.0876	4.578	0.993	21.096	Risk

Table 4.14 Analysis of association for haplotypes of DKK3 with schizophrenia in East UK cohort.

Haplotype frequencies, p-values for association and odds ratio (OR) values were calculated using Golden Helix©. Corrected p-values were also calculated using FDR values. Significant p-values are indicated by * for $p<0.05$.

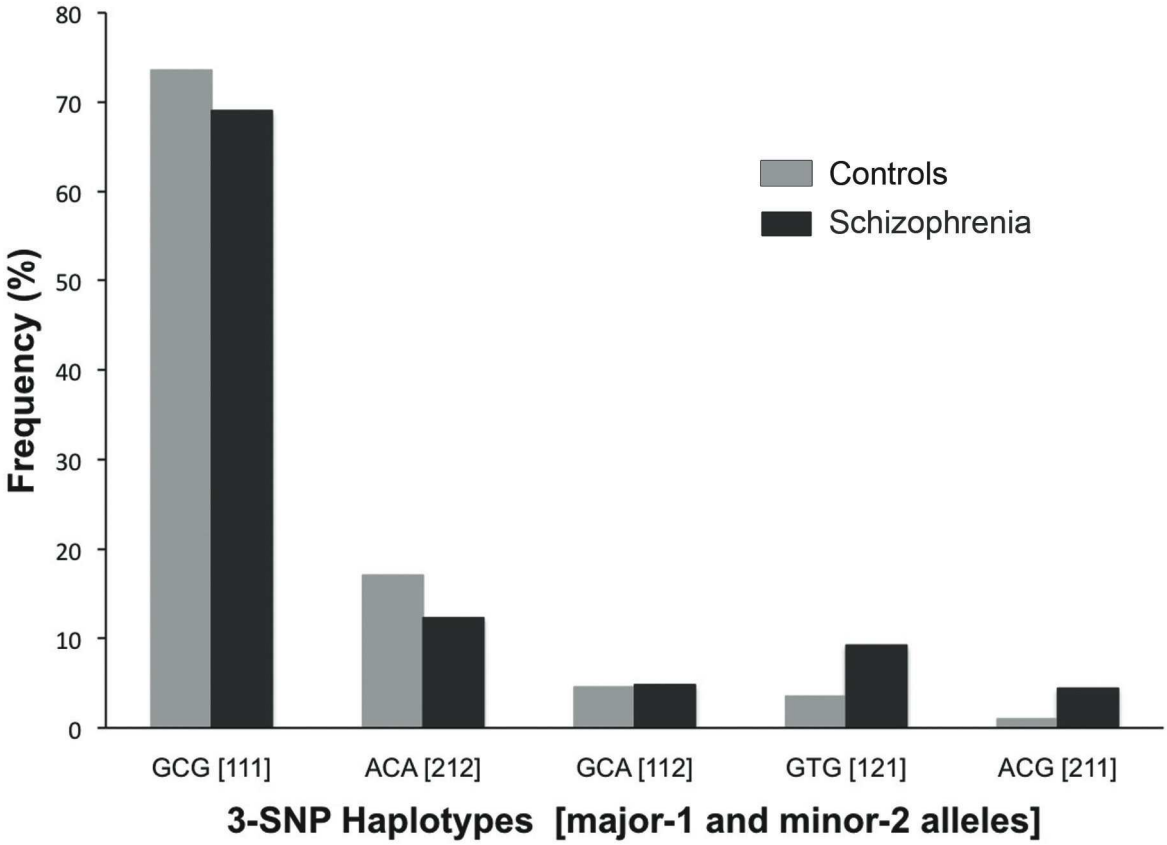


Figure 4.20 Frequencies of three-SNP haplotypes of DKK3 in East UK cohort.

Frequencies of three-SNP haplotypes of DKK3 as calculated by Golden Helix©. No haplotype associations with disease were observed from moving-window analysis.

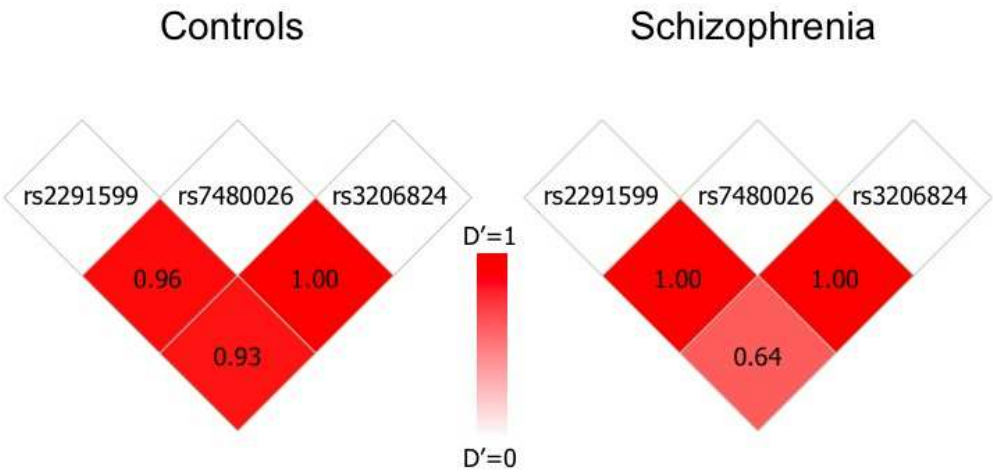


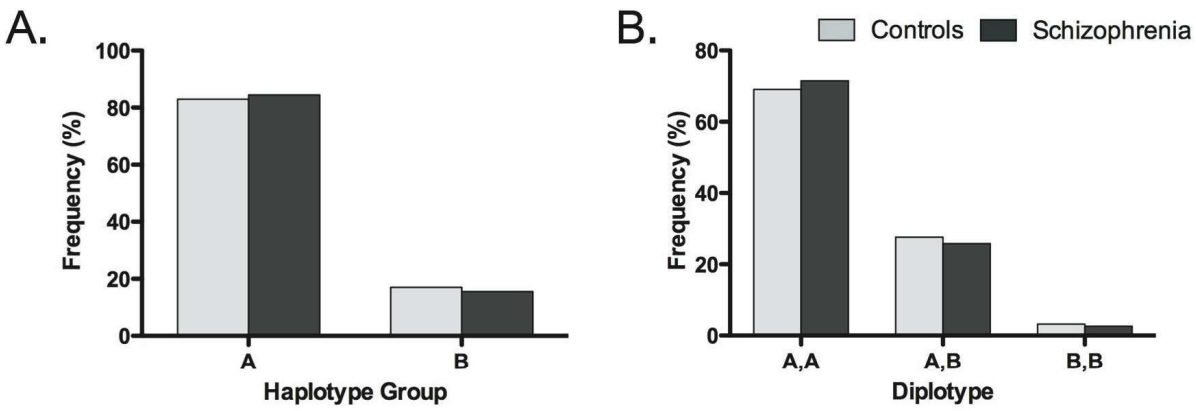
Figure 4.21 LD values for three SNPs of DKK3 in the East UK cohort.

Linkage disequilibrium (LD) diagram obtained using Haploview for controls and cases of the East UK cohort. High D' values (from 0.96 to 1) were seen for SNPs 1 and 2 and SNPs 2 and 3 in both controls and cases. The main differences in LD patterns were observed between SNP 1 and 3 when comparing controls with schizophrenia cases.

	Haplotype	Frequency (%)	
		Controls	Cases
Group A	1-1-1	72.90	69.91
	1-1-2	5.22	5.41
	1-2-1	4.29	8.60
Group B	2-1-1	1.62	4.58
	2-1-2	15.95	11.50

Table 4.15 Haplotype groups defined for DKK3 in the East UK cohort.

Haplotype frequencies in controls and cases calculated using PHASE v 2.1 for two groups of haplotypes of SNPs considering LD patterns calculated from Haploview.



		Frequency (%)				
		Haplotypes		Diplotypes		
	n	Group A	Group B	A,A	A,B	B,B
East UK Schizophrenia	151	84.4	15.6	71.5	25.8	2.7
East UK Controls	123	82.9	17.1	69.1	27.6	3.3

Figure 4.22 Frequencies of two haplotype groups of SNPs in DKK3 in the East UK cohort.

Frequencies of haplotypes in Group A were compared to those in Group B in controls and cases using Fisher's exact test (**A**). Phased data was also used to perform a comparison of diplotype frequencies using χ^2 test (**B**). No significant associations were observed from this analysis.

4.3.4 Analysis of epistatic interaction between genes involved in Wnt signalling pathway

Analysis of epistatic interactions carried out using GAIA resulted in a significant interaction of SNP 1 (rs10206992) in FRZB and SNP 2 (rs1230348) in WISP3 (Figure 4.23). Moreover, although no interactions of DKK3 with any of the other two candidate genes were observed, a significant association with disease of SNP 2 (rs7480026) was obtained from the analysis (p=0.03519). These results are consistent with those obtained from analysis of association of DKK3 with schizophrenia, which revealed significant allelic and genotypic associations of this SNP. Corrections for multiple comparisons in this case were performed using an in-built permutation test, part of the GAIA application. Empirical p values did not remain significant after 1000 permutations.

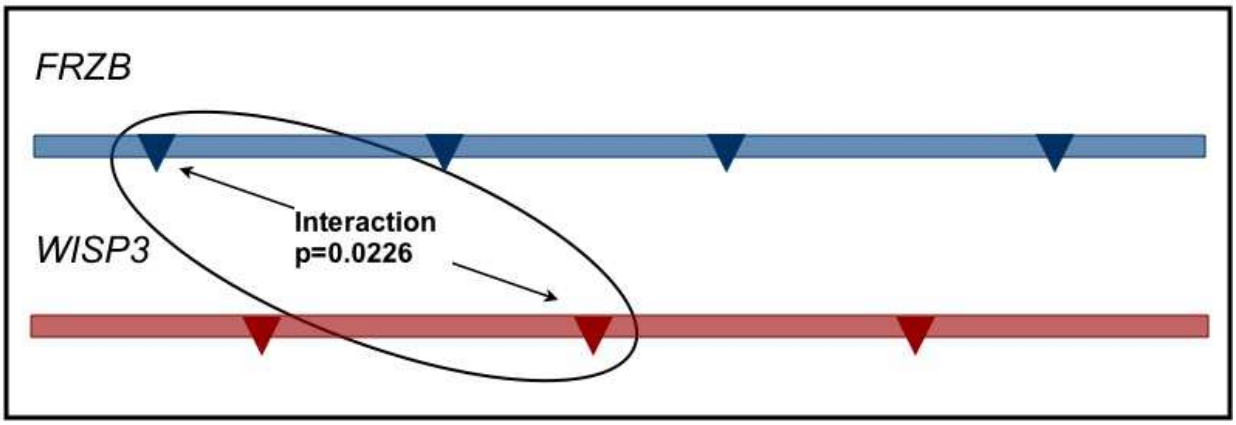


Figure 4.23 Representation of epistatic interactions between polymorphisms in FRZB and WISP3.

Evidence for statistical epistasis was obtained by analysis using GAIA. A significant increased risk for disease was observed for the interaction of SNP 1 in FRZB (rs10206992) and SNP 2 in WISP3 (rs1230348).

4.4 Discussion

4.4.1 Significant differences in expression levels of FRZB for a number of genotypes and diplotypes

Analysis of selected polymorphisms in three candidate genes implicated in the Wnt signalling pathway substantiate the previous findings of significant differences in levels of expression between controls and cases and further extend this analysis to show that this extends to all genotypes and diplotypes for FRZB and WISP3 in the ICPS cohort. In the case of FRZB Bonferroni's post-test comparisons revealed significant association of individual SNPs and diplotypes with levels of gene expression. Further analysis of the effect of four-SNP haplotypes also revealed consistent results. Classification of four-SNP haplotypes into two groups according to their likely evolution revealed a significant interaction of disease status and diplotype with respect to expression of FRZB suggesting that these SNPs might be in strong LD with regulatory variants. In particular SNP 4 (rs16823799) which is located in the 3'-UTR of the gene, 58 bp and 86 bp away from the recognition sequences of the micro RNAs has-miR-629 and has-miR-1323, respectively, according to data from the miRDB database (http://mirdb.org/miRDB/index.html; Wang and El Naqa, 2008; Wang, 2008).

The data used for analysis of DKK3 corresponded to samples from the ICPS cohort, different to those used in the expression study reporting reduced expression of the gene in schizophrenia in BA22 (Ftouh et al., 2005). In spite of the fact that no significant effects of either disease status or genotype/diplotype resulted from the correlation of three SNPs in DKK3 with mRNA levels in ICPS samples, this gene

remains an interesting candidate for analysis of association with disease considering the role it plays in the Wnt signalling pathway.

4.4.2 No association of SNPs or haplotypes in Wnt signalling genes with schizophrenia

Analysis of association of genetic variants with disease revealed significant nominal p values for allelic association of SNP 2 in DKK3 (rs7480026) as well as genotypic association of this SNP when using logistic regression to test an additive model of disease penetrance. However, these associations did not hold up after correction with FDR values, in spite of the large effect denoted by the OR values (2.3 for allelic association and 2.20 for contribution of genotype to risk of disease).

In addition, moving-window analysis revealed association of four haplotypes in DKK3 and three more in WISP3 with schizophrenia. Once more, these associations did not remain significant after correction for multiple testing. An important consideration to make in the case of DKK3, is the low frequency of the four associated haplotypes in both controls and cases. Comparison of LD patterns in controls and cases for all three genes also revealed interesting differences. In the particular case of polymorphisms in DKK3 and WISP3, where strong intermarker LD was observed, the association of haplotypes in spite of the lack of association of individual loci highlights the advantage of multi-locus analysis for detection of association with disease (Morris, 2005; Sanders et al., 2005).

