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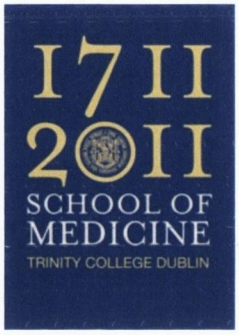

Department of Psychiatry, School of Medicine, Trinity College Dublin

Investigation of S100B Gene as a Risk Factor for Bipolar Disorder and Schizophrenia

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A thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

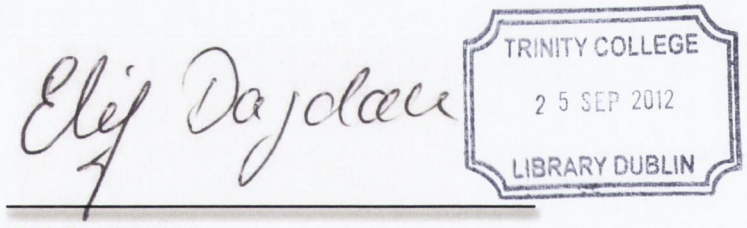
April 2012

DECLARATION

This PhD Thesis titled "Investigation of S100B Gene as a Risk Factor for Bipolar Disorder and Schizophrenia" has not been previously submitted to this or any other university for examination for higher degree. The work represented here is entirely my own except where noted. This thesis may be made available for consultation within the university library. It may be photocopied or lent to other libraries for the purposes of consultation.

This work was undertaken in Smurfit Institute of Genetics, School of Genetics and Microbiology and the Neuropsychiatric Genetics Research Group, Department of Psychiatry, School of Medicine at Trinity College Dublin.

The thesis work was conducted from November 2007 to September 2011 under the supervision of Dr. Siobhan Roche, Dr. Derek Morris and Prof. Michael Gill.



Elif DAGDAN

Dublin, 18 April 2012

Thesis 9585

Dedicated specially to my beloved DAD

and

to all who suffer daily with the symptoms of bipolar disorder and to their families who suffer watching their loved ones valiantly struggling to cope

SUMMARY

Calcium-binding protein S100B has been implicated in the pathology of bipolar affective disorder (BPAD) and schizophrenia (SZP). S100B protein levels are elevated in serum of patients with both disorders compared to controls. Previously, genetic association of a SNP in the promoter of S100B, rs3788266, was reported for a psychotic form of BPAD. The disease-associated G allele of rs3788266 disrupts a Trex/MEF3 consensus recognition, which is bound by Six-family transcription factors, suggesting that it could affect S100B expression.

The functional effect of rs3788266 on S100B promoter activity was studied using the luciferase reporter system in U373MG glioblastoma and SH-SY5Y neuroblastoma cell lines. Allelic effects of rs3788266 on protein complex formation at the S100B promoter were investigated by an electrophoretic mobility shift assay (EMSA). To test for genotypic effects of rs3788266 *in vivo,* S100B serum protein levels were measured in 570 samples comprised of Irish BPAD cases and their unaffected relatives, Irish SZP or schizoaffective disorder (SZA) cases and healthy German controls, all with known S100B genotype. The allelic expression imbalance (AEI) of rs3788266 was also investigated in 41 heterozygote post-mortem brain RNA samples. Several transcription factor databases predicted an allelic effect of rs3788266 on transcription factor binding. Binding of Six-family transcription factors to the site of rs3788266 was investigated with EMSA using nuclear extract from both U373MG glioblastoma and SH-SY5Y neuroblastoma cell lines, and SIX1 recombinant protein and SIX4 antibody.

Luciferase gene expression was significantly increased in the presence of the G allele compared to the A allele in SH-SY5Y (p=<0.0001), and in U373MG (p=<0.0008) cell lines. The binding affinity of both SH-SY5Y and U373MG protein complexes for the S100B promoter was significantly stronger in the presence of G allele compared to the A allele promoter fragments. Consistent with the *in vitro* findings, higher mean serum S100B levels were associated with the risk G allele of rs3788266 in BPAD cases (p=0.0001), unaffected

relatives of BPAD cases (p=<0.0001), SZP or SZA cases (p=0.001) and unrelated controls (p=<0.0001). Allelic expression imbalance was not observed with rs3788266. Transcription factor SIX1, which is known to suppress neuronal gene expression, bound only to the A allele of the SNP. SIX4 bound to both alleles but preferentially to the G allele.

In an endophenotype analysis, the association of rs3788266 with clinical symptomotology and variation in cognitive performance was tested. The clinical symptom factors were investigated in 813 patients with a DSM-IV diagnosis of SZP, SZA or BPAD. Cognitive performance was assessed in 433 patients with SZP or SZA and 232 healthy participants. For this study cognition has been assessed using tests for general cognitive functioning, episodic memory, working memory and attention. Social cognition was also tested. Individuals homozygous for the risk G allele performed worse in general IQ (p=0.01), verbal IQ (p=0.014), performance IQ (p=0.016), verbal working memory (p=0.016) and attention tasks (p=0.035). Furthermore, the risk allele was correlated with severity of psychosis in SZP and SZA (p=0.045). Increased serum S100B protein concentration in SZP and SZA subjects correlated with severity of psychosis (Spearman r= 0.137; p=0.023) and age (Spearman r=0.162, p=0.011). A trend between increased S100B protein concentration and verbal IQ was observed.

The association of rs3788266 with psychosis was performed with 698 SZP or 248 BPAD cases and 2023 controls. There was no evidence of association at rs3788266 with SZP or BPAD. Further the epistatic interaction between the SIX-family genes and S100B was investigated using large collaborative datasets but no strong associations were identified.

Overall, these data support rs3788266 as a functional promoter variant in the S100B gene where the presence of the G allele promotes increased gene expression and is associated with increased serum levels of the protein, which in turn might have impact on psychotic symptom severity and cognitive performance in SZP and BPAD patients. This study provides support for the involvement of the S100B protein in the biology of psychosis.

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This part is the most important one for me, as it marks the completion of a long-term challenge, which I set myself a few years ago when I left home. Of course, preparing a thesis abroad, in a different language, was only possible by having the right people around me.

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PUBLICATIONS

Bergen S, Chen J, Dagdan E, Foon TS, Goes FS, Houlihan LM et al. 2009. Selected summaries from the XVI World Congress of Psychiatric Genetics, Osaka, Japan, 11-15 October 2008. *Journal of Psychiatric Genetics*. Oct;19(5):219-36.

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PUBLICATIONS IN PROGRESS

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AWARDS

Best Presentation Award at Young Science Track Competition, World Congress of Psychiatric Genetics 2008 in Osaka, Japan for Research Project: *"Functional Assessment of S100B as a Susceptibility Gene for Bipolar disorder".*

Rapporteurs Award from the International Society of Psychiatric Genetics at the World Congress of Psychiatric Genetics 2009 in San Diego, USA.

PRESENTATIONS AT INTERNATIONAL MEETINGS

Dagdan E, McKeon P, and Roche S. Functional Assessment of S100B as a susceptibility gene for psychotic bipolar disorder. *16th World Congress of Psychiatric Genetics,* Osaka, Japan, October 2008 (Oral Presentation).

Dagdan E, McKeon P, and Roche S. Functional Assessment of S100B as a susceptibility gene for psychotic bipolar disorder. *EBPS Behavioural Genetics and Neuropsychiatric Disorders*, Cork, Ireland; 25-27 August 2008. (Poster Presentation).

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ABBREVIATION

ABI	Applied Biosystems
AD	Alzheimer's Disease
AEI	Allelic Expression Imbalance
ANOVA	Analysis of Variance
BPAD	Bipolar Affective Disorder
CDCV	Common Disease-Common Variant
CDRV	Common Disease-Rare Variant
CEPH	Centre d'Etude du Polymorphisme Humain
CEU	Caucasian European from Utah
Chr	Chromosome
CNS	Central Nervous System
CNV	Copy Number Variant
COMT	Catechol-O-Methyl Transferase
DISC1	Disrupted-in-Schizophrenia 1 DNA Deoxyribonucelic Acid
DSM-IV	Diagnostic Statistical Manual IV
EMSA	Electrophoretic Mobility Shift Assay
GASP	Genetic Association study of Schizophrenia and related Psychoses
GWAS	Genome-Wide Association Studies
ICD-10	International Classification of Diseases 10
ISC	The International Schizophrenia Consortium
KO	Knock-Out
LD	Linkage Disequilibrium
LOD	Log of Odds
LTP	Long Term Potentiation
MAF	Minor Allele Frequency
MDD	Major Depressive Disorder
MHC	Major Histocompatability Complex
N/A	Not Available
NCBI	The National Center for Biotechnology Information
NS	Not Significant
NRG1	Neuregulin 1
OMIM	Online Mendelian Inheritance in Man

OR	Odds Ratio
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPGI	Resource for Psychiatric Genetics in Ireland
S100B	S100 Calcium-Binding Protein, Beta
SCID-P	Structured Clinical Interview DSM
SIX	Sine Oculis Homeobox
SNP	Single Nucleotide Polymorphism
SZA	Schizoaffective Disorder
SZP	Schizophrenia
TCD	Trinity College Dublin
TF	Transcription Factor
UCSC	University College, Santa Cruz
VCU	Virginia Commonwealth University
WTCCC	Wellcome Trust Case-Control Consortium

ONLINE RESOURCES

Cluster Buster	www.zlab.bu.edu/cluster-buster/cbust.html
Evolutionary Conserved Regions Browser	http://ecrbrowser.dcode.org/
GeneticAssociation Database	http://geneticassociationdb.nih.gov
Genomatix	www.genomatix.de
Haploview	http://www.broadinstitute.org/haploview
JASPAR	http://jaspar.genereg.net/
PLINK	http://pngu.mgh.harvard.edu/~purcell/plink/
PolyPhen-2 Database	http://genetics.bwh.harvard.edu/pph2/dbsnp
SchizophreniaGene Database	http://www.szgene.org
TFsearch	http://www.cbrc.jp/research/db/TFSEARCH
Tfsitescan Database	http://www.ifti.org/Tfsitescan/
The International Schizophrenia Consortium	http://pngu.mgh.harvard.edu/isc/
The International HapMap Project	http://www.hapmap.org
The National Center for Biotechnology Information Gene Database	www.ncbi.nlm.nih.gov/gene
The National Human Genome Research Institute	http://www.genome.gov/26525384
The UCSC Genome Browser	http://genome.ucsc.edu/
TRANSFAC	www.gene-regulation.com

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Chapter 1

Review of the Literature

1.1. Introduction to Bipolar Disorder and Schizophrenia

Bipolar Disorder

The concept of a mood disorder with two extremes has its origin almost 2000 years ago in ancient Greece. Aretaeus of Cappadocia was the first to connect mania and melancholia and to attribute these two affective states to a single condition. "Melancholia is the beginning and a part of mania....the development of a mania is really a worsening of the disease (melancholia) rather than a change into another disease" (Aretaeus of Cappadocia, ca 100 AD).

Bipolar Affective Disorder (BPAD), also known as Manic-depressive Disorder, Manic Depression or Bipolar Disorder is a mental disorder that causes significant morbidity and mortality affecting all populations around the world. BPAD presents with disturbance in mood ranging from extreme elation, or mania, to severe depression usually accompanied by disturbances in thinking and behaviour, which may include psychotic symptoms, such as delusions and hallucinations. Typically it is an episodic illness, often with full recovery between episodes. BPAD is considered as a spectrum of phenotypes ranging from mild depressive and hypomanic symptoms to severe forms of mania and depression (BPAD type I). The high rate of disability and the loss of life through suicide contribute to the economic burden of bipolar illness to society. Disease onset typically occurs during adolescent or early adulthood. An early onset is typically associated with a more severe course of illness. If the onset of symptoms occurs later on, the condition may be secondary to the other medical causes (Goodwin FK & Jamison K, 2007).

Schizophrenia

The first description of Schizophrenia (SZP) dates back to the Pharaonic Egypt of 2000 B.C. Many sign and symptoms of SZP as now recognised have also been described in ancient Greek, Roman, and Chinese scripts. Emil Kraepelin classified the two major mental disorders, BPAD and SZP, into different categories. He used the term "Dementia Praecox" which means early

dementia in the year 1878 for individuals who had symptoms that we now associate with SZP. The term 'Schizophrenia' was first used by Eugen Bleuler in 1911 to describe the 'split mind' meaning split from reality, not as it is still to this day wrongly used to mean a split personality (Kyziridis TC, 2005).

Schizophrenia [OMIM 181500] is a severe mental disorder, which is characterised by positive "psychotic" symptoms, negative "deficit" symptoms, and cognitive impairment (Baron M, 2001). The burdens of SZP on sufferers, their families and society can be severe, and include the early onset of the illness, poor response to medication, frequent relapse and the chronic course of the illness (Williams HJ *et al.,* 2009). Social stigma remains a major problem in the coping and recovery of the patients with Schizophrenia. To prevent the problem of stigma the term for Schizophrenia in Japan was changed from "mind-split-disease" to "integration disorder" in 2002. This change increased the percentage of patients who were informed of their diagnosis from 37% to 70%, over a three-year period (Sato M, 2006).

1.1.1. Clinical Description

Bipolar Disorder

BPAD is a chronic debilitating disease, which results in significant disability. There are higher rates of unemployment, and a high risk of suicide compared to other psychiatric or medical illness. BPAD is defined as a disorder in which a person can experience recurrent episodes of mood disturbances either of mania or depression (Goodwin FK & Jamison K, 2007).

Manic states are characterised typically by heightened or dysphoric mood, more and faster speech, quicker thought, brisker physical and mental activity, greater energy, decreased need of sleep, irritability, perceptual acuity, paranoia, heightened sexuality, and impulsivity. Subclassification of mania, as hypomania or mania, is determined by the degree, types, and chronicity of these affective, cognitive, perceptual, and behavioural changes. Hypomania is less severe than mania (Goodwin FK & Jamison K, 2007).

The depressive state is the contrast of the manic state, and is characterised by a slowing or decrease in most aspects of emotion and behaviour including the rate of thought and speech, energy, sexuality, feeling of sadness, worthlessness, guilt, the ability to experience pleasure and sleep disturbances. The severity of the depression state varies widely. Symptoms can range from mild physical and mental slowing, with very little distortion in cognition and perception, to profound depressive stupors, delusions, hallucinations, and clouding of consciousness. Social isolation, psychomotor retardation, agitation, and other behavioural changes also accompany depression (Goodwin FK & Jamison K, 2007).

Schizophrenia

There is a very significant heterogeneity in the symptomatology and course of SZP. A person who is diagnosed with SZP may experience an admixture of positive, negative, cognitive, mood, and motor symptoms. The severity varies across patients and through the course of the illness. SZP is also characterised by social disability, frequent comorbid substance abuse, and decreased life span (Tandon R *et al.*, 2009).

Positive symptoms involves impaired reality experience and can include delusions, hallucinations and disorganised though and speech. A delusion is a fixed false belief not in keeping with the individuals social or ethnic group. Delusions are often characterised by paranoia which is a believe that others are trying to harm them. An hallucination is a false attribution of a perceptual experience to an external source and can occur in any sense modality. A patient experiencing a hallucination sees, hears, smells, and feels things that are not present in reality (Tandon R *et al.*, 2009; Blashki G *et al.,* 2004).

Negative symptom are characterised by a loss of a range of affective and cognitive functions. Symptoms can include a "flat affect", in which a patient's face does not move and he/she talks in a dull or monotonous voice (Tandon R *et al.*, 2009; NIMH, 2009). Other symptoms in this group include loss of motivation, inability to experience pleasure, lack of initiative, reduction of

speech, lack of interest, and reduce social drive. These symptoms can be mistaken for depression or other conditions. The pathology of negative symptoms is purely understood (NIMH, 2009).

1.1.2. Diagnosis

Diagnosis of BPAD and SZP are both based on sets of symptoms and clinical signs as there are no diagnostic 'tests'. Both disorders are predominantly diagnosed according to the criteria as outlined in the Diagnostic and Statistical Manual of Mental disorders, Fourth Edition (DSM-IV; American Psychiatric Association, 2000) or the International Classification of Diseases, Tenth Revision (ICD-10; the World Health Organisation, 2007).

Table 1.1. DSM-IV main diagnostic categories of psychotic disorders (the table modified from van Os J and Kapur S, 2009).

Non-affective psychotic disorders	• **Schizophrenia** • **Schizoaffective disorder** • **Schizophreniform disorder** • **Delusional disorder** • **Brief psychotic disorder** • **Psychotic disorder not otherwise specified**
Affective psychosis	• **Bipolar disorder with psychotic features** • **Major depressive disorder with psychotic features**
Substance-induced psychotic disorders	• **Alcohol-induced** • **Other substance-induced**
Psychotic disorders due to a general medical condition	

Bipolar Disorder

It is very clear that BPAD is not a homogeneous group. BPI, BPII, cyclothymia, and BPAD-NOS classifications of BPAD are described conditions within the bipolar spectrum. The Figure 1.1 shows the classification of BPAD and its subtypes according to DSM-IV. The Diagnostic criteria for Depressive or Manic Episodes from DSM-IV are summarised in Table 1.2.

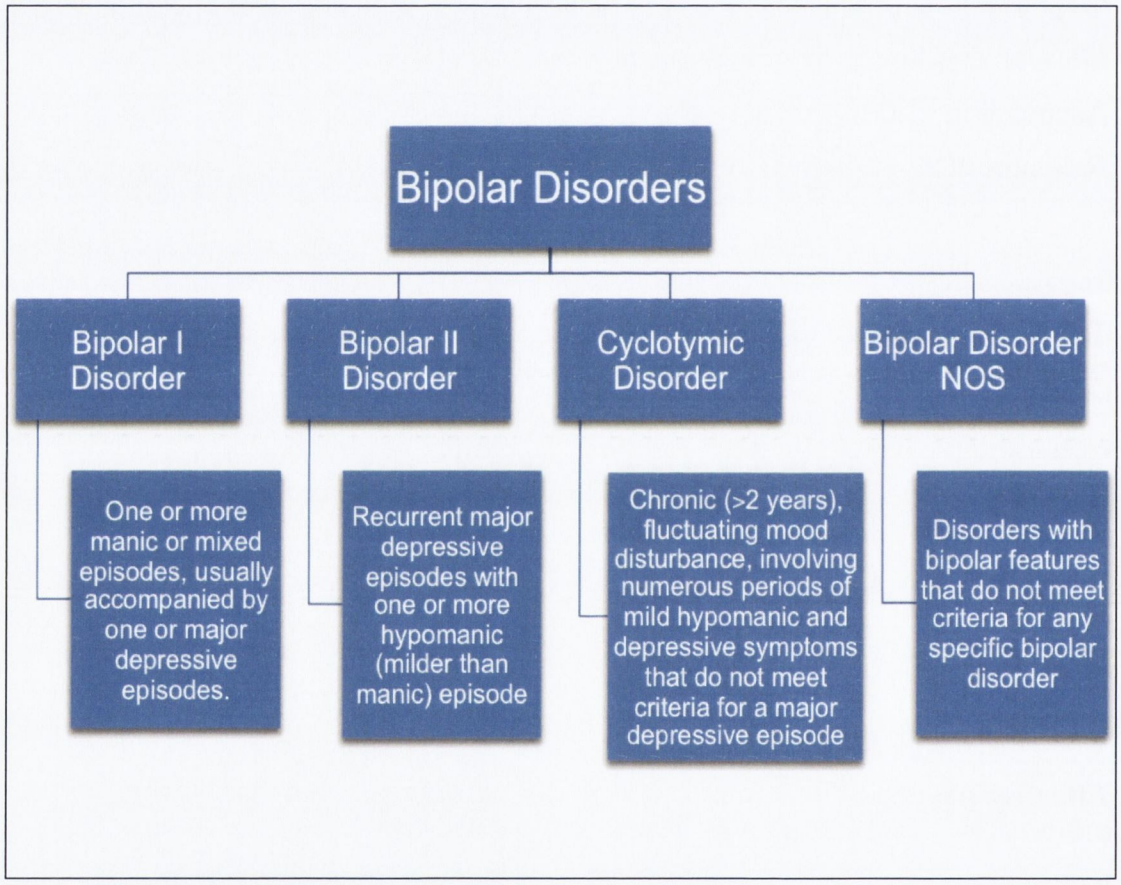


Figure 1.1. DSM-IV classification of Bipolar Disorder. NOS = Not otherwise specifies (The figure is modified from Müller-Oeslinghausen B *et al.*, 2002).

Table 1.2. Diagnostic criteria for Depressive or Manic Episodes (DSM-IV)

Depressive episode	**Manic episode**
A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure. 1. Depressed mood 2. Markedly diminished interest or pleasure 3. Appetite or weight changes 4. Insomnia/hypersomnia 5. Psychomotor agitation/ retardation 6. Fatigue/loss of energy 7. Feelings of worthlessness/ excessive/inappropriate guilt 8. Diminished ability to think/concentrate 9. Recurrent thoughts of death/suicidal ideation B. The symptoms do not meet the criteria for a Mixed Episode. C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning. D. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism). E. The symptoms are not better accounted for by bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than two months or are characterized by marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation.	A. A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least 1 week (or any duration if hospitalization is necessary). B. During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree: 1. Inflated self esteem/grandiosity 2. Decreased need for sleep 3. Talkativeness 4. Flight of ideas/subjective experience that thoughts are racing 5. Distractibility 6. Increased goal-directed activity/psychomotor agitation 7. Excessive involvement in pleasurable activities that have a high potential for painful consequences C. The symptoms do not meet the criteria for a Mixed Episode. D. The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning /in usual social activities/ relationships with others/to necessitate hospitalization to prevent harm to self or others/there are psychotic features. E. The symptoms are not due to the direct physiological effects of a substance.

Schizophrenia

Diagnosis criteria for schizophrenia based on DSM-IV are listed in Table 1.3.

Table 1.3. Diagnostic criteria for schizophrenia (DSM-IV, 1994)

A. Characteristic symptoms: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behaviour or thoughts, or two or more voices are conversing with each other.	Two or more of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated): 1. Delusions 2. Hallucinations 3. Disorganized speech 4. Grossly disorganized or catatonic behaviour 5. Negative symptoms
B. Social/occupational dysfunction:	For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or, when the onset is in childhood or adolescence, failure to achieve the expected level).
C. Duration:	Continuous signs of the disturbance persist for at least 6 months, of which at least one month should be of symptoms that meet Criterion A. The 6 months may include periods of prodromal and residual symptoms.
D. Schizoaffective and mood disorder exclusion:	Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either no major depressive, manic, or mixed episodes have occurred concurrently with the active-phase symptoms, or if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the active and residual periods.
E. Substance/general medical condition exclusion:	The disturbance is not due to the direct physiological effects of a substance or a general medical condition.
F. Relationship to a pervasive developmental disorder:	If there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

1.1.3. Epidemiology

Bipolar Disorder

For BPAD there is a lifetime prevalence of approximately 1% and the rate is similar in males and females (Perala J *et al.*, 2007). Relaxation of the diagnostic criteria to include other affective disorders in the bipolar spectrum, such as BPAD type II and cyclothymia, results in an increased prevalence of almost 8% (Angst J, 1998). An overall prevalence of 3.7% for Bipolar Spectrum Disorders has been described (Hirschfeld RM, 2003). Some studies suggest that women are more likely to be hospitalized during their manic episodes, and that rapid cycling occurs in woman more often than in men. Women are also more likely to have predominantly depressive features over the course of their illness (Goodwin FK & Jamison K, 2007).

The lifetime risk of BPAD in relatives of a bipolar probands is 5-10% for a first-degree relative (Craddock N & Jones I, 1999). Adoption studies have found that the biological parents of bipolar adoptees were more likely to have BPAD (7%) compared to adoptive parents (1.8%) (Craddock N & Jones I, 1999). Twin studies, in general, have agreed with family studies. The twin studies from Danish (Bertelsen A *et al.,* 1977), Swedish (Kendler KS *et al.,* 1995), UK (Cardno AG *et al.,* 1999) and Finnish (Kieseppä T *et al.,* 2004) populations have reported concordance rates for monozygotic twins ranging from 38-75%. The dizygotic concordance rates are comparable to those of sibling recurrence rates ranging from 5-40% across studies (Craddock N & Jones I, 1999). First degree relatives also have a 2-3 fold increased risk of developing unipolar depression and most monozygotic co-twins that do not develop bipolar disorder will develop some form of affective illness in their lifetime. Using the data from twin studies, the heritability estimates for BPAD typically range from 33% to 85% but in some reports are as high as 90% (Goodwin FK & Jamison K, 2007).

Schizophrenia

SZP has been reported as having a prevalence of approximately 1% worldwide (Aleman A *et al.*, 2003). Men tend to have more severe form of the disorder compared to women (Castle DJ *et al.*, 1995). The age of onset for SZP is between late adolescence early adulthood. Men tend to show the first sign of disease during their early 20's, whereas women present during their mid and late 20's (Castle DJ *et al.*, 1995).

First-degree relatives of individuals with SZP have a 10-fold higher risk of developing SZP, suggesting a familial component (Kendler KS & Diehl SR, 1993). Figure 1.2. Shows the lifetime risk of Schizophrenia based on familial relationship. Calculated from twin studies, the heritability of Schizophrenia is estimated at up to 85% (Cardno AG *et al.*, 1999). Heritability is the proportion of phenotypic variation in the population, which is attributable to genetic factors.

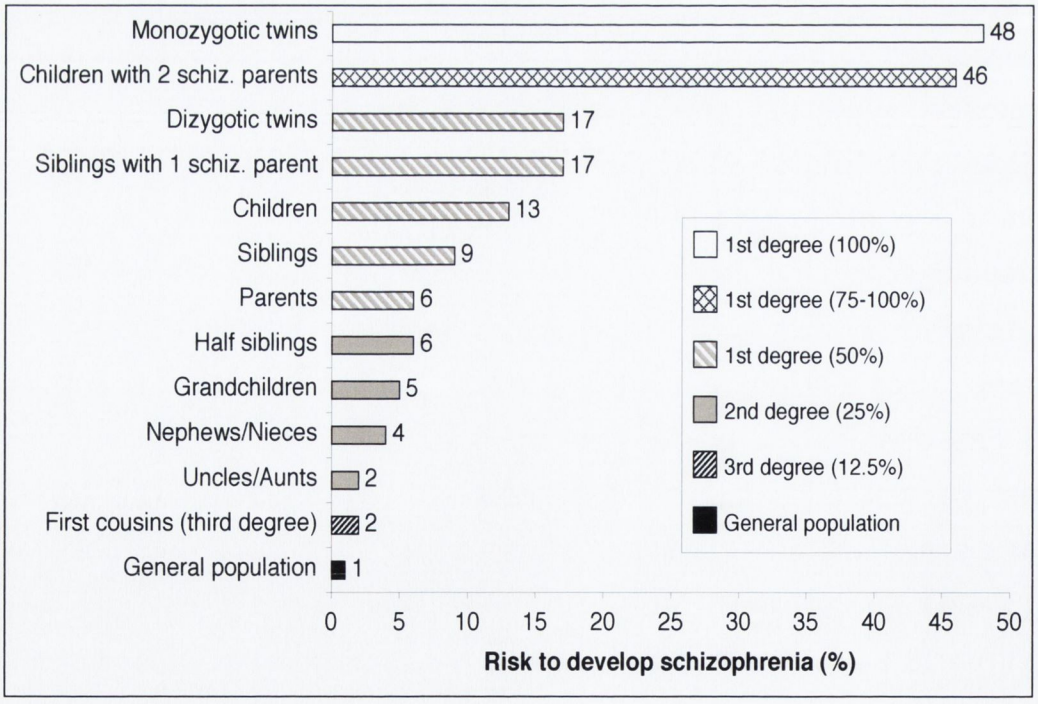


Figure 1.2. Morbid risks for Schizophrenia. Lifetime risk of developing risk Schizophrenia based on relationship to an affected individual, which is presented in percentage. The degree of genetic relationship to the affected in represented in the bar pattern. (The figure is adapted from Gottesman II, 1991).

The MZ twin concordance rates for BPAD and SZP being less than 100% imply that those disorders may be modulated by environmental factors.

1.1.4. Environmental Risk Factors

There is strong evidence for a significant contribution from genetic factors but environmental factors may also make an important contribution to the development of Bipolar Disorder and Schizophrenia (van Os J & Kapur S, 2009; Goodwin FK & Jamison K, 2007).

Bipolar Disorder

Childhood abuse or trauma and loosing loved ones early in life have been suggested as risk factors in developing BPAD (Goodwin FK & Jamison K, 2007; Etain B *et al.,* 2008). Several studies reported association between social class and bipolar disorder, upper class being at higher risk for BPAD (Goodwin FK & Jamison K, 2007). Different prevalence rates have been reported for rural and urban areas. The rate in urban areas was found to be higher for BPAD (Goodwin FK & Jamison K, 2007; Kaymaz N *et al.,* 2006). Other environmental risk factors that have been associated with BPAD are stressful life events (Tsuchiya KJ *et al.*, 2003), viral infections (Salvatore M *et al.,* 1997; Frank O *et al.,* 2005; Hobbs JA, 2006), solar cycle (Davis GE & Lowell WE, 2006), and cannabis use (Henquet C *et al.,* 2006; van Laar M *et al.*, 2007).

Schizophrenia

Prenatal malnourishment, low birth weight and prematurity have shown to increase the risk for SZP (van Os J *et al.,* 2010). A meta-analysis found increased risk of developing SZP in people with a personal or familial history of immigration (Selten JP *et al.,* 2007). The prevalence of SZP is higher in the people born during the winter. There is a higher risk for development of SZP in individuals living in urban sites than the ones living in rural areas (van Os J *et al.,* 2010). Exposure to stress during a critical period of the foetal brain

development is also suggested as an environmental risk factor for SZP (Kinnunen AK *et al.,* 2003; Murray RM *et al.,* 1992). Experimental studies have shown that exposure to dronabinol, the main transient psychotropic metabolite of Cannabis, causes mild transient psychotic states and increases the risk for psychotic disorders (van Os J & Kapur S, 2009). Figure 1.3. lists demonstrated environmental factors for developing SZP.

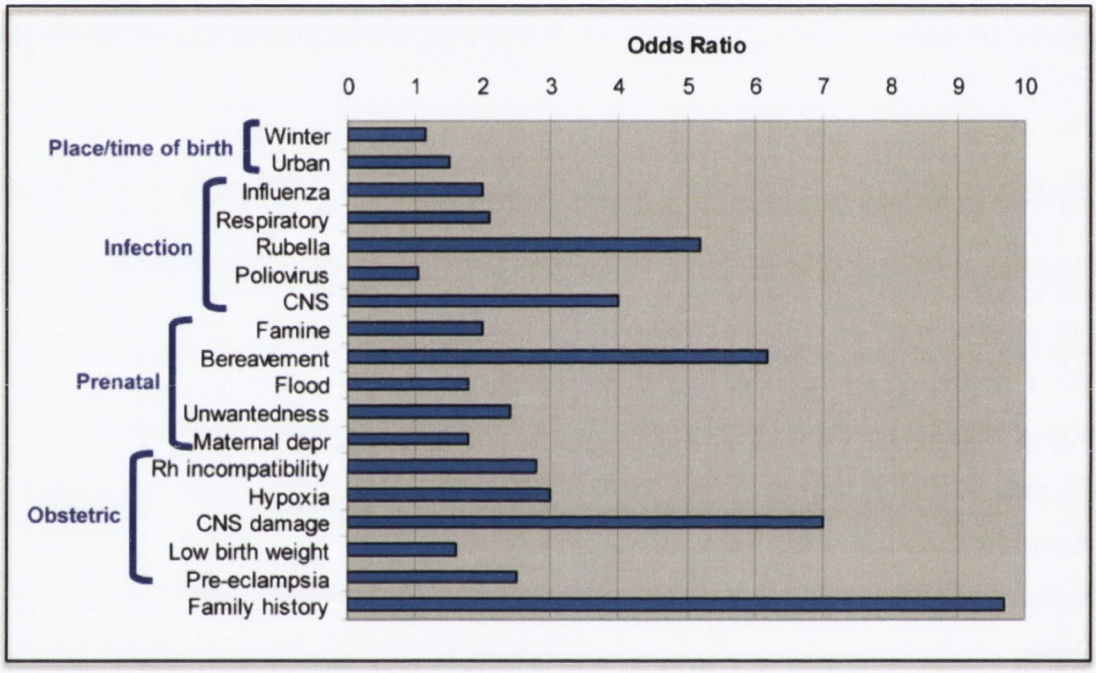


Figure 1.3. Environmental risk factors for developing Schizophrenia. (Abbreviations: CNS, central nervous system; depr, depression; Rh, Rhesus; Sullivan PF, 2005).

1.1.5. Pathophysiology

Many neuropathological studies, in-vivo biochemical studies, and studies using neuroimaging techniques are being conducted to try to understand the cause of BPAD and SZP. Bipolar Disorder and Schizophrenia appear to overlap in many reported pathophysiological abnormalities (Goodwin FK & Jamison K, 2007; Möller HJ, 2003). Early biological theories regarding the pathophysiology of BPAD and SZP focused on various neurotransmitters (Müller-Oerlinghausen B *et al.,* 2002). There are many pathological hypothesis for SZP and BPAD but the findings are often inconsistent.

Bipolar Disorder

There have been many abnormalities reported in BPAD including heightened pro-inflammatory function, hypothalamic-pituitary-adrenal axis dysfunction, structural brain abnormalities, circadian rhythm instability, intracellular signal transduction pathway dysfunction, particularly intracellular calcium signalling (Goodwin FK & Jamison K, 2007). Different kind of pathophysiological abnormalities found in BPAD is shown in Figure 1.4.

Monoaminergic systems received the greatest attention in neurobiological studies of BPAD including the dopamine, serotonin, gama amino acid butyric acid (GABA), norepinephrine (NE) and glutamate neurotransmission systems (Goodwin FK & Jamison K, 2007). Monoaminergic systems dysfunction appears to occur at many levels in the pathophysiology of BPAD, including neurotransmitter synthesis, storage, release, presynaptic autoreceptor function, neurotransmitter reuptake, metabolism and postsynaptic neurotransmitter receptors (Goodwin FK & Jamison K, 2007).

Excessive dopamine neurotransmission has been suggested in the development of manic symptoms, and dopamine D2 antagonists were found to be anti-manic (Berk M *et al.,* 2007; Frey BN *et al.*, 2006). Reduction of dopaminergic activity has been correlated with depression (Kapczinski F *et al.,* 2004). Reduced activity of serotonin has been found in depressed patients. Antidepressants block the reuptake of serotonin, which causes temporary increase in the level of serotonin (Goodwin FK & Jamison K, 2007). Altered levels of glutamate have been observed in CSF, plasma and brain tissue from patients in various mood states (Goodwin FK & Jamison K, 2007; Frye MA *et al.,* 2007; Palomino A *et al.*, 2007). Reduced plasma level of GABA has been found in BPAD patients (Kapczinski F *et al.*, 2004). Increased NE has been suggested to be associated with a switch from depression into mania or hypomania (Salvadore G *et al.,* 2010).