4.4.3 Evidence for modest statistical epistasis between polymorphisms in FRZB and WISP3 contributing to the schizophrenic phenotype

The final analysis of interactions between loci performed using the GAIA application provided evidence for epistatic interactions between SNP 1 (rs10206992) in FRZB and SNP 2 (rs1230348) in WISP3 in the East UK cohort. Although this interaction did not remain significant after correction for multiple testing, it is interesting given the close functional relation of these two genes. Although the mechanisms through which FRZB and WISP3 regulate Wnt signalling pathway are yet to be clearly elucidated, and might be cell type specific, the two proteins have been shown to interact with Frizzled receptors during defined developmental processes, leading to inhibition of Wnt signalling pathway (Leyns et al., 1997; Nakamura et al., 2007). Moreover, the presence of a significant interaction between polymorphisms in these genes in spite of the lack of association of individual SNPs with disease makes further exploration of possible gene-gene interactions worth pursuing.

4.4.4 Conclusion

The results described in this section confirm the significant downregulation of FRZB and the significant upregulaton of WISP3 observed in schizophrenia in BA10 and BA22 respectively, in previous microarray studies. Furthermore, significant association of genotype with expression was observed for individual SNPs in FRZB when comparing mRNA levels in controls and cases of the ICPS cohort. Further analysis of haplotype effect on gene expression supported the previous findings with individual haplotypes significantly associated with lower levels of expression when comparing controls and cases. These findings provide support for a candidate-gene

approach and for the use of available SNPs as markers for potential regulatory variants. Particularly interesting is SNP 4 (rs16823799) located within a hundred base pairs from the recognition sequences of two miRNAs.

The results also support the consideration of FRZB as an important novel candidate gene in schizophrenia aetiology. Although the precise role of FRZB in regulation of Wnt signalling is still subject of investigation, down-regulation of the gene in BA10 of schizophrenic patients is intriguing considering the suggestion of an important role in cell proliferation (Qu et al., 2008; Hirata et al., 2010). This becomes more interesting in the light of reports of an important role of the disrupted in schizophrenia (DISC1) gene in neuronal progenitor proliferation through modulation of the β-catenin/GSK-3β pathway (Mao et al., 2009).

The analysis of genotype association with disease status revealed an allelic association of an intronic SNP (rs7480026) in DKK3 with schizophrenia. In addition, significant association of genotype with disease was also observed for this SNP consistent with an additive model of disease penetrance. Although these associations were not significant after correction for multiple testing it is important to recognise the limitations posed by the low MAF of this polymorphism. It is also important to consider the possibility that this SNP might be in strong LD with an associated variant. Moreover, four haplotypes of DKK3 and three more haplotypes of WISP3 also were associated with disease. Although these associations did not hold up to multiple testing correction they further emphasise the increased power of haplotype analysis, which has been pointed out from previous associations studies of schizophrenia candidate genes such as COMT (Shifman et al., 2002; Handoko et al., 2005)

Investigation of the role of the Wnt signalling pathway in schizophrenia aetiology remains an important subject to explore, not only due to the well established importance of this pathway in developmental processes and the more recent evidence of its involvement in synaptic plasticity. In the case of FRZB and DKK3 it is interesting that although both genes are considered generally as inhibitors of Wnt signalling pathway, data collected from a number of reports suggests that the mechanisms through which these genes modulate Wnt signalling might be cell-type specific and it might depend on stage of development (Kawano and Kypta, 2003; Nakamura et al., 2007; von Marschall and Fisher, 2010).

CHAPTER 5 A method for analysis of CpG island methylation in schizophrenia candidate genes designed using DKK3.

5.1 Introduction

The dynamic nature of epigenetic mechanisms has provided the basis for the theory that these mechanisms might be involved in schizophrenia aetiology. In particular, a number of studies have provided evidence for aberrant methylation patterns in regions or genes previously linked to schizophrenia (Impagnatiello et al., 1998; Guidotti et al., 2000; Knable et al., 2004; Fatemi et al., 2005; Straub et al., 2007; Shifman et al., 2008; Liu et al, 2010). Moreover, a recent epigenomic profiling study (Mill et al., 2008) identified a number of genes differentially methylated in schizophrenia and bipolar disorder. Amongst the most significant changes in methylation patterns observed in major psychosis were those found in the zinc finger 195 (ZNF195) gene and the gene coding for the switching B-cell complex 70 KDa subunit protein (SWAP70). Both of these genes are located in the chromosome 11p region, within 10Mb of DKK3. DKK3 remains the one member of the dickopff family of proteins whose precise role in modulation of Wnt signalling pathway is not yet known. Most of the data available to date on the mechanisms of action of DKK3, as well as its interaction with other proteins, has been gathered through extensive research on the involvement of DKK3 in proliferation of cancer cell lines (Tsuji et al., 2000; 2001; Hsieh et al., 2004, Abarzua et al., 2005; 2007). A number of studies have found evidence for hypermethylation of the promoter region of DKK3 leading to downregulation of the gene in a variety of tumours. (Kobashashi et al., 2002; Roman-Gomez et al., 2004; 2007; Veeck et al., 2008b; Chim et al., 2008; Yue et al., 2008; Lee EJ et al., 2009).

The most common method for analysis of CpG island methylation in these and other candidate gene based studies is direct sequencing after conversion of unmethylated

cytosines to thymines through bisulphite modification of DNA. However, bisulphite sequencing frequently demands very specific conditions and optimisation is often required in order to obtain a good quality sequence where it is possible to determine not only full but also partial methylation (i.e. methylation of only one allele; Figure 5.1).

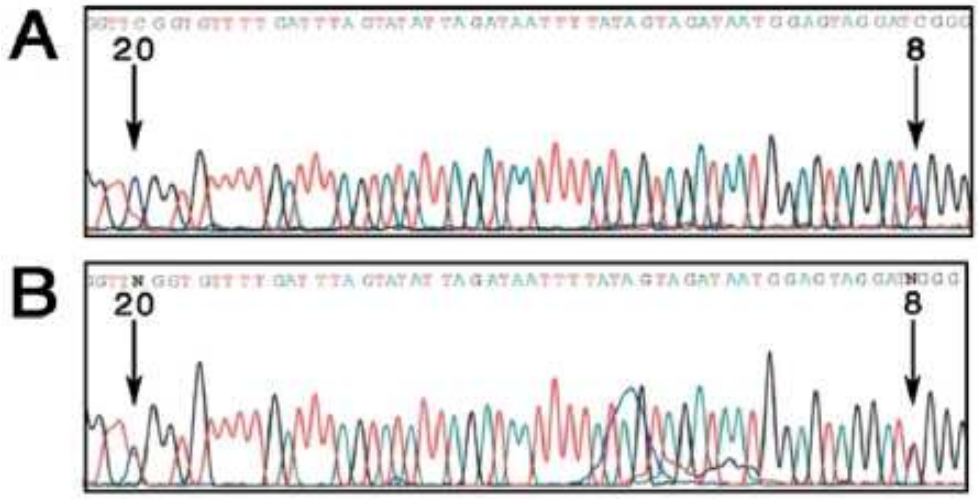


Figure 5.1 Example of partial methylation from Murphy et al. (2008).

Panel A from this figure shows clear methylation at cytosines 8 and 20, with a prominent C signal and only traces of T, while panel B shows partial methylation of the same sites with an ambiguous signal called N**.**

Many of the previously mentioned studies have used methylation specific PCR (MSP) as an alternative or complementary method for the analysis of the methylation status of CpG islands in the promoter region of DKK3 (Roman-Gomez et al., 2004; 2007; Veeck et al., 2008b; Chim et al., 2008; Yue et al., 2008). This method, first described by Herman et al. (1996) consists of designing a pair of primers specific for each one of the two different methylation states based on the principle that only unmethylated CpGs would be affected by bisulphite reaction (Figure 5.2).

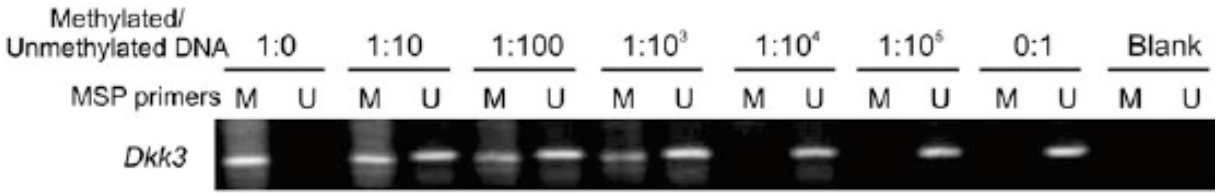


Figure 5.2 Example of methylation specific PCR from Yue et al. (2009).

Bands obtained from MSP analysis of the promoter region of DKK3 using a pair of primers designed for methylated DNA (M) and a second pair designed to amplify unmethylated DNA (U), after bisulphite modification. The authors used different methylated/unmethylated DNA ratios to demonstrate the sensibility of the assay to detect DNA methylation.

The main objective of the last section of this study is to design a MSP based method in order to compare CpG methylation status of the selected candidate genes whose expression in schizophrenia has been found to be changed in schizophrenia cases compared to controls. In order to do this and taking advantage of the extensively documented use of bisulphite sequencing and MSP for the analysis of CpG island methylation of DKK3, this gene was used as the basis of this section in spite of the lack of association of SNPs in DKK3 with levels of expression in the ICPS cohort and the modest evidence for association of these SNPs with schizophrenia in the East UK cohort.

5.1.1 Overview

The initial step in the analysis of methylation of the promoter region of DKK3 in ICPS cohort controls and cases was the identification of the CpG islands in the gene. The online application CpG Island Plot from EMBL-EBI (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html) was used for this purpose. Once the region of interest was identified, optimisation of bisulphite modification was carried out using three different modification kits as well as appropriate controls in order to confirm that transformation was successful. This was a critical step particularly due to the fact

that the DNA used for this analysis was extracted from brain tissue samples corresponding to controls and cases from the ICPS cohort. In addition to the conversion rate of the kit used for bisulphite modification, it was important that the yield of the recovered DNA was as high as possible in order to maximise the possibility of a successful PCR amplification and sequencing of the modified template. Bisulphite modified DNA from tissue samples of controls and cases was then amplified using primers designed for a two round PCR reaction. Samples were subsequently prepared for sequencing at the MRC CSC Genomics Core Laboratory, Imperial College London. Direct sequencing of bisulphite modified DNA is not an easy task, due to the nature of bisulphite modified DNA and the presence of large thymine repeats. Two methods were tried in an attempt to improve the quality of the sequence. The first method was to use PCR to add a common sequencing primer tag (M13(-20)F or M13R) at the 5' end of each CpG island. The second method was to introduce the CpG island fragment into a TA vector for subsequent sequencing. As this method does not allow for identification of partial methylation, at least 10 constructs were sequenced for each sample analysed.

Primers for the CpG island in the promoter region of DKK3 were designed for MSP and were used to amplify and sequence this region in controls and cases of the ICPS cohort. The aim was to compare both assays in order to confirm the efficiency of the MSP method in identifying methylation. As primer design is critical for the success of MSP, the primers used were based on the primers previously validated for this region (Yue et al., 2009) for analysis of the same CpG island in the promoter region of DKK3 as the one chosen for the present study. The overall aim of this section was to establish a quick and reliable method that could be used for analysis of a number of

selected candidate genes whose expression was changed in schizophrenia (Maycox et al., 2009; Barnes et al., 2011).

5.2 Methodology

5.2.1 Identification of CpG islands in the promoter region of DKK3

In order to identify possible CpG islands in DKK3 a fragment from the promoter region of the gene (~ 2Kb) was analysed using the CpG Island Plot from EMBL-EBI (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). From this analysis two regions were recognized as putative CpG islands (Figure 5.3), consistent with the data reported by Yue et al. (2008).

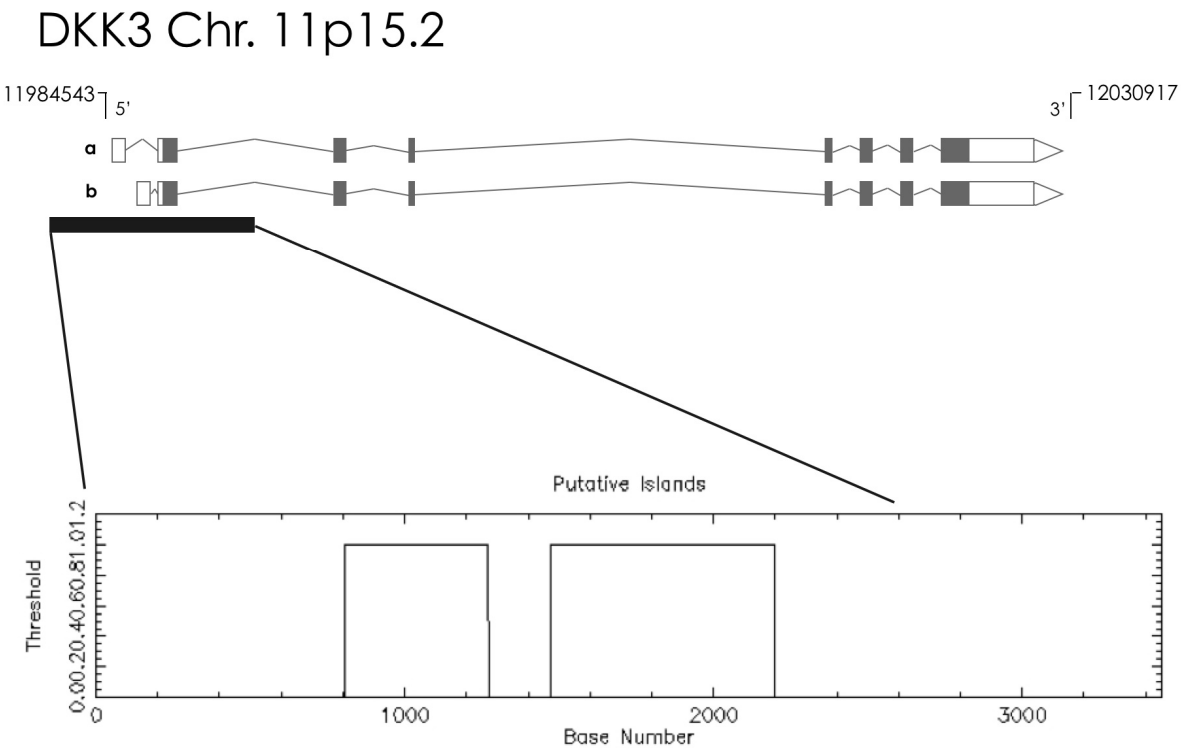


Figure 5.3 Output from the analysis of the promoter region of DKK3 using CpG Island Plot EMBL-EBI.