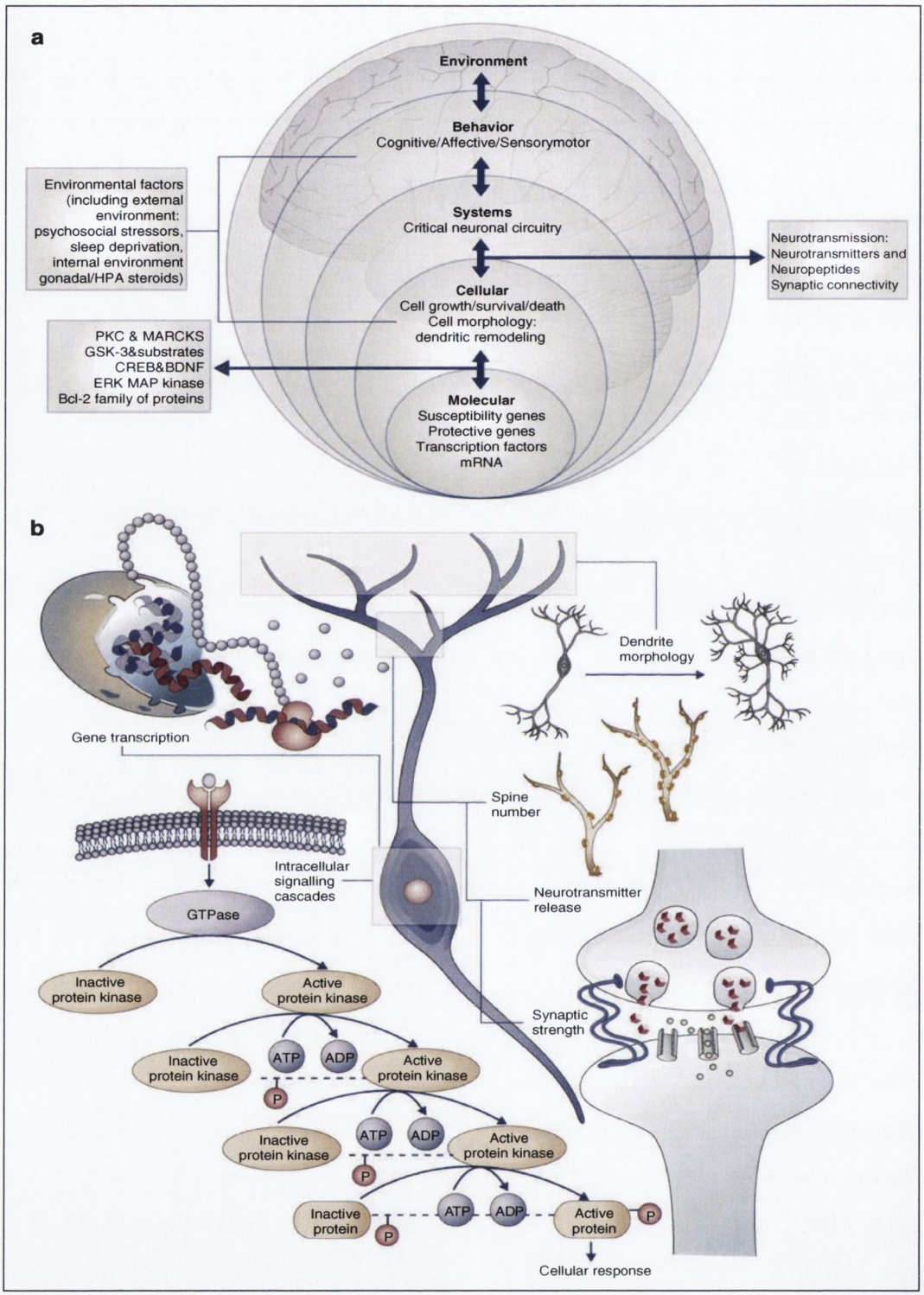


Figure 1.4. (a) A true understanding of the pathophysiology of BPD must encompass different systems on different physiological levels at which the disease manifests: molecular, cellular, and behavioral. **(b)** Biological mechanisms underlying neuroplasticity. The remarkable plasticity of neuronal circuits is achieved through different biological means including alterations in gene transcription and intracellular signaling cascades. These changes modify diverse neuronal properties such as neurotransmitter release, synaptic function and even morphological characteristics of neurons (adopted from Schloesser RJ *et al.*, 2008).

Changes in pathways regulating inflammation, oxidative stress, and neurotropic factors have been found in BPAD patients (for review Berk M *et al.*, 2011). An increased level of dopamine is an important source of oxidative stress in the brain and it is recently suggested that antipsychotic drugs may protect against oxidative stress (Berk M *et al.*, 2011). Many studies found abnormality in oxidative energy generation in BPAD (Kato T, 2007). It has been suggested that brain energy generation may be increased during mania and decreased during the depression phase (Baxter LR *et al.*, 1985).

Several studies suggest mitochondrial dysfunction in BPAD, which might be linked to impaired brain energy generation (Berk M *et al.*, 2011; Kato T, 2007). It has been reported that mitochondrial DNA variants might result in altered mitochondrial calcium regulation (Kato T, 2007). Calcium homoeostasis appears altered across all mood states in BPAD (Langan C & McDonald C, 2009; Kato T, 2008; Warsh JJ *et al.*, 2004). Recently, calcium channels have been strongly associated with BPAD (Kato T, 2008; Sklar P *et al.*, 2008). Calcium channel blockers have been used in the treatment of BPAD. A large body of data has accumulated demonstrating that Lithium modulates intracellular calcium homeostasis (Warsh JJ *et al.*, 2004).

As interruption in normal brain function is a clear feature of BPAD, researchers have examined for structural differences in affected individuals compared to controls. A meta-analysis by Hoge EA *et al.* (1999) reported that while global brain volumes in BPAD patients are normal compared to healthy individuals, regional brain differences are observed. A recent meta-analysis conducted by Bora E *et al.* (2010) found that grey matter reduction in left rostral anterior cingulate cortex and right frontoinsular cortex was associated with BPAD.

Several studies report glial cell abnormalities in BPAD. For example, decreased glial cell number and density has been described (Drevets WC, 1998; Brauch RA *et al.*, 2006; Cotter D *et al.*, 2001). Reduction of glial cell density appears in frontal cortical areas (Öngür D *et al.*, 1998; Rajkowska G, 2000). Alterations have been described histopathologically, particularly for

astrocytes, which are a subtype of glial cells (Miguel-Hidalgo JJ *et al.*, 2000; Si X, 2004).

The hypothesis arising from these studies is that tissue damage, structural and functional changes in the brain's neuronal substrates of mood regulation have the potential to increase the risk of recurrence and reduce the potential treatment response for BPAD (Berk M, 2011). Genome-wide transcriptome and proteome analyses of post mortem brain tissue indicated that altered apoptosis, protein degeneration, mitochondrial function and synaptic function might also contribute to BPAD, and indeed to SZP (Konradi C, 2005). Also, several studies found abnormalities in synaptic and neuronal plasticity cascades, leading to potential aberrant information processing in critical synapses and circuit in BPAD (Schloesser RJ *et al.,* 2008; Goodwin FK & Jamison K, 2007).

Schizophrenia

Many abnormalities identified in BPAD, as described above, are also identified in SZP, which supports a continuum model. The current theories for the underlying biology of SZP mainly include altered neurodevelopment, synaptic dysfunction, neurochemistry and aberrant neuronal connectivity (review Keshavan MS *et al.,* 2011). One of strongest theories on the aetiology of Schizophrenia is one of aberrant neurodevelopment. It proposes that genetic variation in neurodevelomentally important genes interact with environmental events during early periods of life to produce neurodevelopmental abnormalities that will predispose to SZP. Additional environmental insult later in life may cause neurodevelopmentally impaired individuals to develop SZP (Marenco S & Weinberger DR, 2000; Faludi G & Mirnics K, 2011).

At a molecular level, several neurotransmitter systems, particularly dopamine, serotonin, glutamate and GABA have been implicated in SZP patients (for review see Keshavan MS *et al.,* 2011). Most if not all currently available antipsychotics block the dopaminergic D2 receptor (Kapur S, 2003). Alteration

in the excitatory glutamate system, particularly N-methyl-D-aspartate (NMDA) receptor function had been found in SZP patients (Olney JW & Farber NB, 1995; Javitt DC & Zukin SR, 1991). Several lines of evidence indicates altered oxidative stress as contributing to the pathophysiology of SZP (Wood SJ *et al.*, 2009; Bitanihirwe BK & Woo TU, 2011). Increased serum concentrations of several pro-inflammatory cytokines have been implicated, which may reflect the response by microglia (Keshavan MS *et al.,* 2011).

At structural level, several studies report neuroanatomical abnormalities including reduced total brain volume, abnormal hippocampal and grey matter volumes and increased ventricular volumes in SZP patients (Daniel DG *et al.,* 1991; Steen RG *et al.*, 2006; Shenton ME *et al.*, 2001; Ward KE *et al.*, 1996; Wright IC *et al.*, 2000). Consistent grey matter volume reductions have been found, particularly in the thalamus, bilateral insula/frontal lobe, anterior cingulated, and bilateral hippocampal-amygdala regions (Segall JM *et al.*, 2009). In a more recent adaptation of the neurodevelopmental model of the pathology of SZP, impaired synaptic connectivity has been reported in several post-mortem, functional and structural brain imaging studies (Selemon LD & Goldman-Rakic PS, 1999).

1.2. Molecular Genetics

The classical genetics approaches of family, adoptee, and twin studies have established a significant role for genetic inheritance in the aetiology of BPAD and SZP (Riley B & Kendler KS, 2006; Goodwin FK & Jamison K, 2007). Those studies have been remarkably consistent. Nonetheless, finding the genetic variation contributing to BPAD and SZP has proven to be challenging.

Strategies for pinpointing risk genes for psychiatric disorders include linkage and association approaches (Goodwin FK & Jamison K, 2007). Linkage studies look for patterns of cosegregation of a disease phenotype and a DNA variant within families whereas genetic association studies seek evidence for association between a DNA variant and a disease phenotype at the population level (Goodwin FK & Jamison K, 2007).

1.2.1. Linkage Studies

Bipolar Disorder.

From linkage studies, many susceptibility loci for BPAD have been suggested, but not definitively confirmed, including 1q31-32, 4p16, 6p24, 10q25-26, 12q23-24, 13q31-32, 18p11, 18q21-23, 21q22, 22q11-13 and Xq24-28 (Baron M, 2002). The first meta-analysis of BPAD genome scans reported by Badner JA and Gershon ES (2002) suggested susceptibility loci at 13q and 22q, whereas a further meta-analysis by Segurado R *et al.* (2003) failed to identify any chromosomal regions that were of genome-wide significance, with the strongest results for regions 9p22-21, 10q11-22 and 14q24-32.

Schizophrenia

Several studies have reported evidence for linkage on 6p24-22 for SZP (Riley B & Kendler K, 2005). A meta-analysis of genomewide studies by Lewis CM *et al.* (2003) supported a susceptibility locus on chromosome 2p12-q22.1 for SZP. A more recent meta-analysis by Ng MY *et al.* (2009) analyzing 32 linkage studies indicated susceptibility loci on chromosome 2q and 5q for SZP.

Chromosome 21

As there are many linkage studies on BPAD and SZP reported in the literature but for the purpose of this thesis, the following section will focus in chromosome 21. Straub R *et al.* (1994) reported the first study of linkage with BPAD on chromosome 21. Since then there has been many linkage studies reported, some of which support a susceptibility locus 21q21-22 for BPAD and SZP (Detera-Wadleigh SD *et al.*, 1996, 1997; Smyth C *et al.*, 1997; Liu J *et al.*, 2001; Aita VM *et al.*, 1999; Ewald H *et al.*, 2003). A summary of the results for linkage studies on chromosome 21q21-22 is shown in Table 1.4.

Table 1.4. Linkage studies of the chromosome 21q21–22 with the bipolar and related unipolar affective disorders phenotypes. (the table modified from McQuillin A *et al.*, 2006).

Contry of Origin	Reference	Number of families	Max Lod	Admixture Lod	significance
USA/NY/Israel	Straub R *et al.*, 1994	47	3.04		P<0.000001
	Aita V *et al.*, 1999	40		3.35	
	Liu J *et al.*, 2001	16	1.89		
USA/Amish	LaBuda MC *et al.*, 1996	1[a]	0.19		P=0.043
USA/NIMH	Detera-Wadleigh S *et al.*, 1996	22	1.79		P<0.008
UK/Brazil	Vallada H *et al.*, 1996	60		1.21	
UK/Iceland	Smyth C *et al.*, 1997	23	2.25		P=0.001
USA/NIMH genetics initiative	Detera-Wadleigh SD *et al.*, 1997	97			P = 0.0008
Canada	Morissette J *et al.*, 1999	1[b]	1.7		P=0.004
Australia	Kwok JB *et al.*, 1999	12			P<0.001
USA/San Diego	Kelsoe JR *et al.*, 2001	20	2.04[c]		
Denmark	Ewald H *et al.*, 2003	1	1.09		P=0.014

[a]Subset of Amish kindred, unipolar depression not included.
[b]One branch of Sanguenay Lac St Jean kindred.
[c]Sex specific.

Linkage studies of BPAD and SZP demonstrated several features. Complexity and phenotypic overlap, as already predicted on the basis of genetic epidemiology; that there are putative risk loci on almost all chromosomes and that the existence of a highly penetrant risk locus affecting a reasonable proportion of pedigrees is unlikely. The strongest linkage findings can be only proven to be true when disease risk variants are identified. A first step to this identification is the application of genetic association methods to follow up and fine map the regions identified in the linkage studies.

1.2.2. Association Studies

Association studies have more power than the linkage studies to detect risk variants of small effect under certain circumstances. However, because populations are the unit of study, association studies require a higher density of variants. The current hypotheses is that there may be some families where the illness is caused by a single mutation of major effect, but that the majority of cases involve the interplay of multiple genes and more complex genetic mechanism in determining the susceptibility of BPAD and SZP (Craddock N & Sklar P, 2009; Bondy B, 2011). Under this model, much genetic susceptibility is likely to result from a combination of common variants with a small effect in addition to rare mutations with relatively large effect (Owen MJ *et al.*, 2009; Craddock N & Sklar P, 2009).

Bipolar Disorder

The heritability of BPAD is estimated to be as high as 93% (Kiesappä T *et al.*, 2004), which is higher than many common complex diseases. Results from a variety of candidate gene studies found evidence supporting many susceptibility genes for BPAD and the strongest candidate genes are the disrupted in schizophrenia 1 (DISC1), catechol-O-methyl-transferase (COMT), dystrobrevin-binding protein 1 (DTNBP1), Neuregulin 1 (NRG1), gene encoding for monoamino oxidase A (MAOA), serotonin transporter (5HTT), D-amino acid oxidase activator (DAOA) and brain derived neurotropic factor (BDNF) (Craddock N & Sklar P, 2009; Kato T, 2007; Craddock N *et al.*, 2006; Goodwin FK & Jamison K, 2007).

Schizophrenia

At the time of writing of this thesis, the Schizophrenia Research Forum has published a database (SchizophreniaGene, http://www.szgene.org), which includes the summary results of more than 1,727 association studies, which include 8788 variants from 1008 different genes. The DISC1, DTNBP1, DAOA, NRG1, COMT genes are the most promising candidate genes for

SZP, which overlaps with the findings from BPAD summarised above (Craddock N & Sklar P, 2009; Waddington JL *et al.,* 2007).

In general, genetic association studies of SZP and BPAD have implicated numerous genes related to dopaminergic-, serotonergic- and glutamatergic neurotransmission systems; genes involved in brain development, neuronal growth and growth hormones; circadian rhythm; oxidative or other stress, channelopathies and others summarised in Table 1.5. All studies listed in Table 1.5. have found association between the variant within the reported gene and BPAD or SZP.

Table 1.5. Genes that have been tested for association in BPAD and SZP. All listed genes have been associated with SZP or BPAD. Genetic Association Database is used to create this table (http://geneticassociationdb.nih.gov; Goodwin FK & Jamison KR, 2007.)

			References	
Gene	**Location**	**Function**	**BPAD**	**SZP**
Involved in Serotonin System				
5-HTR3A	11q23	Encodes for serotonin receptor gene 3A	Niesler B *et al.*, 2001	Gu B *et al.*, 2008 Schuhmacher A *et al.*, 2009
5-HTR2A	13q14	Encodes for serotonin receptor gene 2A	Chee IS *et al.*, 2001 Yosifova A *et al.*, 2009	Yamanouchi Y *et al.*, 2003 Baritaki S *et al.*, 2004
SLC6A4	17q11.1	Encodes for serotonin transporter which takes the serotonin into the presynaptic neuron and terminates the synaptic action and recycles it into the neurotransmitter pool.	Mellerup E *et al.*, 2001 Masoliver E *et al.*, 2006	Dolzan V *et al.*, 2008 Lin C *et al.*, 2009
MAOA	Xp11.4	Encodes for Monoamine Oxidase A. It is a degradation enzyme.	Lim LCC *et al.*, 1994 Furlong RA *et al.*, 1999	Jönsson EG *et al.*, 2003
TPH1	11p15.1	Encodes for Tryptophan hydroxylase 1. It is a synthesis enzyme.	Serretti A *et al.*, 2001 Chen C *et al.*, 2008	Hong CJ *et al.*, 2001 Saetre P *et al.*, 2010

Gene	Location	Function	BPAD	SZP
Involved in Dopamine System				
DRD1	5q35.2	Encodes for dopamine receptor gene 1	Severino G *et al.*, 2005 Del Zompo *et al.*, 2007	Allen NC *et al.*, 2008 Rybakowski JK *et al.*, 2005
DRD2	11q23.2	Encodes for dopamine receptor gene 2	Pérez de Castro I *et al.* 1995 Massat I *et al.*, 2002	Rybakowski JK *et al.*, 2005 Serretti A *et al.*, 1998a
DRD4	11p15.5	Encodes for dopamine receptor gene 4	Serretti A *et al.*, 1998b López León S *et al.*, 2005	Tang Y *et al.*, 2001 Pal P *et al.*, 2009
SLC6A3	5p15.33	Encodes for dopamine Transporter.	Keikhaee MR *et al.*, 2005 Ohadi M *et al.*, 2007	Pal P *et al.*, 2009
COMT	22q11	Encodes for Catechol-O-methyltransferase which is involved in catalyzing the O-methylation and inactivation of catecholamine neurotransmitter (e.g., dopamine, norepinephrine) and catechol hormones.	Papolos DF *et al.*, 1998 Li T *et al.*, 1997	Shifman S *et al.*, 2002 Neuhaus AH *et al.*, 2009
TH	11p15.5	Encodes for Tyrosine hydroxylase which is involved in the synthesis of Dopamine and Norepinephrine.	Serretti A *et al.*, 1998c	Wei J *et al.*, 1995 Pae CU *et al.*, 2003
Involved in Glutamate System				
DAO	12q24.11	Encodes for amine oxidase that regulates the level of the neuromodulator D-serine in the brain.	NA	Liu X *et al.*, 2004 Wood LS *et al.*, 2007
DAOA (G72/G30)	13q33.2	Encodes for D-amino acid oxidase activator.	Korostishevsky M *et al.*, 2004 Yue W *et al.*, 2006	Bass NJ *et al.*, 2009 Williams NM *et al.*, 2006
DTNBP1	6p22.3	Encodes for dysbindin which is playing role in synaptic vesicle trafficking and in neurotransmitter release.	Breen G *et al.*, 2006 Pae CU *et al.*, 2007	Schwab SG *et al.*, 2003 Riley B *et al.*, 2009
GRIA1	5q33.2	Encodes for glutamate receptor A1	Shi J *et al.*, 2008	Magri C *et al.*, 2006
GRIN2B	12q12	Encodes for NMDA receptor subtype 2 of	Martucci L *et al.*, 2006	Allen NC *et al.*, 2008

		glutamate-gated ion channels with high calcium permeability and voltage-dependent sensitivity to magnesium.		Di Maria E *et al.*, 2004
Gene	**Location**	**Function**	**BPAD**	**SZP**
Involved in Channelopathies				
ANK3	10q21	Encodes for Ankyrin G that play key roles in activities of cell motility, activation, proliferation, contact and the maintenance of specializes membrane domains.	Ferreira M *et al.*, 2008 (GWAS) Schulze TG *et al.*, 2009	Athanasiu L *et al.*, 2010 SPGC *et al.*, 2011
CACNA1C	12q13.3	Encodes for calcium channel voltage dependent alpha 1C which mediates the influx of calcium ion into the cell upon membrane polarization.	Ferreira M *et al.*, 2008 (GWAS) Casamassima F *et al.*, 2010	Moskvina V *et al.* 2009 Green EK *et al.*, 2009
KCNN3	1q21.3	Encodes for calcium-activated potassium channel protein 3, which form a voltage-independent potassium channel activated by intracellular calcium.	Saleem Q *et al.*, 2000	Bowen T et al. 1998
KCNC2	12q14.1	Encodes the Shaw-related voltage-gated, which is a potassium channel.	WTCCC 2007 (GWAS)	NA
NTNG1	1p13.3	Encodes for Netrin G1 proteins that act as axon guidance cues during nervous system development.	NA	Fukasawa M *et al.*, 2004 Ohtsuki T *et al.*, 2008
P2RX7	12q24.31	Encodes for purinergic receptor P2X 7 that is a ATP-binding calcium channel receptor.	Barden N *et al.*, 2006	NA
SCL39A3	19p13.3	Encodes for Zinc transporter ZIP3 protein.	Baum AE *et al.*, 2008 (GWAS)	NA
TRPM2	21q22.3	Encodes for the calcium-permeable cation channel protein that mediates calcium and sodium ion influx in response to oxidative stress.	Xu C *et al.*, 2006 Xu C *et al.*, 2009	NA
Involved in brain development, neuronal growth and growth hormones				
AKT1	14q32	Encodes serine-threonine protein kinase that plays an important role in cell proliferation, apoptosis and	Magno LA *et al.*, 2010	Emamian ES *et al.*, 2004 Ikeda M *et al.*, 2004

		cell migration.		
Gene	**Location**	**Function**	**BPAD**	**SZP**
BDNF	11q14	Encodes for Brain-derived neurotropic factor, which promotes the survival and differentiation of selected neuronal population during the development. It is involved in the axonal growth and regulates synaptic transmission.	Okada T *et al.*, 2006 Neves-Pereira M *et al.*, 2002	Rosa A *et al.*, 2006 Watanabe Y *et al.*, 2007
EGFR	7p12	Encodes for epidermal growth factor receptor.	Sklar P *et al.*, 2008 (GWAS)	Benzel I *et al.*, 2007
NCAM1	11q23.2	Encodes for neural cell adhesion molecule 1 that involved in neuron-neuron adhesion, neurite outgrowth.	Arai M *et al.*, 2004 Atz ME *et al.*, 2007	Atz ME *et al.*, 2007
NRG1	8p21	Encodes for Neuregulin 1 signalling proteinwhich is a important factor glial growth.	Green EK *et al.*, 2005 Goes FS *et al.*, 2009	Stefansson H *et al.*, 2003 Turunen JA *et al.*, 2007
Involved in Circadian Rhythm				
ARNTL	11p15.2	Encodes for a basic helix-loop-helix protein that activates transcription of a number of proteins of the circadian clock.	Nievergelt CM *et al.*, 2006 Mansour HA *et al.*, 2006	Mansour HA *et al.*, 2009
CLOCK	4q12	Encodes for Circadian Locomotor Output Cycles Kaput protein.	Benedetti F *et al.*, 2003	Takao T *et al.*, 2007
NR1D1	17q11.2	Encodes fornuclear receptor subfamily 1 D1 that participates in development and circadian regulation.	Kishi T *et al.*, 2008 Severino G *et al.*, 2009	NA
PER3	1p36.23	Encodes a protein that plays a role in the circadian rhythms of locomotor activity, metabolism, and behavior	Nievergelt CM *et al.*, 2006 Rocha PM *et al.*, 2010	Mansour HA *et al.*, 2006
TIMELESS	12q12-q13	Encodes TIM protein that regulates the circadiam rhythm. Involved in cell survival after DNA damage or replication stress.	Mansour HA *et al.*, 2006 Mansour HA *et al.*, 2009	Mansour HA *et al.*, 2006
Involved in Oxidative or other stress				
MTHFR	1p36.3	Encodes for methylenetetrahydrofolate reductase.	Kempisty B *et al.*, 2007 Ozbek Z *et al.*, 2009	Wei J *et al.*, 1999 Joober R *et al.*, 2000

Gene	Location	Function	BPAD	SZP
NOS1	12q24.2-q24.31	Encodes for nitric oxide synthase 1 which produces nitric oxide and this display many prosperities of Neurotransmitter.	Fallin MD *et al.*, 2005 Yosifova A *et al.*, 2009	Shinkai T *et al.*, 2002 Fallin MD *et al.*, 2005
NOS3	7q36	Encodes for nitric oxide synthase 3 which produces nitric oxide.	Reif A et al. 2005	NA
NDUFV2	18p11.31-p11.2	Encodes for NADH dehydrogenase flavoprotein 2, mitochondrial enzyme.	Washizuka S *et al.*, 2004 Zhang J *et al.*, 2009	Washizuka S *et al.*, 2006
Others				
DISC1	1q42.	Encodes for disrupted in schizophrenia 1 which is involved in the regulation of multiple aspects of embryonic and adult neurogenesis.	Thomson PA *et al.*, 2005 Schosser A *et al.*, 2009	Thomson PA *et al.*, 2005 Zhang F *et al.*, 2006
S100B	21q22.3	Encodes for S100B protein, which is involved in neurite extension, stimulation of Ca2+ fluxes, inhibition of PKC-mediated phosphorylation, astrocytosis and axonal proliferation.	Roche S *et al.*, 2007	Liu J *et al.*, 2005
ZNF804A	2q32.1	Encodes for a transcription factor zinc finger protein 804A.	Williams HJ *et al.*, 2011	Riley B *et al.*, 2010 Zhang R *et al.*, 2011

In conclusion, the wide range of candidate gene association studies published to date reflect the many and varied biological models of SZP and BPAD, reflecting also the suspected pathophysiological overlap. The sample size for case control studies has typically been in the region of 100-300 individuals, and lower for family based approaches. Very few, if any of these findings have been confirmed by meta-analysis or by genome-wide association.

1.2.3. Genome Wide Association Studies

One of the most exciting prospects in the dissection of complex disorders has been the development of the technology enabling genome wide association studies covering a large proportion of the genetic variation in the human genome. GWAS are hypothesis-free; that is they search the entire genome for association rather focusing on candidate areas. The discovery and cataloguing of million of common (minor allele frequency > 5%) SNPs across the human genome (The Human Genome Project and International HapMap Project) and improvement of technologies in high-throughput genotyping SNPs have made GWAS possible (International HapMap Consortium, 2003, 2005; International Human Genome Sequencing Consortium, 2000).

There are several difficulties in evaluating the results of GWAS, particularly the problem of extensive multiple testing (~ 1 million of SNPs are tested simultaneously). Correcting for multiple testing results in a genome wide significance at $p < 5 \times 10^{-8}$. A correction for this multiple testing will reduce the risk of false positive findings but also on the other side it might hamper the detection of genes with small disease risk (Nöthen MM *et al.,* 2010).

GWAS have successfully identified candidate genes many disorders, for example, Crohn's disease (Rioux JD *et al.,* 2007), age-related macular degeneration (Klein RJ, 2005), Type 2 diabetes (Zeggini E *et al.,* 2008), breast cancer (Gold B *et al.,* 2008) or prostate cancer (Eeles RA *et al.,* 2008). There are a limited number of published GWAS to date for BPAD and SZP, although the numbers are expanding rapidly, and large scale collaborative studies are planned (Sullivan PF, 2010).

Bipolar Disorder

Eight GWAS have been reported for BPAD using either pooled (Baum AE *et al.,* 2008a; Cichon S *et al.*, 2011) or individually genotyped samples (the Wellcome Trust Case Control Consortium, 2007; Baum AE *et al.,* 2008b; Ferreira MA *et al.,* 2008; Sklar P *et al.,* 2008; Scott LJ *et al.*, 2009; Smith EN *et al.,* 2009). There is poor consistency across the GWAS regarding their top association findings. The results from GWAS of BPAD are summarized in Table 1.6. Only 3 genes reached genome wide significance; those are DGKH, ANK3 and NCAN. None of the top association findings have been clearly replicated. The odd ratios (OR), the measure of effect size, are small ranging generally between 1.1-1.5.

DGKH encodes for *diacylglycerol kinase*, which is a key protein in the lithium-sensitive phosphatidyl inositol pathway and has also been associated with SZP and BPAD in independent samples (Zeng Z *et al.,* 2011). ANK3 encodes for a large protein *ankyrin-3*, whose neuronal-specific isoforms are found in the axon initial segments that have been shown to regulate the ion channels and cell adhesion molecules (Ferreira MA *et al.,* 2008; Kordeli E *et al.,* 1995). NRCAN encodes for a *Neurocan core protein*, which is thought to be involved in cell adhesion and migration. Cichon S *et al.* (2011) found that expression of this gene is localized to cortical and hippocampal areas in mice. Those areas are reported to be involved in cognition and emotion regulation (Cichon S *et al.*, 2011).

Table 1.6. Top genes or genomic regions identified in recent genome-wide association studies of BPAD.

Reference	case/control subjects	Top hits	Lower *p*-value	OR
WCCC, 2007	1868/2938	*PALB2*	6.3×10^{-8}	1.2
Baum AE *et al.*, 2008a	1233/1439	*DGKH*	1.5×10^{-8}	1.59
Baum AE *et al.*, 2008b	3101/4377	*JAM3* *SLC39A3*	1.0×10^{-6} 5.0×10^{-6}	1.27 1.35
Sklar P *et al.*, 2008	1461/2008	*MYO5B*	1.7×10^{-7}	1.51
Ferreira MA *et al.*, 2008	4387/6209	*ANK3* *CACNA1C*	1.7×10^{-9} 7.0×10^{-8}	1.45 1.18
Scott JL *et al.*, 2009	23683/14507	*1p31.1 (no gene)* *NEK4; ITIH1* *MCTP1*	2.0×10^{-7} 1.8×10^{-7} 1.3×10^{-7}	1.12 1.20 1.09
Smith EN *et al.*, 2009	1001/1033(EA) 345/670(AA)	*ROR1* *RGS5* *BTBD16*	1.4×10^{-6} 4.1×10^{-6} 4.5×10^{-6}	2.05 2.11 2.85
Cichon S *et al.*, 2011	8441/35362	*NCAN*	2.14×10^{-9}	1.17

Abbreviation: European ancestry (EA); African American (AA)

Schizophrenia

Several GWAS have been reported for the SZP phenotype (Lencz T *et al.*, 2007; Sullivan PF *et al.*, 2008; O'Donovan MC *et al.*, 2008; Need AC *et al.*, 2009; Purcell SM *et al.*, 2009; Stefansson H *et al.*, 2009; Shi J *et al.*, 2009) and results are summarized in Table 1.7. Only 4 genes/regions appear to have reached genome wide significance; those are ZNF804, NRGN, TCF4 and the MHC region. Only the MHC finding has been replicated.

TCF4 encodes for *transcription factor 4* and ZNF804A encodes for *zing finger protein 804A*. Both are transcription factors that might regulate the expression of many genes. Most top hits from SZP GWAS are from intergenic SNPs that might affect risk through transcription of genes either cis-regulatory

sequences, like enhancers, or through non-coding RNAs transcribed from intergenic regions, like enhancer RNAs (Duan J *et al.*, 2010). The MHC region is very gene-dense, containing over 200 genes. Those genes have a role in immunity and self-recognition (Duan J *et al.*, 2010). NRGN (neurogranin) encodes for postsynaptic protein kinase substrate that binds calmodulin (CaM) in the absence of calcium. NRGN is expressed mainly in brain regions important for cognitive function (Stefansson H *et al.*, 2009).

Table 1.7. Top genes or genomic regions identified in recent genome-wide association studies of SZP (the table modified from Duan J *et al.*, 2010).

Reference	**case/control subjects**	**Gene or Region**	**Lower *p*-value**	**OR**
Lencz T *et al.*, 2007	178/144	*CSF2RA, SHOX*	3.7×10^{-7}	3.23
Sullivan PF *et al.*, 2008	738/733	*AGBL1*	1.71×10^{-6}	6.01
O'Donovan MC *et al.*, 2008	Discovery: 479/2937; Follow up: 6829/9897	*ZNF804A*	9.96×10^{-9}	1.12
Need AC *et al.*, 2009	Discovery: 871/863 Follow up: 1460/12995	*ADAMTSL3*	1.35×10^{-7}	0.68
Purcell SM *et al.*, 2009	3322/3587	MHC region *MYO18B*	9.5×10^{-9} 3.4×10^{-7}	0.82
Stefansson H *et al.*, 2009	Discovery: 2663/13,498 Follow up: 4999/15,555	MHC region *NRGN* *TCF4*	1.4×10^{-12} 2.4×10^{-9} 4.1×10^{-9}	1.16 1.15 1.23
Shi J *et al.*, 2009	2681/2653 (EA) 1286/973 (AA)	MHC region *CENTG2* (in EA) *ERBB4* (in AA)	9.5×10^{-9} 4.59×10^{-7} 2.14×10^{-6}	0.88 1.23 0.73

Abbreviation: European ancestry (EA); African American (AA)

The GWAS findings suggest that ion channel abnormalities might be involved in the pathogenesis of BPAD and imbalance in the regulation of gene expression might be involved in pathogenesis of SZP (Ferreira MA *et al.,* 2008; Duan J *et al.,* 2010). However, the majority of the heritability of SZP and BPAD is unexplained by GWAS (Duan J *et al.,* 2010; Rudan I, 2010). The GWAS approach has been successful in the identification of susceptibility genes for diabetes type 2 or for phenotypes such as human height and body mass index (Rudan I, 2010). The lack of similar success in finding susceptibility genes for BPAD and SZP might be explained by diagnosic difficulties and genetic heterogeneity (Schulze TG, 2010). The sample size is another factor in GWAS of BPAD and SZP. These studies to date have involved only low number thousands of cases and controls whereas the sample size for Type 2 diabetes has reached 50000 cases and samples for the study of human height and body mass index have reached more than 250000 participants (Rudan I, 2010; Nöthen MM *et al.,* 2010).

The relative few findings reaching the genome wide significance and the lack of replication suggests that SZP and BPAD might be influenced by the effect of multiple rare variants. GWAS test for association with common SNPs assuming that these variants have an effect, albeit small, in all or most individuals with the disorder. However BPAD and SZP are likely to be of polygenic origin and may be profoundly heterogeneous despite sharing similar phenotypic characteristics. In addition there is the possibility of mitochondrial gene variation, epigenetic variation, rare highly penetrant variants and copy number variation (CNVs) either rare or common. It is anticipated that improvement in GWAS with the ability to examine rare variants (MAF 1%); better detection of CNVs using dedicated arrays, and the application of next generation sequencing will yield tangible results in the near future. The next section will describe the gene that was the subject of this thesis, S100B and the process by which this gene was the one that was chosen for intensive study.

1.3. S100B Protein

1.3.1. S100 protein family

The term S100 refers to member of a multigenic family of calcium-modulated proteins, the S100 family, mostly of low molecular mass (9-13 kDa), that were first identified as a protein fraction detectable in brain but not in non neural extract and called S100 because of their solubility in a 100% saturated solution of ammonium sulphate. At present, there are about 20 proteins identified as belonging to the S100 family, the members of which are characterized by the presence of a pair of so called EF-hand (i.e., helix-loop-helix) calcium binding motifs, first discovered in the crystal structure of parvalbumin, that induce conformational change of the protein after binding with calcium. In addition, some S100 members have been shown to bind Zn+ and Cu+, suggesting the possibility that their biological activity in some cases might be regulated by Zn+ and/or Cu+, rather than Ca+ (Rothermundt M *et al.*, 2004b). S100B protein is one member of the S100 family.

1.3.2. S100B

S100B is an acidic protein with a molecular mass of 21 kDa existing as a homodimer consisting of a two β subunits. The two monomers are configured in a twofold axis of rotation and held together by disulfide bonds. The disulfide-linked form of S100B appears to be required for fully functional neurotrophic action, whereas S100B mutants lacking one or both of the cysteine residues induce the activation response in glia but not in neurons. A monomer of S100B protein is shown at Figure 1.4. Within cells S100B exist as a homodimer but may be found as a heterodimer coupled with S100A1. The S100B is a Ca^{2+}-binding protein with four Ca^{2+}-binding sites. It also binds Cu^{2+} at four binding sites and Zn^{2+} at six to eight binding sites, and the binding of the later two ions influences the Ca^{2+} binding capacity of the protein (Rothermundt M *et al.,* 2004b). The Protein S100B consists of 92 amino acids (Sequence: MSELEKAMVA LIDVFHQYSG REGDKHKLKK SELKELINNE LSHFLEEIKE QEVVDKVMET LDNDGDGECD FQEFMAFVAM VTTACHEFFE HE; www.ncbi.nlm.nih.gov/gene).