Two CpG island were predicted from the analysis of a 2Kb region comprising the promoter region of both isoforms of DKK3.

5.2.2 Bisulphite Modification

Modification of DNA with bisulphite salts is useful in the identification of methylated sites in CpG islands. The bisulphate reaction with cytosine results in their transformation to uracil, a subsequent PCR amplification then replaces these uracils with thymines. Methylation of cytosines prevents their reaction with bisulphite and therefore their transformation into thymines after PCR amplification (Figure 5.4)

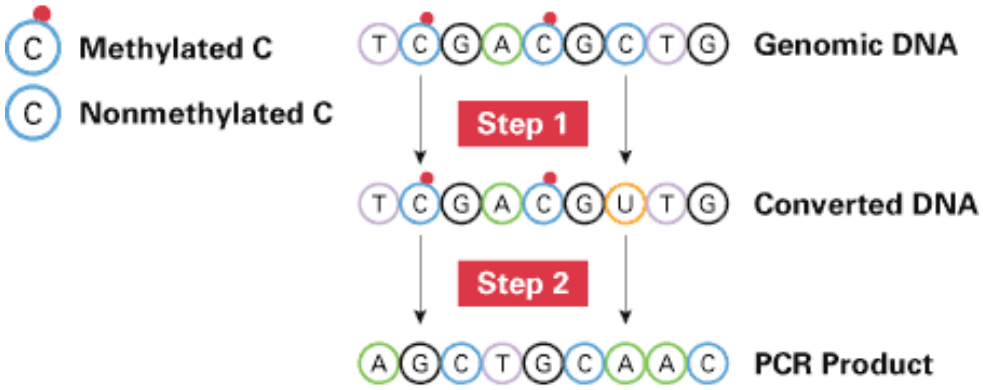


Figure 5.4 Schematic representation of DNA bisulphite modification.

Bisulphite treatment of DNA leads to conversion of Cytosine (C) to Uracil (U) through reaction with bisulphite salts. Methylated Cytosines are protected from transformation and remain unchanged after PCR amplification while nonmethylated sites are converted to thymines. The figure shows the complementary strand of the PCR product where guanines (G) are paired with cytosines and adenines (A) are paired with thymines.

Tissue samples from controls and cases from the ICPS cohort were used for analysis of possible methylation of CpG islands in DKK3. Methylated controls were prepared using a CpG methyltrasferase (NEB). Three bisulphite modification kits were tested. Two column-based kits were used for modification of DNA: the Imprint kit (Sigma-Aldrich) and the Methylation Gold kit (Zymo Research). Modifications were carried out according to the instructions from the manufacturers. A total amount of 1μg of

template was used with herring sperm DNA (Promega) used as a carrier for some samples.

The MethylEasy kit (Human Genetic Signatures) was also tested. More steps are used for DNA modification using this kit and instead of a column it uses precipitation for cleaning-up the modified DNA, ensuring a better product yield. The kit also includes two control samples, C1 (unmodified) and C2 (modified) and two pairs of control primers for nested PCR.

5.2.3 Bisulphite Sequencing

After transformation PCR was carried out for amplification of the selected CpG island in DKK3. Two pairs of primers were designed in order to perform a nested PCR amplification. In addition, two additional primers were designed to introduce the sequence from the M13 pair of primers, commonly used as sequencing primers for some plasmids (Table 5.1). For convenience, primers were designed for the sense strand.

Each PCR amplification was performed using 38 cycles of 20 seconds at 95°C followed by 30 seconds at 55°C (the recommended temperature for this set of primers), and finally 90 seconds at 72°C. The amount of GoTaq polymerase (Promega) used was 2 units per reaction. Bands were excised from the gel and purified using QIAquick gel extraction kit (QIAGEN). Forward and reverse second round primers or M13 primers were used for sequencing at the MRC CSC Genomics Core Laboratory, Imperial College London.

In order to increase the quality of the sequence obtained from bisulphite modified DNA was cloning the amplified fragments corresponding to the CpG island was

cloned into a pCR 2.1 TA vector (Invitrogen, Figure 5.5). Preparation, cloning and purification of constructs were carried out as described in sections 2.3.1 and 2.3.2.

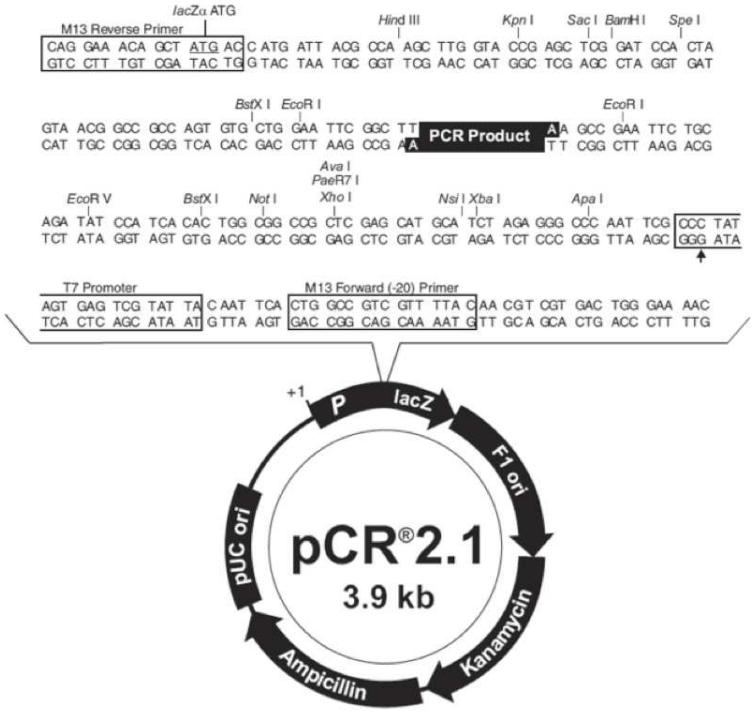


Figure 5.5 Map of the pCR2.1 TA vector.

pCR2.1 vector (Invitrogen) was used for cloning bisulphite modified DNA fragments corresponding to the second CpG island in the promoter region of DKK3. Plasmid DNA for at least ten colonies for each sample was recovered and sequenced using an M13 sequencing primer (Invitrogen)

Bisulphite Sequencing			
	Forward (5' to 3')	**Reverse (5' to 3')**	**Fragment Size (bp)**
First round	GGTTGAGAGAGGTTTGAGGTG	ATCCTCCATCAATTCCTCAAC	631
Second round	GGATAGAGTTTAGGTGAGTTG	AACAACAAACACAACAAAATAAC	413
Tagged Primers			
M13(-20)F	GTAAAACGACGGCCAGAGTGTGCTGGAATTCGGCGGATAGAGTTTAGGTGAGTTG		
M13R	CAGGAAACAGCTATGACAGTGTGATGGATATCTGCAACAACAAACACAACAAAATAAC		

Table 5.1 Primers used for amplification and sequencing of bisulphite modified DNA.

Primers used for amplification of a bisulphite modified DNA fragment corresponding to the second CpG island identified in DKK3.

5.2.4 Methylation specific PCR (MSP)

Three pairs of primers were designed for MSP corresponding to methylated DNA, unmethylated DNA and unmodified DNA, this last pair of primers were used as controls (Table 5.2). These primers were designed following the general recommendations for this type of assay (Herman et al., 1996; Clark et al., 2006) and particularly those from a previous study where the methylation status of DKK3 was analysed (Yue et al., 2009). All primers were designed for amplification of the sense strand, after bisulphite modification. Amplification of the CpG island of DKK3 for controls and cases was performed in two parts. First round sequencing primers were used to amplify the entire CpG island, using the MSP primers for a second round PCR. Each sample was amplified using both primer pairs, for methylated and unmethylated DNA. After performing a gradient to determine the annealing temperature that produced sharp bands, each amplification was performed using 38 cycles of 20 seconds at 95° C followed by 30 seconds at 55° C, and finally 90 seconds at 72° C with 2 units of GoTaq polymerase (Promega). PCR products were subsequently analysed by gel electrophoresis, using a 2% agarose gel.

MSP			
	Forward (5' to 3')	**Reverse (5' to 3')**	**Fragment Size (bp)**
Methylated	GGCGTTAGGGGCGGGCGGC	GCTCTACGCCCGCAACCGCCG	119
Unmethylated	GGGTGTTAGGGGTGGGTGGTG	CCACTCTACACCCACAACCACCA	122
Unmodified	AAGAGGGGCGCCAGGGGCGGGC	CTCCGCTCTGCGCCCGCAGCCG	130

Table 5.2 Primers used for methylation specific PCR of the promoter region of DKK3

Primers were designed based on the recommended primers from Yue et al., 2009.

5.3 Results

For this section, analysis of one of two predicted CpG island in the promoter region of DKK3 was performed, which was the CpG island closest to the coding region of the gene. Bisulphite modification was achieved successfully using the MethylEasy kit (Human Genetic Signatures). Direct bisulphite modification was not successful, even after using the common sequencing primers M13(-20) and M13R to tag the CpG island fragments to be analysed. The only method that yielded a completely readable sequence involved cloning the bisulphite modified CpG island fragment into a TA vector before sequencing. This procedure, however, has the important drawback that only one allele of the gene is selected for each construct and therefore is not possible to know whether partial methylation is occurring. For this reason it was necessary to sequence more than one colony for each sample analysed, making it difficult to analyse a large number of samples. For comparison with MSP results, five controls and five cases from the ICPS cohort were sequenced, with 10 colonies per sample.

All of the analysed samples presented some level of methylation, with the exception of one schizophrenia case (sample 73). In general, a low level of methylation was observed in this CpG island of DKK3, in both controls and cases. However, more methylated colonies were observed in cases compared to controls in the region between CpG sites 16 to 19. MSP was performed on the same samples that were cloned and sequenced. The assay proved to be successful in identifying methylation and most results seem to be consistent with those obtained from sequencing data, although some failed PCR samples and the presence of non-specific bands, together

with the low level of methylation found in DKK3 made it hard to determine the sensitivity of the MSP assay.

5.3.1 Optimisation of Bisulphite modification

Initially, the Imprint kit (Sigma-Aldrich) was used to modify four control samples and four schizophrenia samples from the ICPS cohort. In order to confirm that transformation was successful, all modified samples were amplified using primers designed for methylation specific PCR (MSP) for both methylated and unmethylated DNA. Unmodified DNA was also amplified using a pair of primers for the same fragment but without modified cytosines. Amplification was carried out using the standard conditions previously described (Section 5.2.3). For unmodified samples a band of the expected size was observed but no bands were present for modified DNA with either one of the three pairs of primers, after amplification (Figure 5.6).

In order to assess the quality of DNA, PCR was also performed with a pair of primers previously used successfully. It was observed that unmodified DNA was successfully amplified with the pair of primers used for the SNP2 of DKK3, which indicated that the template was good enough to perform PCR. As expected, these primers were not successful in amplifying the samples after modification (Figure 5.7). However, further attempts to amplify modified samples using MSP and bisulphate sequencing primers did not yield any bands.

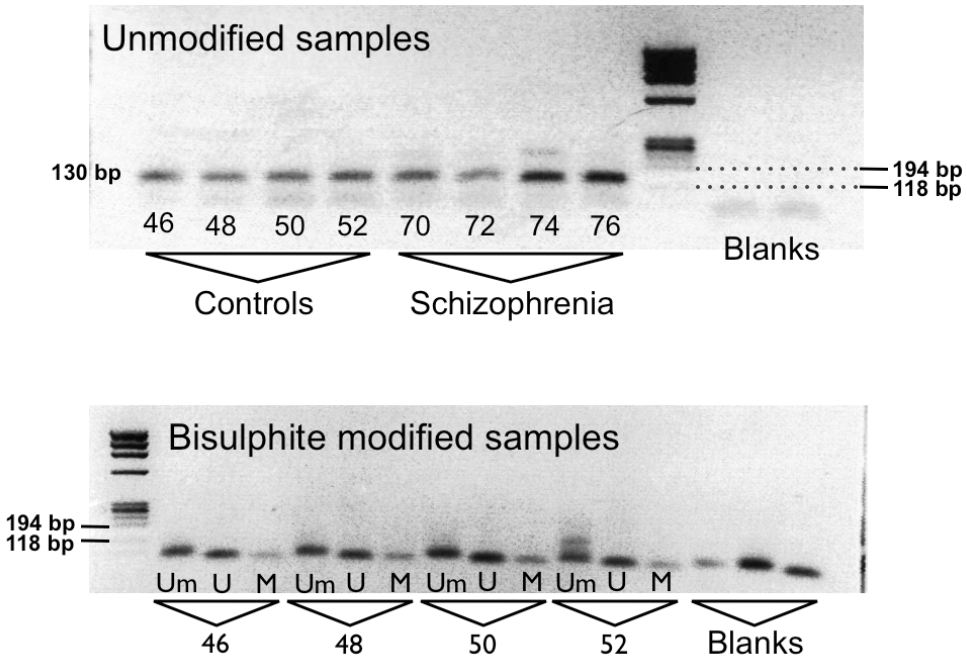


Figure 5.6 PCR amplification of samples before and after modification with the Imprint kit (Sigma-Aldrich).

Three pairs of primers were designed to amplify the same region within the second CpG island. The pair of primers designed for unmodified DNA was also used in modified samples as a negative control. We observed bands of the expected size when amplifying unmodified samples but not for samples after modification.

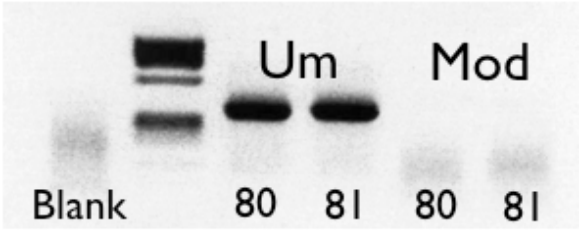


Figure 5.7 Amplification of unmodified and modified DNA with a control primer pair.