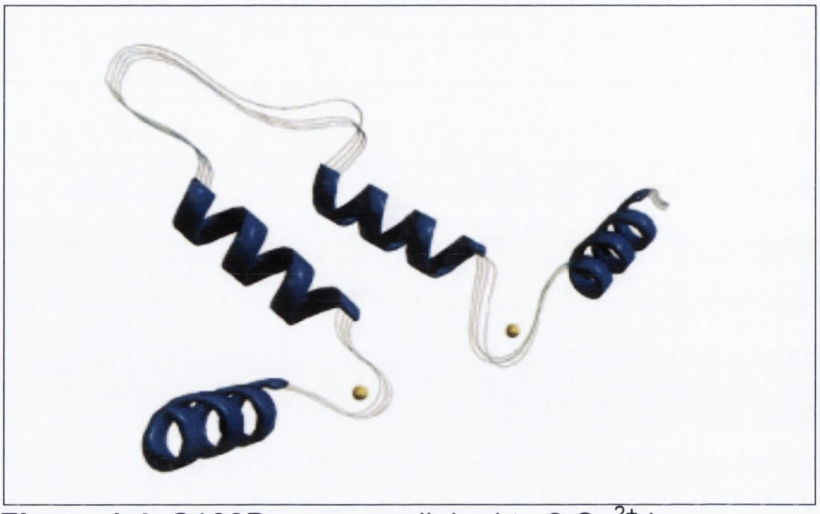


Figure 1.4: S100B monomer linked to 2 Ca^{2+} ions.

S100B is abundant in glial cells of the central and peripheral nervous system where it reaches a concentration of 10 µmol, predominantly in astrocytes, and is also expressed in nonneuronal tissues including melanocytes, chondrocytes, Langerhans cells, folliculostellate cells of the adenohypophysis, adrenal gland satellite cells, Leydig cells, and interdigitating reticulum cells, whereas adipose tissue constitutes a site of concentration for the protein comparable to nervous tissue. The majority of astrocytic S100B is located within the cytoplasm, with 5-7% membrane bound. At least 80-90% of the total S100B pool is found within the brain (Rothermundt M *et al.*, 2004b).

The gene encoding for S100B in humans is located on chromosome 21q22.3 at a distance of 100-140 kb from the chromosome terminus. The human S100B gene is composed of three exons, the first of which specifies the 5' – untranslated region, while the second and third each encode a single EF-hand, Ca2+-binding domain (Figure 1.5.). The promoter region contains several potential regulatory transcription elements including the amp-responsive elements CRE and AP-2, SP1 binding site and GC box (Allore RJ *et al.*, 1988; Castets F *et al.*, 1997).

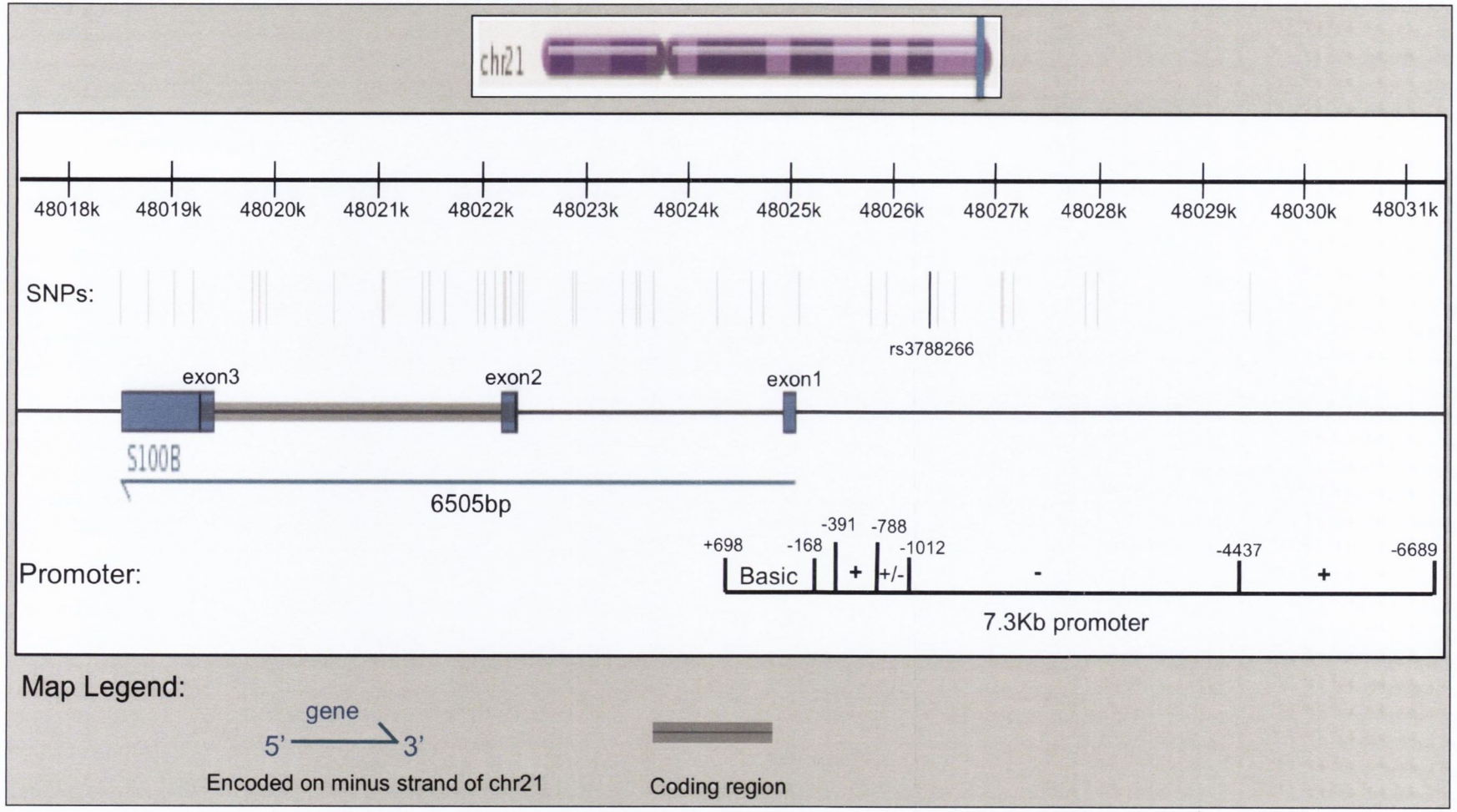


Figure 1.5. Genomic organization of the 6.5-kb S100B gene and SNPs (www.ncbi.nlm.nih.gov/gene; www.appliedbiosystems.com). A schematic representation of the minimal S100B promoter, and the upstream positive and negative regulatory domains are shown (Castets F *et al.*, 1997; Tsoporis JN *et al.*, 2003).

1.3.2.1. S100B Function

S100B involved in many intracellular and extracellular regulatory processes which are summarised in Figure 1.6. Intercellularly S100B is involved in signal transduction via inhibition of protein phosphorylation, regulation of enzyme activity and Ca^{2+} homeostatsis. Moreover S100B is functionally involved in the regulation of cell morphology by interaction with elements of the cytoplasmic cytoskeleton. S100B also has extracellular functions and is actively secreted by cells (Donato R *et al.*, 2009; Rothermundt M *et al.*, 2004b). Calcium is an universal intracellular second messenger that plays a regulatory role in processes such as conduction and transmissions of the nerve impulse, muscle contraction, cell motility, cell growth and differentiation, gene expression, communication between various enzyme systems, apoptosis, and necrosis (Berridge MJ *et al.*, 2000). The major intracellular function of S100B is to block the phosphorylation of protein kinase C (PKC) target proteins in a Ca2+ concentration-dependent manner. S100B inhibits the phosphorylation of several neuronal proteins, which are involved in transmitter release, ion channel modulation, and plasticity as shown in Table 1.8 (Donato R *et al.*, 2009).

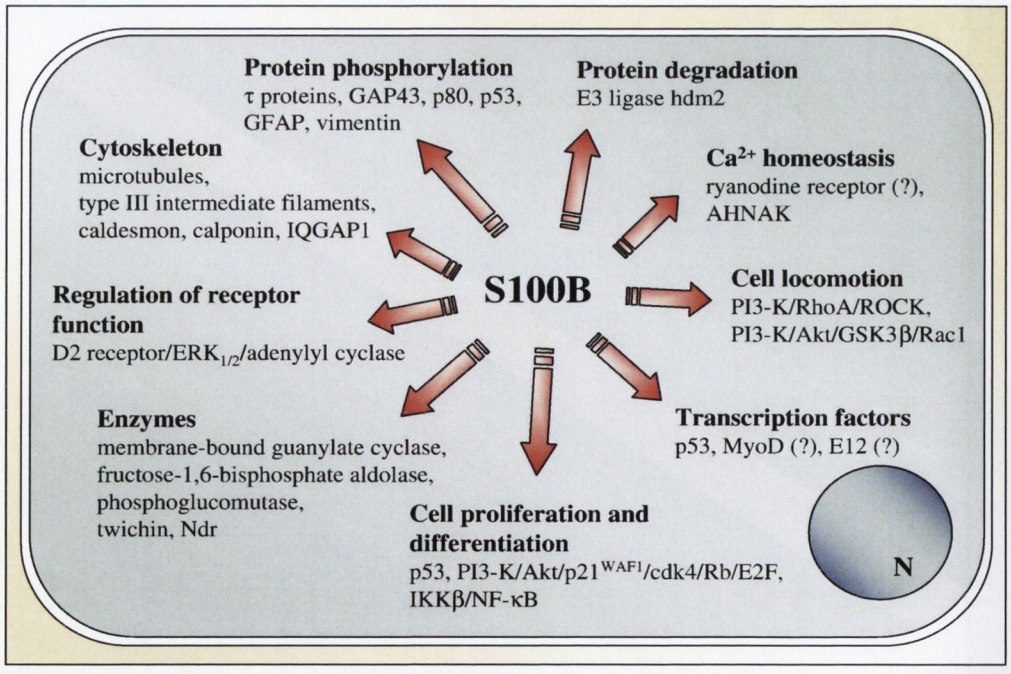


Figure 1.6. Schematic representation of proposed intracellular regulatory effects of S100B (Donato R *et al.*, 2009).

S100B is involved in the regulation of the energy metabolism of the brain cells with stimulation of the enzyme activity as shown in Table 1.9 (Donato R *et al.*, 2009). S100B also interact with cytoskeletal elements. S100B seems to play a role in the assembly of important components of cell cytoarchitecture (Rothermundt M *et al.*, 2004b).

Table 1.8. S100B Protein-Dependent Inhibition of Protein Phosphorylation by PKC (modified from Rothermundt M, 2004b).

Target Protein	Functional correlates (in vitro)	References
Annexin VI	Blocks the ability of S100B to inhibit intermediate filament assembly	Garbuglia M *et al.*, 2000
Caldesmon	Reversal of cadesmon-dependent inhibition of actomyosin ATPase activity	Pritchard K & Martson SB, 1991; Skripnikova EV & Gusev NB, 1989
GAP-43 (neuromodulin)	Unknown	Lin LH *et al.*, 1994; Sheu FS *et al.*, 1994
GFAP, vimentin	Inhibits intermediate filament assembly	Ziegler DR *et al.*, 1998
MARCKS MARCKS-like retinal phosphoprotein p80	Unknown	Albert KA *et al.*,1984; Pozdnyakov N *et al.*, 1998; Sheu FS *et al.*, 1995
Neurogranin	Unknown	Sheu FS *et al.*, 1995
p53	Protection of p53 from thermal denaturation and aggregation, stimulation of p53-dependent cell growth arrest and apoptosis, inhibition of p53-dependent transcription activation via disruption of the p53 tetramer.	Baudier J *et al.*, 1992; Rustandi RR *et al.*, 1998; Scotto C *et al.*, 1998; Wilder PT *et al.*, 1998
τ proteins	disrupts the Wnt pathway	Baudier J *et al.*, 1987; Esposito G *et al.*, 2008

Table 1.9. S100B Protein-Dependent Regulation of Enzyme Activity (addopted from Rothermundt M, 2004b).

Enzym	Effect	Suggested function	Reference
Fructose-1, 6-biphosphate aldolase	Stimulation	Regulation of energy metabolism	Zimmer DB & Van Eld LJ, 1986
Membrane-bound guanylate cyclase	Stimulation	Dark adaptation of photoreceptors	Duda T *et al.*, 199 Margulis A *et al.*, 199 Pozdnyakov N *et a* 1998; Rambotti MG *al.*, 1999
Phosphoglucomutase	Stimulation	Regulation of energy metabolism	Landar A *et al.*, 1996

S100B protein is secreted by astrocytes (Donato R *et al.*, 2009; Sen J & Belli A, 2007). Nothing is known to date about its mechanism of secretion, except that orthodox routes are excluded (Sen J & Belli A, 2007). S100B expression is stimulated by interleukin-1 (IL-1), by cyclic-AMP and serotonin receptors (Rothermundt M *et al.*, 2004b; Donato R *et al.*, 2009). Glial cell secreted S100B exerts tropic or toxic effects depending on its concentration. At nanomolar concentration S100B stimulates neurite outgrowth although perhaps not in serotonergic neurons as was once thought. It enhances survival of neurons during development and after injury, prevents motor neuron degeneration in newborn rats after sciatic nerve section. Local administration of S100B stimulates regeneration of injured rat sciatic nerve in vivo (Sen J & Belli A, 2007).

The effect of S100B on cell proliferation seems to be dose-dependent with cells increasing in number at lower doses of S100B but when concentration is increased, the effect is reversed, resulting in cell death. S100B is also thought to play a role in apoptosis. These observations point to a physiological role of secreted S100B as a neurotrophic factor, which could be important during both development and nerve regeneration (Rothermudt M, 2004b).

Astrocytes with high concentration of S100B result in a potent activation of nitric oxide synthase and a subsequent generation of nitric oxide, an ubiquitous biological messenger molecule that can lead to astrocytic cell death in cultures. Changes in nitric oxide synthase activity have also been associated with psychological stress, motor activity, sexual regulation and aggressivity (Sen J & Belli A, 2007).

As mentioned above, nothing is known about its mechanisms of secretion. However, in cultured astrocytes the release of S100B can be observed within a few minutes of receptor activation and may last for up to 10 hours (Rothermundt M *et al.*, 2004b). Depending on its concentration, secreted glial S100B exerts tropic as well as toxic effects. In a concentration within the nanomolar range S100B has neurotrophic effect, e.g. stimulating neuronal outgrowth, enhancing the survival of neurons during development and after injury. On the other hand at micromolar concentration levels S100B may have deleterious effects by inducing apoptotic neuronal cell death. The mechanism how S100B is neurotropic and apoptotic is shown in Figure 1.7 for neurons and Figure 1.8. for astrocytes and microglia.

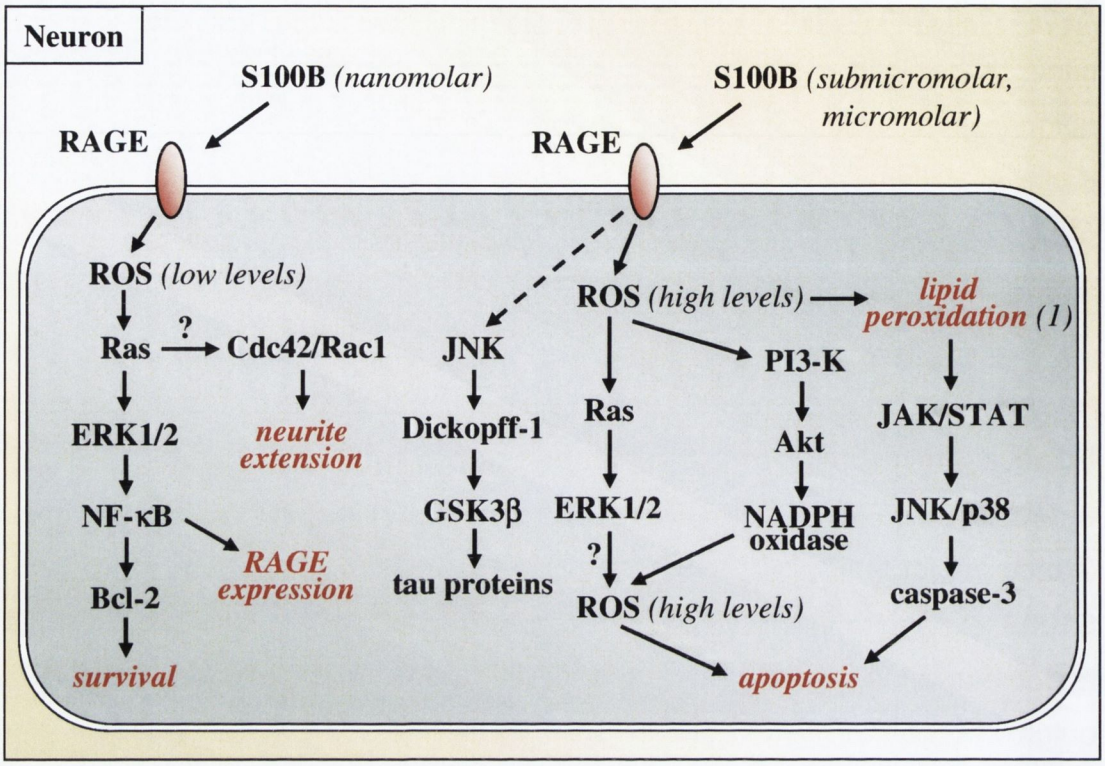


Figure 1.7. Proposed model of effects of extracellular S100B on neurons. At low doses, S100B activates a RAGE-dependent production of moderate amounts of ROS and, probably, activation of Srctyrosinekinase with ensuing activation of a Ras-MEK-ERK1/2-NF-kB pathway leading to upregulation of expression the anti-apoptotic factor Bcl-2, and a Rac1-Cdc42 pathway leading to neurite extension. At high doses, S100B activates a RAGE-dependent overproduction of ROS and excessive stimulation of the Ras-MEK-ERK1/2 pathway with accompanying cytochrome-C release from mitochondria and activation of caspase-3, leading to neuronal apoptosis. The target(s) of ERK1/2 under these latter conditions have not been elucidated. Schemes do not take into account multimerization of extracellular S100B and S100B-induced RAGE oligomerization and or RAGE oligomer stabilization. (1) S100B- RAGE-ROS-dependent lipid peroxidation has been described in neural progenitors from Down syndrome brain only. One cannot exclude that this mechanism can operate in any RAGE-expressing cell type exposed to high S100B concentration, however one should also take into account that responses of human Down syndrome neural progenitor cells to S100B might be strongly dependent on the altered transcriptional regulation of a subset of genes throughout the entire genome in consequence of the increased levels of expression of genes on chromosome 21 (adopted from Donato R, 2008).

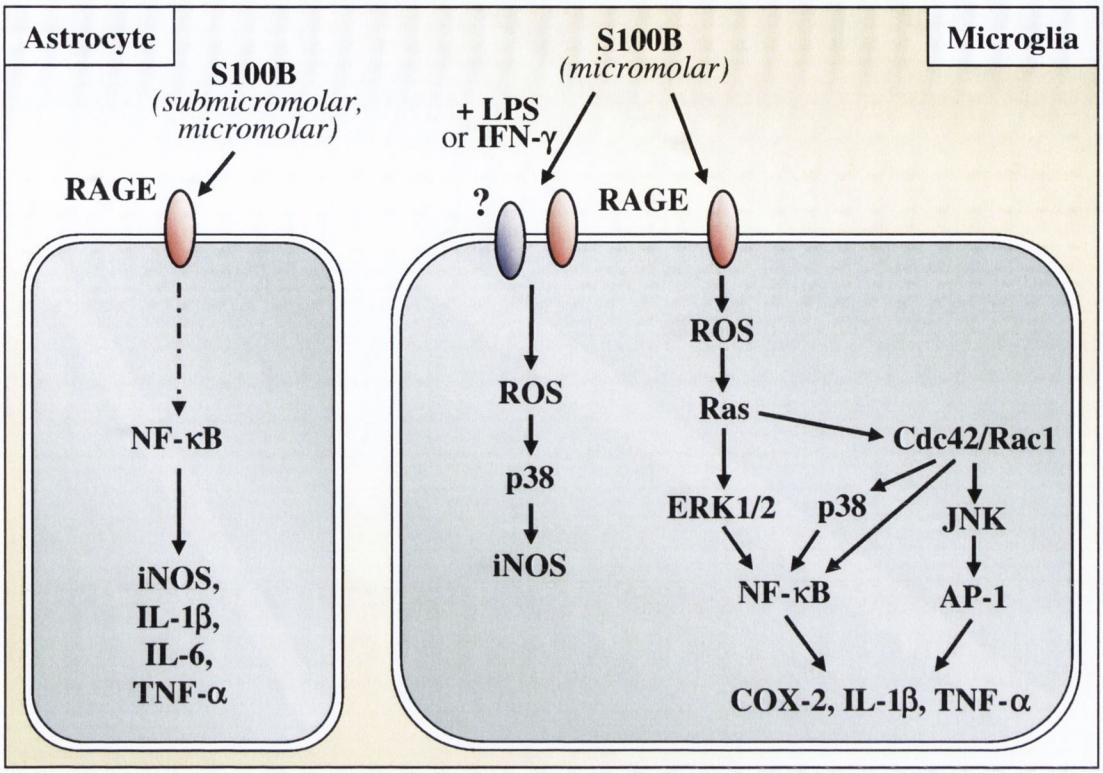


Figure 1.8. Proposed model of effects of S100B on astrocytes and microglia. (Left) RAGE engagement by S100B results in NF-kB-dependent upregulation of IL-1ß, IL-6, TNF-α and iNOS expression. (Right) At high doses S100B synergizes with IFN-γ to upregulate iNOS expression and NO production in microglia. The molecular mechanism by which S100B synergizes with IFN-γ is not known. At high doses S100B also causes a RAGE-dependent (over)production of ROS likely via activation of the NADPH oxidase complex. In turn, ROS activate a Ras-Rac1-Cdc42-MKK4/7-JNK-AP-1 pathway and a Ras-Rac1-NF-kB pathway with ensuing upregulation of expression of COX-2, IL-1ß and TNF-α expression. A Ras-MEK-ERK1/2- NF-kB pathway and a Ras-Rac1-Cdc42-MKK6-p38 MAPK-NF-kB pathway might contribute to upregulation of IL-1ß and TNF-α expression. Schemes do not take into account multimerization of extracellular S100B and S100B-induced RAGE oligomerization and or RAGE oligomer stabilization (Donato R *et al.*, 2009).

1.3.2.2. Animal Studies

The role of S100B has been found using different models such as S100B knockout mice, S100B over-expression mice and infusing S100B or S100B antiserum to mice. Hydén H and Lange PW (1970) reported the first observation that S100B protein is involved in learning and behaviour. They injected rats intraventriculary antiserum against the S100 protein during the course of the training and shown that antibodies against S100B can inhibit memory trace consolidation but did not affect the motor function of the animals. Those findings were in agreement with later finding by Lewis D and Teyler TJ (1986) and Fazeli MS *et al.* (1990), they demonstrated that anti-S100B antibodies could influence induction of long-term potentiation (LTP) in cultured hippocampal slices. The LTP is a measure of synaptic activity and is widely accepted to mediate hippocampus-controlled memory processes (Bliss TV & Collingridge GL, 1993). An other experiment by O'Dowd BS *et al.* (1997) reported that application of S100B-antiserum in chicks before or immediately after training on an avoidance task caused amnesia. On the other had, infusion low level of S100B protein in rats after training in a step-down inhibitory task facilitated long-term memory in a dose dependent manner (Mello e Souza T *et al.*, 2000).

Friend WC *et al.* (1992) reported the first S100B transgenic mice carrying 10-100 copies of the human S100B gene and over-expressing the gene in a gene dose-dependent manner in the brain of the mice. Several lines of evidence suggest that S100B transgenic mice exhibit impaired hippocampal function (Gerlai R *et al.*, 1994, 1995; Janus C *et al.*, 1995; Roder JK *et al.*, 1996; Whitaker-Azmitia PM *et al.*, 1997; Winocur G *et al.*, 2001). S100B transgenic mice also appear to have impaired short-term memory since they demonstrate differences in spatial and temporal exploratory pattern compared to the control mice (Roder JK *et al.*, 1996; Winocur G *et al.*, 2001). S100B over-expressing mice (Friend transgenic mice; Friend WC et al., 1992) showed behavioural abnormalities including female specific hyperactivity, lack of habituation to novelty and reduced T-maze spontaneous alteration rate (Gerlai R *et al.*, 1993; Gerlai R & Roder J, 1993; Gerlai R & Roder J, 1995).

Gerlai R and Roder J (1995) reported S100B over-expressing mice behavioural abnormalities were in a gene dose-dependent manner (8, 10, 70 and 110 copies of human S100B gene). Same transgenic mice exhibited impaired hippocampus LTP and spatial ability in the Morris water test (Gerlai R *et al.*, 1995).

Other experiments by Whitaker-Azmitia PM *et al.* (1997) showed loss of dendrites in S100B over-expressing transgenic mice (Friend transgenic mice; Friend WC et al., 1992). Another strain of transgenic mice with over expression of murine S100B gene (S100B level correlated with transgene copy number) demonstrated alterations in astrocytes morphology and axonal spouting, especially in the dentate gyrus, which is a part of hippocampal formation (Reeves RH *et al.*, 1994).

S100B knockout mice cognitive characterization is insufficient so far. S100B knockout mice were viable, fertile and did no exhibit any behavioral abnormalities up to 12 months of age (Xiong Z *et al.*, 2000). These mice did not show any alteration in the distribution and morphology of the astrocytes or neurons. However, Nishiyama H *et al.* (2002) demonstrated enhanced LTP in the S100B knockout mice as compared to wild-type animals. Applying the physiological concentration of the S100B protein could reverse this effect. S100B knockout mice had also in addition to enhanced LTP, enhanced spatial memory in the Morris water maze test and enhanced fear memory in the contextual fear conditioning. This data seems rather contradictory but a study by Schulte-Herbrüggen O *et al.* (2008) reported significant increase hippocampal BDNF in S100B knockout mice and suggested that BDNF may represent an endogenous attempt to compensate trophic effect of S100B.

Overall, the findings of animal studies indicate that S100B is involved in the cognitive functions such as spatial and nonspatial memory and learning. The evidence suggest that a balanced S100B level in the synapses and the extracellular space is needed for the normal memory and learning abilities (Rothermundt M *et al.*, 2004b). The process of acquiring and recalling information is disturbed by too much or too little S100B protein.

1.3.2.3. Association studies with S100B variants

There are several association studies performed for S100B, described in detail in chapter 3. Briefly, studies have shown some evidence for association between S100B gene SNPs and several psychiatric disorders including SZP, BPAD and major depressive disorder (MDD). Liu J *et al.* (2005) reported association between schizophrenia and S100B gene SNP rs11542311 and a haplotype spanning rs11542311 and rs9722 and discussed a genetic predisposition for increased S100B expression as a relevant pathophysiological mechanism. Roche S *et al.* (2007) reported an association between psychotic BPAD and 2 SNPs (rs3788266 and rs2839350). Kun Yang MD *et al.* (2009) has reported 2 SNPs (rs9722 and rs11911834) which did not display a significant association with MDD, but there were significant differences in age of onset according to the genotypes of S100B rs9722 and they found that the variants rs9722 and rs11911834 were related to the reoccurrence of depression. Lambert *et al.* (2007) has shown association between SNPs within the S100B gene and low cognitive performance and dementia in the elderly. Yosifova A *et al.* (2009) did not find association with S100B gene SNP rs3788266 in a Bulgarian sample.

1.3.2.4. Functional studies in BAPD and SZP

Many studies have found that the level of S100B protein in serum and cerebrospinal fluid is altered in psychiatric disorders. It is well accepted that an increased level of S100B is seen predominantly in the acute phase of SZP (Rothermundt M *et al.,* 2009). The severity of depression is positively correlated with S100B protein level, and antidepressive treatment reduces this level in parallel with improvement in depressive symptoms (Schroeter ML *et al.*, 2008). These authors hypothesized that elevated serum S100B during depressive and manic episodes of mood disorders may indicate alterations in astrocytes, which are reversed by antidepressive treatment. The study by Machado-Vieira R *et al.* (2002) also showed elevated serum S100B protein in drug-free bipolar patient during the first manic episode. There was no significant difference between serum S100B level and the number of previous

depressive episodes. Increased serum S100B has been also described in schizophrenia. Studies on S100B serum levels in patients and controls are shown in Table 1.11 for SZP and Table 1.12 for mania and depression.

Table 1.11. Previous studies investigating serum S100B in Schizophrenia (modified from Schroeter ML *et al.*, 2009).

Reference	**Serum S100B in ng/L (*N*)**		**Effect Size[a]**	**Medicated (N/Drugs)**
	Patients	**Controls**		
Wiesmann M *et al.*, 1999	165±138 (20)	54±31 (20)	3.58	All
Gattaz WF *et al.*, 2000	440±270 (23)	550±140 (23)	-0.79	All
Lara DR *et al.*, 2001	120±140 (20)	66±67 (20)	0.81	None
Rothermundt M *et al.*, 2001b	98±76 (26)	34±18 (26)	3.66	None
Schroeter ML *et al.*, 2003	241±152 (16)	113±53 (19)	2.4	All
Rothermundt M *et al.*, 2004a	65±31 (21)	38±8 (21)	3.38	None
Rothermundt M *et al.*, 2004c	73±32 (98)	44±15 (98)	1.93	None[b]
Schmitt A *et al.*, 2005	132±43 (41)	61±26 (23)	2.74	37
Steiner J *et al.*, 2006	90±30 (12)	80±20 (17)	0.5	10
Rothermundt M *et al.*, 2007	85±70 (12)	38±8 (12)	5.88	3
Ling SH *et al.*, 2007	119±59 (57)	67±22 (60)	2.36	None[c]
Schroeter ML *et al.*, 2009	111±32 (14) 73±72 (20)	42±70 (19)	-0.03 0.45	None 15

[a]According to Cohen J, 1988.

[b/c]At least 1 or 2 weeks before measurements. Generally mean +/- SD is reported. If studies examined several time points (Ling SH *et al.*, 2007; Rothermundt M *et al.*, 2001b, 2004c; Schroeter ML *et al.*, 2009), values of the first measurement are reported.

Table 1.12. Previous studies investigating serum S100B in depression and mania (modified from Schroeter ML *et al.*, 2008; Schroeter ML *et al.*, 2010).

Reference	Disorder	Illness duration in years	Serum S100B in ng/l (N)		Effect Size[a]	Medicated (N/Drugs)
			Patiens	Cantrols		
Rothermundt M *et al.*, 2001a	Major Depressive Episode in MDD	ns	91 ± 63 (28)	47 ± 23 (28)	1.91	24 (SSRI, TCA)
Machado-Vieira R *et al.*, 2002	Manic Episode of BPAD	ns	65 ± 68 (20)	18 ± 29 (20)	1.62	None
Schroeter ML *et al.*, 2002	Major Depressive Episode (3 BPD, 6 MDD)	4.1 ± 7.1	96 ± 173 (9)	< 100(12)	ns	2 (ns)
	Manic Episode of BPAD	9.6 ± 7.3	183±126 (11)			7 (ns)
Arolt V *et al.*, 2003	Major Depressive Episode in MDD	6.8 ± 10.4	95 ± 65 (25)	48 ± 24 (25)	ns	21 (SSRI, TCA)
Dietrich DE *et al.*, 2004	Remitted MDD	ns	106 ± 52 (12)	46 ± 14 (12)	1.96	All (ns)
Hetzel G *et al.*, 2005	Major Depressive Episode in MDD	ns	67 ± 48 (18)	34 ± 10 (18)	4.29	None
Andreazza AC *et al.*, 2007	Manic Episode of BPAD	12.8 ± 9.6	70 ± 33 (32)	44 ± 18 (32)	3.3	All (MS)
	Depressive Episode of BPAD	22.1 ± 10.7	76 ± 48 (21)		1.44	
	Remitted BPAD	17.3 ± 11.9	58 ± 25 (32)		0.78	
Schroeter ML *et al.*, 2008	Major Depressive Episode in MDD	6.0 ± 6.9	78 ± 53 (10)	18 ± 24 (10)	2.5	5 (AA, MS, SNRI, SSRI, TCA)

Yang K *et al.*, 2008	Major Depressive Episode in MDD	1.1±1.0	1440±620 (54)	1180±270 (35)	0.96	none
Zhang Y *et al.*, 2009	Remitted MDD Remitted MDD	ns ns	140±50 (6) 70±10 (6)	50±20 40±10 (6)	4.5 3.0	3 (Mirt, SSRI) 3 (Mirt, SSRI)

[a]According to Cohen J, 1988. Generally mean ± standard deviation. If studies examined several time points (Hetzel G *et al.*, 2005; Schroeter ML *et al.*, 2002, 2008), values of the first measurement are reported. Abbreviation: MDD major depressive disorder; BPAD bipolar affective disorder ; ns not specified; Mirt Mirtazapine; MS mood stabilizers; SNRI selective noradrenaline re-uptake inhibitors; SSRI selective serotonin re-uptake inhibitors; AA alpha-2-antagonists; TCA tricyclic antidepressants.

1.4. Background of the project

Our group performed a whole genome linkage scan in a sample of 60 BPAD families multiply affected with the disorder. The samples were genotyped using the Marshfield Human STRP Screening Set 16 comprising 401 microsatellite markers across the 24 chromosomes with a mean marker spacing of 8.03 cM (Cassidy F *et al.*, 2007; details also available at http://researchmarshfieldclinic.org/genetics/sets/combo.html). Our group identified some evidence for linkage to chromosomal regions 6p23, 9p21, 12q24, 16p13, and 21q22. A multi-point non-parametric lod score (NPL) score of 1.42 (P=0.078) was obtained at marker D21S1446/47 Mb chromosome 21q22 for a bipolar type I (BPI) disorder phenotype (personal communication, Dr. Siobhan Roche). An NPL score is a likelihood ratio score that measures the strength of linkage. Although this result was not significant the evidence for linkage observed at 21q22 was located 12Kb upstream of the S100B gene (Figure 1.9). This gene has been implicated in the pathology of psychiatric disorders (for review Rothermundt M *et al.*, 2004b).

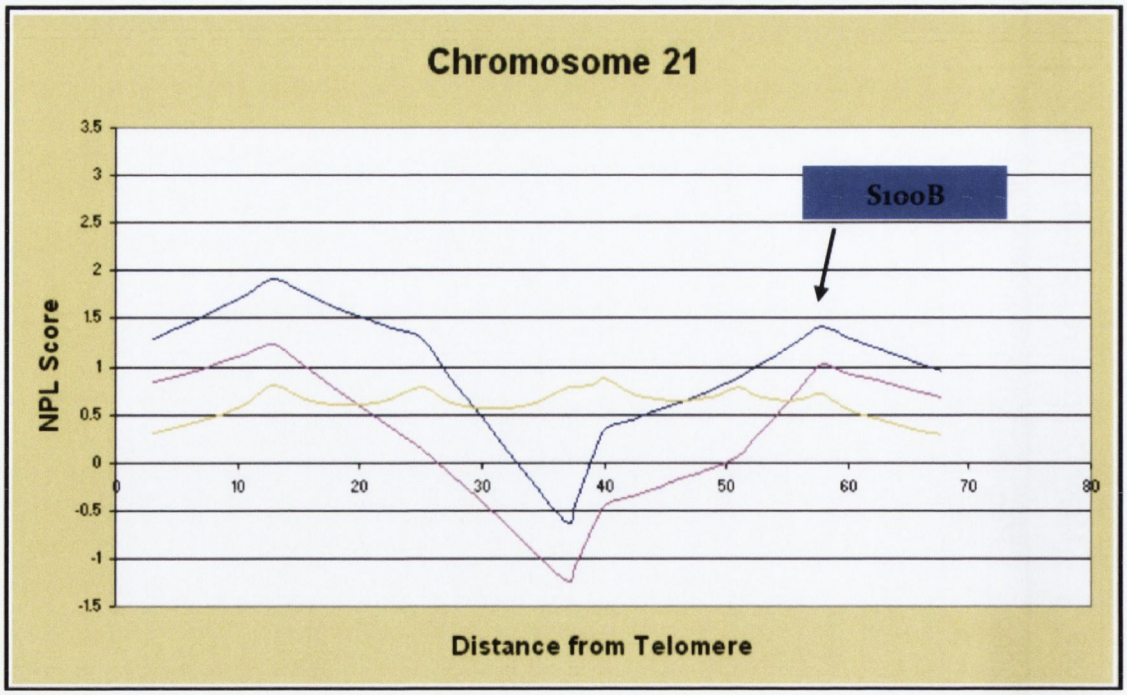


Figure 1.9. NPL scores across chromosome 21 (Dr. Siobhan Roche, personal communication)

As this gene was very interesting, our group decided to perform a follow up study. This study tested if the linkage signal at 21q22 could result from risk variants within the S100B gene and a family-based association analysis of S100B gene variants was performed in a collection of 151 BPI trios (families containing a proband and both parents). The Transmission Disequilibrium Test (TDT), which compares the transmission vs. non-transmission of marker alleles from parents to affected offspring, was used. Preferential transmission of a particular allele indicates that either this allele, or one in LD with it, is associated with disease susceptibility. This association study suggested that S100B SNPs within the promoter (rs3788266, P=0.031) and 3'UTR (rs2839350, P=0.022) regions of the gene were associated with BPAD (Figure 1.10). Evidence for association was also observed at the haplotype level (Roche S *et al.*, 2007).