The pair of primers used for amplification of SNP2 of DKK3 used to verify the quality of the template and the success of the modification

A number of possible reasons were considered to explain the absence of bands when attempting to amplify the bisulphite-modified samples. The first possibility was that the bisulphite reaction was incomplete or unsuccessful. This seemed particularly likely as a previous PCR of six bisulphite modified samples using primers for unmodified, methylated and unmethylated DNA, yielded a band of the expected size only when using the pair of primers for unmodified samples (Figure 5.8). A second possibility was that PCR reaction conditions required optimisation. For this we chose to use the primer pairs designed for sequencing to perform a gradient of annealing temperatures using one sample from the ICPS cohort. A band of ~631 bp was observed with annealing temperatures between 60.3 and 65.2 °C (Figure 5.9). A new PCR was performed using 10 μl of the bisulphite modified samples and an annealing temperature of 64°C. We observed bands for three out of eight samples (Figure 5.10). The bands were cleaned-up for sequencing, however the amount of DNA recovered was low and the resolution of the sequence obtained was very poor.

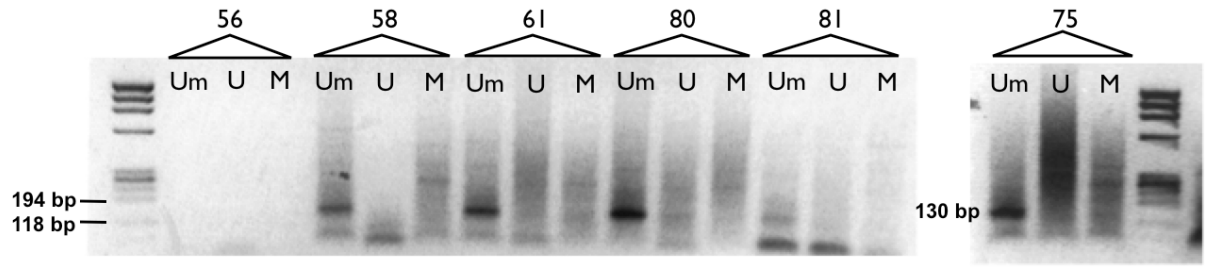


Figure 5.8 PCR performed with samples after modification with Imprint (Sigma-Aldrich).

Bands of the expected size were only observed when amplified with primers used for unmodified DNA.

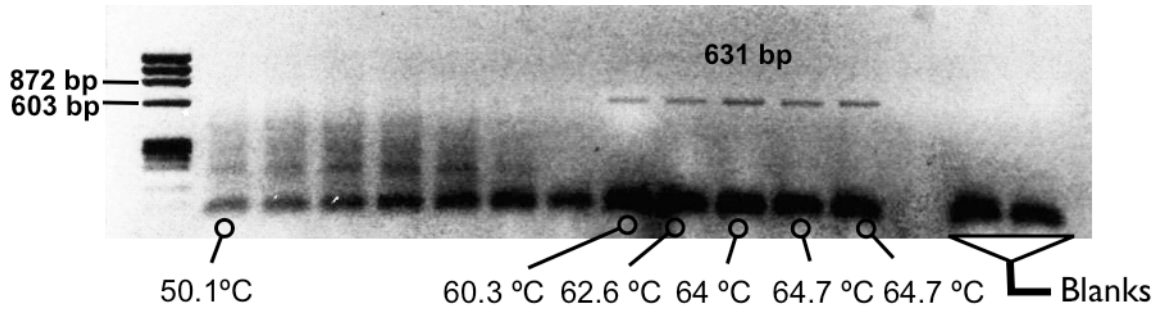


Figure 5.9 Annealing temperature gradient of a modified control DNA sample.

PCR amplification was carried out after modification with the Imprint (Sigma-Aldrich) kit using the pair of primers designed for sequencing.

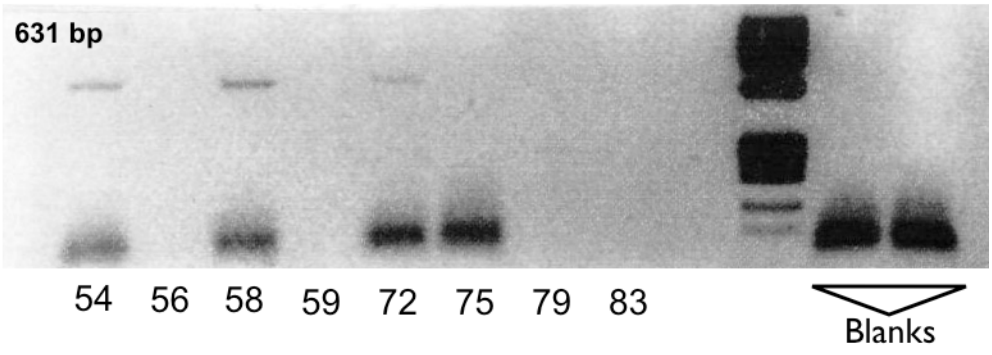


Figure 5.10 Amplification of eight samples modified with the Imprint kit.

PCR was performed using primers designed for sequencing (annealing temperature of 64ºC)

A new bisulphite modification was performed on a set of 10 samples, this time using the Methylation Gold kit (Zymo Research). In addition to the samples used above, two methylated controls were prepared using a CpG methyltransferase (NEB). No bands were observed and this result was persistent even after applying an annealing temperature gradient and semi-nested PCR amplification. Following this result and considering the low amount of DNA that was recovered from column-based kits a new modification was made using the MethylEasy kit (Human Genetic Signatures), which involves a precipitation clean-up step that ensures a better product yield. Initially four modified samples and two controls were successfully amplified with the control primers provided with intense bands observed after the second round PCR. Nested PCR for DKK3 using bisulphite sequencing primers was also successful for amplification of ICPS tissue samples as well as the controls provided by the kit (Figure 5.11).

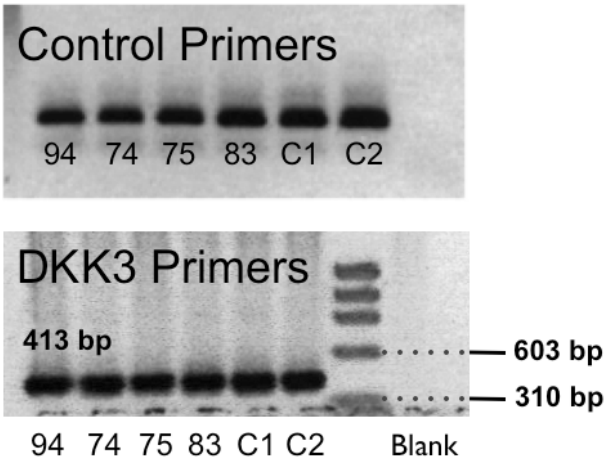


Figure 5.11 Bands obtained for amplification four ICPS samples after modification with MethylEasy (HGS) kit.

Two controls, provided with the kit, were amplified as well. C1 is an unmodified control that was treated along with the samples while C2 is a positive control. Amplification was successful for control primers and DKK3 primers for bisulphite sequencing after second round PCR at an annealing temperature of 55º C.

5.3.2 Direct bisulphite sequencing was only possible from TA constructs

Following the successful amplification of the desired fragment of the promoter region for DKK3 containing the CpG island of interest, bands from controls and cases were excised from the gel and cleaned up for sequencing. Although the amount of DNA recovered was sufficient for sequencing, the quality of the sequence obtained from many attempts was very low and it was impossible to clearly identify any possible methylated sites.

In order to improve the quality of the sequence new primers were designed to introduce the sequence from the M13 pair of primers, commonly used as sequencing primers for some plasmids. The bands obtained from this amplification were excised from the gel and cleaned-up for sequencing. However, the quality of the sequence only improved enough to enable the reading of very few initial nucleotide bases and the majority of the CpG island remained illegible (Figure 5.12).

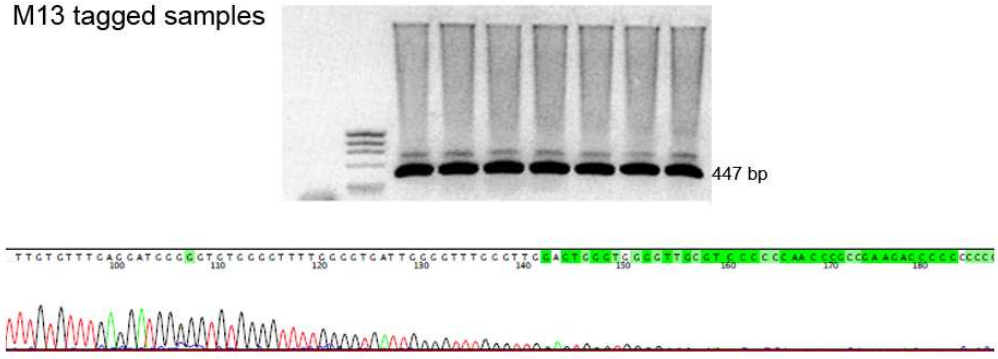


Figure 5.12 DKK3 CpG island fragment tagged with an M13 sequencing primer.

Sequence from the M13 sequencing primers was used to tag CpG fragments as an attempt to improve the quality of the sequence fluorogram. A slight improvement was observed but it was not possible to read most of the sequence.

A further attempt to obtain sequence of better quality involved cloning the amplified fragment corresponding to the CpG island of DKK3 into a pCR 2.1 TA vector (Invitrogen). One important limitation of this approach over direct sequencing is the fact that this method effectively implies the selection of one of the two copies of the gene and therefore it is not possible to identify whether a particular CpG site could be partially methylated. For this reason at least ten colonies were sequenced for each one of the tissue samples that were analysed using an M13 reverse primer (Invitrogen).

Five cases and five controls from the ICPS cohort were selected for the study. The sequence obtained from the clones prepared confirmed that the DNA was successfully modified and revealed that methylation was indeed occurring in the second CpG island, in the promoter region of DKK3 (Figure 5.13). A number of methylated sites were observed in both controls and cases. A region between CpG sites 15 and 28 was identified where the degree of methylation was particularly high. Moreover this region appears to be more heavily methylated in cases compared to controls (Figure 5.14).

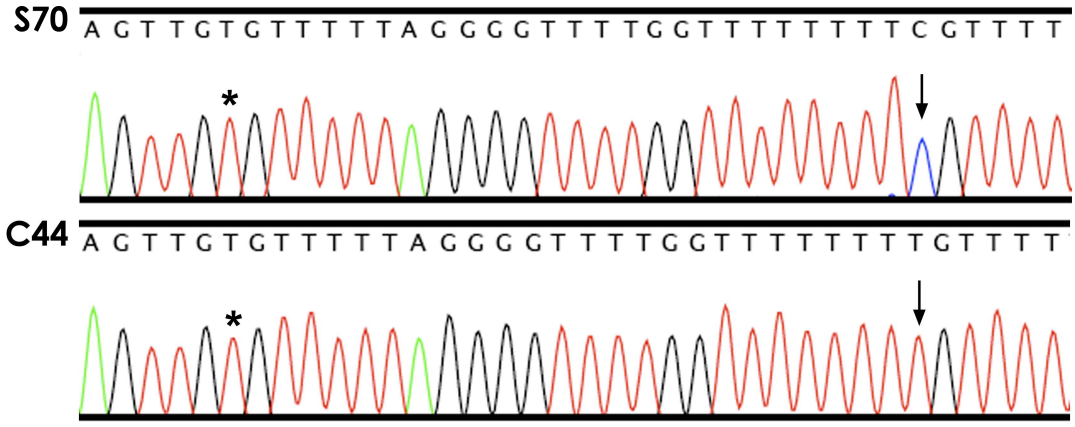


Figure 5.13 Sequence fluorograms of one colony from sample 70 (schizophrenia) and sample 44 (control) from the ICPS cohort.

Arrows indicate a CpG site, which was methylated in S70 and appears as a cytosine (C), but not in C44 where it is shown as a tyrosine (T). Particular to this sequence was the site marked by asterisk, which appears to be an unmethylated CpG site but was in fact T from the original sequence.

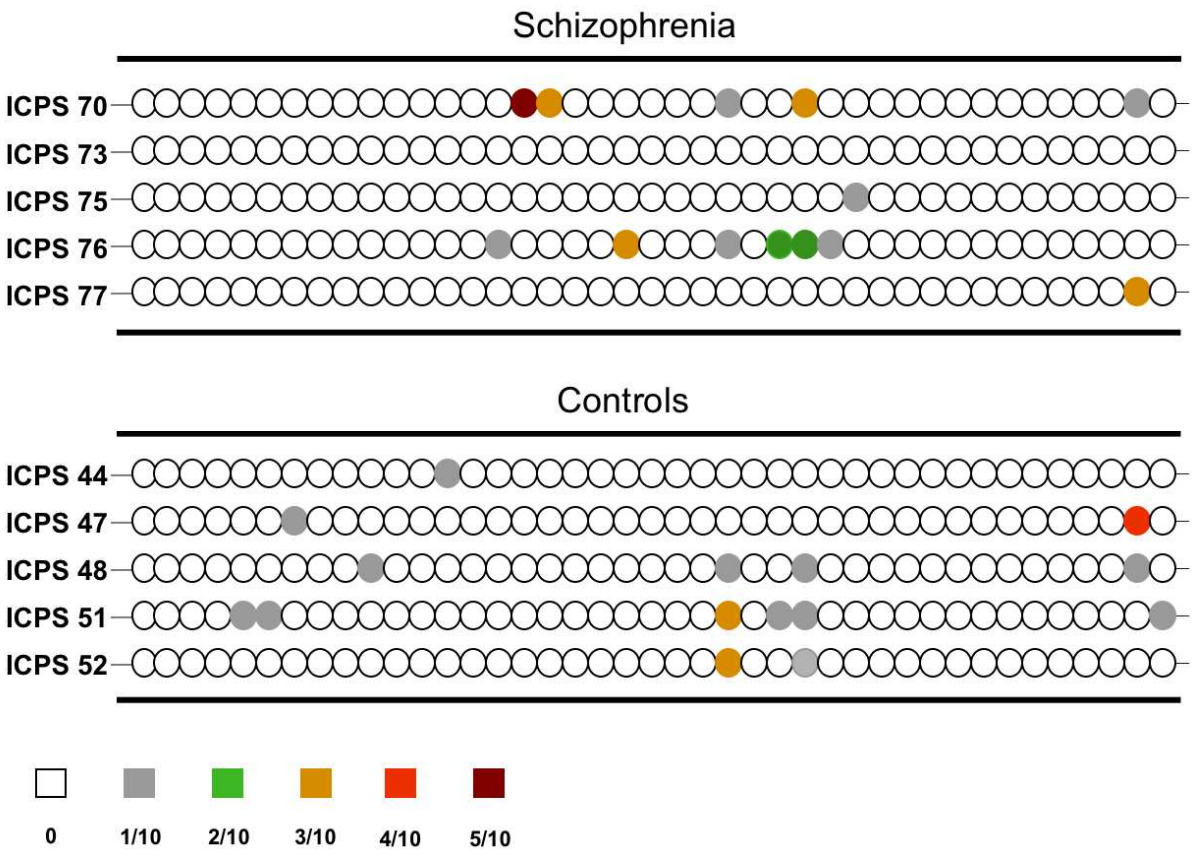


Figure 5.14 Schematic representation of the methylation status of the 41 CpG sites in the analysed fragment from DKK3.