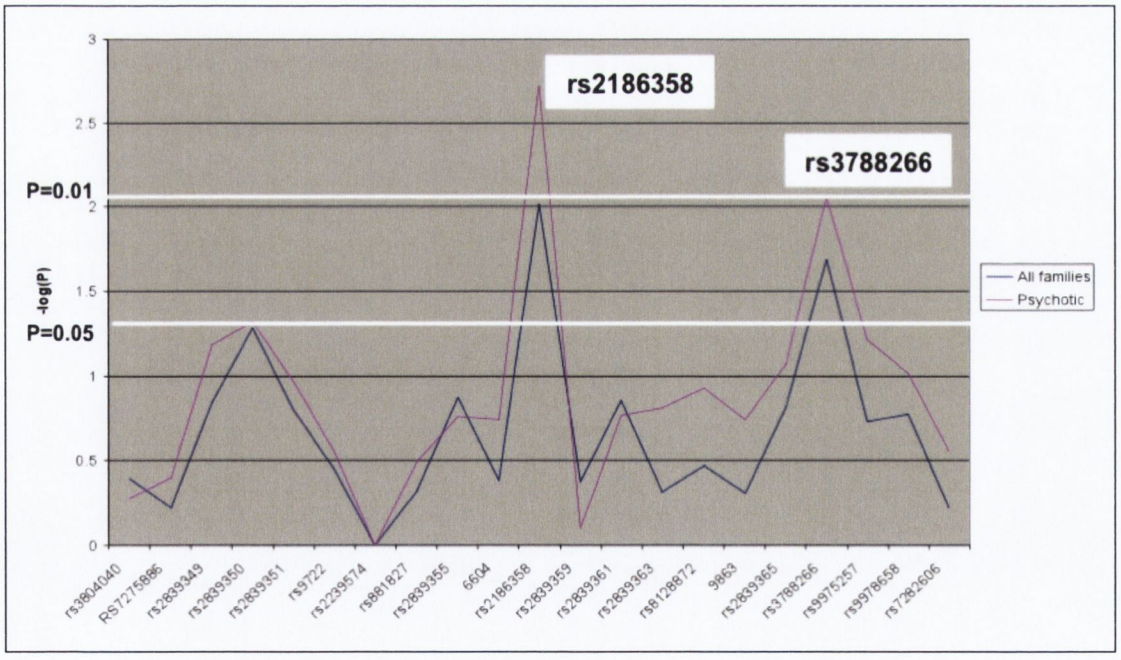


Figure 1.10. Association Analysis of S100B SNPs (Roche S *et al.*, 2007)

Furthermore, the association increased in significance, particularly for the promoter SNP, when the analysis was restricted to 86 trios with a psychotic

proband (rs3788266 G-allele P=0.0088, rs2186358: P=0.0159, Figure 1.9). This result was very interesting in light of the association of S100B with schizophrenia and suggests that disease variants within the gene may confer susceptibility to psychosis, a shared phenotype between BPAD and schizophrenia.

The location of the associated SNPs within non-coding regions of the gene suggests that they may affect gene expression. Bioinformatics analysis revealed that the disease-associated G allele of rs3788266 disrupts a Trex/MEF3 consensus recognition site (MACCYGA, position of rs3788266 A/G SNP underlined) located 1.4Kb upstream of the transcription start site (Roche S *et al.*, 2007). The Trex/MEF3 site is bound to by the Six-family of transcriptional activator proteins, suggesting that it could directly affect S100B expression. Six-family proteins are expressed in the brain and regulate brain development and possibly differentiation and maturation of neuronal cells (Ohto H *et al.*, 1998, Kawakami K *et al.*, 2000). Furthermore, there is a cluster of Six-family genes at chromosomal location 14q23, which has been also identified as a BPAD susceptibility locus by the same group (Kealey C *et al.*, 2005), suggesting that Six-family genes could also confer susceptibility to BPAD. Indeed, Six5 has been implicated in the pathophysiology of myotonic dystrophy, a neuromuscular disorder that also includes neuropsychiatric impairment (Hanson IM, 2001; Sato S *et al.*, 2002).

Further support for the location of the actual disease variant within the non-coding regions of the gene was obtained after re-sequencing of the entire S100B gene in 48 BPI probands. Only a single non-synonymous amino acid change was identified (Gln72Lys) which occurred in a single proband, thus ruling out altered biochemical function/post-translational modification as a general disease mechanism for S100B (Roche S, Personal Communication). Therefore, this thesis focused on the contribution of altered S100B gene expression and not biochemical function, to the pathogenesis of BPAD.

1.5. Aim of the project

This research sought to test the hypothesis that the S100B gene promoter variant rs3788266 is related to the altered expression of the protein that has been implicated in the aetiology of bipolar disorder and schizophrenia and to assess its impact on neurocognitive performance and symptom severity in SZP cases and controls.

The studies that make up this thesis are outlined below:

Study 1. Functional Assessment of a Promoter Polymorphism in S100B, a Putative Risk Variant for Bipolar Disorder: Functional effect of S100B promoter SNP rs3788266 are investigated using reporter gene assay and electrophoretic mobility shift assay in two different cell lines. Serum level of S100B protein is measured and investigated for association with rs3788266 variants in BPAD, unaffected relatives and healthy subjects. Further, Allelic expression imbalance of S100B gene is investigated.

Study 2. Investigation of Transcription Factor Binding to S100B Gene Promoter at SNP rs3788266: Putative transcription factor binding on rs3788266 is investigated. Further, the effect of rs3788266 on Six-family transcription Factor binding is investigated.

Study 3. The Effect of the S100B Promoter SNP rs3788266 on Measures of Clinical Symptoms, Neurocognitive Performance and Serum S100B protein levels: Impact of rs3788266 variant and increased S100B serum level on cognitive performance and symptom severity in SZP, SZA or healthy individuals is investigated.

Study 4. Association and Epistatic Analysis of S100B and SIX-Family Genes Variants with Schizophrenia and Bipolar Disorder: An association study is performed to investigate the association between rs3788266 and BPAD or SZP. SNPs at SIX-family genes are investigated. Finally, epistatic interaction between S100B and SIX-family genes are investigated.

Chapter 2

Functional Assessment of a Promoter Polymorphism in S100B, a Putative Risk Variant for Bipolar Disorder

2.1. Introduction

S100B is a calcium (Ca^{2+})-binding protein produced and secreted mainly by glial cells. It modulates the proliferation and differentiation of neurons and glia in addition to a number of intracellular functions (Rothermundt M *et al.*, 2004b). Intercellularly, S100B is involved in signal transduction via inhibition of protein phosphorylation, regulation of enzyme activity and of Ca^{2+} homeostasis. The S100B protein is functionally involved in the regulation of cell morphology via its interaction with elements of the cytoplasmic cytoskeleton. S100B also has extracellular functions and is actively secreted (Rothermundt M *et al.*, 2004b). The interaction of S100B with the Receptor for Advanced Glycation Endproducts (RAGE) implicates S100B as having important functions during development, tissue homeostasis and even in inflammatory, degenerative and tumour processes (Donato R, 2007).

Rothermundt M *et al.* (2003) and Sen J and Belli A (2007) reviewed what is known of S100B involvement in the regulation of degenerative and regenerative processes in the central nervous system. S100B exerts a protective effect if it is kept within normal physiological levels (nanomolar range) and this supports a role for S100B as a neurotrophic factor during development and nerve regeneration. In contrast, elevations of the protein into the micromolar range, as occurs following neurological traumas, can induce apoptosis and neurodegeneration (Donato R *et al.*, 2009). S100B is best known as a highly sensitive biochemical marker for brain damage and can signal glial cell activation, neural death or blood-brain-barrier dysfunction (Rothermundt M *et al.*, 2004b).

Levels of S100B have been found to be elevated in the CSF and/or serum of patients with various neuropsychiatric diseases including bipolar affective disorder (BPAD), major depression, schizophrenia (SZP) and Alzheimer's Disease (Rothermundt M *et al.*, 2004a, 2007; Schroeter ML *et al.*, 2002; Steiner J *et al.*, 2006; Arolt R *et al.*, 2003; Marchado-Viera R *et al.*, 2002; Andrezza AC *et al.*, 2006; Peskind ER *et al.*, 2001; Wiesmann M *et al.*, 1999). Elevated levels of S100B have been shown to be associated with negative

symptomotology and slower remission upon treatment in SZP (Rothermundt M *et al.*, 2001, 2004c; Ling SH *et al.*, 2007; Schroeter ML *et al.*, 2003).

The gene encoding S100B in humans is located on chromosome 21q22.3 (Allore R *et al.*, 1988). Evidence for genetic linkage to BPAD has been found in this region (Straub RE *et al.*, 1994; Cassidy F *et al.*, 2007; McQuilin A *et al.*, 2006; Lui J *et al.*, 2001; Badner JA *et al.*, 2002; Baron M, 2002). Linkage to this region has also been reported for SZP (Demirhan O & Tatemir D, 2003), Down's syndrome (Demirhan O & Tatemir D, 2003), and familial late-onset Alzheimer's disease (Kehoe P *et al.*, 1999). Our group previously performed a whole genome linkage scan in 60 Irish BPAD affected sib-pairs (ASPs) and obtained very modest evidence for linkage at 21q22, at a region more distal to previously reported linkage. The strongest evidence was observed for a marker at 21q22 (D21S1446/47 Mb, multipoint NPL = 1.42, P = 0.078, BPAD Type I disease model), which is located 12Kb upstream of the S100B gene (Cassidy F *et al.*, 2007).

It was hypothesized that the weak linkage signal at 21q22 could result from risk variants within the S100B gene and given the prior evidence implicating increased serum levels of S100B in various psychiatric disorders, our group performed a family-based association analysis of this gene in 125 BPAD Type I affected individuals and their parents. S100B single nucleotide polymorphisms (SNPs) within the promoter (rs3788266, P=0.031) and 3'UTR (rs2839350, P=0.022) regions of the gene were found to be associated with BPAD. A more significant association was detected for the promoter SNP when the analysis was restricted to 86 trios with a psychotic proband (rs3788266, P=0.0088) (Roche S *et al.*, 2007).

Re-sequencing of the entire S100B gene in 48 BPAD cases identified only a single non-synonymous amino acid change (Gln72Lys) that occurred in one case. This suggested to us that the associated SNPs were not tagging a common amino-acid changing polymorphism. *In silico* analysis with the Tfsitescan database (http://www.ifti.org/Tfsitescan/) revealed that the disease-associated G allele of rs3788266 disrupts a Trex/MEF3 consensus

recognition site (M**A**CCYGA, (position of rs3788266 A/G SNP underlined) located 1.4Kb upstream of the transcription start site. The Trex/MEF3 site is bound by the Six-family of transcriptional activator proteins (Himeda CL *et al.*, 2004), suggesting that rs3788266 could directly affect regulation of S100B gene expression. Six-family proteins are expressed in the brain and regulate brain development and possibly differentiation/maturation of neuronal cells (Ohto H *et al.*, 1998, Kawakami K *et al.*, 2000). In this study, we sought to test the hypothesis that the risk G allele of rs3788266 contributes to the increased expression of the S100B protein that has been implicated in the aetiology of BPAD and other psychiatric disorders.

2.2. Material and Methods

2.2.1. Bioinformatics Analysis of S100B Promoter Region

The association of rs3788266 genotype with S100B protein levels in serum suggested that the variant, which is located within a transcriptional regulatory site for Six-family proteins within the S100B promoter, might influence gene expression via control of promoter activity. We performed a multi-species comparison of the promoter region of the S100B gene using the Evolutionary Conserved Regions (ECR) browser (http://ecrbrowser.dcode.org/; Ovcharenko I *et al.*, 2004) to determine whether the SNP is located within a conserved region. We identified 8 conserved promoter regions between the human and Rhesus genomes (Figure 2.1., panel 4, ECRs 1-8). A minimal S100B promoter necessary for luciferase reporter gene expression in multiple cell types, including glial, overlaps with the most highly conserved region of ECR 1 (Castets F *et al.*, 1997; Tsoporis JN *et al.*, 2003). The S100B promoter deletion analyses performed by Castets F *et al.* (1997) and Tsoporis JN *et al.* (2003) also revealed a complex transcriptional regulation of S100B. Cell type-specific positive regulatory elements were identified which map to ECRs 2 and 5-8. Negative regulatory elements were also localized to a broad region extending from ECR 2 to a region just prior to ECR 5. A promoter region encompassing all 8 ECRs (6.6Kb promoter: 5'UTR, exon 1 and 627bp intron 1) induces maximal luciferase expression in cardiac myocytes and accurate

spatial distribution of a GFP reporter gene in transgenic mice. The *in silico* analysis revealed that the Trex/MEF3 site encompassing the rs3788266 variant is contained within the ECR 2 thus supporting a role for this region in regulation of gene expression.

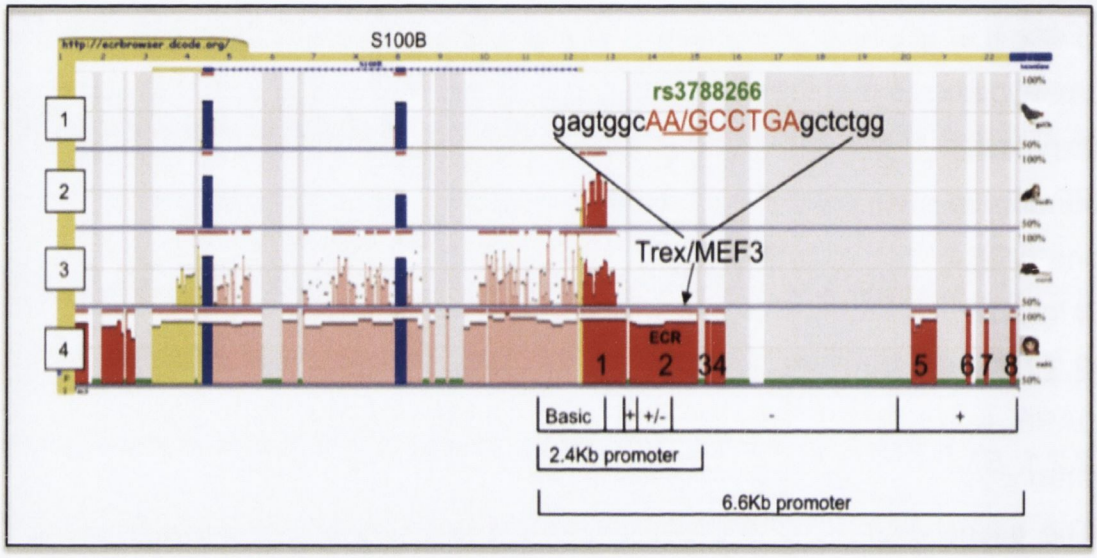


Figure 2.1. Evolutionary Conserved Regions in the S100B Gene: The S100B gene, depicted by the blue line in the top panel, is transcribed in the reverse orientation. Conserved coding regions are depicted in blue, intronic in pink, UTR in yellow and intergenic in red. Each panel represents alignment of the human gene to the chicken (1), dog (2), mouse (3) and Rhesus monkey (4) S100B genes (Ovcharenko I *et al.*, 2004).

2.2.2. Vector Construction

The pGL4 Vectors are luciferase reporter gene vectors that express firefly luciferase or Renilla luciferase. The Evolutionary Conserved Region 2 (ECR 2) (Ovcharenko I *et al.*, 2004) of the S100B promoter, which is 1.1 kb in length and contains the Trex/MEF3 regulatory site, which contains the rs3788266, (Figure 2.1.), was subcloned into the pGL4.23 minimal promoter-luciferase vector (Figure 2.2., Cat#E8411, Promega). Only the ECR 2 region of the promoter was included in order to restrict functional analysis to the portion of the promoter containing rs3788266.

First, the 1.1kb ECR 2 fragment was PCR-amplified using the primers 5'ECR2 XhoI (5'- GAGATACTCGAGGGGAAGCTGGTGAGGTTGTA) and 3'ECR2 BglII (3'- ATGCATAGATCTTGTTTTACAGAGGCGTGTGG) and genomic DNA from individuals homozygous for the G allele of rs3788266. PCR amplification resulted in insertion of the recognition sites for the restriction endonucleases XhoI and BglII at the termini of the ECR 2 fragment. The PCR fragment was then digested with XhoI and BglII and ligated to XhoI and BglII-digested PGL4.23. Positive subclones were initially screened for the presence of inserts by XhoI and BglII restriction digests and were sequenced using the pGL4.23F (AGTGCAGGTGCCAGAACATT) and pGL4.23R (AACAGTACCGGATTGCCAAG) primers, in addition to S100B primers spanning the ECR 2 sequence, to ensure that no mutations had occurred during PCR amplification. The resulting pGL4.23-ECR 2(G) subclone was then used as a template for the site directed mutagenesis experiments.

To create an alternate allelic version of the pGL4.23-ECR 2(G) subclone to include the rs3788266 allele A, the QuikChange-Site-Directed-Mutagenesis Kit (Cat#200518) and primers 5'-CCAGAGCTCAGGTTGCCACTCTGGGG-3' and antisense 5'-CCCCAGAGTGGCAACCTGAGCTCTGG-3' were used. The mutagenesis was performed according to the manufacturer's recommendations. The resulting pGL4.23-ECR 2(A) vector was sequenced, to ensure that the only difference between the A and G subclones occurred at the rs3788266 SNP site (sequencing as described at 2.2.3).

The purified pGL4.23-ECR 2 (A) and (G) plasmids were transformed into $CaCl_2$ competent XI1-Blue cells (Cat#200130, Strategene) using the standard heat shock method and plasmid DNA was isolated using the Qiagen MaxiPrep Kit (cat#12162, QIAGEN).

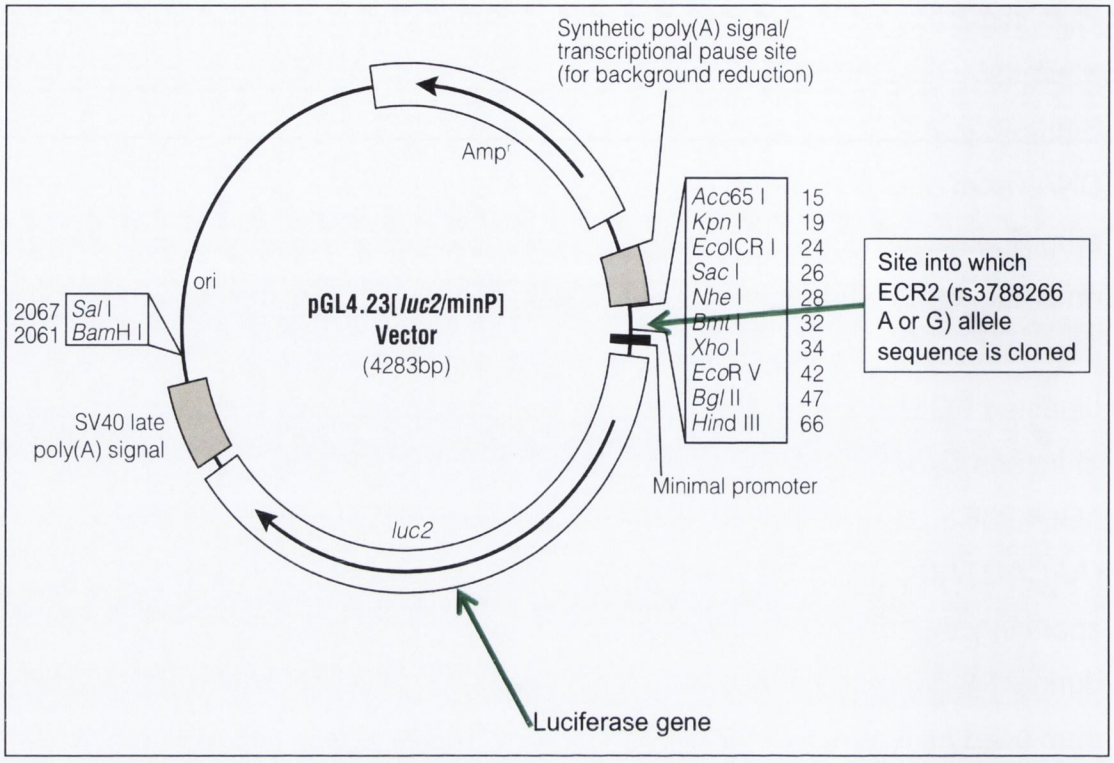


Figure 2.2. Diagram of the GL4.23[luc2/minP] - ECR2 Promoter vector used in this study. luc2: A synthetic version of the wild-type firefly luciferase gene that has been codon-optimized for mammalian expression and cleared of a C-terminal peroxisome target signal, two potential N-glycosylation sites, internal restriction sites and consensus sequences for genetic regulatory binding proteins. minP: A minimal promoter with a TATA box.

2.2.3. Sequencing

The Site ECR2, which contains A or G allele (1.2kb), was sequenced. Sequencing was carried out with approximately 400 ng of DNA, several primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit (#4337455, ABI). Cycle sequencing involves successive repeats of denaturation, annealing, and extension resulting in linear amplification of extension products by sequential addition of 3' fluorescent dye-labelled dideoxynucleotide triphosphates (dye terminators; ddNTPs) to the 3' end of a primer annealed to the template DNA. Four different dyes are used for sequencing to identify the adenosine, cytosine, guanine, and thymine bases (A,C,G and T), so all four bases can be detected and distinguished in a single capillary injection using ABI 310 Genetic Analyser. After terminating the growing chain and labelled with the dye that corresponded to the specific base. The sequencing reaction

consisted of a sequencing primer (3.2pmols), 1x sequencing buffer, BigDye reaction mix (AmpliTaq DNA polymerase and all four ddNTPs) and PCR fragments about 400ng in total volume of 10µl. The sequencing protocol was as follows: 96°C for 4 min, and than 96°C for 30 sec, 50°C for 15 sec and 60°C 4 min cycled 27 times.

The extension product was then purified using sodium acetate/ethanol precipitation method. 3µl 3M NaOAc, 62.5µl 100% (v/v) ethanol, and 14.5µl distilled water was added. After 15 min room temperature, the tubes were centrifuged at max speed for 20 min. 250µl of 70% (v/v) ethanol was added and the tubes were centrifuged at max speed for 5 min. After removing the supernatant and allowing the tube to fully air dry, the DNA was re-suspended in 25µl of template suspension reagent. Samples were heated to 95°C for 5 min and than incubated on ice until run on the ABI 310 sequencer.

2.2.4. Luciferase Reporter Gene Expression

The SNP rs3788266 is located within the transcriptional regulatory site for Sixfamily proteins suggesting that the variant might have a direct effect on S100B expression. To test this hypothesis we used the Dual-Luciferase Reporter Assay System. Genetic reporter systems are widely used to study eukaryotic gene expression and cell physiology. Applications include the study of receptor activity, transcription factors, intracellular signalling, mRNA processing and protein folding. Dual reporters are commonly used to improve experimental accuracy. The term "dual reporter" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the 'experimental' reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected "control" reporter provides an internal control that serves as baseline response (Adapted from Promega Inc.).

The DLRTM assay system utilises both the firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferase reporters, which can be measured

sequentially in a single sample, permitting one of the reporters to be used as a transfection control. For example, the S100B promoter constructs were generated using the firefly luciferase reporter and were co-transfected into cells along with a thymidine kinase (TK): Renilla luciferease control plasmid. The firefly luciferease activity level of each sample was normalised (firefly/rennila, 1:10) to the corresponding Renilla luciferase activity to control for between-well variations in transfection efficiency.

The U373MG glioblastoma and SH-SY5Y neuroblastoma cell lines were used to test for direct allelic affects of rs3788266 *in vitro.* Both cell lines are known to express S100B and therefore, should also express the required regulatory proteins. U373MG cells from ECACC (Cat#89081403) were grown in 10ml of Minimum Essential Medium Eagle (M5650, Sigma) supplemented with 10% fetal bovine serum (Cat#10270, Invitrogen) and 1x L-Glutamine (Cat#25030, Invitrogen) in T25 flasks to a density of ~ $2.1x10^6$ at 37°C and 5% CO_2. The SH-SY5Y neuroblastoma cell line from a four-year old female and are commonly used for neuronal cell functional studies (Biedler JL *et al.,* 1973). The SH-SY5Y neuroblastoma cell line was grown in 10ml of RPMI-1640 Medium (Cat#R0883, Sigma) supplemented with 10% fetal bovine serum (Cat#10270, Invitrogen) and 1x L-Glutamine (Cat#25030, Invitrogen) in T25 flasks to a density of ~ $2.1x10^6$ at 37°C and 5% CO_2. The cells were trypsinised with 1x Trypsin (Sigma).

The reporter constructs were introduced by Lipofectamine-mediated transfection (cat#11668-019, Invitrogen) into the U373MG or SH-SY5Y cell lines. Each construct was transfected in duplicate and cell extracts were assayed for luciferase activity assay using the Dual Luciferase Reporter (DLRTM) Assay System from Promega (Cat#E1910), according to the manufacturer's recommendations. The pGL4.74 vector (Cat#E6921, Promega), which expresses renilla, was used as internal control and was transfected at a ratio of 1:10 pGL4.74/pGL4.23-ECR 2 (A/G). The firefly luciferase activity level of each sample was normalised to the corresponding renilla luciferase activity to control for between-well variations in transfection

efficiency. Each vector assayed in duplicate per experiment and the entire experiment was performed in triplicate. Differences in the fold induction of reporter transgene activity between the A versus G versions of the Vector were statistically tested using an unpaired *t* test using GraphPad Prism 5.

2.2.5. Electrophoretic Mobility Shift Assay

Binding of protein complexes to the Trex/MEF3 site in the S100B promoter was investigated using a LightShift chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) kit from PIERCE (Cat#E3050) and according to manufacturer's recommendations. The LightShift chemiluminescent EMSA kit utilises biotin labelled DNA probes with visualisation of the label by streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate.

The EMSA is a technique (Figure 2.3.) used to observe DNA-protein interactions. The technique exists in many variations but all follow the principle of incubating a labelled oligomer, typically labelled with P32, and fluorescent moiety or biotin, with a nuclear extract (NE), presumably containing transcription factors and other regulatory proteins. This DNA-protein complex, along with an uncomplexed labelled DNA sample, is then subject to gel electrophoresis, either with poly-acrylamide or agarose, transferred to membrane (blotting) and visualised using a technique appropriate to the method of labelling. If the labelled DNA binds proteins contained in the extract its migration through the gel will be slower than that labelled DNA alone. In the context of understanding the role of polymorphisms it may be used to compare the DNA-protein interactions of different alleles, e.g., the creation/elimination of a transcription factor binding sites. Furthermore, the identification of protein may be detected with specific antibody.

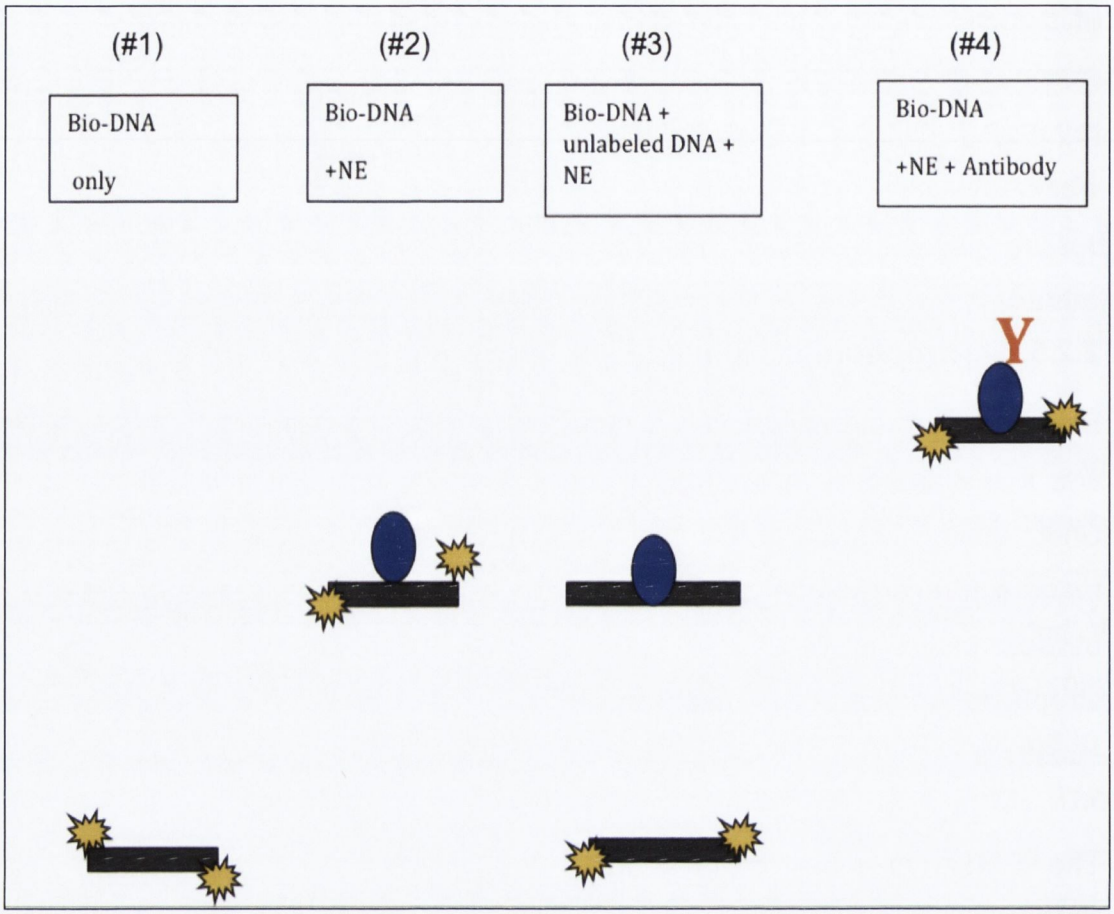


Figure 2.3. Methodology of the Electrophoretic Mobility Shift Assay. Reaction 1 (#1) defined the migration distance for the labelled probe in the absence of nuclear extract while reaction 2 (#2) defined the 'shift' caused by complexing with nuclear protein. Reaction 3 (#3) defined the specificity of the complex as determined by incubating labelled probe and nuclear extract with a 400M excess of unlabelled oligonucleotide of same sequence. Reaction 4 (#4) defined the migration distance for the labbled probe in the presence of the specific protein and antibody.

The complementary biotin labelled and unlabeled oligonucleotides corresponding to a 50bp sequence 5'- BIO -ACCACTTCCC CAGAGTGGCA **A/G**CCTGAGCTC TGGATTAACC CTTCAGTGTC-3' (Trex/MEF3 site underlined, they were synthesised by MWG Biotech, Ebersberg, Germany). The LightShift system is supplied with a positive control probe containing the DNA sequence for the Epstein-Barr nuclear antigen and corresponding biotin-labelled DNA. Labelled oligonucleotides were annealed to create the double-stranded DNA probe using a thermal cycler under the following conditions: oligonucleotides were diluted to a concentration of 1 pmol/µl in annealing

buffer (10mM Tris, 1mM EDTA, 50mM NaCl) and incubated at 95°C for 5 mins followed by a cooling regime of 70 x 1 min cycles with the temperature being reduced by 1°C at each cycle. Annealed oligonucleotides were stored at -20°C.

The U373MG and SH-SY5 cell lines were used for nuclear protein extraction. Each cell line was grown as described previously. Nuclear extract from the U373MG and SH-SY5Y cell lines was purified using the nuclear extract kit from Active Motive (cat # 40010) according to the manufacturer recommendations. Each cell line was cultured for 3 weeks prior to extract preparation. Approximately $8x10^7$ cells were harvested for nuclear extraction, and the resulting nuclear extract was stored frozen at -80°C. The total protein concentration of the nuclear extracts was determined using the BCA Protein Assay Kit from Thermo Scientific (cat#23227).

Protein-DNA binding reactions were performed according to the manufacturer's protocol. A total reaction volume of 20µl was used. Three reactions were performed for each oligonucleotide set (Figure 2.3). 50 fmol of biotin labelled double stranded DNA probes homozygous for the A or G alleles of rs3788266 were incubated with 1 µg poly(dI.dC), 1 µl 10x Binding Buffer, 40 pmol unlabelled DNA or 7 µg (SH-SY5Y) and 5 µg (U373MG) nuclear extract. The reaction was incubated for 20 min at room temperature. 5x loading dye was added and the samples were then loaded onto 7% polyacrylamide gel (made in 0.5X Tris boric acid EDTA, pH 8.0 (TBE) and electrophoresed until the dye front had migrated approximately ¾ the length of the gel. Polyvinyldifluoride (PVDF) membranes (cat#IPVH15150, SIGMA) were briefly washed in methanol and rinsed in 0.5X TBE prior to their use in blotting. The PVDF membrane and the gel were assembled in an electrophoretic transfer assembly and dry transferred at 380 mA (~100V) for 30 min in 0.5X TBE. Membranes were then placed face down on a UV (312nM) transluminator for 15 min in order to cross link the transferred DNA to the membranes. Cross-linked membranes underwent biotin-labelled chemiluminescent detection as described by the manufacturer. The

membrane was then placed in a film cassette and exposed to X-ray film for 1-3 mins and developed according to the manufacturer's instruction. Experiments were performed in triplicate for nuclear extracts from both the U373MG and SH-SY5Y cell lines. This experiment was repeated at both passages 12 and 18 to test for potential effects of cell line maturity.

The density of the bands was measured using the ImageJ 1.43 Software. The density of the each shifted band was calibrated by subtracting the background level of the signal. The percentage increase of the shifted band was calculated by comparison to the alternate allele's shifted band signal density.

2.2.6. Sample Preparation and Genotyping

Irish samples: The collection of the Irish BPAD family samples and the preparation of DNA for genetic analyses have been described previously (Cassidy F *et al.*, 2007). Serum was isolated from coagulated whole blood within one hour of venipuncture using a standard procedure consisting of a 10-minute centrifugation at 3000 rpm. Aliquots of the serum supernatant were stored frozen at -80°C. For Irish samples, the time of day when blood was taken for serum collection was as follows: BP cases (data available on 83/87); morning = 15 (19%), afternoon = 32 (38%), evening = 36 (43%). Unaffected relatives (data available on 65/67); morning = 6 (10%), afternoon = 32 (49%), evening = 27 (41%).

German samples: DNA samples and corresponding serum S100B measurements from German healthy control individuals (n=196) were kindly provided by Christa Hohoff from the Department of Psychiatry, University of Münster, Germany (Hohoff C *et al.*, 2010). Blood samples were centrifuged within 4 h after collection (morning, fasted), aliquoted, and frozen at -80°C until analysis.