Summary of the data obtained from sequencing ten colonies for each sample. A colour grade is used to represent different degrees of methylation for each site from 0/10 to >5/10.

5.3.3 MSP results compared to bisulphite sequencing findings

In order to meet the main objective of this section, MSP was performed using the same five controls and five cases from the ICPS cohort that were cloned into the TA vector and sequenced, in order to compare the results from both analyses. Methylated controls prepared using a CpG methyltransferase (NEB) were also used and sequenced to confirm that all CpG sites contained within the analysed fragment were methylated (Figure 5.15). First round sequencing primers were used to amplify the entire CpG Island, using the MSP primers for a second round PCR. The fragment obtained from this procedure only includes CpG sites from 20 to 41, which coincide with the region identified as being more heavily methylated from the sequencing of colonies from controls and cases (Figure 5.14).

All samples used for MSP, were amplified with the two pair of primers specific for each methylation status. Although non-specific bands were observed in both methylation cases and for all amplified samples, analysis of the unmethylated and methylated control samples produced a band of the expected size only present in the appropriate lane (Figure 5.16). When considering that the analysed CpG island was not completely methylated for any of the samples that we sequenced, it would be expected that both bands would be present in our MSP analysis. This was the case for most of the samples analysed. It is important to mention that this procedure was performed at least three times for each sample and the results were not consistent in all cases. One exception was the schizophrenia sample number 70 for which, in every attempt, a clear band was observed only when using the primer pair for the unmethylated case.

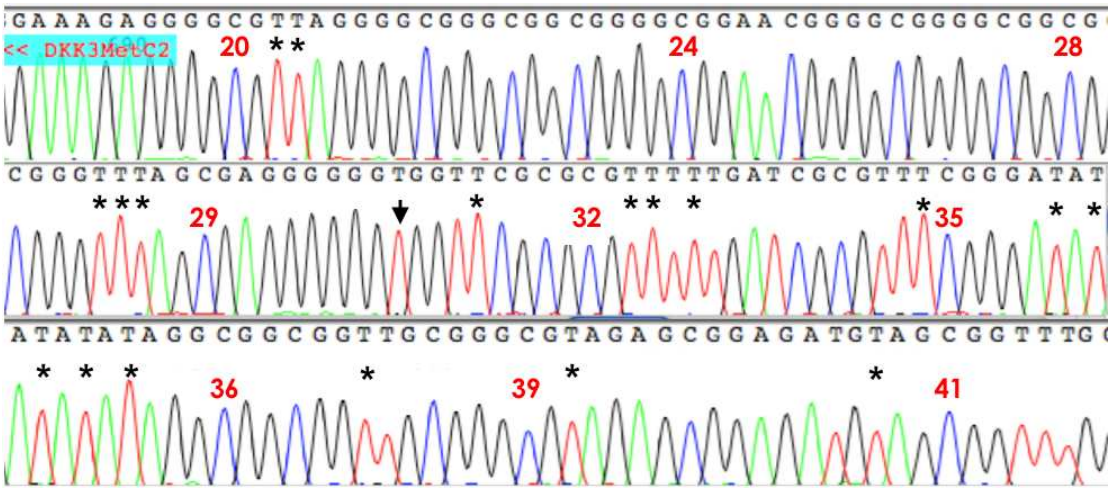


Figure 5.15 Sequence from a methylated control sample prepared by treatment with a CpG methyltransferase (NEB).

Marked CpG sites are included in the fragment amplified by MSP primers. Non-CpG, unmethylated cytosines were converted and are marked with asterisks. The arrow indicates a thymine present in the fragment even before bisulphite treatment.

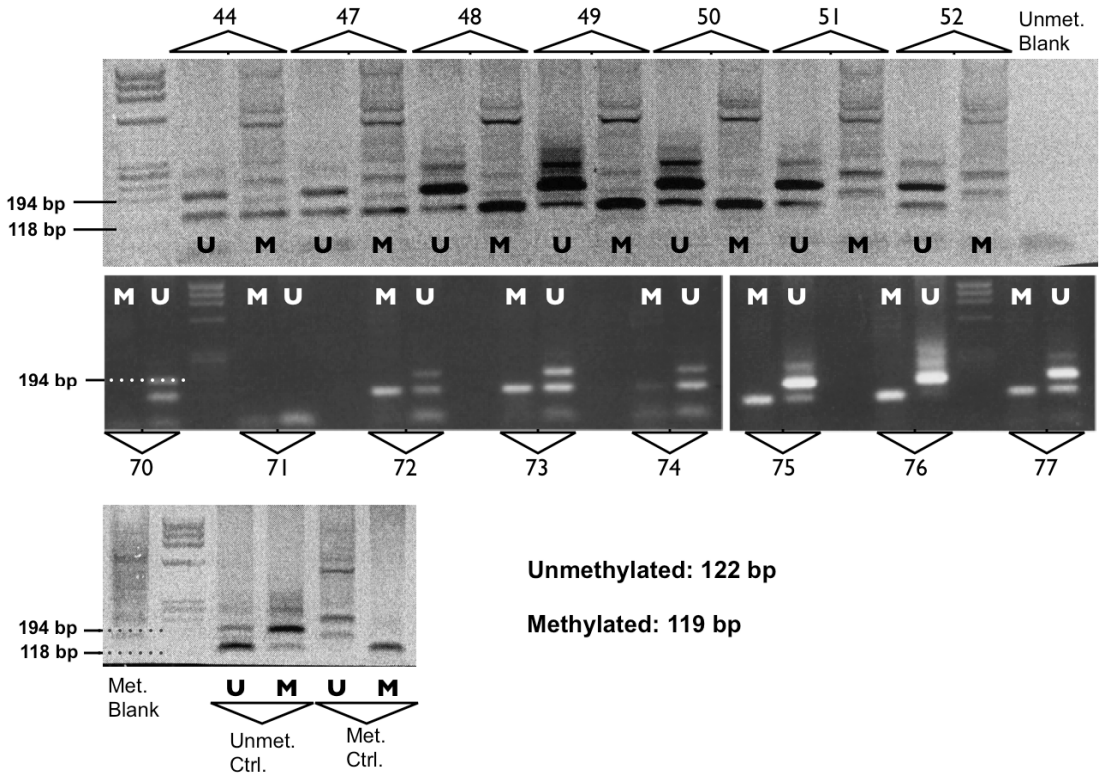


Figure 5.16 MSP of controls and cases of the ICPs cohort.

Bands obtained from methylation specific PCR (MSP) of seven control samples and eight schizophrenia cases from the ICPS cohort, including the ones previously sequenced. A control sample was included for each methylation status.

5.4 Discussion

5.4.1 Successful bisulphite modification of DNA from brain tissue samples

A successful modification of DNA was a critical step for the analysis of CpG island methylation of DKK3. An important consideration to make is that after modification the two strands of DNA are no longer complementary and therefore primers have to be design for each individual strand. It is important to consider that in general DNA isolated from tissue, as is the case of the samples from the ICPS cohort, might be more resistant to bisulphite conversion than DNA isolated from cultured cells (Warnecke et al., 2002). This was the main reason why three different kits had to be tested for the present analysis. It was determined that the MethylEasy kit (Human Genetic Signatures) was the most effective particularly because it involves a clean-up procedure that is based on precipitation of DNA after bisulphite modification and this results in a better yield of product.

5.4.2 Methylation of a CpG island in the promoter region of DKK3 in schizophrenia cases and controls detected through bisulphite genomic clonal sequencing analysis

We report methylation of a CpG island in the promoter region of DKK3 in schizophrenia cases and controls of the ICPS cohort. Although the degree of methylation observed was not extensive for any of the samples analysed, overall there was a larger number of prominently methylated sites in cases when compared to controls. Of particular interest was the region comprising CpGs 16 to 29 in which methylation appeared to be much heavier in cases compared to controls. These differences in methylation of specific sites in cases compared to controls is especially

noteworthy when considered in the context of the "critical site" model originated from reports where methylation of one or two cytosines was observed to be sufficient for downregulation of gene expression (Robertson et al., 1995; Pogribny et al., 2000).

Even though limitations associated with the methodology made it hard to include a larger number of samples in the analysis, our data confirm the presence of CpG island methylation of DKK3 in samples from the ICPS cohort. Moreover, the level of detail achieved by using a clonal sequencing method allows for the identification of methylation in each CpG site at a single-molecular level. The resolution obtained from clonal bisulphite sequencing also allows for the identification of allele specific methylation, which is recently a subject of investigation in relation to complex disorders (Polesskaya et al., 2006; Meaburn et al., 2010). This is an advantage over direct bisulphite sequencing and any other methods available to date, which can only give a semi-quantitative measure of the level of methylation of the target sequence. To date, bisulphite clonal sequencing is the only method that has been recognised to be able to achieve this degree of resolution (Warnecke et al., 2002; Clark et al., 2006).

5.4.3 MSP as a rapid method for detection of methylation in brain tissue samples

The results obtained from the MSP analysis of controls and cases appear to be consistent with the degree of methylation observed from those same samples from the clonal sequencing analysis. Moreover, the use of methylated and unmethylated control samples demonstrates that the primers designed are able to successfully differentiate each methylation status. However, there are important discrepancies

that together with the presence of non-specific bands suggest that mispriming might be occurring, causing false positives or false negatives. This made it difficult to assess the sensitivity of the method. It is also important to note that very few CpG sites were methylated in all samples analysed through bisulphite sequencing. This represents a drawback for the use of MSP because the primers designed for the assay only cover CpG sites from 20 to 23 (forward primers) and from 37 to 40 (reverse primers). This could explain why control samples 51 and 52 are only amplified with the pair of primers specific for unmethylated DNA.

5.4.4 Further investigation of the effect of DKK3 in β-catenin translocation in neuronal cells

In addition to the notion of the implication of the canonical Wnt signalling pathway during developmental stages in the aetiology of schizophrenia, the important role of β-catenin in synaptic plasticity and memory consolidation (Maguschak and Ressler, 2008) increases the interest for further investigation of this subject. Reports of NMDA-receptor activation leading to generation of more stable forms of β-catenin in hippocampal neurons (Abe and Takeichi, 2007) might provide the basis for further studies. In a recent study, Mao et al. (2009) provide evidence for an important role of the disrupted in schizophrenia (DISC1) gene in neuronal progenitor proliferation through this β-catenin/GSK-3β pathway. In a sophisticated analysis the authors demonstrate that DISC1 knock down significantly increases phosphorylation of β-catenin, affecting progenitor cell proliferation in vivo and in vitro. Additionally, they showed that normal expression of DISC1 overrides this effect. Further investigation led to identification of a particular peptide of DISC1 that interacts directly with GSK-3β with a strong inhibitory effect. Finally, GSK-3β has been proposed as a candidate

gene for psychotic disorders, due to the multiple functions of this enzyme in distinct metabolic pathways and its involvement in a number of processes in the CNS (Emamian et al., 2004; Beaulieu et al., 2007; Peineau et al., 2008; Freyberg et al., 2010).

DKK3 remains the one member of the dickopff family of proteins whose precise role in modulation of Wnt signalling pathway is not yet known (Wu et al., 2000; Brott and Sokol, 2001; Mao et al., 2001; 2002; Davidson et al., 2002; Mao and Niehrs, 2003). Data collected from a number of studies suggests that the roles of DKK3 in Wnt signalling regulation are varied and may be cell type specific (Caricasole et al., 2003; Hoang et al., 2004; Nakamura Y et al., 2007; Nakamura REI et al., 2010). Moreover, a number of reports suggests that the mechanisms through which DKK3 and other related genes modulate Wnt signalling might depend on whether they exert their function in developing or adult organisms (Kawano and Kypta, 2003; Nakamura et al., 2007; von Marschall and Fisher, 2010).

Most of the data available to date on the mechanisms of action of DKK3, as well as its interaction with other proteins, has been gathered through extensive research on the involvement of DKK3 in proliferation of cancer cell lines (Tsuji et al., 2000; 2001; Hsieh et al., 2004, Abarzua et al., 2005; 2007). A number of studies have found evidence for hypermethylation of the promoter region of DKK3 leading to downregulation of the gene in a variety of tumours. Data from these studies also suggests that this decrease in expression of DKK3 leads to translocation of β-catenin into the cell nucleus and stimulation of cell proliferation (Kobashashi et al., 2002; Hoang et al., 2004; Roman-Gomez et al., 2004; 2007; Arjen et al., 2008; Veeck et al., 2008b; Chim et al., 2008; Yue et al., 2008; Lee EJ et al., 2009).

In an attempt to gain an insight into the functional relevance of down-regulation of DKK3 in the pathophysiology of schizophrenia, particularly with respect to the function of this gene in processes related to the Wnt signalling pathway an additional analysis was carried out based on reports of DKK3 being able to obstruct b-catenin translocation to the cell nucleus after activation of Wnt signalling pathway (Yue et al., 2008). Mouse neuroblastoma N2a cells were used as a neuronal cell line and COS7 cells were chosen as controls. However, this study could not be completed due to extensive optimisation required for this analysis. Preliminary results are presented in Appendix E as the basis for future investigation.

Cells were transfected with GFP-DKK3 and an RFP-β-catenin constructs in order to observe the effect of expression of DKK3 in the nuclear translocation of β-catenin for gene transduction through TCF/Lef in the context of activation of the Wnt signalling pathway. This last objective was achieved by luciferase reporter assay using a TCF activated reporter (TOPFlash) and a second construct with the same TCF domain but containing mutations for inactivation was used as a negative control (FOPFlash).

The distribution of GFP-DKK3 was observed to be cytosolic in both N2a and COS7 cells (Supplementary Figure 1) consistent with other reports (Nakamura et al., 2007; Untergasser et al., 2008). Western blot analysis confirmed the expression of the secreted glycosylated form of DKK3. Expression of the RFP-β-catenin fusion protein was also confirmed by western blot, whilst its subcellular localization was observed to be primarily nuclear (Supplementary Figure 2) in both cell lines. Analysis of the luciferase activity of TOP and FOPFlash reporters later confirmed that transfection of the RFP-β-catenin construct induced activation of Wnt signalling in N2a cells and that

co-transfection of GFP-DKK3 resulted in significant inhibition of this activation (Supplementary Figure 3).

These preliminary findings are a starting point for the investigation of the precise role of DKK3 in the regulation of β-catenin mediated gene transcription in neurons. The importance of elucidating the mechanisms through which schizophrenia candidate genes involved in the Wnt signalling pathway interact becomes evident when considering previous reports of increased Wnt activity in schizophrenia (Miyaoka et al., 1999; Kozlovsky et al., 2000; 2004; Beasley et al., 2001). Moreover, the data presented in this preliminary analysis leads to the formulation of interesting questions for future investigation. It can be hypothesised, for example, that through modulation of the Wnt signalling pathway, DKK3 might be playing a role in homeostasis and synaptic plasticity in the adult CNS. This idea becomes more intriguing when considering the important role of DKK3 and the canonical Wnt pathway in cell proliferation (Chen et al., 2001; Kobashashi et al., 2002; Yue et al., 2008), which is in concordance with the evidence of a role of DISC1 in neuronal progenitor proliferation (Mao et al., 2009) and adequate integration of adult-born neurons in hippocampus (Duan et al., 2009).

Finally, in the context of the growing evidence for the involvement of the β-catenin/Wnt signalling pathway in modulation of synaptic function and memory consolidation in adult CNS in mammals (Hirabayashi et al., 2004; Chen et al., 2006; Beaumont et al., 2007; Wexler et al., 2008; Avila et al., 2010), extension of the investigation of the role of DKK3 in the canonical Wnt pathway would be worth perusing. One particular idea for the extension of the present study is based on recent evidence of the initiation of gene expression by β-catenin through a

mechanism involving activation of NMDA-receptor activation in hippocampal neurons (Abe and Takeichi, 2007). Specifically, the investigation of DKK3 modulation of canonical Wnt signalling as a response to activation or inhibition of NMDA receptors could be carried out using NSC34 cells. NSC34 have a close similarity to developing motor neurons and they are known for the expression of NMDA receptors and their ability to model early synapse formation (Cashman et al., 1992).

5.4.5 Conclusion

The use of MSP for analysis of CpG island methylation has been used successfully for the analysis of DKK3 and other genes in cancer cell lines and tumour tissue (Herman et al., 1996; Roman-Gomez et al., 2004; 2007; Veeck et al., 2008b; Chim et al., 2008; Yue et al., 2008). In these cases, the density of methylation across the CpG island was greater than that observed for DKK3 in the ICPS cohort. The fact that the pair of primers designed for umethylated DNA failed to produce a band of the correct size for the methylated controls (and vice versa) seems to support the idea that a denser pattern of methylation is needed for MSP to be successful. Therefore, this method must not be excluded as a viable and rapid alternative for the investigation CpG island methylation of other candidate genes whose expression has been reported as changed in schizophrenia.

Although, these findings might not be enough evidence to conclude that epigenetic mechanisms playing a part in the downregulation of DKK3 reported in schizophrenia (Ftouh et al., 2005), the location of the gene in a chromosomal region whose hypermethylation has been associated with major psychosis is encouraging (Mill et al., 2008). Further investigation of methylation status in the first CpG island, which was not included in the present study, could provide a more complete analysis.

CHAPTER 6 General Discussion

6.1 Significant genotype and haplotype correlation with levels of expression for ZnT3, CACNA1E and FRZB in the ICPS cohort

Analysis of the effect of SNP genotype and haplotype on levels of expression of the selected schizophrenia candidate genes revealed interesting correlations for three genes in particular. For all three genes ANOVA revealed a significant association of disease status with levels of expression. In the case of ZnT3, a significant overall effect was observed for genotypes and two-SNP haplotypes on levels of expression of the gene in BA10. This effect contributed to a significant interaction between disease status and genotype (or haplotype) with respect to gene expression. Unlike ZnT3, no overall significance was observed of SNPs in CACNA1E or FRZB with expression levels of the genes in BA22 and BA10, respectively. However, for these two genes, significant associations of individual genotypes and haplotypes with mRNA levels resulted from *post-hoc* Bonferroni comparisons.

The fact that these associations of genotype with expression levels were present for CACNA1E and FRZB, in spite of their lack of genetic association with disease, brings up important questions. One of them is whether the changes in regulation of expression of these genes observed in schizophrenia are a result of the disease or rather a consequence of other factors (e.g. neuroleptic treatment). This is particularly intriguing considering the role of CACNA1E and FRZB in glucose methabolism and chronic inflammation (Matsuda et al., 2007; Bi et al., 2009; Hirata et al., 2010; Ekström et al., 2011). Extensive research is being conducted in order to elucidate the implications of both of these processes in the methabolic syndrome observed in schizophrenia patients subject to chronic antipsychotic treatment

(Thakore, 2005; Remington, 2006; Heald, 2010). Thus the SNPs in CACNA1E and FRZB identified as markers of expression, rather than markers of disease, might be correlated with endophenotypes representing metabolic disfuction. This may contribute to a better understanding of the mechanisms underlying the significant changes in the expression of these genes in schizophrenia cases compared to controls.

Even though none of the SNPs for which association with expression was observed was a functional polymorphism, these SNPs are reported here as markers for regions within the genes that could be implicated in regulation of expression. Moreover, when considering LD patterns obtained across polymorphisms within these three genes, an interesting possibility for future studies is that one or more of the selected SNPs could be in strong linkage disequilibrium with other variants directly involved in regulation of gene expression (Skelly et al., 2009). Notably, there has been a suggestion that genetic variants that have an impact in mRNA or protein levels are likely to have an important contribution to disease susceptibility in complex disorders (Bray et al., 2003). This idea has been supported by recent studies, which have shown that amongst polymorphisms with a high probability of being also expression quantitative trait loci (eQLT) are enriched amongst those identified from GWAS as marginally associated with schizophrenia (Richards et al., 2011) and in general with complex traits (Nicolae et al, 2010).

6.2 Genetic association of the synaptic vesicle specific zinc transporter (ZnT3) with schizophrenia

From the analysis of genetic association of genes involved in synaptic function and cellular communication with schizophrenia, ZnT3 gene was the most consistently associated with the disorder. Significant allelic association was observed for four SNPs with disease status in the East UK cohort. Also, genotypic associations were observed for all four SNPs, consistent with a dominant model for disease penetrance. Haplotype analysis further supported these associations with five haplotypes associated with risk for disease, even after multiple comparison correction.

Our results are supported by a recent patent reporting a number of polymorphisms significantly associated with schizophrenia (Chissoe et al., 2008). This patent describes a study where polymorphisms within or in proximity of genes coding for proteins that could potentially be used as targets for drug design were analysed in two cohorts of controls and schizophrenia cases of Caucasian ancestry. The first of these cohorts comprised 511 schizophrenia cases and 500 controls collected from Munich. The second cohort was conformed of 546 and 669 controls collected from Aberdeen. Amongst the polymorphisms identified as significantly associated with disease in the Munich cohort, were rs2083363 and rs2485506. The first of these polymorphisms is located close proximity of ZnT3 at approximately 2.1 Mb from the 3' end of the gene. Both allelic (p=0.01926; OR=1.3) and genotypic (p=0.01993; OR=2.28) associations with schizophrenia were reported for this SNP. The second SNP (rs2485506), located in an intronic region of CACNA1E, was also found to be associated with disease in the Munich cohort at thte genotypic (p= 0.0462; OR= 1.44) and allelic level (p= 0.0323; OR= 1.22). Furthermore, SNP rs7775, a non-

synonimous variant in exon 6 of FRZB, was one of the polymorphisms that presented the most significant genotypic (p= 0.0042; OR= 1.82) and allelic (p= 0.0044; OR= 1.67) association with schizophrenia in the Aberdeen cohort.

It is interesting that three of the six selected candidate genes for the present study were reported as associated with schizophrenia in the previously described patent, particularly considering that the methodology followed for selection and analysis of these genes was different from the one used here. This provides support for the candidate gene approach used for this study, in which genes were selected for analysis on the basis of their function and their already established differential expression in schizophrenia. In spite of this, no significant associations of SNP genotype with disease were identified for any of the candidate genes, other than ZnT3. For some of the markes in these genes, and particularly for those in CACNA1E, the small size effects observed might result in a reduced power of the study for detecting significant associations.

Furthermore, it is important to consider other SNPs identified as associated with schizophrenia from previous GWAS studies and which are located in genes that might be functionally related to those analysed here. Examples of these include SNPs located in or close to, zinc finger and SCAN containing domain 23 (ZSCAN23), zinc finger, BED-type 4 (ZBED4) and the zinc finger and BTB domain containing 9 (ZBTB9), as well as 22 (ZBTB22) proteins and a number of zinc finger proteins. Amongst the most significant are ZNF311, ZNF490, ZNF284, ZNF204, ZNF391, ZNF184 and ZNF274. Especially notable are ZNF322A, which was replicated in two studies (ISC, 2009; Shi et al., 2009) and ZNF804, which like ZNT3 is located in chromosome 2 (Williams et al., 2011; Zhang et al., 2011). Also relevant were two

more SNPs located in the CACNA1C gene with schizophrenia (ISC, 2009; Moskvina et al., 2009), which is different to the one reported previously as associated with bipolar disorder (Ferreira et al., 2008). In addition, association of genetic variants with schizophrenia has also been reported for polymorphisms in the secreted Wnt ligand Frizzled-3 (FZD3) in Chinese and Japanese populations (Yang et al., 2003; Zhang et al., 2004; Katsu et al., 2003), although attempts to replicate these findings and or reproduce them in other populations have not been successful (Ide et al., 2004; Wei and Hemmings, 2004; Hashimoto et al., 2005; Jeong et al., 2006). Finally, association of a SNP in a region close to the WNT4 gene was reported in one of these GWAS (ISC, 2009).

6.3 Modest evidence for statistical epistasis and insights into the possible disruption of particular functional pathways in schizophrenia

The analysis of SNP association with gene expression levels and disease status presented here has its base on previous microarray studies where a number of genes were identified as significantly changed in schizophrenia. Candidate genes belonging to two major functional groups were analysed, genes implicated in synaptic function and cellular communication and genes involved in Wnt signalling pathway. This approach for the selection of the genes analysed was used as an attempt to identify not only particular genes associated with schizophrenia, but to highlight particular functional pathways that might be implicated in the aetiology of the disorder. In spite of the fact that consistent significant associations with schizophrenia were observed for only one of the candidate genes analysed here the evidence for statistical epistasis between genes within both functional groups provides support for further

investigation functional interactions between genes within each of these categories that might have important implications for biological function in schizophrenia, as has been suggested for the case of interactions between the GAD1 and COMT genes (Straub et al., 2007; Marenco et al., 2010).

6.4 A method for investigation of possible CpG island methylation of DKK3 for application to other schizophrenia candidate genes

The complex genetics known to be involved in the aetiology of schizophrenia as well as the prevailing onset of the disease during the adult life have made the involvement of epigenetic mechanisms in the pathophysiology of the disorder an exciting subject of investigation. Furthermore, the increasing evidence collected on the effect of promoter region methylation on down-regulation of schizophrenia candidate genes, such as GAD1 and REELN (Dong et al., 2005) makes this investigation worth pursuing. The analysis of CpG island methylation in candidate genes for schizophrenia will benefit from developments in high throughput techniques such as the use of microarrays and DNA-pooling techniques (Mill et al., 2008; Docherty et al., 2010; Gu et al., 2010).

For this study, it was considered important to determine the methylation status of the promoter regions of the selected candidate genes whose expression was changed in schizophrenia (Ftouh et al., 2005; Maycox et al., 2009; Barnes et al., 2011). DKK3 was used as the basis of the analysis in order to establish a method that could be used for analysis of methylation of CpG islands of other candidate genes. The method was based on clonal bisulphite sequencing, which is the only

method available that allows for the determination of the methylation status of each CpG site (Clark et al., 2006). Results from sequencing of the promoter region of DKK3 in controls and schizophrenia cases were later compared to those obtained from MSP analysis. The objective of this was to establish whether MSP could be used as a rapid method for detection of CpG island methylation in candidate genes using post-mortem tissue samples from schizophrenia cases and controls.

It was concluded from this analysis that while MSP is a sensitive method for detection of CpG island methylation using DNA from post-mortem brain tissue samples. However this technique seems to be more reliable in cases where the density of methylation is high. Since this was not the case of DKK3, there was the need to use clonal bisulphite sequencing as a complementary method. The laborious nature of this process meant that the number of samples that could be used was reduced, however this technique allows for the identification of the methylation status of each CpG site, which is not possible to achieve with any other method at the moment. Although from this study, it was not possible to conclusively state that epigenetic mechanisms are indeed responsible for the down-regulation of DKK3 observed in schizophrenia, MSP might still be a suitable method for the analysis of other schizophrenia candidate genes showing more robust changes in expression in cases compared to controls. Moreover, the possibility of CpG "critical site" (Robertson et al., 1995; Pogribny et al., 2000) regulating expression of DKK3 in a subset of schizophrenia cases is an intriguing possibility and one that might be worthy of future investigation.