All 350 Irish and German samples were genotyped for rs3788266 using the predesigned Taqman® Assay (ID: C_25800807_10) from Applied Biosystems

(Warrington, UK). Amplification and allelic discrimination for the assay were performed using either an Applied Biosystems 7300 Real-Time PCR system or an Applied Biosystems 7900HT Sequence Detection System.

2.2.7. Quantification of Serum S100B Protein

Irish samples: Serum samples from 87 Irish BPAD Type 1 cases (49 of which had psychotic symptoms) and 67 unaffected first-degree relatives of BPAD cases were used in this experiment. Only one case sample and one unaffected first degree relative (where available) from each family was included in these two samples. Serum S100B levels in the Irish samples were quantified using the Immunoassay S100 from COBAS (cat #: 03175243 190, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions (performed at NKI-AVL, Amsterdam, the Netherlands). The electrochemiluminescence immunoassay (ECLIA) is intended for use on Elecsys and COBAS immunoassay analyzers. The detectable limit of this Kit was < 0.02 µg/L. The principle of this assay is that 20 µL of sample, a biotinylated monoclonal S100-specific antibody, and a monoclonal S100-specific antibody labelled with a ruthenium complex react to form a sandwich complex. After addition of streptavidin-coated micro particles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the micro particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier. Results are determined via a calibration curve, which is instrument-specifically generated by 2-point calibration, and a master curve provided via the reagent barcode.

German samples: S100B concentrations were determined by applying the LIAISON Sangtec 100 assay (AB Sangtec Medical, Bromma, Sweden), a quantitative automated luminometric immunoassay according to the manufacturer's instructions. Briefly, the two-site sandwich assay was based

on paramagnetic particles coated with two monoclonal antibodies and a monoclonal tracer antibody labeled with an isoluminol derivative. The magnetic particles, assay buffer and sample were incubated together and washed to remove unbound material. Tracer was added and after a second incubation, unbound tracer was removed by a second washing cycle. Starter reagents were added and the S100B concentrations were determined from the chemiluminescence reaction induced (with light signal measured in relative light units (RLUs) directly proportional to the amount of S100B in the samples and the antibody highly specific for the S100ß monomer). The intra-assay (within-run) imprecision (CVs) is 6.4% at 0.11 µg/l, 3.1% at 0.31 µg/l, 2.8% at 1.6 µg/l, 3.9% at 2.6 µg/l, 2.8% at 9.9 µg/l, and 3.6% at 18.4 µg/l. The inter-assay variation (CVs) is 10.7% at 0.11 µg/l, 8.2% at 0.3 µg/l, 3.7% at 1.6 µg/l, 2.2% at 2.6 µg/l, 6.3% at 9.9 µg/l, and 3.2% at 18.4 µg/l. Analytical recovery ranges between 91 and 100% (Adapted from Hohoff C *et al.*, 2010).

To investigate genotypic influences on S100B protein expression, affected Irish BPAD Type I individuals, their unaffected relatives and German controls were grouped according to their rs3788266 genotype and between-group differences in S100B protein levels were statistically tested by an ANOVA test (Bonferroni corrected $P < 0.05$) using GraphPad Prism 5.

2.2.8. Allelic Expression Imbalance at S100B Gene

Measurement of mRNA allelic expression imbalance (AEI) has emerged as a powerful method for identification genetic variants that influence the expression of mRNAs (Yan H *et al.*, 2002; Bray NJ *et al.*, 2003). For the AEI method, relative levels of mRNA expressed from each of two alleles are measured using RNA isolated from a sample that is heterozygote for a SNP within the mRNA. To be able to detect a difference in mRNA expression from each allele requires use of a marker SNP located in transcribed regions. The allelic ratios are compared in genomic DNA and mRNA (cDNA). This method can identify variations when the differences between the expression levels of the two alleles differ by more than 20%. In this AEI method, each allele acts

as the control of the other. To be able to determine an AEI, which is indicative for a *cis*-acting polymorphism affecting the gene expression a deviation from 50:50 expression of each allele must be evidence (Quinn EM *et al.*, 2010). Because only single samples of mRNA isolated from the same tissue are used, variation between individuals that may arise from differences in environment factors, physiological states, or trans-acting factors are minimized (Lim JE *et al.*, 2007).

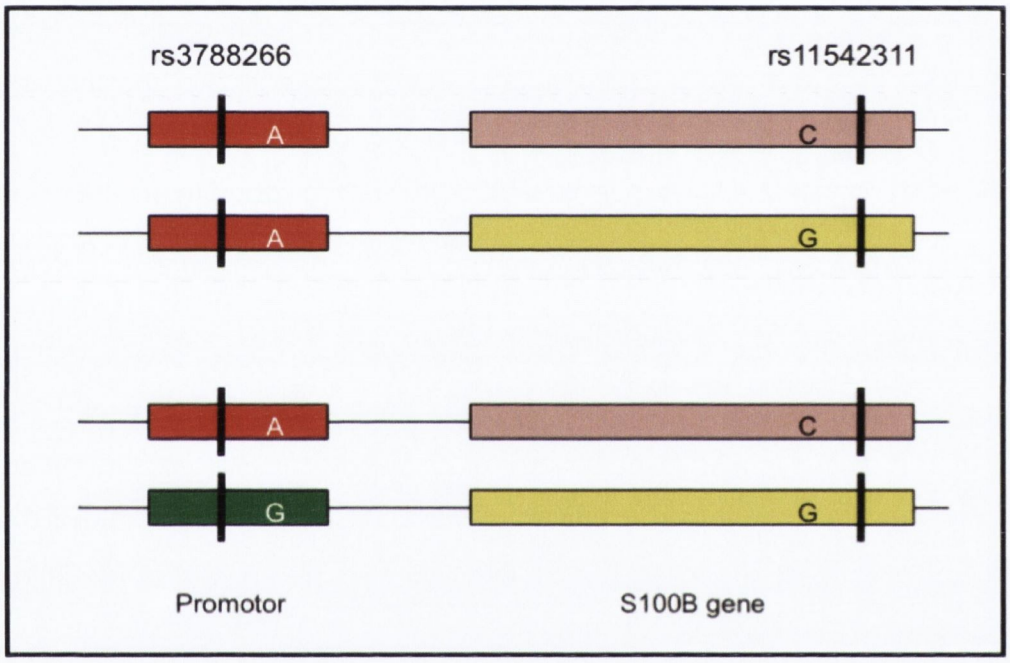


Figure 2.4. Design of Allelic Expression Imbalance experiment. The bases of the allelic expression imbalance is that mRNA from each of two copies of a gene from a heterozygote sample measured to determine if there is a difference in expression of the copies. To test a present allelic expression imbalance a SNP in an exon is needed. As rs3788266 is located in the promoter region of the gene, we used the SNP rs11542311 (C/G) in exon 2 that is in LD with rs3788266 (D'= 0.554 and r2=0.116). To be able to determine an imbalance at a promoter SNP there must be a deviation from 50:50 expression of each allele in samples heterozygote for both SNP rs3788266 and rs11542311 (bottom half) and there must be no deviation from 50:50 expression of each allele in samples heterozygote for exonic SNP rs11542311 and homozygote for promoter SNP rs3788266 (top half).

For this experiment 105 DNA samples isolated from the pre-frontal cortex (PFCx, Brodmann's area 46) of post-mortem brains from individuals with

BPAD, schizophrenia and normal controls obtained from the Stanley Foundation were genotyped for rs3788266 and rs11542311 with TaqMan genotyping assays from Applied Biosystems. From 105 brain DNA samples, 41 were heterozygote for rs11542311; 3 of those samples were AA homozygotes, 16 GG homozygotes and 22 AG heterozygotes for promoter SNP rs3788266.

Forty-one rs11542311 heterozygote samples total pons RNA were first reverse transcribed to cDNA with QuantiTect reverse transcription kit from Qiagen according to manufacturer's recommendations. The real-time PCR quantification is carried using a custom designed TaqMan® SNP genotyping assay for rs11452311 (C_2447155_20, Applied Biosystems, Inc., Foster City, CA, USA). Each sample was measured in triplicate for cDNA (2µl) and genomic DNA (20ng). Threshold cycles (Ct) for both gDNA and cDNA were determined using the SDS software of 7900HT fast real-time PCR-System (Applied Biosystems, Inc.). To be able to calculate the relative allele frequencies (rF) I used the formula: $rF = [1/(2\ \Delta Ct'+1)]$ where ($\Delta Ct' = \{[Ct^{Allele\text{-}1}$ (cDNA) – $Ct^{Allele\text{-}2}$ (cDNA)] – (ΔCt gDNA)}. Ct for cDNA is therefore normalized to that of gDNA representing a 1:1 ratio of both alleles. An AEI result of 0.5 indicates a 50:50 ratio of alleles in the cDNA sample as described by Quinn EM et al. (2010).

2.3. Results

2.3.1. Luciferase Reporter Gene Expression

I analysed the effect of the rs3788266 variant on luciferase reporter expression in U373MG and SH-SY5Y cell lines by cloning a segment of the S100B promoter encompassing the Trex/MEF3 site (i.e. ECR 2) into a test vector and found that the promoter activity was significantly increased in the presence of the risk G allele compared to A allele in both the U373MG (t= 4.701, *P*=0.0008; Figure 2.5. panel A) and SH-SY5Y (t= 5.67, *P*=<0.0001; Figure 2.7. panel B) cell lines.

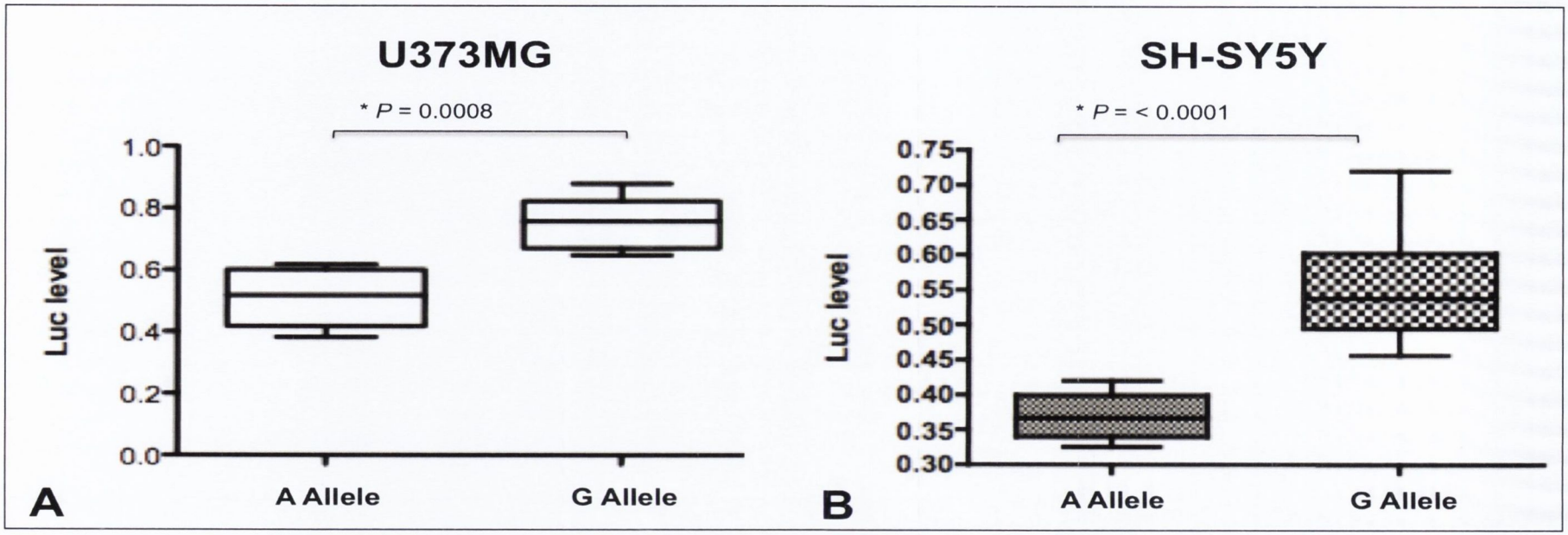


Figure 2.5. Luciferase Reporter Assay results for rs3788266 in U373MG glioblastoma cell line (A) and SH-SY5Y neuroblastoma cell line (B). The expression of constructs containing the risk G allele of rs3788266 were significantly increased compared to A allele in both cell lines.

2.3.2. Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were performed to determine whether the observed allelic effects of rs3877266 on S100B promoter activity were due to altered protein complex formation at the site, which contains rs3788266. EMSAs were performed in U373MG and SH-SY5Y cell lines in triplicate with nuclear extract prepared from cells at passage 12. We observed a similar pattern of shifted bands in both cell lines. Figure 2.6. displays an example of the EMSA results for the SH-SY5Y cell line at passage 12. Figure 2.7. displays an example of the EMSA results for the U373MG cell line at passage 12. For both cell lines, the same 4 shifted bands were detected at the G allele and A allele. However there were differences in the intensity of bands 1 and 3, with both bands showing stronger intensity for the G allele compared to A allele (Table 2.1 and Table 2.2.).

Table 2.1. SH-SY5Y cell line nuclear extract proteins binding density with -G-Allele (left) and -A-Allele (right) of rs3788266 for band 1 and 3 using EMSA.

	EMSA 1		**EMSA 2**		**EMSA 3**	
Band 1	-G-	-A-	-G-	-A-	-G-	-A-
	1.076	0.846	0.606	0.345	1.249	1.048
	21.4%		43.1%		16.1%	
	Average 26.8 %					
Band 3	-G-	-A-	-G-	-A-	-G-	-A-
	0.614	0.520	0.741	0.443	0.765	0.588
	15.3%		40.2%		23.1%	
	Average 26.2 %					

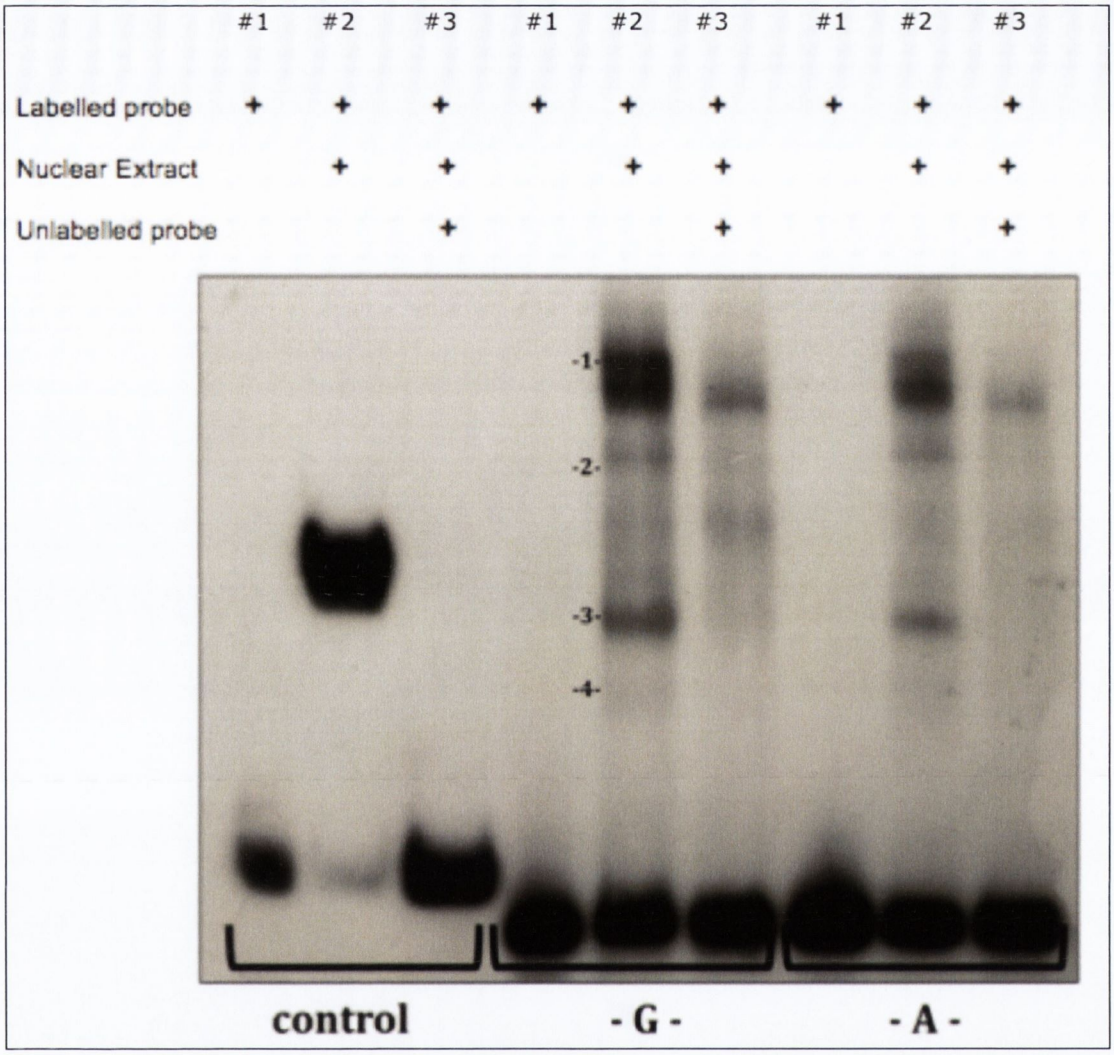


Figure 2.6. SH-SY5Y cell line nuclear extract proteins binding with G/rs3788266 (left) and A/rs3788266 (right) fragments using EMSA. For both alleles four shifted bands (labelled 1-4) were detected when reactions 2 and 3 were compared, which indicated specific binding to the S100B promoter fragment. The intensity of the bands 1 and 3 was stronger for the risk G allele compared to the A allele.

Table 2.2. U373MG cell line nuclear extract proteins binding density with -G-Allele (left) and -A-Allele (right) of rs3788266 for band 1 and 3 using EMSA.

	EMSA 1		EMSA 2		EMSA 3	
Band 1	-G-	-A-	-G-	-A-	-G-	-A-
	0.398	0.157	0.630	0.413	0.535	0.388
	60.6%		34.4%		27.5%	
	Average 40.8 %					
Band 3	-G-	-A-	-G-	-A-	-G-	-A-
	0.526	0.210	0.718	0.500	0.543	0.408
	60.1%		30.4%		24.9%	
	Average 38.4 %					

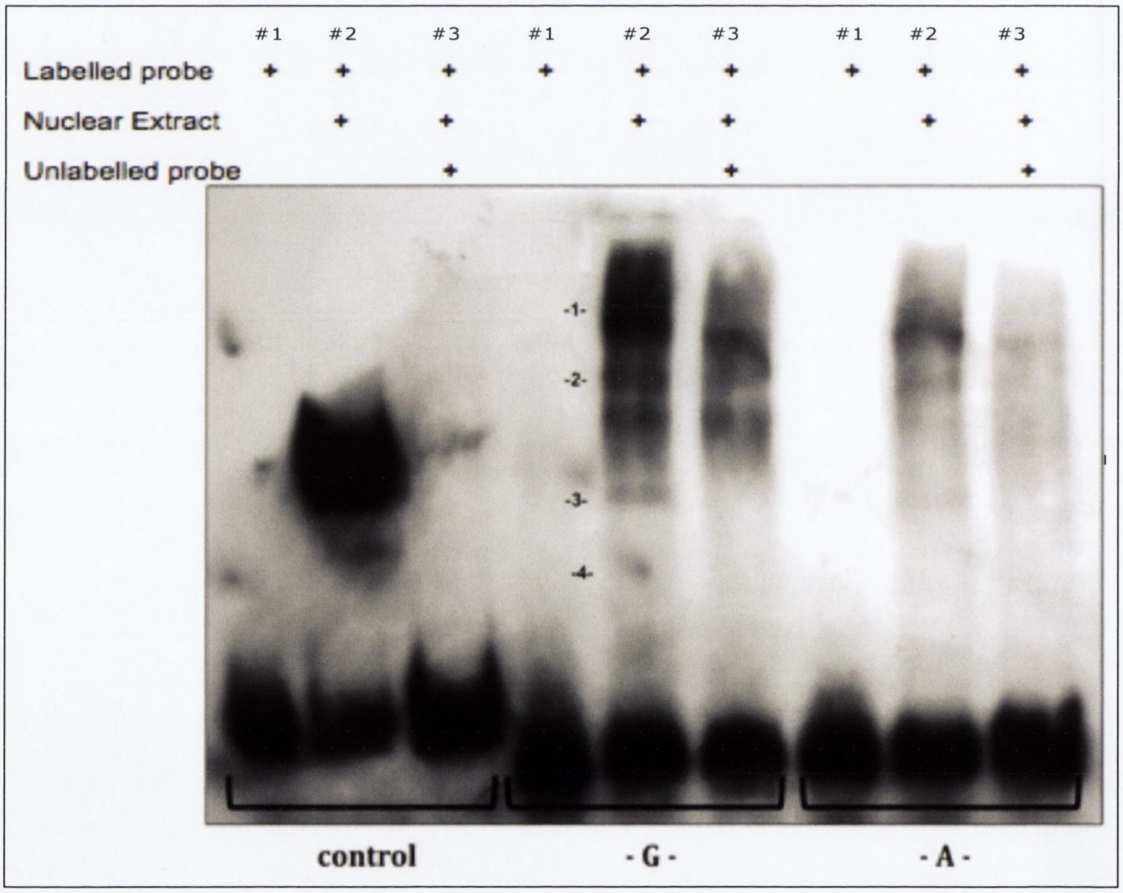


Figure 2.7. U373MG cell line nuclear extract proteins binding with G/rs3788266 (left) and A/rs3788266 (right) fragments using EMSA. For both alleles four shifted bands (labelled 1-4) were detected when reactions #2 and #3 were compared, which indicated specific binding to the S100B promoter fragment at passage 12. The intensity of the bands 1 and 3 was stronger for the risk G allele compared to the A allele.

To investigate the effect of cell maturity on the EMSA analysis we performed the experiments for a second time using nuclear extract from both cell lines at passage 18. For the SH-SY5Y cell line we observed the same shifted bands as before (Figure 2.8). For the U373MG cell line we observed a different result. As shown in Figure 2.9., bands 1 and 3 are not present for either allele. Two new bands are observed for the G allele which are not present for the A allele and one new band is observed for the A allele which is not present for the G allele. Figure 2.10. shows the differences between passage 12 and 18 for the U373MG cell line.

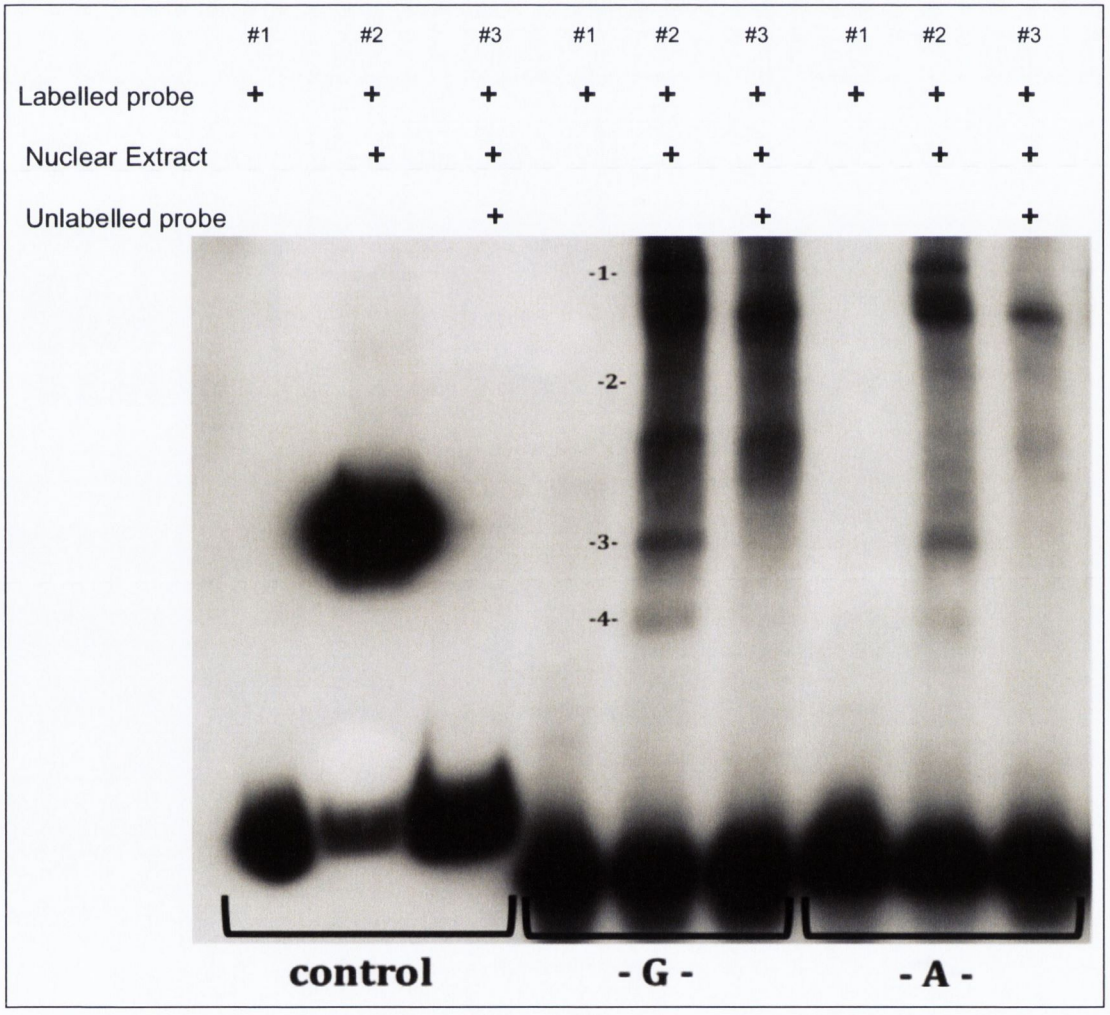


Figure 2.8. Analysis using nuclear extract from the SHSY5Y cell line at passage 18 did not identify any new bands for the G or A allele fragments compared with the nuclear extract from passage 12.

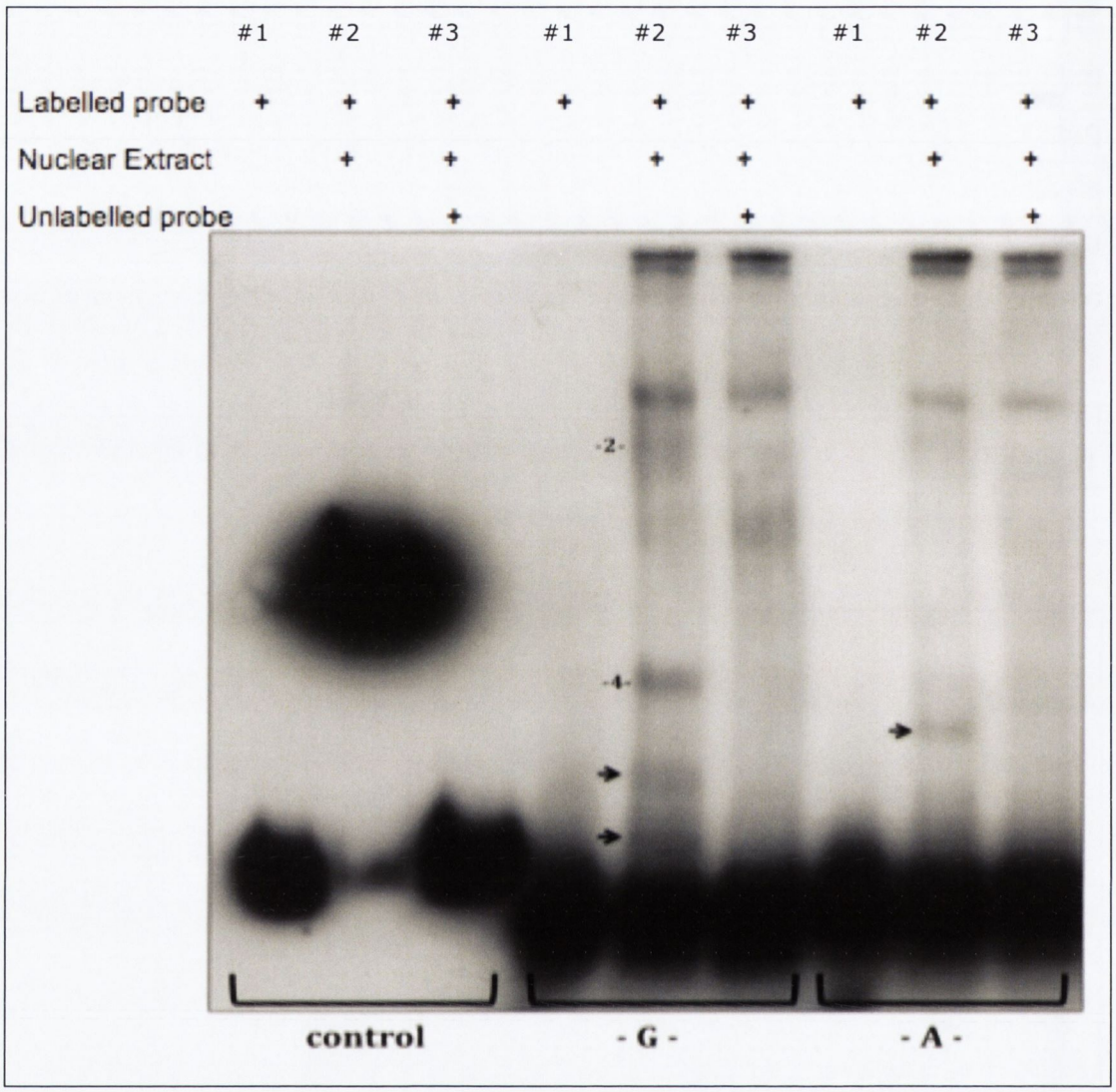


Figure 2.9. Analysis using nuclear extract from the mature U373MG cell line identifies 2 new bands for the G allele fragment and one new band for the A allele (arrows) at passage 18. These new bands were not observed when EMSA was performed using nuclear extract from the mature SH-SY5Y cell line (Figure 2.8).

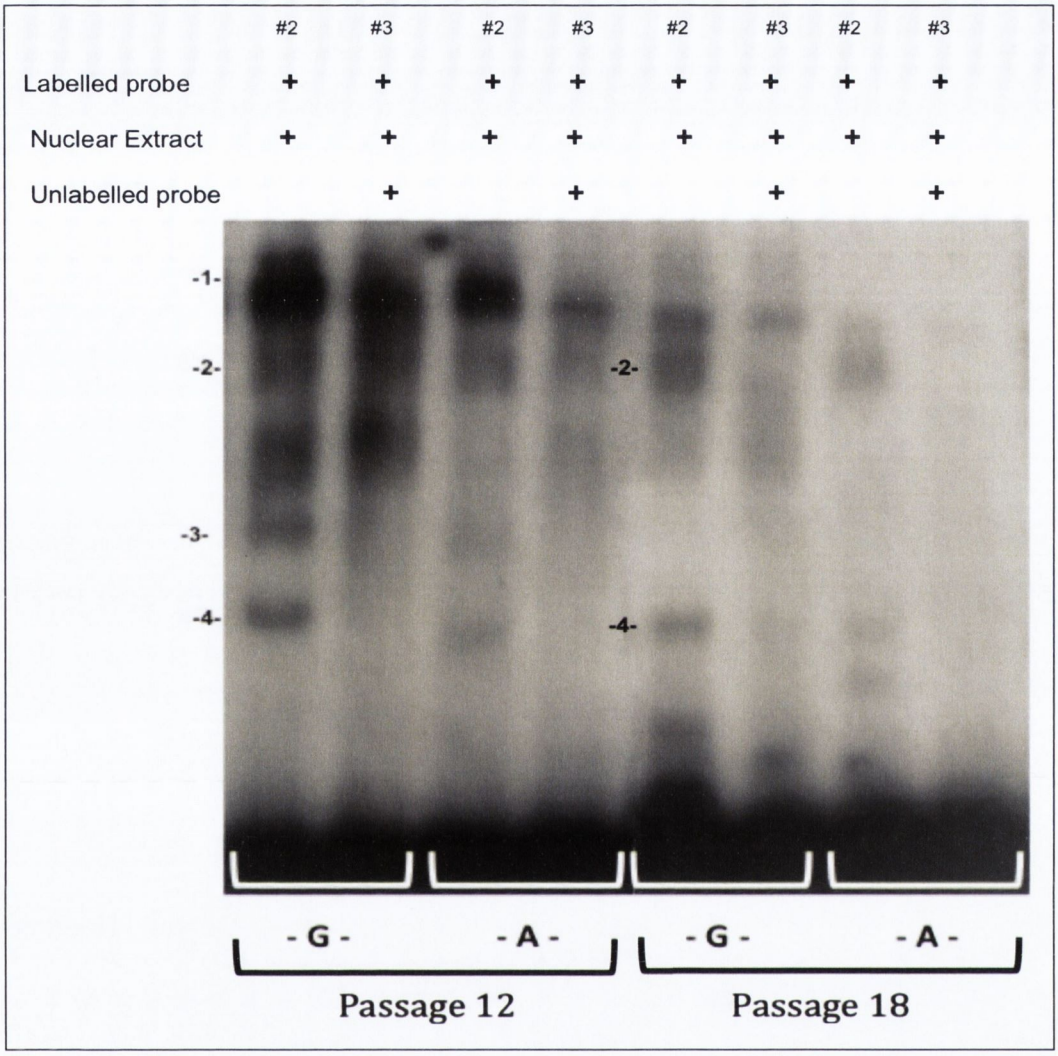


Figure 2.10. U373MG cell line nuclear extract proteins from passage 12 (left) and passage 18 (right) binding with G/rs3788266 and A/rs3788266 fragments using EMSA. For both nuclear extract two shifted bands (labelled 2 and 4) were detected to be common.

It is worth noting that we did observe a reduction in the proliferation rate of the U373MG cell line but not for the SH-SY5Y cell line between passages 12 and 18. For U373MG, this was evident from the longer time required to passage cells at passage 12 (~3 days) compared to passage 18 (~7 days). For passage 12, one quantity of medium was used to cultivate the cells but for passage 18, the medium was changed once during cultivation due to a change in medium colour that was likely due to a drop in PH. In addition, the concentration of nuclear extract derived for the SH-SY5Y cell line was constant across passages, but for the U373MG cell line, it dropped by ~34%

when comparing passage 18 to passage 12. This change in cell line performance for the U373MG cell line but not for the SH-SY5Y cell line between passages 12 and 18 may explain the different EMSA results observed for the two cell lines at passage 18. Overall, the results of the gel shift experiments suggest that the binding of proteins to the Trex/MEF3 site of the S100B promoter is influenced by the rs3788266 SNP.

2.3.3. Quantification of Serum S100B Protein

To investigate possible functional effects of rs3788266 *in vivo*, an analysis of S100B serum level by rs3788266 genotype was performed in 87 Irish BPAD cases, 67 of their unaffected relatives and 196 German control samples. The rs3788266 SNP was in Hardy-Weinberg Equilibrium in each of the 3 samples (P>0.05). The demographic information are summerised in Table 2.3.

Table 2.3. Demographic information for test samples

	Irish BP Type I Cases	**Irish Unaffected Relatives**	**German Healthy Controls**
Number	87	67	196
Age (s.d.)	38.91 (13.1)	56.3 (13.6)	30.3 (8.2)
% Male	32%	51%	63.3%
Age at Onset (s.d.)	25.2 (9.1)	n/a	n/a

As shown in Figure 2.11., there is a strong effect of rs3788266 genotype on S100B serum levels in each of the three sample sets tested. Importantly, the direction of the effect is the same for each sample with carriers of the G risk allele showing significantly higher levels of S100B than carriers of the non-risk A allele. Association analysis of S100B serum concentration with the rs3788266 variant is summerised in Table 2.4.

Because the method of S100B serum level measurement was different for the Irish cases and German controls, we did not perform a direct analysis to test for differences between these two groups.

Table 2.4. Association Analysis of S100B Serum Consentration With rs3788266 Variant in the Serum Samples (N=350).