6.5 Conclusion

Investigation of the genetic risk factor contributing to the aetiology of schizophrenia has been a challenging task. Very few loci associated with disease have been identified so far, in spite of the availability of data from genome-wide studies even with the use of large numbers of samples in some studies. Amongst the reasons proposed to explain this phenomenon is the fact that the individual effect of common variants in the risk of complex disorders tends to be relatively small. Genome-wide linkage and association studies have been extensively used for the elucidation of genetic factors contribution to the risk of schizophrenia. However, there are a number of limitations associated with the genome-wide approach. One of these limitations is that the interpretation of data becomes difficult due to the large number of loci analysed resulting in the need to adjust the threshold of significance depending on the conditions, in order to minimise the occurrence of false positive reports (Lander and Kruglyak, 1995; Morris, 2008). Other factors affecting the ability of GWAS to yield information about genetic factors contributing to the heritability of schizophrenia are population structure and the assumption that common variants will make the greatest contribution. Moreover, the tag-SNPs generally included in GWAS are not representative of haplotype blocks and therefore do not fully represent the genetic variation present in the population (Morris, 2005).

The approach used in the present study consisted in selecting a number of candidate genes based on strong biological plausibility. Selected genes showed robust changes in expression in schizophrenia in brain regions implicated in functional abnormalities occurring in this condition (Maycox et al., 2009; Barnes et al., 2011). This approach has been used successfully in a number of studies (Voisey et al 2010a; Voisey et

2010b; Burdick et al 2010). Furthermore, in the paper by Burdick et al (2010), which was also highlighted in an Editorial (Cannon 2010), a cohort of 173 schizophrenia cases and 137 controls (310 individuals in total) was used for a candidate gene association study. This is a similar size cohort to that used in the present study for analysis of association with schizophrenia (320 individuals in East UK cohort).

Significant correlations of expression with schizophrenia in all selected candidate genes arising from our previous microarray study were identified. A significant interaction between genotype and disease status with respect to gene expression was identified for ZnT3 using 3-way ANOVA. Significant association of particular genotypes and haplotypes with gene expression was also observed for CACNA1E and FRZB. A subsequent association study was carried out in a case-control cohort from East UK (East Anglia and East Yorkshire) using the same widely used analytical approach with correction for multiple tests that have been successfully used by Voisey et al (2010a: 2010b) in their studies of similar cohort size (160 schizophrenia cases and controls) and OR values.

The results from the present study particularly substantiate ZnT3 as strong candidate gene for schizophrenia and provide support for the use of a candidate gene approach for the analysis of association with disease. The exclusive localization of ZnT3 to synaptic vesicles (SV) in different neuronal populations serving as the only source of metallic zinc (Paoletti et al., 2009) makes this gene a particularly interesting candidate from the functional point of view. Zinc enriched neurons are also glutamatergic neurons (Sindreu et al., 2003; Frederickson et al., 2005). Consistent with this is the finding that vesicular zinc uptake is not only regulated by ZnT3 but also by expression of the vesicular glutamate transporter type 1 (Vglut1) in PC12

cells (Salazar et al., 2005). There is evidence of zinc-mediated modulation of excitatory synapses through regulation of glutamate and γ aminobutiric acid (GABA) receptors (Xie et al., 1994). It has also been suggested that zinc might have an important role in modulatinon of synaptic plasticity through inhibition of NMDA receptors (Vogt et al., 2000; Sindreu et al., 2003). In spite of these findings, the precise role of vesicular zinc in glutamatergic synapse is still not well understood. However, the localization of zinc enriched neurons somata to either cortical or limbic structures suggests an important role of these neurons in linking these regions (Sindreu et al., 2003; Frederickson et al., 2005; Paoletti et al., 2009).

Two recent studies have provided data supporting the role of vesicular zinc in hippocampus-dependent memory and learning processes (Sindreu et al., 2011; Pan et al., 2011). Using the ZnT3 KO mouse model, Sindreau et al. (2011) show that the absence of the ZnT3 gene contributes to important deficits in spatial working memory and contextual discrimination. These findings are particularly interesting in the context of the downregulation of ZnT3 in the prefrontal cortex (PFC) of schizophrenia cases (Maycox et al., 2009). Functional interaction between hippocampus and PFC is considered to have important implications in memory and learning processes (Yoon et al., 2008; Mölle and Borne, 2009). Furthermore, there is extensive evidence suggesting a disruption in connectivity between the hippocampus and PFC underlying schizophrenia pathophysiology (Harrison, 2004; Sigurdsson et al., 2010). In this study four SNPs in ZnT3 were found to be significantly associated with schizophrenia. Correlations of SNP genotype with cognitive endophenotype in a lager cohort of samples might be performed in the future, in order to further investigate the contribution of the genetic variation of ZnT3 to the disease.

The pathway approach taken in this study was supported by the evidence for statistical epistasis between polymorphisms in both groups of genes analysed. In consideration to this, it is perhaps important to draw attention to the polymorphisms that have been identified as risk factors for schizophrenia from GWAS in other genes related to synaptic processes or to modulation of the Wnt signalling pathway (ISC, 2009; Moskvina et al., 2009; Stefansson et al., 2009; Shi et al., 2009). Particularly interesting were results reported in the study by Chissoe et al. (2008), where a number of polymorphisms were identified as significantly associated with schizophrenia. Amongst the more significantly associated polymorphisms were two SNPs located in ZnT3, one SNP located in CACNA1E and one more located in FRZB.

Finally, in order to explore the possible epigenetic regulation of expression of the selected candidate genes, clonal bisulphite sequencing and MSP were used. The objective of the approach was to provide a quick and reliable method for determination of CpG island methylation. For the purpose of designing the method, DKK3 was used as MSP primers had already been used successfully to determine methylation in cancer cell lines (Yue et al., 2008). It was concluded from this analysis that MSP is a sensitive method for detection of methylation. However, the presence of some false positives and negatives resulted in the need to use clonal bisulphite sequencing as a complementary method. Additionally, the density of methylation in DKK3 was low in the ICPS cohort and therefore it was not possible to conclusively state that epigenetic mechanisms are indeed responsible for the down-regulation of the gene observed in schizophrenia. However, once established the conditions for successful bisulphite modification of post-mortem brain tissue samples, it is possible to use this method for analysis of CpG island methylation of other candidate genes whose expression has been identified as significantly changed in schizophrenia.

Glossary

Allele	A variant of polymorphism at a given locus.
Diplotype	A specific combination of two haplotypes in an individual.
Disease penetrance	The risk of disease in a given individual. Genotype-specific penetrances reflect the risk of disease with respect to genotype.
Epistasis	A phenomenon where the effect of one gene are modified by one or more other genes to produce a given phenotype.
Exon	A section of a gene that is represented in the mature form of the mRNA transcript
False discovery rate	The proportion of non-casual or false positive significant SNPs in a genetic association study.
Genotype	The combination of two alleles across both chromosomes at a given locus in an individual.
Haplotype	A consecutive series of alleles at different loci on a single chromosome in an individual.
Hardy-Weinberg equilibrium	Given a minor allele frequency of p, the probabilities of the three possible unordered genotypes (a/a, A/a, A/A) at a biallelic locus with minor allele A and major allele a, are $(1 - p)^2$, $2p (1 - p)$, p^2. In a large, randomly mating, homogenous population, these probabilities should be stable from generation to generation.
Intron	A noncoding section of a gene that is removed from the mature mRNA sequence by a process called splicing.

Linkage disequilibrium (LD)	The population correlation between two allelic variants (usually nearby) on the same chromosome. The variants are said to be in LD if they are inherited together more often than expected by chance.
Moving-window haplotype analysis	For this analysis haplotype blocks are generated according to specific settings. For example, in an analysis comprising four SNPs, one might opt for a case/control comparison of two, three or four SNP haplotype frequencies. The number of markers selected defines the size of the 'window' that slides across the LD spine to generate the haplotype blocks. A χ^2 test is used subsequently to identify significant differences in haplotype block frequencies between controls and cases.
Odds ratio	A measure of association derived from case-control studies; it is the ratio of the odds of disease in the exposed group compared with the non-exposed.
Population stratification or structure	The mixture of individuals with heterogeneous genetic backgrounds. It refers to the presence of two or more subgroups with distinct genetic ancestry in a given population.
Regulatory region	A section of the DNA sequence that directly or indirectly affects the expression of a gene.
TagSNP	A SNP in a region of the genome featuring high LD, which is a proxy for others in close proximity and which can be used

	to genotype individuals at a reduced cost, while maintaining power.
Type I error	In a hypothesis test, a type I error occurs when the null hypothesis H_0, is rejected when it is in fact true.
Type II error	In a hypothesis test, a type II error occurs when the null hypothesis H_0, is not rejected when it is in fact false.
Untranslated region	A section of the mRNA transcript that is not translated to amino-acid sequence.

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Appendix A

Gene	Assay	Probe	BA22 Fold	p value
CACNA1E	Taqman	242410_s_at	1.24	0.003
S100A8	RT-PCR	202917_s_at	3.16	0.007
WISP3	RT-PCR	210861_s_at	2.5	0.024

Supplementary Table 1 Data from Barnes et al. (2011)

Represented in the table are the changes in expression obtained for CACNA1E, S100A8 and WISP3 from the microarray study by Barnes et al. (2011). Validated fold values of each gene in BA22 of cases compared to controls is provides, as well as p values for those changes. Also in the table are specified the type of assay used for validation of the microarray data and the probe ID.

Appendix B

ZnT3 (SLC30A3)			
	Forward (5' to 3')	**Reverse (5' to 3')**	**Fragment Size (bp)**
SNP1 rs6547521	ACAGGATGCTTCCACCTGTTCC	ACTTCATCACCATGAGGTGAG	380
SNP2 rs11126936	CTGAGAATTGGACCAGAATG	GTGGAGTGAAGTGTGGAGTG	442
SNP3 rs2083363	AGATTACAGGGGAAACATTCAC	TGTAATCCTGGCTACTCGGGAG	388
SNP4 rs11126931	TCAATGATTGCTGCTTCCTG	TGGTTCTGCTCTGCCTTTAC	233
CACNA1E			
SNP1 rs17743547	GTCAGGGGAAAGGGTACA	GATGTCTCTGCAGCCCAC	294
SNP2 rs2280865	GGAAAGACCTTTGACCTTGA	GGACATCTGCATGTGCTTCC	483
SNP3 rs35737760	CATGTGTCCTGCCCTCTCACA	GAAGAGGACTCCTTGCCTTCT	600
SNP4 rs4652678	CCCCTATAACCAGCTATTGAA	CTGCCTGTGTCACCTTCTCAA	599
SNP5 rs704326	GGCTGAATGTGACAAATG	CGGGCATAAACTAAGAGG	656
CAMKK2			
SNP1 rs3817190	TCATTGTGGTCACCGAGTGT	CAAGGTTCCCTGGAGTCAAC	460
SNP2 rs1132780	GGAGTGGAAGGTTGGAAAAGG	CTAGCCAAGCCTGAAGCCTAC	493
S100A8			
SNP1 rs3795391	GAGAAACCAGAGACTGTAGCAAC	CCCTGTAGACGGCATGGAAATTC	746
SNP2 rs3006488	GGCTTCTTCCTCCTCAATC	CAAGAGCCACATCTAAGAC	590

Supplementary Table 2 Primers pairs for analysis of SNPs in genes involved in synapsis and intracellular signalling.

	Forward (5' to 3')	Reverse (5' to 3')	Fragment Size (bp)
FRZB			
SNP1 rs10206992	ACCTGATGACTGAGATTGCC	ACTGATGCACTCTGGCACTG	218
SNP2 rs7607737	TAATGGTCAGGATGGAACCC	TCAGTGGGAAGTGATAGTTCAC	349
SNP3 rs288329	AGCATCCATCATTGACTAAG	TGAGTCAATGAGTAAACATGG	366
SNP4 rs16823799	AGGCCTGTATTTTAATCCACC	GCCTGATTGAGAAGCACAAC	872
WISP3			
SNP1 rs2280153	ATGGAGAGACACTGTTTCCCG	TCTCCACTCTTCTGCTTGCTG	366
SNP2 rs1230348	ACCTACTGGACATCTCCACC	AAGGCATGGAAGGAGAGTGTG	468
SNP3 rs1230345	ACTCCAGTCTCGTACCTAGG	TTAGAGGCCTTATCCATGGG	607
DKK3			
SNP1 rs2291599	GCTCTGCTCTCCATTGACAC	CAAACAACATGACCAGAGGA	388
SNP2 rs7480026	GACAGTGAGTGCTGTGGAGA	GGCTTTTGCATCCTGAAGT	371
SNP3 rs3206824	GCAGGCTCCCACGTTTAGAG	GCTATTTCTATTGCACATCTACCC	293

Supplementary Table 3 Primers pairs for analysis of SNPs in genes involved in the Wnt signalling pathway.

Appendix C

ZnT3 (SLC30A3)					
		Frequency (%)			
	n	Genotype			Minor Allele
SNP1 rs6547521:5'					
		G/G	G/C	C/C	C
ICPS schizophrenia	30	46.7	46.7	6.7	30.0
ICPS controls.	36	38.9	47.2	13.9	37.5
SNP2 rs11126936: Intron 1					
		G/G	G/T	T/T	T
ICPS schizophrenia	36	61.1	30.6	8.3	23.6
ICPS controls.	36	44.4	44.4	11.1	33.3
SNP 3 rs2083363: 3'					
		G/G	G/C	C/C	C
ICPS schizophrenia	35	62.9	28.6	8.6	22.9
ICPS controls.	31	45.2	45.2	9.7	32.3
SNP 4 rs11126931: 3'					
		C/C	C/T	T/T	T
ICPS schizophrenia	33	60.6	30.3	9.1	24.2
ICPS controls.	34	41.2	47.0	11.8	35.3
CACNA1E					
SNP1 rs17743547: Intron 10 (Splice Site)					
		G/G	G/A	A/A	A
ICPS schizophrenia	36	69.4	27.8	2.8	16.7
ICPS controls.	37	73.0	27.0	0.0	13.5
SNP2 rs2280865: Intron 22					
		T/T	T/C	C/C	C
ICPS schizophrenia	36	38.9	47.2	13.9	37.5
ICPS controls.	37	43.2	48.6	8.1	32.4
SNP3 rs35737760: Exon 23 [D/E]					
		T/T	T/A	A/A	A
ICPS schizophrenia	34	88.2	11.8	0.0	5.9
ICPS controls.	35	80.0	17.1	2.9	11.4
SNP4 rs4652678: Exon 32					
		T/T	T/C	C/C	C
ICPS schizophrenia	36	58.3	36.1	5.6	23.6
ICPS controls.	36	56.8	36.1	11.1	29.2

Supplementary Table 4 Genotype and allele frequencies for ICPS samples.