	Genotype	**N (%)**	**S100B (ng/ml)**	***P*-Value**
Irish BP Type I cases	AA	18 (20.7)	0.039	**0.0001**
	AG	40 (46)	0.052	
	GG	29 (33.3)	0.067	
Irish unaffected-relatives	AA	13 (19.4)	0.045	**< 0.0001**
	AG	31 (46.3)	0.052	
	GG	23 (34.3)	0.076	
German healthy control	AA	48 (24.5)	0.049	**< 0.0001**
	AG	98 (50)	0.068	
	GG	50 (25.5)	0.074	

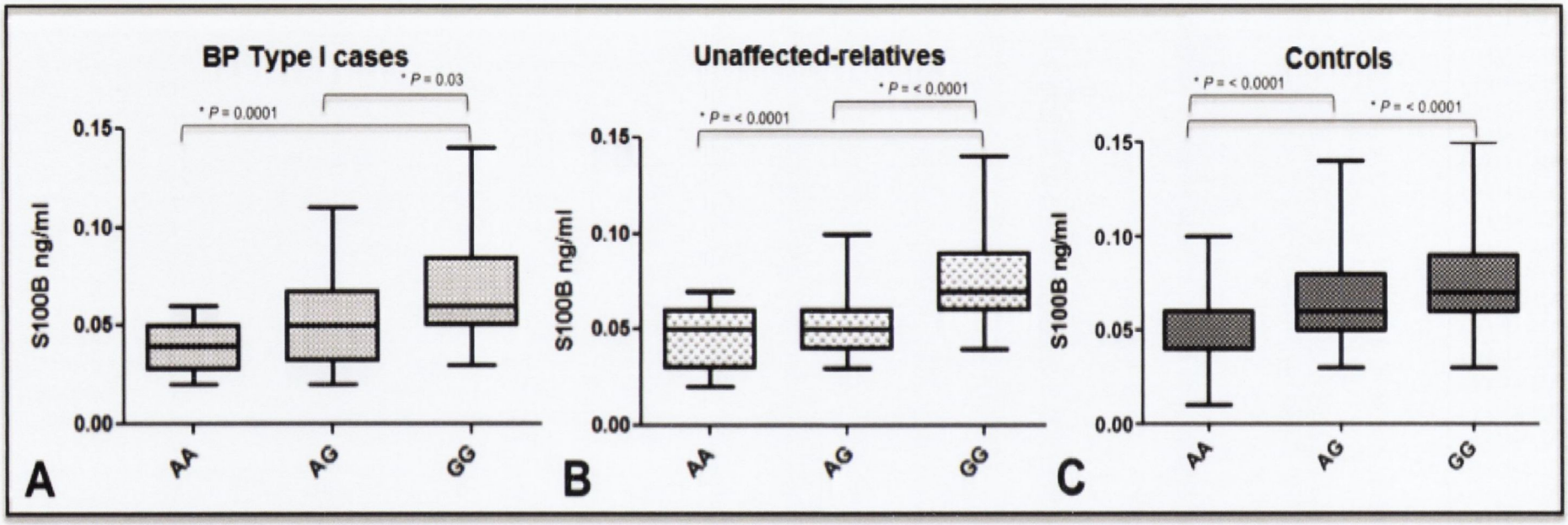


Figure 2.11. Analysis of S100B serum levels by rs3788266 genotype. The S100B protein level in serum was measured with ELISA. Graph **A** is BP Type 1 cases, graph **B** is the unaffected relatives and graph **C** is controls. Each graph is divided in 3 genotypic group AA, AG and GG. Carriers of the G risk allele show significantly higher levels of S100B than carriers of the non-risk A allele. The dose-dependent effect of G-allele was tested using an ANOVA followed by post-hoc pairwise analysis as displayed in the figure.

2.3.4. Allelic Expression Imbalance at S100B Gene

To be able to detect a difference in mRNA expression level from each allele of rs3788266 we used the AEI method. As the rs3788266 variant was in the promoter region we used SNP rs11542311 at Exon 2 to test for AEI. As shown in Figure 2.12., allelic expression imbalance was not observed at S100B.

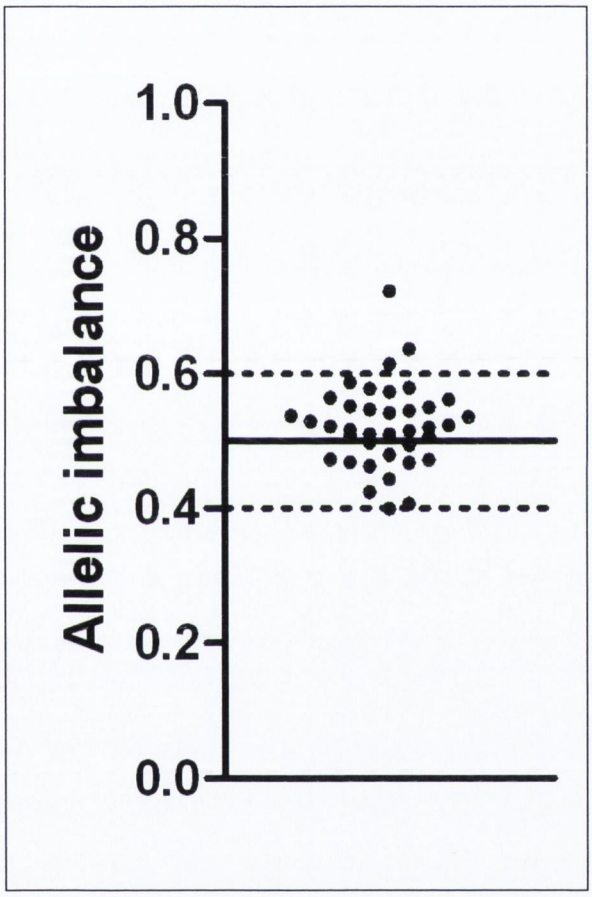


Figure 2.12. The Allelic expression imbalance analysis of rs3788266. The y-axis represent the allele frequencies at coding marker SNP in the complementary DNA corrected by the genomic DNA ratio. A total of 41 heterozygote individuals were examined only 3 showed an allelic imbalance > 0.6.

2.4. Discussion

A study by our group identified a linkage peak on chromosome 21q22 with a genome-wide scan in BPAD. The peak marker was located upstream of S100B. I hypothesized that the linkage may result from pathogenic disease variant within S100B and performed an association analysis, which revealed 2 SNPs (rs2839350 and rs3788266) significantly associated with BPAD. Interestingly, rs3788266 was located within the consensus-binding site for transcription factors and suggested that this variant may directly affect S100B gene expression. Using both *in vivo* and *in vitro* experimental approaches, we set out to investigate whether the rs3788266 promoter variant exerts functional effects on the activity of the S100B gene.

A direct influence of this variant on S100B promoter activity was supported by the luciferase reporter assay which demonstrated increased promoter activity in the presence of the disease associated G allele versus A allele of rs3788266 in both U373MG and SH-SY5Y cell lines. The *in vitro* expression data reported here are supported by the EMSA results where the binding affinity of U373MG and SH-SY5Y protein complexes to the S100B promoter was stronger for the G compared to the A allele promoter fragment. There was allele specific binding when we used the protein complex from U373MG cells at a later stage of passage. Differential affinity and allelic effect on binding of transcription factors found at this study suggest that altered transcription factor binding may be the mechanism for the altered expression of S100B seen in BPAD and SZP subjects.

In-Silico analysis by our group (Roche S *et al.*, 2007) suggested Six-family protein binding to the site, which contains the SNP rs3788266. Other In-Silico analysis is required to find other possible transcription factors binding to rs3788266. We cannot exclude the possibility that transcription factors other than those binding to rs3788266 are binding to the oligonucleotide during the EMSA. A limitation of the EMSA method is that the nuclear extract proteins are not at chemical equilibrium during the electrophoretic steps which might also lead to denaturalizing of possible transcription factors which might bind to

rs3788266. Other limitations of EMSA are that rapid dissociation during electrophoresis can prevent detection of complexes, while even slow dissociation can result in underestimation of binding density (Hellman LA & Fried MG, 2007). ChIP analysis would be a better method for determining the analysis of protein complex formation at promoter sites but requires specific antibodies to known transcription factors. The advantage of EMSA as used as a discovery tool here is that you do not need to know what is binding but can still show a functional allele-specific effect. What is relevant to this study is allele-specific binding by transcription factors indicating the potential for a functional impact of the SNP on gene regulation therefore EMSA is a better method to investigate the affect of the rs3788266 on transcription factor binding.

We have also demonstrated that the G allele of rs3788266, which is associated with increased risk of developing BPAD, is also associated with increased S100B protein levels *in vivo* in three different samples. However, there are some limitations to the use of serum samples. The clinical status and levels of medication at the time of collecting the serum might influence the level of S100B. As I did not have information about the clinical and medication status of the patients, I would not use them in this analysis. The relationship between S100B levels and illness severity/treatment response of BPAD patients remains to be elucidated. Studies have shown that serum measures of S100B are an accurate reflection of levels in the cerebrospinal fluid and the brain (Rothermundt M *et al.*, 2004a; Reiber H, 2001). The protein is only ~10kD and is believed to freely cross the blood-brain-barrier into CSF and serum. The underlying causes of elevated levels of S100B in neurological and psychiatric disorders are unknown. It may result from identified glial cell abnormalities (Webster MJ *et al.*, 2005; Brauch RA *et al.*, 2006; Benes FM *et al.*, 1991; Cotter D *et al.*, 2001) or possibly an increased secretion from glial cells as part of a neuroprotective response, as supported by a preliminary study of first-onset schizophrenic patients (Steiner J *et al.*, 2006). In patients with major depression or SZ, S100B protein levels positively correlate with symptom scores/severity and tend to normalise in treatment responders yet remain elevated in non-responders (Rothermundt M *et al.*, 2004c; Schroeter

ML *et al.*, 2002; Arolt V *et al.*, 2003). These studies have identified S100B as a potential biomarker for illness severity and therapeutic response in psychiatric patients. The observation that rs3788266 has an effect on S100B serum level in independent case and control samples supports this variant as a functional SNP. The mechanism by which this SNP may increase BPAD risk is still to be determined but it is likely to be in combination with other genetic and environmental factors.

The comparison of serum levels between Irish case and German control samples, at first glance, indicates higher S100B levels in the control samples. This would be at odds with the literature. Because the method of serum collection and measurement differed for the two samples, we have not performed a direct comparison. The serum data was only used for within sample analysis of genotype effect on S100B levels. Our group also investigated genotypic effect of rs3788266 on S100B gene expression in BPAD post-mortem brain samples (n=105) obtained from the Stanley Foundation (pre-frontal cortex region BA46). Analysis showed a non-significant trend towards higher mean mRNA levels in the presence of the G compared to A allele. The lack of a significant effect of rs3788266 genotype on S100B brain mRNA expression in this preliminary study could be attributed to the limited statistical power to detect allelic effects or could result from genetic heterogeneity between the Irish and US populations the later being the source population of the Stanley samples. As it is not possible to obtain brain tissue from the BPAD cases used in the Roche et al. study, we instead focussed our investigation on possible indirect allelic effects of the variant on S100B protein levels in serum samples isolated from the same cases as used in the original genetic association study. But mRNA concentration can be different in different part of the brain as the study by Dean B *et al.* (2006) has shown.

Animal models provide further support for a role for S100B in psychiatric disorders and point towards possible biological consequences of elevated S100B in patients. Transgenic mice over-expressing S100B are hyperactive and exhibit attention and memory defects while mutant mice lacking S100B

exhibit enhanced synaptic plasticity, learning and memory (Rothermund M *et al.*, 2004b). Interestingly, S100B protein levels are also increased in the ouabain-induced rat model of mania (Machado-Viera R *et al.*, 2004).

Further supporting a role for S100B in the aetiology of psychiatric disorders is that several genetic studies have shown a relationship between SNPs in the S100B gene and SZP, BPAD and MDD (Liu J *et al.*, 2005, Roche S *et al.*, 2007, Yang K *et al.*, 2009). Also, Lambert JC *et al.* (2007) has shown association of SNPs within the S100B gene with low cognitive performance and dementia in the elderly. Finally, Rothermundt M *et al.* (2003) suggested that S100B levels are at least partly regulated by the serotonergic system, which is has an important role in the pathophysiology and treatment of many psychiatric disorders, such as anxiety, MDD, and SZP (Hohoff C *et al.*, 2010).

It is worth to mention that other SNPs in LD with rs3788266 might influence the expression levels of S100B. We didn't investigated the effect of the other SNP or haplotypes on gene expression rs9722 is a good candidate, as it is located 3'-UTR of S100B, 3'UTR typically contain important regulatory elements described to modulate gene expression, for example, U-rich motifs (Fu L *et al.*, 1999), AU-rich elements (Wilusz CL *et al.*, 2001), or microRNA target sites (Valencia-Sanchez MA *et al.*, 2006; Vasudevan S *et al.*, 2007), which might be the case for rs9722. The SNP rs9277 is in strong LD with rs3788266 (D'= 1.0 and r2=0.09). Our group recently found an association between psychotic BPAD and S100B gene SNP rs2186358 (data not published), which in located in intron. Gene expression may also be influenced by SNPs, which are located at intron of the gene as it is reported by Queen EM *et al.* (2010).

The allelic expression imbalance of rs3788266 was also investigated in 41 heterozygote post-mortem brain RNA samples. Allelic expression imbalance was not observed with rs3788266. This study had certain limitations that need to be taken into account. This experiment had small sample size and would

not have the power to detect small differences. Another limitation of this experiment is the SNP rs11542311 is not in strong LD with rs3788266.

In summary, our results demonstrate that rs3788266 influences S100B promoter activity whereby the presence of the G allele promotes increased gene expression and is associated with increased serum levels of the protein. This promoter SNP may be a functional susceptibility variant for BPAD by contributing to the increased S100B levels observed in BPAD patients. The location of the SNP within a Trex/MEF3 site suggests that the complexes could be comprised of Six-family transcription factors. Given that other transcription factors may potentially bind to this site, further molecular work is required to determine the exact binding site and transcription factor function disrupted by this SNP. It will be necessary to examine larger psychiatric GWAS datasets as they become available because they may provide further support for association between this SNP and a BPAD/psychosis phenotype.

Chapter 3

Investigation of Transcription Factor Binding to S100B Gene Promoter at SNP rs3788266

3.1. Introduction

A transcription factor is a protein that binds to a specific DNA sequence (enhancer or promoter region) and interacts to activate or repress the transcription of a particular gene. The activation of transcription by a transcription factor can be diverse and complicated (Figure 5.1.). The binding of transcription factors guide the RNA polymerase enzyme that will perform the transcription of genetic information from DNA to RNA into place at the transcription initiation start site. RNA polymerases also require activator, mediator and chromatin modifying proteins (Alberts B, 2002). Multiple factors that bind to several enhancers and promoter elements within a gene regulatory region can interact with each other to fine-tune the level and timing of transcription initiation (Klug WS *et al.*, 2007).

Based on similarities in the sequence of their target DNA binding sites, transcription factors can be grouped together. Some transcription factors are expressed in specific tissues and regulate expression of their target gene. Some transcription factors are only expressed during a specific time in the cell, e.g. during development or in response to external physiological signals. Some transcription factors can only be active when modified structurally, e.g. by phosphorylation or by binding to a co-activator such as a hormone. Different transcription factors may bind to a specific DNA sequence or one transcription factor might bind to several overlapping sequences. Some transcription factors may target different DNA binding sites in different tissues (Klug WS *et al.*, 2007).

Transcription factors have two different domains. These are clusters of amino acids that carry out specific functions to activate or repress transcription. The first domain binds to the specific DNA sequence in a regulatory side, known as the DNA-binding domain, and the second domain binds to the so called *trans*-activating or *trans*-repression domain which activates or represses the transcription of the gene through protein-protein interaction.

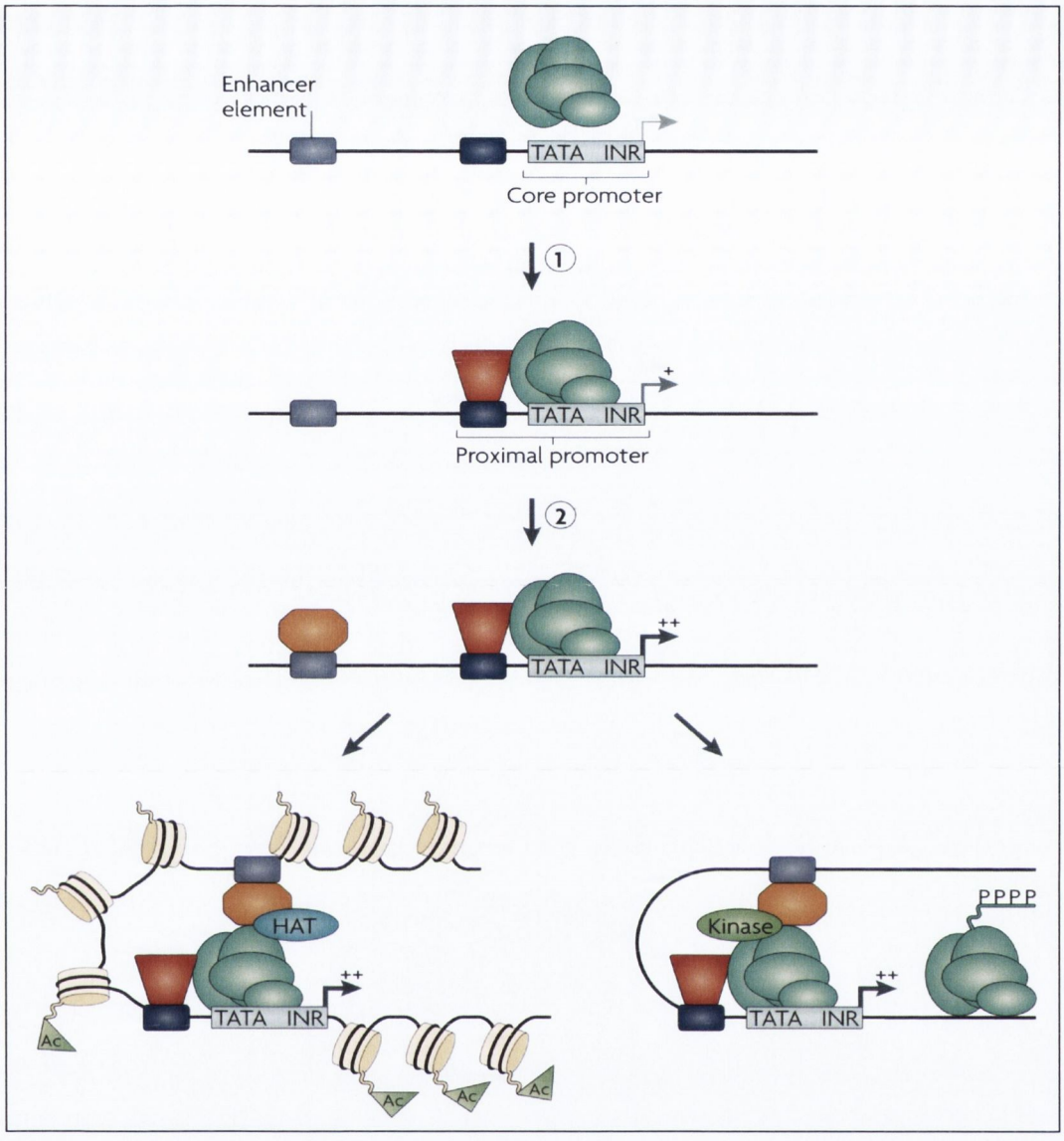


Figure 3.1. Transcriptional regulation by promoters and enhancers. General transcription factors (green ovals) bind to core promoter regions through recognition of common elements such as TATA boxes and initiators (INR). However, these elements on their own provide very low levels of transcriptional activity owing to unstable interactions of the general factors with the promoter region. Promoter activity can be increased (represented by +) by site-specific DNA-binding factors (red trapezoid) interacting with *cis* elements (dark blue box) in the proximal promoter region and stabilizing the recruitment of the transcriptional machinery through direct interaction of the site-specific factor and the general factors (step 1). Promoter activity can be further stimulated to higher levels by site-specific factors (orange octagon) binding to enhancers (step 2). The enhancer factors can stimulate transcription by (bottom left) recruiting a histone-modifying enzyme (for example, a histone acetyltransferase (HAT)) to create a more favourable chromatin environment for transcription (for example, by histone acetylation (Ac)) or by (bottom right) recruiting a kinase that can phosphorylate (P) the carboxy-terminal domain of RNA polymerase II and stimulate elongatio (figure taken from Farnham PJ, 2009).

Mutations in transcription factor DNA binding sites have been associated with several human diseases, e.g. cancer and psychiatric disorders (Jimenez-Sanchez G *et al.*, 2001; Hannenhalli S, 2008; Shah AK *et al.*, 2010; Kim CH *et al.*, 2006; Campbell DB *et al.*, 2006). Shah AK *et al.* (2010) reported two rare variants at the NRXN1 (neurexin 1) promoter in patients with schizophrenia and found that several transcription factors may be affected. Kim CH *et al.* (2006) reported a NET (norepinephrine transporter) gene promoter SNP, which is associated with attention-deficit hyperactive disorder and alters the activity of the promoter. This SNP creates a new consensus motif that is bound by a transcription factor and down-regulates the expression of the gene. Campbell DB *et al.* (2006) reported a MET (Mesenchymal epithelial transition factor) gene promoter SNP that is associated with autism and alters the activity of the promoter by influencing the binding of specific transcription factor complexes.

The S100B promoter deletion analysis performed by Castets F *et al.* (1997) and Tsoporis JN *et al.* (2003) revealed a complex transcriptional regulation of S100B. Cell type-specific positive and negative regulatory elements were identified which map to Evolutionary Conserved Regions 2 (ECR2), where my SNP of interest (rs3788266) is located (Figure 2.1. Page 51). Allore RJ *et al.* (1990) also reported that the promoter region of the S100B gene contains several potential regulatory elements including the cAMP-responsive elements CRE and AP-2.

It is important to investigate the transcription factor DNA binding sites in order to understand the transcription regulatory mechanism. Transcription factor databases are particularly useful for identification of transcription factor binding sites (Elkin PL, 2003). Databases like TRANSFAC (Matys V *et al.*, 2006) and JASPAR (http://jaspar.genereg.net/) are typically used to predict transcription factor binding sites *in silico*. The most common genome-wide technique for *in vivo* identification of binding sites for a specific transcription factor is chromatin immunoprecipitation (ChIP) of DNA bound to the transcription factor followed by hybridization to a pre-designed array (ChIP-chip; Hannenhalli S, 2008). More recently, this method has been advanced

using next-generation sequencing where the ChIP DNA is sequenced and the identity of target sites for the transcription factor is determined by mapping the sequence data back to the genome (ChIP-seq; Farnham PJ, 2009). *In vitro* techniques to investigate transcription factor binding sites include Electro Mobility Shift Assay (EMSA) and Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Hannenhalli S, 2008; Yang VW, 1998).

In chapter 2, I described my study of the rs3788266 SNP in the promoter of S100B. I found that this SNP affected expression *in vitro* and affected serum levels of the protein *in vivo*. Using EMSA, I identified that proteins from nuclear extract, most likely transcription factors, had binding affinity at the site of rs3788266 in U373MG and SH-SY5Y cell lines and found allele-specific transcription factor binding in both cell lines. In this chapter I describe my follow-up study of this EMSA work. I wanted to investigate which transcription factors are binding to the rs3788266 site in order to better understand the regulatory control of S100B expression.

3.2. Material and Methods

3.2.1. Bioinformatics Analysis

To identify *in silico* which transcription factors possibly bind to the A or G allele of rs3788266 in the S100B promoter, the following databases were used:

- TRANSFAC (Matys V *et al.*, 2006, www.gene-regulation.com);
- TFSEARCH (www.cbrc.jp/research/db/TFSEARCH);
- cluster buster (www.zlab.bu.edu/cluster-buster/cbust.html);
- Genomatix (www.genomatix.de);
- UCSC Genome Browser (www.genome.ucsc.edu).

3.2.2. Electrophoretic Mobility Shift Assay

EMSA methodology is as described in section 2.2.4. Briefly, nuclear extracts were obtained from the U373MG and SH-SY5Y cell lines. Both nuclear extracts and an EMSA Kit from PIERCE (Cat#E3050) were used for this experiment. SIX1 and SIX4 antibodies were used (Abcam, Cambridge UK) and SIX1 recombinant antibody (Abnova, Taiwan) to detect SIX1 and SIX4 transcription factor binding in the cell lines. The only difference from the previously described protocol is that 50% glycerol (1 µl), 100 mmol/L MgCl2 (1 µl) and 1% NP-40 (1 µl) were included in the reaction mix for the SIX4 antibody experiment. Those co-factors are included to protect the transcription factors from degrading during the presses of EMSA and/or to stabilize the DNA-protein binding.

3.3. Results

3.3.1. Bioinformatics Analysis

3.3.1.1. TRANSFAC Database Analysis

TRANSFAC compares a given DNA sequence with the known recognition matrices for a large number of known transcription factors within its database. The investigation of transcription factor binding for sequence containing the A or G allele of rs3788266 (5'-ACCACTTCCCCAGAGTGGCA**A/G**CCTGAGCTCTGGATTAACCCTTCAGTG TC -3') revealed that there are 6 putative transcription binding sites for the A allele (matrix names MEF3, C_EBP2, RFX1, C_EBPBETA1, FLI1 and RBP_Jk) and 5 putative transcription factor binding for the G allele (matrix names SZF1, MEF3, SMAD4, SMAD3 and FLI1). Details of the transcription factors that bind at these sites are provided in table 3.1.

Table 3.1. A list of putative transcription factor binding sites at the A or G allele of rs3788266 using TRANSFAC database (Fogel GB *et al.*, 2005).

Sequence	Matrix	Transcription Factors	P-value*
A-Allele:			
agctcagg**T**tgcc	MEF_3_0	Six1, Six2, Six4, Six5	5.74E-05
cagagtggca**A**c	C_EBP_2	CCAAT/enhancer binding protein	0.00016
ccccagagtggca**A**cct	RFX1_0	X-box binding protein RFX1	0.00030
agg**T**tgccactctg	C_EBPBETA_1	C/EBP-β	0.00067
cagg**T**tgccac	FLI1_0	FLI1	0.00086
Ttgccact	RBP_JKAPPA_0	RBP-J kappa	0.00086
G-Allele			
ccagagtggca**G**cct	SZF1_1_0	ZNF589	9.47E-05
agctcagg**C**tgcc	MEF_3_0	Six1, Six2, Six4, Six5	0.00014
ctcagg**C**tgccactc	SMAD4_0	SMAD4	0.00031
tggca**G**cct	SMAD3_0	SMAD3	0.00083
cagg**C**tgccac	FLI1_0	FLI1	0.00086

**p*-value is based on the relative measure of similarity. Binding site similarity is considered strong where $p<0.05$.

3.3.1.2. TFSEARCH Database Analysis

The investigation of transcription factor binding with the TFSEARCH database for sequence containing the A or G allele of rs3788266 (5'-ACCACTTCCCCAGAGTGGCA**A/G**CCTGAGCTCTGGATTAACCCTTCAGTG TC -3') did not reveal any putative transcription factor binding at either allele (Table 3.2).

Table 3.2. Results from the TFSEARCH database.

A-Allele	TFMATRIX entries with High-scoring: 1 GTGCTGACCA CTTCCCCAGA GTGGCAACCT GAGCTCTGGA TTAACCCTTC entry score <------- <A MZF1 87.8 <------ <A AML-1a 85.4
G-Allele	TFMATRIX entries with High-scoring: 1 AGTGCTGACC ACTTCCCCAG AGTGGCAGCC TGAGCTCTGG ATTAACCCTT entry score <-------- <A MZF1 87.8 <------ <A AML-1a 85.4

3.3.1.3. Cluster Buster Database Analysis

Cluster buster is a program to find clusters of pre-specified motifs in nucleotide sequence. The main function of the database is to find sequences (motifs) that regulate gene transcription such as enhancers and silencers. Cluster Buster did not detect any motifs at the rs3788266 site that were predicted to bind regulatory proteins.

3.3.1.4. Genomatix Database Analysis

Genomatrix (© 1998-2011 Genomatix Software GmbH, Munich, Germany) has different databases and web-based tools to investigate the transcription regulatory regions. The Genomatix matrix library currently contains more than 1,100 weight matrices each representing the DNA-binding sites of a transcription factor or a transcription factor family. To investigate the

transcription factors binding to the rs3788266 site two tools were used: SNPInspector and Matinspector (Cartharius K *et al.*, 2005). For those analyses Matrix Family Library Version 8.3 was used and the vertebrates (0.75/Optional) group was selected.

SNPInspector analyzes the potential effect of a SNP on a regulatory site by detecting transcription factor binding sites either deleted or generated by the nucleotide exchange. The investigation of transcription factor binding with SNPInspector for SNP rs3788266 revealed that the A/G substitution results in the loss of a transcription factor-binding site for insulator protein CTCF (V$CTCF.02, table 3.3). The A/G substitution results in the gain of a transcription factor-binding site for activating transcription factor 6 (ATF6; V$ATF6.01, table 3.3).

Table 3.3. A list of putative transcription factor binding sites modified by rs3788266 using SNPInspector in the Genomatix database

Inspecting sequence rs3788266 [rs3788266] (1 - 501):

[gnl|dbSNP|rs3788266 rs=3788266|pos=251|len=501|taxid=9606|mol="genomic"|class=1|alleles="A/G"|build=132]

SNP Position: 251

Allele: A -> G

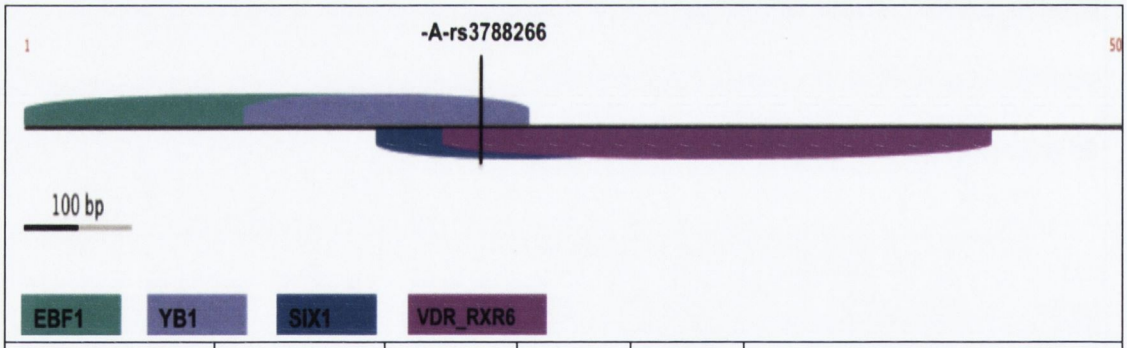
	Name of family/matrix	[a] RE	Opt.	Position	Strand	Core sim.	[b] Matrix sim.
Lost Sites	V$CTCF/CTCF.02	0.00	0.69	230-256	-	0.750	0.693
New Sites	V$EBOX/ATF6.01	1.19	0.93	239-251	-	1.000	0.978

[a]Random expectation (matches per 1,000 bp).
[b]Matrix similarity: A perfect match to the matrix gets a score of 1.00—a "good" match to the matrix usually has a similarity ≥ 0.80.

The investigation of transcription factor binding with Matinspector for sequence containing the A or G allele of rs3788266 revealed that there are 4 putative transcription binding sites for the A allele (matrix names V$EBF1.01, V$YB1.01, V$SIX.01 and V$VDR_RXR.06; table 3.4a) and 5 putative transcription factor binding sites for the G allele (matrix names V$EBF1.01, V$YB1.01, V$ATF6.01, V$MEF3.01 and V$VDR_RXR.06; table 3.5b).

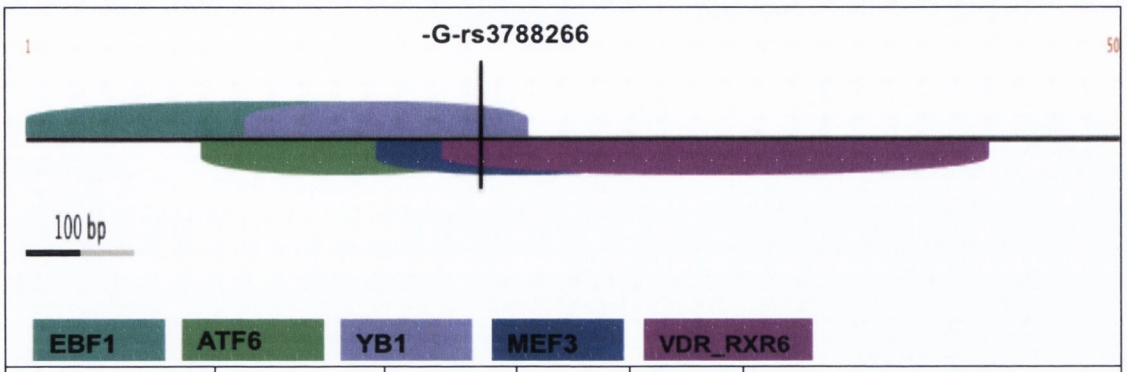
V$EBF1.01 is a matrix representing a DNA-binding site for the transcription factor Early B-cell factor 1 (Ebf1). V$YB1.01 is a matrix representing a DNA-binding site for the transcription factor Y box binding protein 1 (YB-1). V$SIX.01 is a matrix representing a DNA-binding site for the transcription factor SIX1, SIX4, and SIX5. V$VDR_RXR.06 is a matrix representing a bipartite binding site of VDR/RXR heterodimers, DR5 sites. The VDR is a member of the class of nuclear hormone transcription factors that binds as a heterodimer complex with RXR to bipartite vitamin D response elements (VDREs) to activate transcription of downstream target genes (Bi C & Rogan PK, 2004). V$ATF6.01 is a matrix representing a DNA-binding site for the transcription factor ATF6. V$MEF3.01 is a matrix representing a DNA-binding site for the transcription factors SIX1, SIX2, SIX4, SIX5 and SIX6.

Table 3.4a. A list of putative transcription factors that bind to A-Allele using Matinspector in the Genomatix database (bold and underlined is the SNP rs3788266 allele).



Matrix Family Information	Matrix[a]	TFs	Strand	Matrix sim.[b]	Sequence[c]
Neuron-specific olfactory factor	V$EBF1.01	Ebf1	+	0.928	accactTCCCcagagtggca**a**cc
Y-box binding transcription factors	V$YB1.01	YB-1	+	0.895	cagagTGGCa**a**cc
MEF3 binding sites	V$SIX.01	Six1, Six4 Six5	-	0.997	agcTCAGg**t**tgcc
RXR heterodimer binding sites	V$VDR_RXR.06	VDR/ RXR	-	0.765	gaaggg**t**taatccagAGCTcagg**t**t

Table 3.4b. A list of putative transcription factors that bind to G-Allele using Matinspector in the Genomatix database (bold and underlined is the SNP rs3788266 Allele).



Matrix Family Information	Matrix[a]	TFs	Strand	Matrix sim.[b]	Sequence[c]
Neuron-specific olfactory factor	V$EBF1.01	Ebf1	+	0.928	accactTCCCcagagtggca**g**cc
E-box binding factors	V$ATF6.01	ATF6	-	0.979	**c**tgCCACtctggg
Y-box binding transcription factors	V$YB1.01	YB-1	+	0.88	cagagTGGCa**g**cc
MEF3 binding sites	V$MEF3.01	Six1, Six2, Six4, Six5, Six6	-	0.906	agcTCAGg**c**tgcc
RXR heterodimer binding sites	V$VDR_RXR.06	VDR/ RXR	-	0.765	aagggttaatccagAGCTcagg**c**t

[a]Matrix name: V$ indicates vertebrates followed by an acronym for the factor the matrix refers to, and a consecutive number discriminating between different matrices for the same factor.
[b]Matrix similarity: A perfect match to the matrix gets a score of 1.00—a "good" match to the matrix usually has a similarity ≥ 0.80.
[c]red = ci-value >60. CAPITALS = core sequence. The maximum Ci-value of 100 is reached by a position with total conservation of one nucleotide, whereas the minimum value of 0 only occurs at a position with equal distribution of all four nucleotides and gaps. The core sequence of a matrix is defined as the (usually 4) highest conserved, consecutive positions of the matrix.