Summary of genotyping of four SNPs located in or near the ZnT3 gene, in controls and cases from the ICPS cohort used for correlation with expression.

	n	Frequency (%) Genotype			Minor Allele
SNP5 rs704326: Exon 47 [A/T]					
		G/G	G/A	A/A	A
ICPS schizophrenia	35	20.0	60.0	20.0	50.0
ICPS controls.	36	25.0	41.7	33.3	54.2
S100A8					
SNP1 rs3795391: Intron 1					
		A/A	A/G	G/G	G
ICPS schizophrenia	38	78.9	21.1	0.0	10.5
ICPS controls.	27	70.4	29.6	0.0	14.8
SNP2 rs3006488: 3' UTR					
		T/T	T/C	C/C	C
ICPS schizophrenia	38	76.3	21.1	2.6	13.2
ICPS controls.	29	75.9	24.1	0.0	12.1
FRZB					
SNP1 rs10206992: Intron 1					
		T/T	T/G	G/G	G
ICPS schizophrenia	34	50.0	47.1	2.9	26.5
ICPS controls.	34	52.9	35.3	11.8	29.4
SNP2 rs7607737: Intron 1					
		C/C	C/T	T/T	T
ICPS schizophrenia	34	73.5	17.6	8.8	17.6
ICPS controls.	31	87.1	12.2	0.0	6.5
SNP 3 rs288329: Intron 3					
		C/C	C/T	T/T	T
ICPS schizophrenia	33	63.6	30.3	6.1	21.2
ICPS controls.	33	84.8	15.2	0.0	7.6
SNP 4 rs16823799: 3'					
		G/G	G/A	A/A	A
ICPS schizophrenia	34	79.4	20.6	0.0	10.3
ICPS controls.	33	84.8	15.2	0.0	7.6
WISP3					
SNP1 rs2280153: Intron 2					
		C/C	C/T	T/T	T
ICPS schizophrenia	31	35.5	61.3	3.2	33.9
ICPS controls.	33	42.4	54.6	3.0	30.3
SNP2 rs1230348: Intron 2					
		C/C	C/T	T/T	T
ICPS schizophrenia	33	54.5	39.4	6.1	25.8
ICPS controls.	31	48.4	45.2	6.5	29.0
SNP 3 rs1230345: Exon 3 [Q/H]					
		G/G	G/T	T/T	T
ICPS schizophrenia	33	33.4	63.6	3.0	34.8
ICPS controls.	32	46.8	53.1	3.1	29.7

Supplementary Table 4 *(Continued)*

DKK3					
		Frequency (%)			
	n	Genotype			Minor Allele
SNP1 rs2291599: Intron 5					
		G/G	G/A	A/A	A
ICPS schizophrenia	36	77.8	19.4	2.8	12.5
ICPS controls.	37	64.9	32.4	2.7	18.9
SNP2 rs7480026: Intron 6					
		C/C	C/T	T/T	T
ICPS schizophrenia	38	86.8	13.2	0.0	6.6
ICPS controls.	37	89.2	10.8	0.0	5.4
SNP 3 rs3206824: Exon 8 [R/G]					
		G/G	G/A	A/A	A
ICPS schizophrenia	37	73.0	24.3	2.7	14.9
ICPS controls.	36	44.4	52.8	2.8	29.2

Supplementary Table 4 *(Continued)*

Appendix D

Model Associated Odds ratios	Generic SNP Models			SNP1			SNP2			SNP3			SNP4		
	Ma/Mi	Mi/Mi		$OR_{Ma/Mi}$	$OR_{Mi/Mi}$		$OR_{Ma/Mi}$	$OR_{Mi/Mi}$		$OR_{Ma/Mi}$	$OR_{Mi/Mi}$		$OR_{Ma/Mi}$	$OR_{Mi/Mi}$	
			Observed values	1.68	2.19		1.70	1.90		2.06	3.10		2.02	1.57	
Additive	$OR_{Ma/Mi}$	$2(OR_{Ma/Mi})$	Expected values for each model	1.68	3.37		1.70	3.40		2.06	4.15		2.02	4.03	
Multiplicative	$OR_{Ma/Mi}$	$(OR_{Ma/Mi})^2$		1.68	2.83		1.70	2.89		2.06	4.23		2.02	4.06	
Recessive	1	$OR_{Ma/Mi}$		1.00	1.68		1.00	1.70		1.00	2.06		1.00	2.02	
Dominant	$OR_{Ma/Mi}$	$OR_{Ma/Mi}$		1.68	1.68	1	1.70	1.70	1	2.06	2.06	1	2.02	2.02	1
Exponent n so that: $(OR_{Ma/Mi})^n$ =observed OR for Mi/Mi Genotype under a multiplicative model				n			n			n			n		
				1.5			1.2			1.6			0.7		

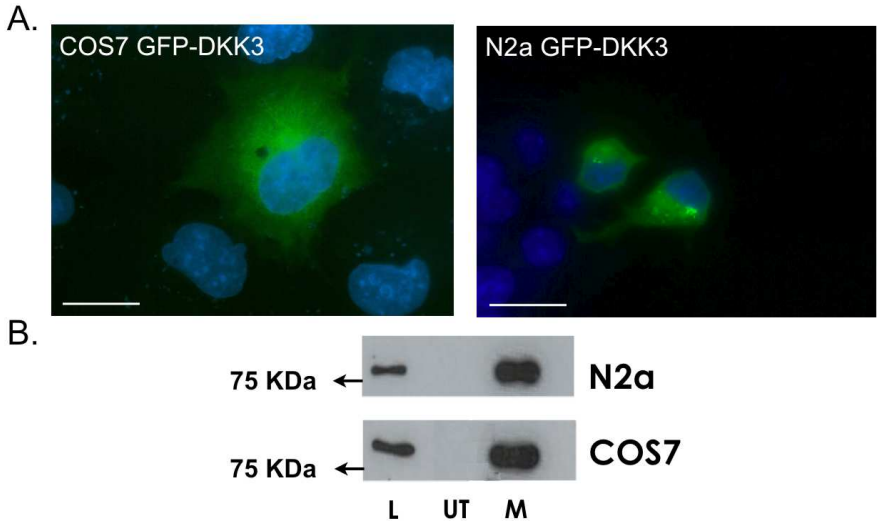
Supplementary Table 5 Best fit of data from ZnT3 with models of disease penetrance.

Odds ratio for genotypes of the minor allele (Ma/Mi and Mi/Mi) with respect to unassociated genotype (Ma/Ma) are presented. Comparison of observed and expected values for four SNPs in ZnT3. The number 1 indicates that the preceding pair is nearest fit to model shown in the same row. For all four polymorphisms the dominant model was the best fit. For a perfect fit to the dominant model, the exponent n=1. The fact that for the four polymorphisms n<>1, indicates a small multiplicative effect.

Appendix E

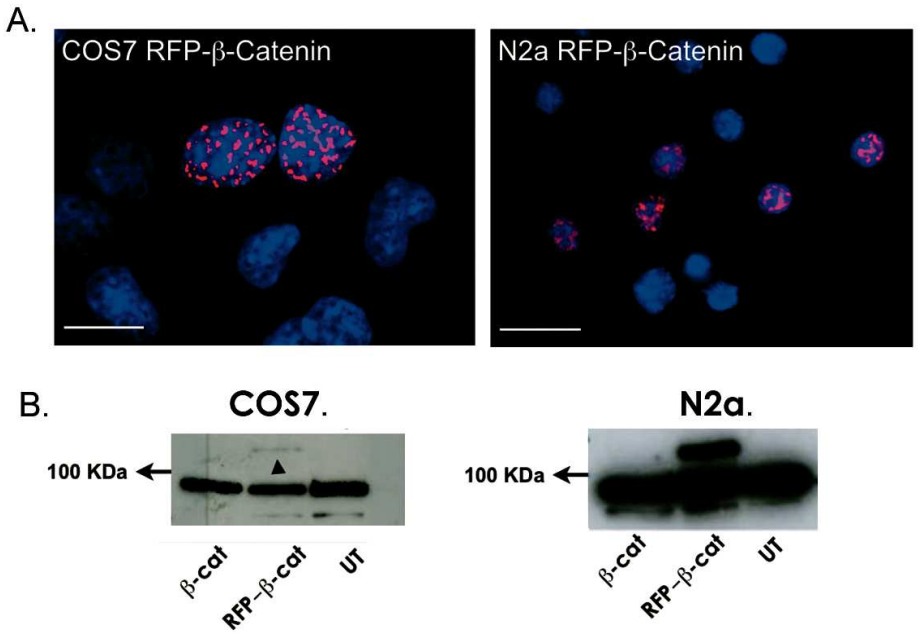
There is growing evidence of the involvement of the canonical Wnt signalling pathway in the pathogenesis of schizophrenia. In this study, the effect of overexpression of DKK3 was investigated on nuclear translocation of β-catenin in neuronal cells, based on previous reports carried out using cancer cells (Yue et al., 2008). However, extensive optimisation was required for the analysis, and the study was not completed as intended. The preliminary results are presented here as the possible basis of future investigation.

A GFP-DKK3 construct was prepared using cDNA from human DKK3 (NM_013253; OriGene) cloned into a pCDNA3.1/NT-GFP-TOPO vector (Invitrogen) and an RFP-β-catenin construct was made by cloning β-catenin cDNA (kindly provided by Dr. Magdalena Sastre) into a pDsRed2-C1 vector (Clontech Laboratories). Transfection of the constructs was carried out as described in section 2.3.3 using N2a cells and COS7 cells as a control. Cellular localization of the GFP-DKK3 fusion protein was observed in the cytosolic region of cells, whilst RFP-β-catenin construct had an exclusive nuclear distribution. The secreted glycosylated form of the DKK3 fusion protein (85 KDa) was observed from western blot analysis (described in section 2.3.5) using a rabbit anti-GFP antibody (Abcam). Expression of the RFP fusion protein (119 KDa) and endogenous β-catenin (94KDa) was also confirmed by Western blot analysis using a rabbit anti-β-catenin antibody (Sigma-Aldrich).



Supplementary Figure 1 Expression of the glycosylated form of DKK3 in COS7 and N2a cells.

Cytosolic distribution of the GFP-DKK3 fusion protein is observed in both cell lines (A). Western blot analysis (B) confirmed expression of the glycosylated form (85 KDa) in lysates (L) and media (M) of both cell lines but not in untransfected controls (UT). Scale bar in (A) is 20μm

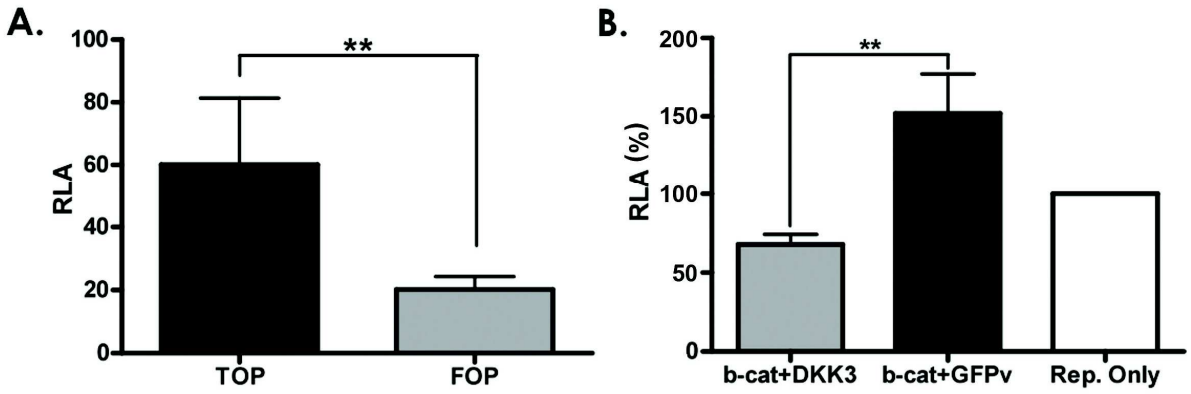


Supplementary Figure 2 Expression of the RFP-β-catenin fusion protein in COS7 and N2a cells

Nuclear localization of the RFP-β-catenin fusion protein was observed in COS7 and N2a cells (A). Western blot analysis shows the 119 KDa band corresponding to the RFP-β-catenin protein only for cells transfected with the tagged construct (B). A second band of 94 KDa band, presumably from endogenous β-catenin, was also present in these cells, as well as cells transfected with an untagged β-catenin construct and untransfected cells (UT). Scale bars in (A) are 20 μm.

In order to quantify the effect of the expression of DKK3 on activation of β-catenin/Wnt signalling luciferase reporter assays were carried out using the TCF activated TOPFlash reporter and the mutant FOPFlash plasmid as a negative control. A Dual-Luciferase Reporter Assay system (Promega) was used and experimental conditions are described in section 2.3.6. Firefly luciferase activity values obtained from the reporter plasmids were normalised to renilla luciferase activity values obtained from the vector pRL-CMV (Promega) used as a transfection control. Due to the variation observed between experiments, and the need for optimisation of the experimental conditions, only N2a cells were used.

A significant decrease in β-catenin mediated activation of the TOPFlash reporter relative to transfection of the GFP empty vector was observed when GFP-DKK3 was co-expressed (p=0.0022, Supplementary Figure 3B).



Supplementary Figure 3 Luciferase reporter assays showing significant inhibition of Wnt signalling by DKK3

The data presented bellow is the result of a series of assays (n=6) conducted on N2a cells 48 hours after transfection. Relative luciferase activity (RLA) units are used to represent the activation of the TOPFlash reporter (A). Although some non-specific activation of the mutant FOPFlash reporter was observed this was significantly minor to the response observed from TOPFlash (p=0.008). Transfection of the GFP-DKK3 construct resulted in inhibition of β-catenin mediated TOPFlash activation (B) to a statistical significant level (p=0.002).