To compare the differences between the MEF3 and SIX1 matrices the MatBase 8.3 database from Genomatrix was used (www.genomatrix.de). The results for MEF3 is based on the publications by Spitz F *et al.* (1998) and

Parmacek MS *et al.* (1994). The results for SIX1 matrix is based on the publication by Himeda CL *et al.* (2004), Chai L *et al.* (2006) and Spitz F *et al.* (1998). There were differences between the matrix consensus of the 2 matrices (Figure 3.2.). The matrix length of MEF3 is 13 bp and SIX1 is 11bp. The difference between both matrices is that MEF3 would probably also bind SIX2 and SIX6 transcription factors in addition to the SIX1 matrix. The SIX1 matrix only contains the A allele of rs3788266 but the matrix MEF3 contains both SNP alleles. The matrix similarity for SIX1 (0.979) is higher than the MEF3 (0.906).

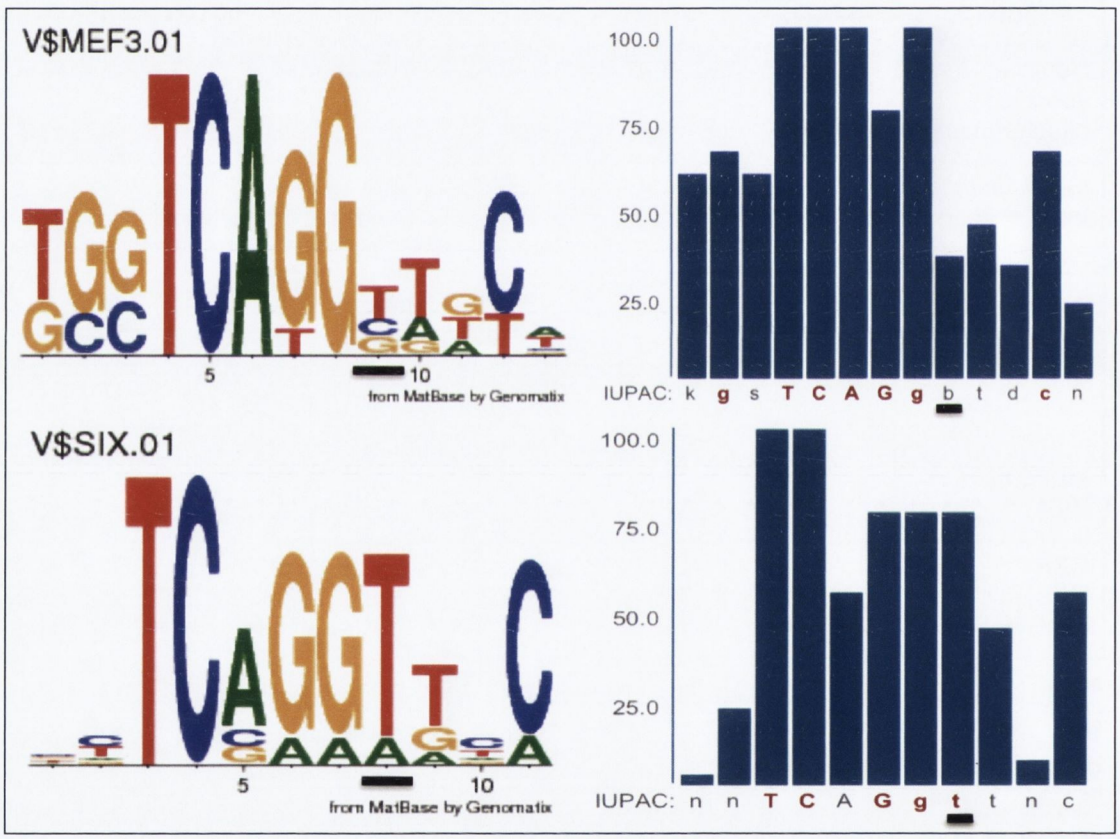


Figure 3.2. Comparison of Matrix MEF3 with SIX1. The location of SNP rs3788266 is underlined. Left represents the sequencing logo and right the IUPAC profile. The sequence logo represents matrix consensus, which is generated using the algorithm described in Crooks GE *et al.* (2004) and Schneider TD and Stephans RM (1990). The IUPAC string consensus is a representation of the matrix based on the following rules (Cavener DR, 1987): A single nucleotide (A,C,G,T) is shown if its frequency is greater than 50% and at least twice as high as the second most frequent nucleotide. Nucleotides marked **red** show a high information content, i.e. the matrix exhibits a high conservation (ci-value > 60) at this position. Nucleotides in capital letters denote the core sequence used by MatInspector.

3.3.1.5. UCSC Browser Analysis

The investigation of transcription factor binding using the UCSC Genome Browser is based on ENCODE transcription factor ChIP-seq - chromatin immunoprecipitation with antibodies specific to the transcription factor followed by sequencing of the precipitated DNA. The UCSC Genome Browser Human Mar. 2006 (NCBI36/hg18) genome assembly was used for this analysis. This analysis revealed 3 putative transcription factors (PU.1, EBF and NF-κB) binding to rs3788266, which are located 1,320 bases upstream of the S100B gene (Figure 3.3). It is unknown if the binding of the transcription factors is allele specific.

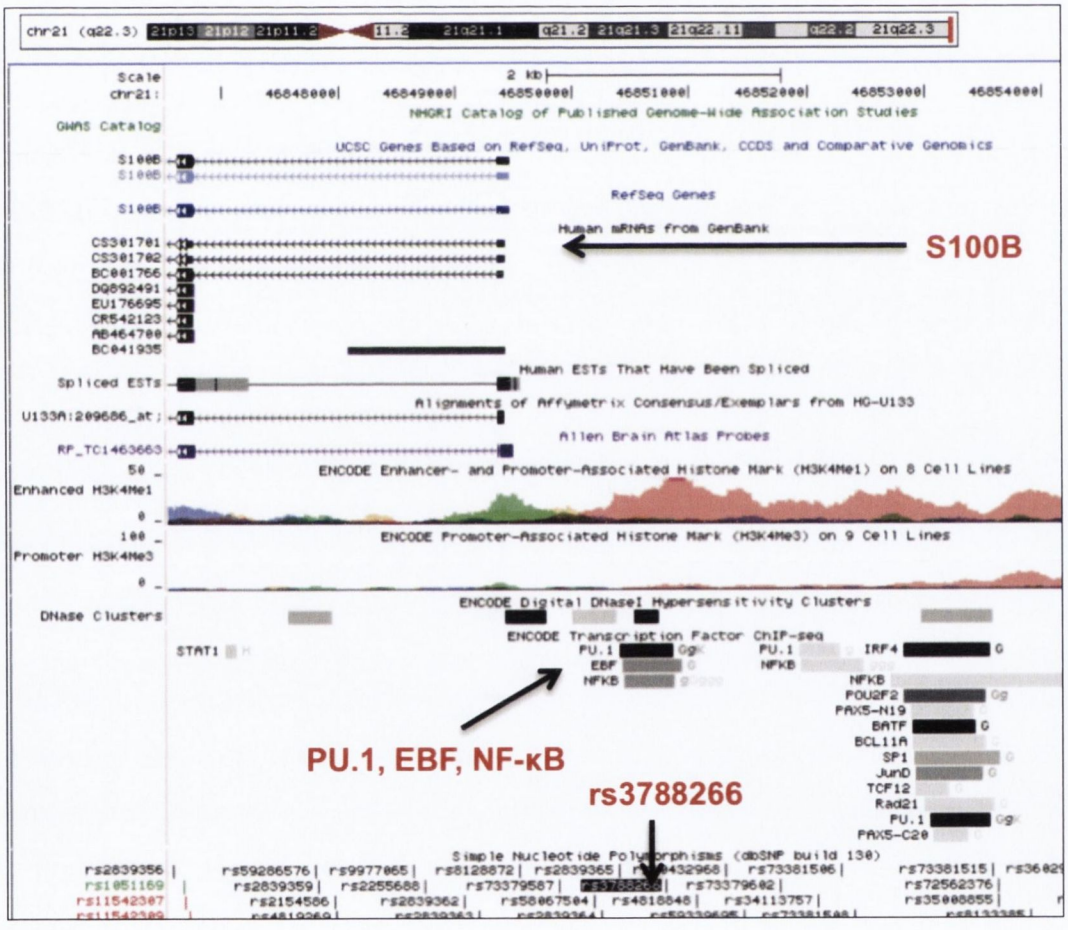


Figure 3.3. ENCODE transcription factor ChIP-seq to rs3788266 site.

Overall, the *in silico* analysis described above revealed a number of transcription factors that are likely to bind to the rs3788266 site, a proportion

of which have potential allele-specific binding. Although not all analyses produce the same results, there are some consistencies across the analyses. Specifically, the SIX-family transcription factor proteins have the strongest probability to bind to rs3788266 as the TRANSFAC analysis revealed the strongest binding for SIX-family proteins by MEF3 matrix (p-value = 5.74E-05) and the analysis by Genomatrix revealed strongest binding again to SIX-family protein by matrix SIX1 (matrix similarity 0.979). The matrix SIX1 also suggests allele specific binding as the G allele does disturb the consensus sequence. Those results directed me to investigate the SIX-family proteins further using molecular *in vitro* methods.

3.3.2. Electrophoretic Mobility Shift Assay

The literature also supports SIX-family members as strong candidates to investigate further. The antibodies and recombinant proteins for SIX-family transcription factors (SIX1, SIX2, SIX4 and SIX5) have been reported to bind to transcription factor binding sites, which are similar to one, which contains the rs3788266 using EMSA. (Hu S *et al.,* 2008; Yu Y *et al.,* 2006; Himeda CL *et al.*, 2004, Chai L *et al.,* 2006; Spitz F *et al.* 1998; Parmacek MS *et al.*, 1994). Kawakami K *et al.* (2000) and Boucher CA *et al.* (1995) reported that SIX-family transcription factors are expressed within the brain. The expression of SIX1 has been reported within the SH-SY5Y neuroblastoma cell line (Plant KE *et al.*, 2009), which was available in our lab.

The SIX-family proteins consist of 3 subgroups (SIX1/2, SIX3/6 and SIX4/5) on the basis of their amino acid sequence (Hu S *et al.*, 2008). The strongest results from *in silico* analysis by TRANSFACT (MEF_3_0) and Genomatrix (V$SIX.01) were for SIX1, SIX2, SIX4 and SIX5 transcription factor binding at S100B and they represent 2 of the 3 different subgroups. Therefore, I decided to investigate SIX1 and SIX4, 1 from each of the 2 subgroups, for binding to S100B promoter using EMSA. This was done using the nuclear extract from both SH-SY5Y and U373MG cell lines as used in previous studies in chapter 2.

3.3.2.1. SIX4 Antibody

An antibody for SIX4 protein was used along with nuclear extract from U373MG and SH-SY5Y cell lines to investigate SIX4 transcription factor binding to the site containing the SNP rs3788266. Each experiment was repeated 3 times. The results from the U373MG cell line revealed a shift by the antibody for SIX4 (Figure 3.4). The intensity of the band's shift by SIX4 antibody was on average only 19% stronger for the risk G allele compared to the A allele. I could not identify any shift by the SIX4 antibody using nuclear extract from the cell line SH-SY5Y.

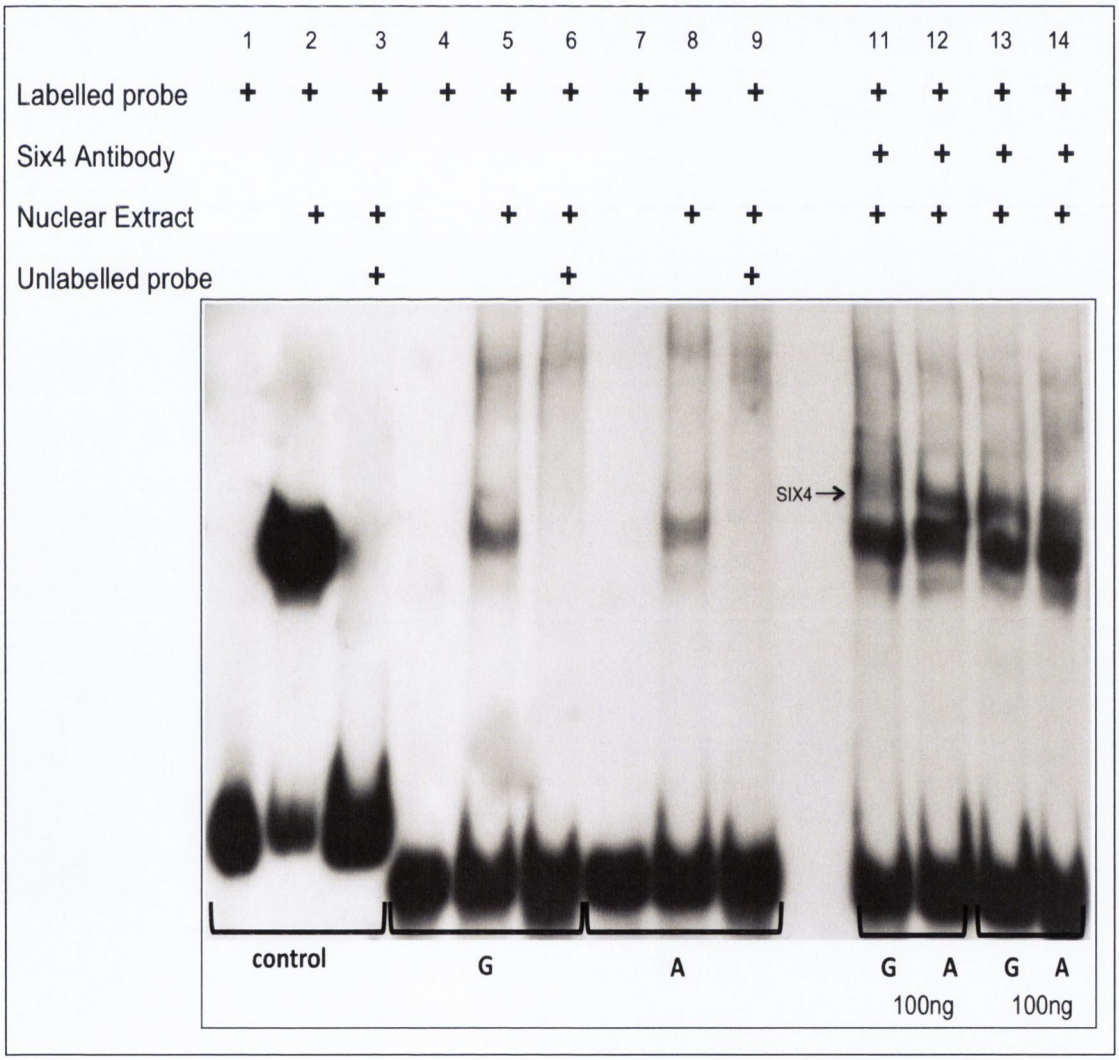


Figure 3.4. The U373MG cell line nuclear extract proteins binding with G/rs3788266 (Lane 4-6) and A/rs3788266 (Lane 7-9) fragments using EMSA. SIX4 antibody was added to lanes 11-14. SIX4 transcription factor binding is detected by the shift present in lanes 11-14.

3.3.2.2. SIX1 Antibody

An antibody for the SIX1 protein and nuclear extract from the U373MG and SH-SY5Y cell lines was used to investigate SIX1 transcription factor binding at the site of SNP rs3788266. Each experiment was repeated 3 times. A shift by the SIX1 antibody was not detected in nuclear extracts from either cell lines. Figure 3.5. is an example for the SIX1 antibody investigation using nuclear extract from SH-SY5Y. As the binding of the SIX1 antibody was not detected, I decided to use a recombinant protein SIX1 to investigate the binding of this transcription factor.

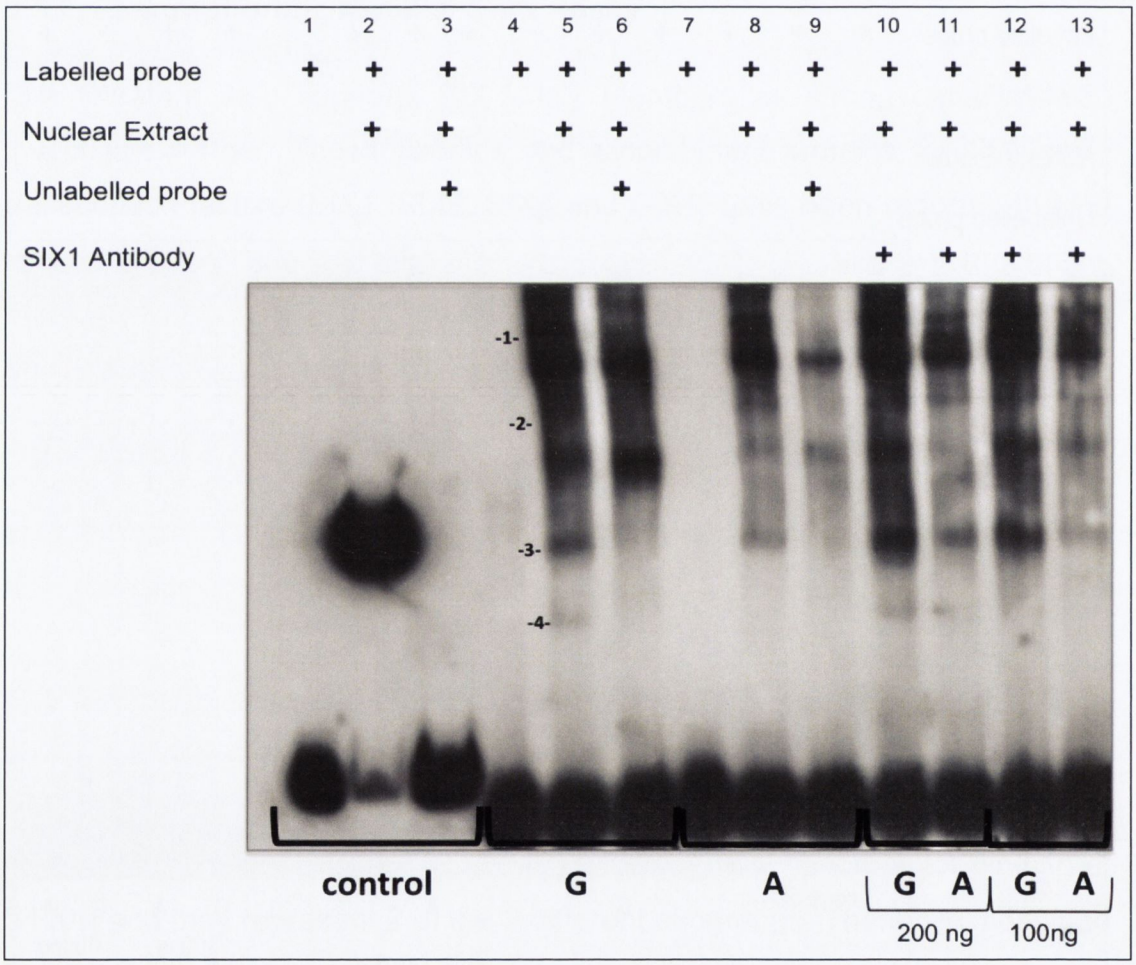


Figure 3.5. SH-SY5Y cell line nuclear extract proteins binding with G/rs3788266 (Lane 4-6) and A/rs3788266 (Lane 7-9) fragments using EMSA. For both alleles four shifted bands (labelled 1-4) were detected. However, no new band was detected in lanes 10-13 containing antibody for the SIX1 protein.

3.3.2.3. SIX1 Recombinant Protein

As I could not detect SIX1 transcription factor using SIX1 antibody, a SIX1 recombinant protein from Abnova was used to investigate SIX1 transcription factor binding at the site containing the SNP rs3788266. This recombinant protein has been used to detect transcription factor binding site using EMSA (Yu Y *et al.,* 2006). The experiment was repeated 3 times and at different concentrations for SIX1 recombinant protein (80ng/ul, 160ng/ul and 320ng/ul). A shift for the SIX1 recombinant protein was detected for the A-Allele but not for the G-Allele (Figure 3.6.).

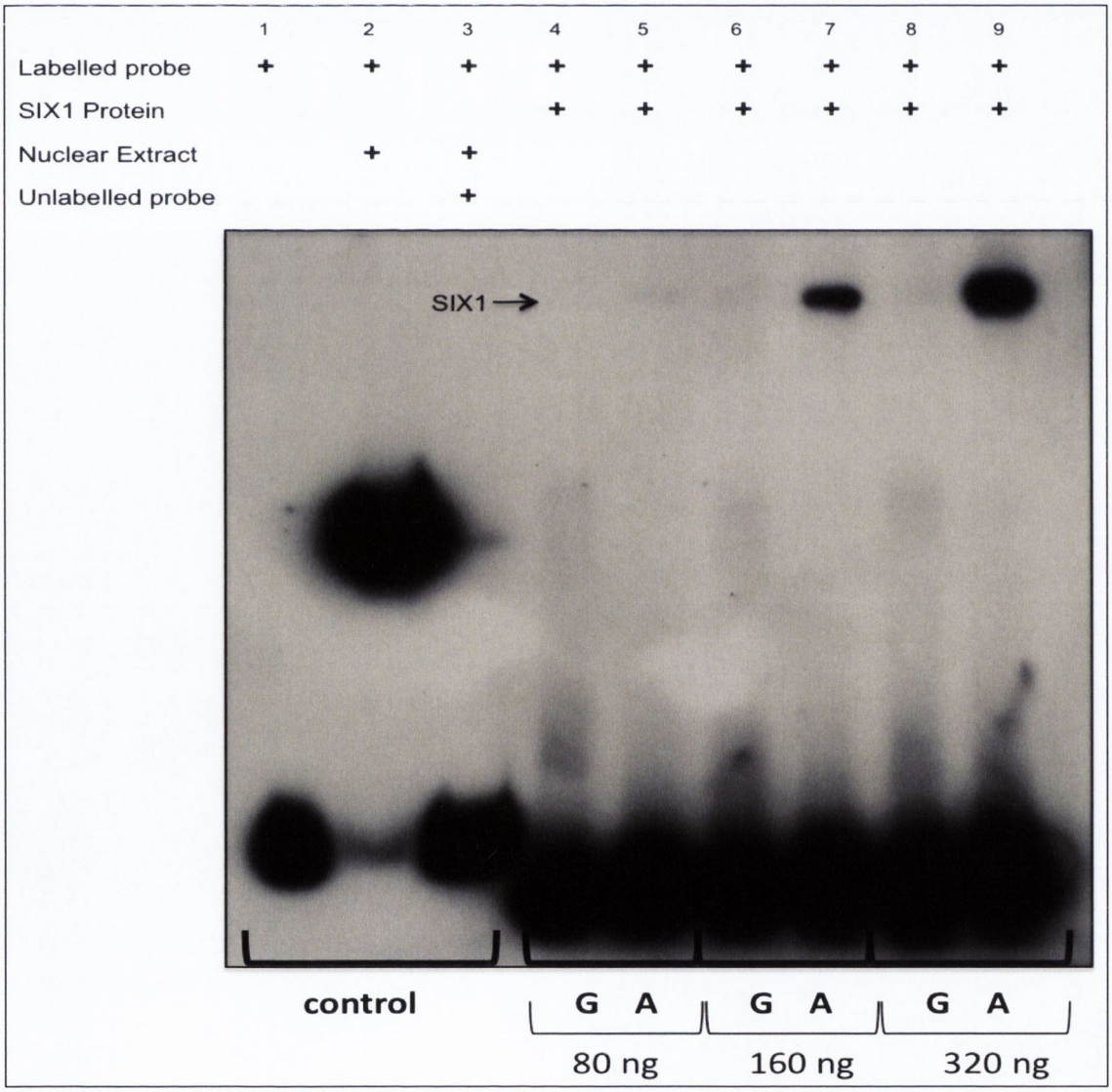


Figure 3.6. A shift for the SIX1 recombinant protein was observed at concentrations of 160ng and 320ng. The shift was only detected for the A-allele and not the G-allele (lanes 5, 7 and 9).

3.3.2.3. Further Studies

I wanted to investigate why the SIX1 antibody did not detect the SIX1 protein in the nuclear extracts from SH-SY5Y and U373MG cell lines (Figure 3.5). It is possible to fail to observe any shift even if the protein is present. Here the binding of SIX1 antibody to SIX1 recombinant protein is investigated. Again the SIX1 recombinant protein bound only to A-allele and not to G-allele. The binding of the SIX1 antibody to SIX1 recombinant protein was not detected using EMSA (Figure 3.7, lanes 10-13). These results indicate that the SIX1 antibody used in previous experiments was not suitable for EMSA because it could not bind to its target protein.

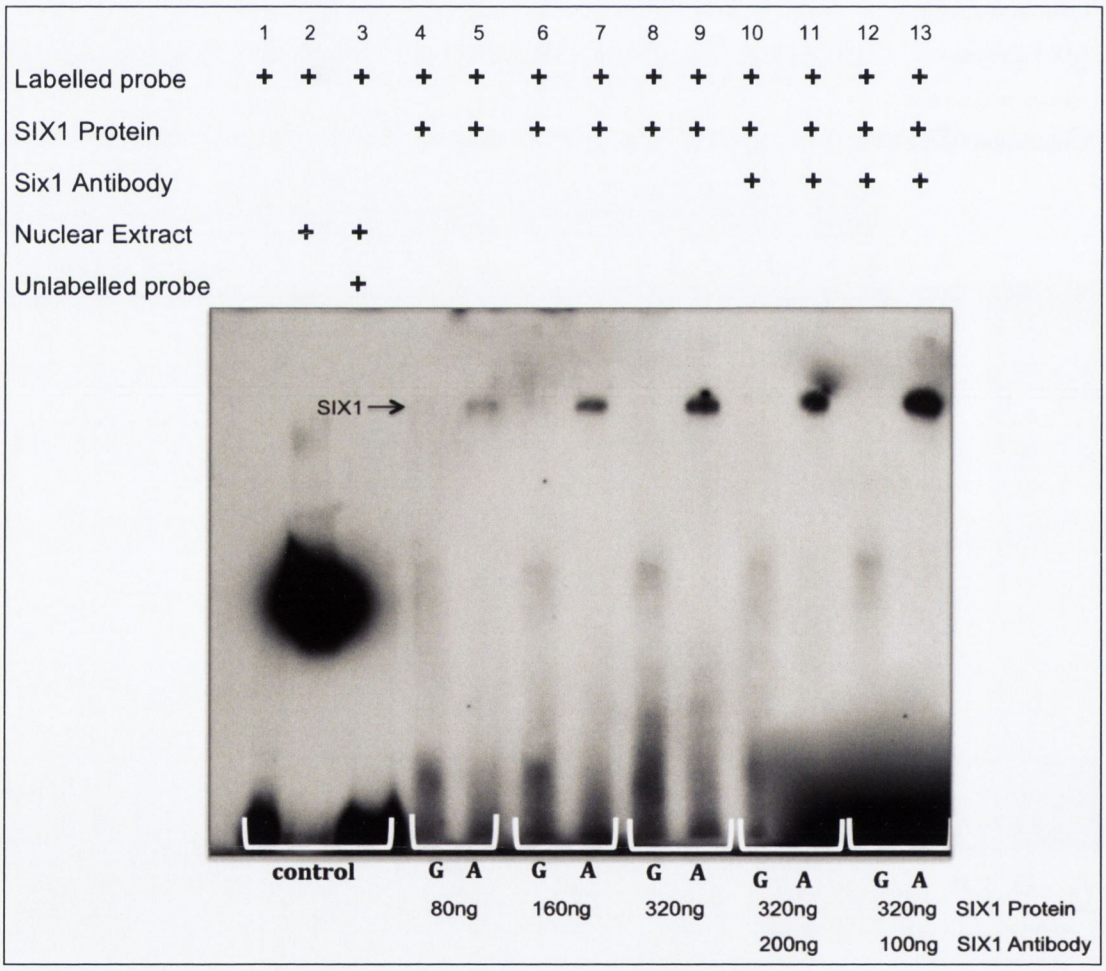


Figure 3.7. A shift for the SIX1 recombinant protein was again observed at different concentrations (80ng, 160ng and 320ng). The shift was only detected by A-Allele and not G-Allele (Lane 5, 7, 9, 11 and 13). No new band was observed in lanes 10-13 where the SIX1 antibody has been added, indicating that it is not binding its target SIX1 protein.

To find out if the SIX1 protein is one of the 4 transcription factors previously identified by in the nuclear extracts of both cell lines (Figure 3.5), I performed an EMSA experiment with U373MG cell line nuclear extract and the SIX1 recombinant protein. The SIX1 protein is not one of the 4 transcription factors as detected before. The SIX1 protein is smaller than the transcription factor marked as -1- and bigger than the transcription factor marked as -2-.

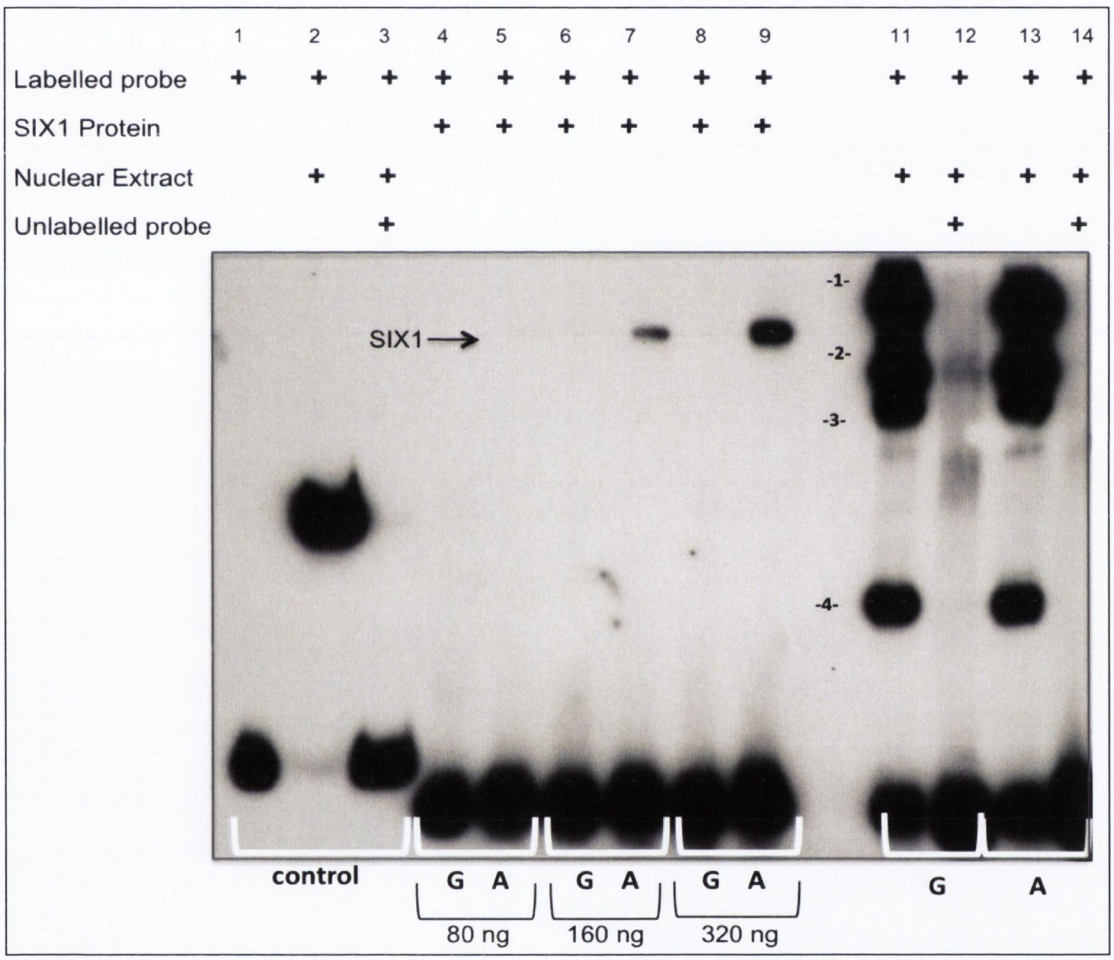


Figure 3.8. A shift for the SIX1 recombinant protein was observed at different concentration (80ng, 160ng and 320ng). The shift was only detected by A-Allele and not G-Allele. The lanes 11-14 contain the U373MG nuclear extract and for both alleles four shifted bands (labelled 1-4) were observed but not at the same position as the band for the SIX1 recombinant protein.

3.4. Discussion

I used two different approaches to identify, which transcription factors are binding to the site of rs3788266 in the S100B promoter and if this binding is allele-specific. Firstly, an *in silico* search was made for possible transcription factors binding using several programs and databases. Secondly, following on from the *in silico* search, binding of the transcription factors SIX1 and SIX4 to at rs3788266 was investigated using EMSA.

The investigation of potential transcription factor binding *in silico* revealed potential differences in transcription factor binding to the A and G allele of rs3788266. The putative transcription factors that bind to the A-allele are SIX1, SIX2, SIX4, SIX5, CCAAT/enhancer binding protein, C/EBP-β, FLI1, Ebf1, YB-1, VDR/RXR PU.1, EBF and NF-κB. The transcription factors that bind to the G-allele are ZNF589, SIX1, SIX2, SIX4, SIX5, SIX6, SMAD4, SMAD3, FLI1, Ebf1, ATF6, YB-1, VDR/RXR, PU.1, EBF and NF-κB. There is also a loss of an insulator protein CTCF binding and a gain of a transcription factor-binding site for activating transcription factor 6 at the SNP rs3788266 by the substitution of an A to G. This type of database analysis has limitations. Most databases rely on *in silico* techniques which focus on creating statistical models to determine the transcription factor consensus-binding site such as position weight matrices of the transcription factor. These methods make assumptions about the transcription factor binding sites by using pre-determined algorithms that may not be correct.

The investigation of the putative transcription factor binding using these databases suggested strong binding of SIX-family proteins to transcription factor binding site containing rs3788266. TRANSFAC database detected the SIX1, SIX2, SIX4, and SIX5 as best candidates (p=5.74E-05 for A-allele and p=0.00014 for G-allele). Genomatix database detected the transcription factors SIX1, SIX4 and SIX5 as best candidates binding to A-allele of rs3788266 at a binding site similarity of 99.7% and for G-allele of the rs3788266 the database detected SIX1, SIX2, SIX4, SIX5, and SIX6 transcription factors at a binding site similarity of 90%. Another bioinformatics

analysis by Roche S *et al.* (2007) reported that the BPAD associated G-Allele of rs3788266 disturbs the Trex/MEF3 consensus recognition site, which is bound by SIX-family transcription factors. For that analysis they used Tfsite Scan database (http://www.ifti.org/Tfsitescan/). The literature also suggests the SIX-family transcription factors as putative transcription factors to bind to the consensus sequence where rs3788266 is located. SIX1, SIX2, SIX4 and SIX5 show similar binding specificity to the MEF3 (GGTGTCAGG**T**TGA) and ARE (TCAGG**T**T) binding site, which have been identified using the molecular techniques DNA footprinting and EMSA (A allele of rs3788266 is underlined; Kawakami K *et al.*, 1996; Spitz F *et al.,* 1998; Ohto *et al.*, 1999; Himeda CL *et al.*, 2004). The SIX1, SIX2, SIX4, SIX5 and SIX6 are also reported to bind to Trex/MEF3 sequence TC[G/A]GG**T**[G/T] (A allele of rs3788266 is underlined, Hu S *et al.*, 2008). The SIX-family transcription factors were the best candidates from the *in silico* analysis and SIX1 and SIX4 were chosen for further molecular work using EMSA.

The investigation of the transcription factors SIX1 and SIX4 binding to the transcription factor binding site containing the SNP rs3788266 revealed allelic differences in the binding of both transcription factors. The binding of the transcription factors SIX1 and SIX4 were investigated using SH-SY5Y and U373MG nuclear extracts and EMSA. The binding density of SIX4 transcription factor to G-allele of rs3788266 was on average 19% increased compared to A-allele using SIX4 antibody and U373MG nuclear extract. A shift by SIX4 antibody was not detected using SH-SY5Y nuclear extract. The investigation of SIX1 transcription factor binding to the transcription factor binding site containing the SNP rs3788266 did not reveal any shift by SIX1 antibody using both cell lines. Therefore, the binding of SIX1 transcription factor was investigated using a SIX1 recombinant protein that did reveal allele-specific binding. SIX1 recombinant protein only bound to the A-Allele and not to G-allele, even at very high concentrations of the protein.

The finding that the SIX1 protein only bound to the A-allele of rs3788266 is in line with the previous finding that the G-allele of this SNP disrupts the binding site motifs of MEF3, ARE and Trex/MEF3 sequences (Kawakami K *et al.*,

1996; Spitz F *et al.,* 1998; Ohto *et al.*, 1999; Himeda CL *et al.*, 2004, Hu S *et al.*, 2008). Interestingly, SIX1 has been recently reported to repress the transcription of target genes in neuronal tissue (Bricaud O & Collazo A, 2011). This suggests that my previous findings of increased serum levels and gene expression associated with the G-allele could be also due to repression of S100B expression by SIX1, which binds in the presence of the A-allele. (chapter 2). In contrast, SIX4 was found to bind to both alleles. This might be due to structural differences of SIX1 and SIX4 transcription factors, each of which belong to different subfamily (Kawakami K *et al.*, 2000).

The reason why no shift was detected using the SIX1 antibody was investigated. First the SIX1 antibody did not bind to SIX1 recombinant protein. This might be because the epitope of SIX1-antibody was binding to DNA and thus unavailable to the antibody, or the SIX1 antibody degraded during the process of EMSA. Second, the data generated here showed that the SIX1 transcription factor was not one of the 4 transcription factors found binding to transcription factor binding site containing the SNP rs3788266 using SH-SY5Y and U373MG nuclear extract. This could be because the SIX1 transcription factor might not be expressed in either cell lines or the expression is so weak so as to be undetectable. But this would be at odds with a previous report that SIX1 is expressed in the SH-SY5Y cell line (Plant KE *et al.,* 2009). The expression of SIX1 transcription factor could also be temporal. Indeed, SIX1 has been reported to be expressed during the development (Ikeda K *et al.,* 2007; Ikeda K *et al.,* 2010) another possibility is that the SIX1 transcription factor might degrade during the process of EMSA.

The SIX (Sine oculis homeobox) family proteins are transcription factors involved in embryonic cell fate determination (Kawakami K *et al.*, 2000). The SIX-family proteins are classified into three major groups. The subgroups are SIX1/SIX2, SIX3/SIX6 and SIX4/SIX5. Mutations of SIX family genes have been associated with developmental disorders such as Branchio-otic syndrome (Ruf RG *et al.,* 2004; Hu S *et al.,* 2008). SIX-family transcription factors are reported to be involved in eye development and are expressed in the brain (Kawakami K *et al.*, 2000; Pham YC *et al.*, 2005). SIX1 is highly

expressed in cranial ganglia during the embryogenesis in Xenopus and in mice (Pandur PD & Moody SA, 2000; Laclef C *et al.*, 2003) and loss of sensory neurons in cranial ganglia has been reported in SIX1 deficient mice (Zou D *et al.*, 2004). Otho H *et al.* (1998) suggested that SIX4 is involved in neuronal cell fate decision or common differentiation or maturation process of neurons.

The function of SIX-family proteins may be relevant to SZP and BPAD. The clusters of SIX-family genes are located at 14q23 and linkage to this region was also observed in the study that identified the linkage between BPAD and the S100B region of chromosome 21 (Cassidy F *et al.*, 2007). Plant KE *et al.* (2009) reported that the mood stabilizers lithium chloride and sodium valproate up-regulate the SIX1 transcription factor. SIX5 has been implicated in the pathophysiology of myotonic dystrophy, a neuromuscular disorder that also includes neuropsychiatric impairment (Hanson IM, 2001; Sato S *et al.*, 2002). SIX3 transcription factor is involved in Wnt signaling which has been reported to be involved in the etiology of BPAD (Gould TD & Manji HK, 2002). I will explore the SIX-family genes as possible risk loci for SZP and BPAD, including epistatic interaction with S100B, in chapter 5.

This research identified the SIX1 and SIX4 transcription factors as transcriptional regulators of S100B. An allele specific binding of these transcription factors was observed at rs3788266, which might explain the differences in expression observed at S100B based on SNP genotype.

Chapter 4

The Effect of the S100B Promoter SNP rs3788266 and Serum Levels of S100B on Measures of Clinical Symptoms and Neurocognitive Performance

4.1.Introduction

Schizophrenia (SZP) and Bipolar Affective Disorder (BPAD) are genetically complex mental disorders that cause considerable morbidity and affect all human populations (Craddock N *et al.,* 2005). SZP is a psychiatric disorder or group of disorders that is characterised by positive "psychotic" symptoms, such as delusions and hallucinations, negative "deficit" symptoms, and variable cognitive impairment (Yudofsky SC & Hales RE, 2008). BPAD is characterised by disturbance in mood ranging from extreme mania (elevated or irritable mood, increased psychomotor activity, distractibility, diminished need for sleep), to severe depression (dysphoric mood, diminished psychomotor activity, decreased concentration, sleep and appetite disturbances and often suicidality), usually accompanied by disturbances in thinking and behaviour, which may include psychotic symptoms, such as delusions and hallucinations (Craddock N *et al.*, 2005; Yudofsky SC & Hales RE, 2008).

BPAD and SZP are similar to each other or overlap in many respects including: lifetime prevalence, genetic susceptibility, age of onset, familial aggregation, course of illness, worldwide distribution, risk for suicide, comparable concordance rate for mono- and dizygotic twins, and the symptoms of psychosis which represent a shared phenotype between both disorders (Maier W *et al.*, 2006; Berrettini WH, 2000). Genetic linkage studies have provided some support for an overlap in genetic risk between the two disorders (Maier W *et al.*, 2006; Berrettini WH, 2000). Recent genome-wide association studies (GWAS) have confirmed that there is overlap between genetic risk factors for BPAD and SZP at several loci including ZNF804A, ANK3 and CACNA1C (Ferreira MA *et al.*, 2008; Schulze TG *et al.*, 2009; Williams HJ *et al.*, 2011; Nyegaard M *et al.*, 2010). There are also neuroimaging and neuropathological similarities between the disorders (Maier W *et al.*, 2006) as well as common neuropharmacological treatments, such as the use of chlorpromazine and lithium in the treatment of both disorders (Maier W *et al.*, 2006; Murray RM *et al.*, 2004).

Cognitive impairments are thought to be part of the core pathology of SZP. Several studies reported deficits in a multitude of neurocognitive functions in SZP, which includes attention and vigilance, working memory, verbal memory and learning, visual memory and learning, reasoning and problem solving, speed of processing, and social cognition (Heinrichs RW & Zakzanis KK, 1998). BPAD patients in general are thought to perform better in tests of neurocognitive performance than patients suffering from SZP (Murray RM *et al.*, 2004).

Patients suffering from BPAD, major depression and SZP are reported to have elevated level of S100B protein in their peripheral blood, cerebral spinal fluid (CSF), and cortical brain tissue (Wiesmann M *et al.*, 1999; Lara DR *et al.*, 2001; Peskind ER *et al.*, 2001; Schroeter ML *et al.*, 2002; Steiner J *et al.*, 2006; Rothermundt M *et al.*, 2007; Steiner J *et al.*, 2008; Schroeter ML *et al.*, 2009). Serum concentration reliably reflects CSF concentration in not only healthy subjects but also in SZP patients (Nygaard O *et al.*, 1997; Steiner J *et al.*, 2006; Rothermundt M *et al.*, 2009). It has been hypothesized that increased level of S100B might be due to passive release from injured astrocytes or neurons but several studies reported that there no evidence of a destruction of astrocytes, oligodendrocytes or neurons, therefore S100B protein appears to be increased by an active secretion from astrocytes (Steiner J *et al.*, 2006; Schroeter ML *et al.*, 2009).

Several studies have reported the correlation between the increased level of S100B protein and clinical features of SZP, particularly with negative and deficit symptoms, and slower remission upon treatment in schizophrenia (Rothermundt M *et al.*, 2001; Schroeter ML *et al.*, 2002; Rothermundt M *et al.*, 2001a; Schmitt A *et al.*, 2005; Ling SH *et al.*, 2007; Tan Y *et al.,* 2010). Increased S100B protein concentration caused behavioural disturbances and cognitive deficits (Donato R, 2001; Heizmann CW *et al.,* 2002; Rothermundt M *et al.*, 2003; Rothermundt M *et al.*, 2004b). Ehrenreich H *et al.* (2007) reported improvement of cognitive function in chronic schizophrenic patients by recombinant human erythropoietin treatment and this treatment and

cognitive improvement were associated with a decline of S100B serum concentration.

The correlation between high level of S100B and attentional processes in patients with mood disorders has been reported (Dietrich DE *et al.,* 2004; Hetzel G *et al.,* 2005). A study by Pedersen A *et al.* (2008) revealed that chronic SZP patients with significantly higher S100B levels were impaired in verbal memory performance. Several studies also showed a correlation between S100B level and cognitive function in patients with brain trauma and cardiac surgery. (Herrmann M *et al.,* 2001; Linstedt U *et al.*, 2002; Connolly ES *et al.*, 2001). A study by Yadavalli S *et al.* (2008) revealed an association between an increased level of S100B protein and poorer cognitive function in neurologically healthy older adults.

Animal studies also found evidence that S100B modulates cognitive performance. Hydén H *et al.* (1970) demonstrated for the first time that learning is associated with the enhanced synthesis of various proteins including S100B, and that intraventricular injection of antibodies against S100B can inhibit memory trace consolidation. Gerlai R *et al.* (1995) reported that transgenic mice overexpressing the human S100B exhibit impaired hippocampal long-term potentiation (LTP) and spatial learning. Hippocampal LTP is thought to be a physiological correlate of long-term memory (Swanson LW *et al.,* 1982). Nishiyama H *et al.* (2002) demonstrated that mutant mice lacking S100B exhibit enhanced synaptic plasticity, learning and memory.

Furthermore, S100B protein levels are increased in the ouabain-induced rat model of mania (Machado-Vieira R *et al.,* 2004). Increased concentrations of S100B protein predict hippocampus dysfunction in animals (for review see Rothermundt *et al.,* 2004b). For normal memory and learning, a balanced concentration of S100B protein in the synapses and extracellular space appears to be required. Too much or too little S100B protein in the synapses and extracellular space appear to disturb the process of acquiring and recalling information (Rothermundt M *et al.*, 2004b).

The functional effect of S100B rs3788266 G-allele has been described in Chapter 2. S100B has a potential role in cognitive performance particularly with spatial and verbal memory and attention as evidenced by clinical and animal studies and the association of S100B level in SZP with negative and deficit symptoms (Rothermundt M *et al.*, 2001b, Schroeter ML *et al.*, 2003). It is unknown if the functional SNP rs3788266 might also effect cognitive function in SZP and BPAD. In this chapter, I investigated if the G allele of rs3788266 is associated with cognitive performance or clinical symptoms in SZP, SZA, BPAD and healthy subjects. Furthermore, I tested if serum levels of S100B are associated with cognitive performance or clinical symptoms in SZP and SZA patients.

4.2. Material and Methods

4.2.1. Sample Characteristics

Cases consisted of clinically stable patients with a DSM-IV diagnosis of SZP, SZA or BPAD recruited from five sites across Ireland. Inclusion criteria required that participants were aged 18 to 65 years, had no history of co-morbid psychiatric disorder or substance abuse in the preceding six months, or prior head injury with loss of consciousness or a history of seizures. Diagnosis was confirmed using the Structured Clinical Interview for DSM-IV Axis 1 Diagnoses (SCID; First MB *et al.*, 1995).

The healthy control sample was recruited through local media advertisements. The control subjects were included only if they were aged between 18 and 65 years and based on clinical interview, satisfied the criteria of having no history of major health problems, intellectual disability, acquired brain injury, or substance misuse in the preceding 6 months based on self-report. In addition to satisfying the criteria above, control participants were excluded from the study if they reported having a first degree relative with history of psychosis. All patients and controls were of Irish ancestry (i.e. four grandparents born in Ireland) and all provided written informed consent. All cases and control assessments were conducted in accordance with the relevant ethics committee's approval from each participating site.

4.2.2. Assessment of Clinical Symptomatology

Clinical symptoms were investigated in a large sample of Irish patients with psychoses (Cummings E *et al.* 2010). The sample consisted of 813 patients with DSM-IV diagnosis of SZP (N=565), SZA (N=124) or BPAD (N=124). Additional diagnostic details and clinical sample characteristics ascertained at time of interview include symptom severity, using the Scale for the Assessment of Positive Symptoms (SAPS) and the Scale for the Assessment of Negative Symptoms (SANS) as described elsewhere (Andreasen NC, 1984a; Andreasen NC, 1984b). The scores of SAPS and SANS yielded 3

factors; positive, negative, and disorganized symptoms, and these were investigated.

As clinical symptoms can vary over time, two measures of clinical symptomatology were also analysed; a lifetime severity scale; the Bipolar Affective Disorder Symptom Scale (BADDS; Craddock N *et al.*, 2004). The Scores for BADDS yielded 4 factors of mania, depression, psychotic, and incongruence and these were investigated. For the later an exploratory factor analysis using the 60 items relating to clinical signs and symptoms from the Operational Criteria Checklist for Psychotic Illness was conducted. Items with loadings of 0.4 or more were then used to produce a five factor solution that included depression and mania factors, and factors for positive, negative and disorganized symptoms. These factors were then analysed with respect to S100B rs3788266 genotype groups.

4.2.3. Assessment of Cognition

The samples consisted of 433 patients with SZP or SZA, 75 patients with BPAD, and 232 healthy participants (Donohoe G *et al.* 2009), who were sub-sample of the patients described in session 4.2.2. All samples are assessed in areas of cognition known to be affected in SZP and BPAD. For this study cognition has been assessed using tests for general cognitive functioning, episodic memory, working memory, attention and social cognition.

General cognitive functioning (IQ): IQ was measured using selected subtests (Vocabulary, Similarities, Block Design and Matrix Reasoning) from the Wechsler Adult Intelligence Scale, 3rd edition (WAIS-III; Wechsler D, 1997) yielding full-scale, verbal, performance IQs.

Episodic memory: Verbal episodic memory was assessed using the logical memory subtest from the Wechsler Memory Scale, 3rd edition (WMS-III) and visual memory was assessed using the WMS-III Faces subtest (Wechsler D, 1998).

Working memory: Verbal working memory was measured using the WMS-III Letter Number Sequencing task (Wechsler D, 1998). Spatial working memory was assessed using the Spatial Working Memory Task from the Cambridge Neuropsychological Test Automated Battery (CANTAB SWM), Expedio Version (Cambridge Cognition, 2003).

Attentional control: Attentional control was assessed using the Continuous performance task, identical pair's version (CPT-IP; Cornblatt BA, 1988). Accuracy was calculated in terms of the signal detection index d', providing a response sensitivity index to the target stimuli. The Cantab IDED task was assesses as described elsewhere (Robertson IH *et al.,* 1997).

Social cognition: Social cognition was assessed using the internal personal and situational attributes questionnaire (IPSAQ; Kinderman P & Bentall RP, 1996) and the 'Reading the Mind in the Eyes' test ('Eyes task'; Baron-Cohen S *et al.*, 2001).

4.2.4. Genotyping of Samples

All samples were genotyped for S100B SNP rs3788266 using the predesigned Taqman® Assay (ID: C_25800807_10) from Applied Biosystems (Warrington, UK). Amplification and allelic discrimination for the assay were performed using an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, California). A number of HapMap CEU DNA samples were also genotyped for rs3788266 for quality control and all were consistent with online HapMap data for this SNP (ww.hapmap.org). All cases and control samples were in Hardy-Weinberg equilibrium ($P > 0.05$).

4.2.5. *Quantification of Serum S100B Protein*

The serum levels of S100B protein in 220 SZP and SZA samples were measured as described in chapter 2 (section 2.2.7. Quantification of Serum S100B Protein, Page 60). All samples for this experiment were sub-sample of the patients described in session 4.2.3.

4.2.6. Statistical Analysis

To confirm that cases and controls were adequately matched for age and gender, t-test and chi-square analysis were performed respectively. Association tests between S100B rs3788266 genotypes (AA, AG and GG) and demographic variables were investigated with one-way analyses of variance, ANOVA, (current age, age at onset, medication) and Chi-squared test (gender, diagnostic groups). In the case of symptoms severity, the association between S100B rs3788266 genotypes and SAPS, SANS and BADDS Scores were analysed using ANOVA. Those statistical analyses were carried out using the statistical software package SPSS version 16.0 (SPSS Inc, Chicago, Illinois). Factor score coefficients were then also calculated in SPSS 16.0 using the regression method. Performance on a task was classified as impaired if 1.5 standard deviations (S.D.) below the normative mean for the task (Chan RC *et al.*, 2006).

The association between genotypes of rs3788266 and measures of cognitive function (IQ, episodic memory, working memory, attention and social cognition) were tested by ANOVA using a statistical software SPSS version 14.0 (SPSS Inc, Chicago, Illinois). For analyses of the neuropsychological performance variables, sex, age and medication at the time of testing were investigated as potential confounding covariates. The cases and controls samples were in Hardy-Weinberg Equilibrium. The rs3788266 genotype (AA vs AG vs GG) and diagnosis (cases and controls) were entered as fixed effects.

Association between rs3788266 genotypes and S100B serum concentration was tested by ANOVA using the statistical software package SPSS version 18.0 (SPSS Inc, Chicago, Illinois). The correlation between the WAIS full-scale IQ, WAIS performance IQ, WMS letter-number sequence task, IDED 8 shapes adjusted task, BADDS Psychosis Score and S100B serum concentrations were analysed using Spearman's rho.

4.3. Results

4.3.1. Assessment of Clinical Symptomatology

The association between the S100B rs3788266 genotype and BADDS scores, or the factor co-efficient scores were investigated. The clinical and demographical characteristics by rs3788266 genotype appear in Table 4.1 and Table 4.2. Association between the rs3788266 genotypes and current age, age at onset, gender, medication (Chlorpromazine equivalent dose), and diagnosis were examined. There were no significant differences observed between the rs3788266 genotype groups by current age, age at onset, gender, medication (Chlorpromazine equivalent dose), and diagnosis.

Table 4.1. Relationship between S100B rs3788266 genotype groups and demographic variables

Genotypes	Age Mean	Age at onset (years)	Mean Chlorpromazine (mg)	Male (%)
AA	42.62	23.13	484.00	66.66
AG	43.49	24.42	480.22	62.46
GG	45	24.07	477.25	69.09
F	1.77	1.262	0.009	
***P* value**	0.17	0.284	0.991	0.231

Table 4.2. Sample Size and DSM-IV Diagnosis of Subjects by S100B rs3788266 genotypes

Genotypes	SZP	SZA	BPAD	Total	X^2 value	p-value
AA	117	25	27	169		
AG	296	56	63	415		
GG	152	43	34	229		
Total	**565**	**124**	**124**	**813**	3.337	0.503

An ANOVA looking at the relationship between S100B rs3788266 genotypes and BADDS dimension scores, or the factor co-efficient scores did not show any significant association between the S100B rs3788266 genotypes and any clinical features in SZP and SZP subjects (Table 4.3). There were no statistically significant associations between BADDS assessed mania, depression or incongruence dimension scores, or the factor co-efficient scores. However, the BADDS psychosis score was observed to be associated with the disease associated G-allele of rs3788266 (p=0.045) in BPAD subjects. It is worth to mention that it was not possible to carry out correction for multiple testing as the measures are not independent.

The BADDS psychosis score approached significance in the SZA group (p=0.057) but the direction was different to that seen in the BPAD subjects. Likewise, the negative symptom factor score did approach significance (p=0.059) and, as in BPAD subjects, GG carriers did have more severe negative symptoms compared AA and AG carriers.

Table 4.3. Multivariate analysis of variance of rs3788266 genotype with BADDS dimension scores and factor co-efficient scores.

Diagnosis / Genotypes	BADD Mania	BADD Depression	BADD Psychotic	BADD Incongruence	Manic Factor	Depression Factor	Positive Symptoms[a]	Negative Symptoms[b]	Disorganized Symptoms[c]
SZP									
AA	22.60	38.68	47.97	66.45	-.1989	-.223	.3416	.2801	.2275.
AG	17.74	37.25	41.29	62.62	-.2237	-.140	.0755	.1028	.0666
GG	22.07	39.01	43.66	65.51	-.3537	-.062	.1117	.2234	.1638
F	2.497	.115	2.127	.992	1.558	.854	2.651	1.16	1.569
***P* value**	.83	.892	.120	.372	.211	.426	.71	.312	.209
SZA									
AA	48.84	48.20	39.88	58.04	.4134	.3958	.132	-.2605	-.0005
AG	56.82	41.61	40.89	62.75	.6748	.1291	.1040	-.776	.2422
GG	43.58	51.51	29.51	50.77	.2584	.2533	.2428	-.837	.0833
F	2.00	.71	2.93	1.813	1.768	.626	2.16	.335	.553
P value	.139	.494	.057	.168	.184	.537	.120	.716	.576
BPAD									
AA	61.81	52.96	20.11	37.89	1.322	.2506	-.3362	-.1115	-.5522
AG	59.93	51.66	19.60	34.67	1.168	.2352	-.5142	-.4967	-.3847
GG	67.74	48.53	27.97	45.82	.9640	.3287	-.3137	-.4971	-.0514
F	1.232	.097	3.181	1.618	1.278	.119	1.657	2.905	1.896
P value	.295	.908	**.045**	.203	.282	.898	.195	.059	.155

[a]Scores from the Scale for the Assessment of Positive Symptoms.
[b]Scores from the Scale for the Assessment of Negative Symptoms.
[c]Scores from the Structured Clinical Interview for DSM-IV Axis I Disorders
*the mean difference is significant at the level of < 0.05.

4.3.2. Assessment of Cognition

The association between the S100B rs3788266 genotype groups and 5 cognitive domains of IQ, working memory, episodic memory, attention and social cognition was investigated. For this analysis SZP and SZA cases (n=433) are combined together. Mean scores for each of the 5 cognitive domains by S100B rs3788266 genotype groups for SZP and SZA cases and controls are presented in Table 4.4. The combined SZP and SZA cases performed significantly below controls on all cognitive tests (P=0.001 for all) with the exception of social cognition. It is worth to mention that it was not possible to carry out correction for multiple testing as the measures are not independent.

There were only 75 BPAD cases available for the assessment of the cognition. As this number is unlikely to have sufficient power to detect association between S100B rs3788266 genotypes and cognitive impairments, these were excluded from analysis.

Table 4.4. Cognitive performance according to S100B rs3766288 genotype in SZP + SZA cases and controls.

Cognitive function	Test or Subscale	Sample	N	AA Mean (SD)	AG Mean (SD)	GG Mean (SD)	$F_{Case\ vs\ Controls}$ (P Value)[a]	$F_{Main\ effect}$ (P Value)[b]	$F_{Simple\ effect}$ (P Value)[c]	$F_{Interaction\ effect}$ (P Value)[d]
IQ	WAIS full-scale IQ	Patient	204	90.9 (19.8)	92.3 (16.3)	85.8 (15.9)	285.7	3.9	3.34	0.17
		Controls	117	122.0 (18.7)	124.9 (13.8)	119.6 (12.4)	(<0.001)	**(0.02)***	**(0.037)***	(0.84)
	WTAR (adult reading test)	Patients	204	94.1 (13.7)	97.7 (12.0)	95.5 (9.9)	128	2.30	1.26	0.5
		Controls	117	108.2 (6.6)	100.8 (4.1)	109.1 (5.1)	(<0.001)	(0.29)	(0.296)	(0.95)
	WAIS verbal IQ	Patients	204	91.7 (20.4)	94.3 (16.2)	89.9 (16.1)	257	4.17	1.29	0.34
		Controls	117	123 (17.7)	127.7 (14.1)	119.8 (12.8)	(<0.001)	**(0.016)***	(0.277)	(0.71)
	WAIS performance IQ	Patients	204	91.7 (19.7)	91.4 (18.1)	83.8 (17.4)	182	1.74	4.671	1.36
		Controls	117	117.8 (21.1)	118.1 (16.9)	117.4 (16.6)	(<0.001)	(0.18)	**(0.01)***	(0.26)
Working Memory	WMS letter-number sequence	Patients	292	8.10 (3.63)	7.25 (3.19)	6.63 (3.03)	285.25	2.91	3.65	0.715
		Controls	156	13.3 (3.32)	13.4 (3.19)	12.64 (3.25)	(<0.001)	**(0.055)**	**(0.027)***	(0.49)
	CANTAB spatial-working memory (Between error)	Patients	288	-1.07 (1.30)	-1.15 (1.39)	-1.12 (1.23)	122.61	0.223	0.067	0.211
		Controls	146	0.337 (0.83)	0.332 (0.72)	0.171 (0.78)	(<0.001)	(0.768)	(0.936)	(0.810)
	CANTAB spatial-working memory (strategy standart)	Patients	288	-0.35 (1.83)	-0.35 (1.70)	-0.55 (1.62)	20.7	0.615	n/a	0.080
		Controls	146	0.479 (1.22)	0.33 (1.02)	0.222 (1.17)	(<0.001)	(0.541)		(0.923)

Cognitive function	Test or Subscale	Sample	N	AA Mean (SD)	AG Mean (SD)	GG Mean (SD)	$F_{Case\ vs\ Controls}$ (P Value)[a]	$F_{Main\ effect}$ (P Value)[b]	$F_{Simple\ effect}$ (P Value)[c]	$F_{Interaction\ effect}$ (P Value)[d]
Episodic Memory	WMS Logical Memory Immediate	Patients	298	6.28 (3.57)	6.31 (3.31)	5.79 (2.97)	346.4	1.27	0.813	1.53
		Controls	154	11.7 (2.45)	12.9 (2.75)	12.8 (3.25)	(<0.001)	(0.70)	(0.444)	(0.21)
	WMS Logical Memory Delayed	Patients	298	6.84 (3.22)	7.11 (3.29)	6.77 (2.85)	348.2	3.14	0.359	1.41
		Controls	154	12.1 (2.37)	13.7 (2.48)	13.0 (2.89)	(<0.001)	**(0.044)***	(0.699)	(0.24)
	WMS Faces 1	Patients	284	8.21 (2.05)	8.31 (2.75)	8.61 (2.76)	101.1	1.19	0.82	0.30
		Controls	155	10.9 (2.57)	11.6 (2.97)	11.6 (2.54)	(<0.001)	(0.30)	(0.441)	(0.74)
	WMS Faces2	Patients	284	8.26 (2.91)	9.18 (2.59)	8.81 (2.92)	69.57	4.91	2.37	0.15
		Controls	155	10.5 (2.40)	11.8 (2.94)	11.4 (2.33)	(<0.001)	**(0.008)***	(0.95)	(0.86)
Attentional Control	IDED (Adjusted standard score)	Patients	220	-3.48 (3.67)	-3.05 (3.47)	-4.33 (4.75)	80.17	1.72	n/a	0.523
		Controls	107	0.30 (0.55)	0.29 (1.09)	-0.72 (1.55)	(<0.001)	(0.537)		(0.59)
	IDED (6 shapes adjusted)	Patients	220	-2.44 (2.76)	-2.35 (3.08)	-3.18 (3.83)	48.99	2.08	1.25	0.13
		Controls	107	0.07 (0.68)	0.27 (0.91)	-0.49 (3.38)	(<0.001)	(0.386)	(0.287)	(0.98)
	IDED (8 shapes adjusted)	Patients	245	8.79 (10.38)	11.29 (10.6)	13.90 (11.7)	8.105	1.98	3.39	1.06
		Controls	143	8.21 (10.09)	8.66 (9.30)	9.38 (9.92)	(0.005)	(0.068)	**(0.035)**	(0.346)
	CPT_IP (2 letters), d'[d]	Patients	197	2.68 (1.23)	2.82 (1.04)	2.52 (1.02)			1.45	
									(0.236)	
	CPT_IP (3 letters), d'[d]	Patients	197	1.78 (1.09)	2.07 (0.94)	1.66 (0.95)			3.46	
									(0.033)*	

Cognitive function	Test or Subscale	Sample	N	AA Mean (SD)	AG Mean (SD)	GG Mean (SD)	$F_{Case\ vs\ Controls}$ (P Value)[a]	$F_{Main\ effect}$ (P Value)[b]	$F_{Simple\ effect}$ (P Value)[c]	$F_{Interaction\ effect}$ (P Value)[d]
	CPT_IP (4 letters), d'[d]	Patients	197	0.98 (0.83)	1.12 (0.79)	0.80 (0.64)			3.20 **(0.056)***	
Social Cognition	Hinting Task	Patients	74	15.28 (3.22)	16.63 (2.22)	16.61 (1.70)	0.690	2.703	0.955	0.560
		Controls	45	16.00 (0.70)	17.33 (1.10)	16.38 (2.02)	(0.408)	(0.615)	(0.386)	(0.573)
	IPSAQ (externalising bias)	Patients	195	0.545 (3.44)	1.62 (3.83)	0.661 (3.43)	0.061	0.621	2.14	3.607
		Controls	115	0.980 (3.67)	0.447 (3.79)	2.14 (4.37)	(0.805)	(0.538)	(0.12)	**(0.028)***
	IPSAQ (personalising bias)	Patients	195	0.460 (0.26)	0.542 (0.26)	0.521 (0.29)	10.8	0.69	1.3	0.52
		Controls	115	0.650 (0.21)	0.634 (0.23)	0.55 (0.23)	(0.001)	(0.5)	(0.26)	(0.59)
	IPSAQ (situational positive)	Patients	195	5.15 (3.41)	5.07 (3.40)	5.33 (3.90)	0.818	1.014	n/a	1.762
		Controls	115	5.00 (2.81)	5.23 (2.74)	3.86 (2.48)	(0.442)	(0.441)		(0.173)
	IPSAQ (external positive)	Patients	195	4.20 (2.82)	3.84 (2.33)	4.20 (3.39)	7.840	0.394	n/a	0.348
		Controls	115	4.72 (2.26)	5.03 (2.47)	5.28 (2.96)	(0.005)	(0.821)		(0.707)

Abbreviations: CANTAB, Cambridge Neuropsychological Test Automated Battery, Expedio Version; CPT-IP, continuous performance test, identical pairs version; WAIS, Wechsler Adult Intelligence Scale; WMS, Wechsler Memory Scale; WTAR, Wechsler Test of Adult Reading; IDED, Intra-dimensional/estra-dimensional; IPSAQ, Internal, Personal, and Situational Attribution Questionnaire; ellipses, not applicable.

[a]The values of $F_{cases\ vs\ controls}$ indicate the difference associated with diagnosis.
[b]The value of $F_{simple\ effect}$ indicate the effect of genotype in cases only.
[c]The values of $F_{main\ effect}$ indicate the main effect of genotype in population (combined cases and controls subjects).
[d]The values of $F_{interaction\ effect}$ indicate the genotype X diagnosis interaction.
[d]$F_{cases\ vs\ controls}$ and $F_{interaction\ effect}$ were not calculated for CPT-IP scores as data were not available for controls in this measure.
*the mean difference is significant at the level of < 0.05.

4.3.2.1. General Cognitive Abilities

The patients performed lower than the control samples on 4 tasks measuring the current IQ ($p < 0.001$). No genotype X diagnosis group interaction was observed in any of the 4 tasks used to measure the current IQ (Table 4.4).

The analysis of full-scale IQ revealed a significant overall association with S100B rs3788266 genotypes ($F_{main\ effect}$: 3.85, p=0.02, Table 4.4.). Tukey post hoc analysis revealed that this association was driven by differences between the AA+AG and GG groups (p=0.01), with homozygous G carriers performing least well in case and control groups (Figure 4.1.).

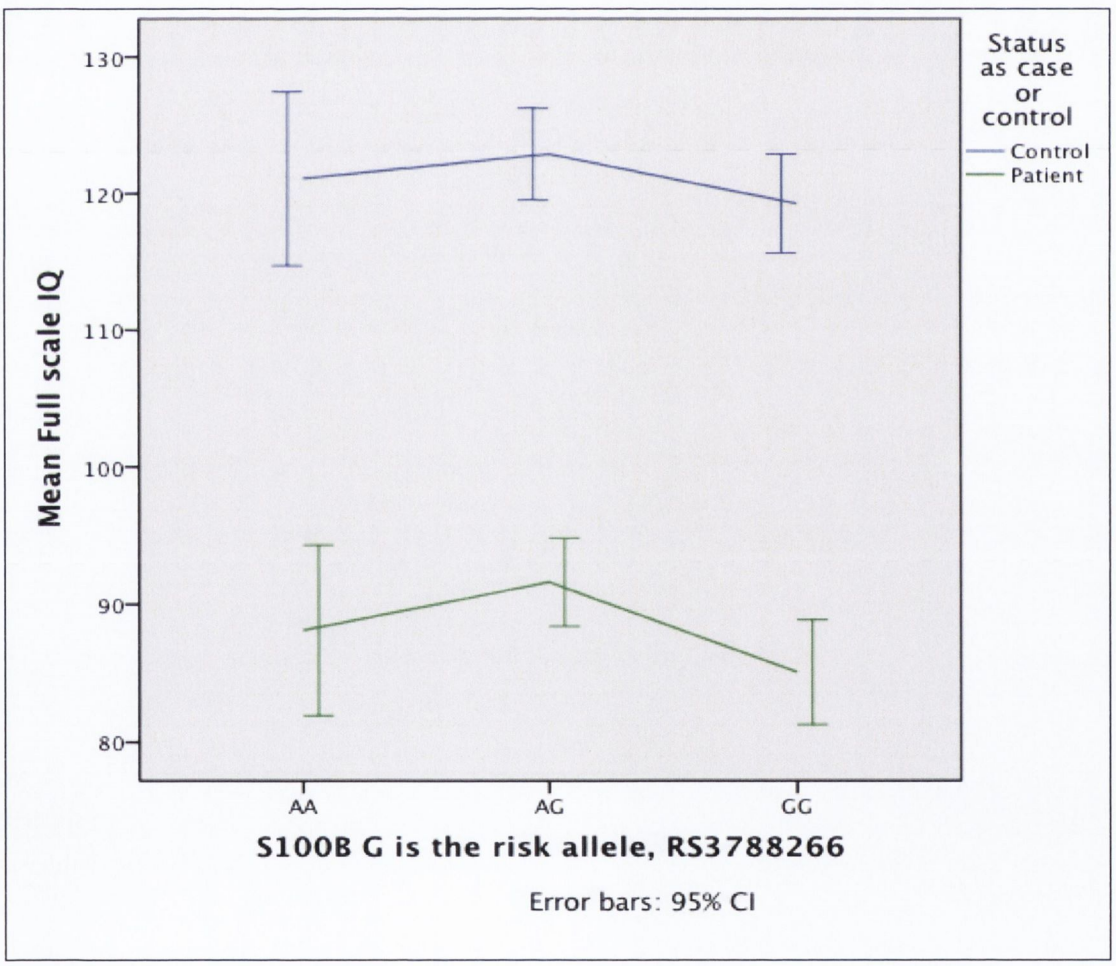


Figure 4.1. Differences in WAIS full-scale IQ associated with S100B rs3788266 genotype in cases and controls. The same effect for the rs3788266 genotypes was observed in cases and controls.

No association between WTAR (adult reading test) and S100B rs3788266 genotypes were observed. An association was found between rs3788266 and verbal IQ ($F_{main\ effect}$: 4.17, p=0.016, Table 4.4.). Tukey post hoc analysis showed that this effect was driven by differences between the AA+AG vs GG groups (p=0.014), with the AA and AG carriers outperforming the GG carriers in verbal memory across both case and control groups (Figure 4.2.)

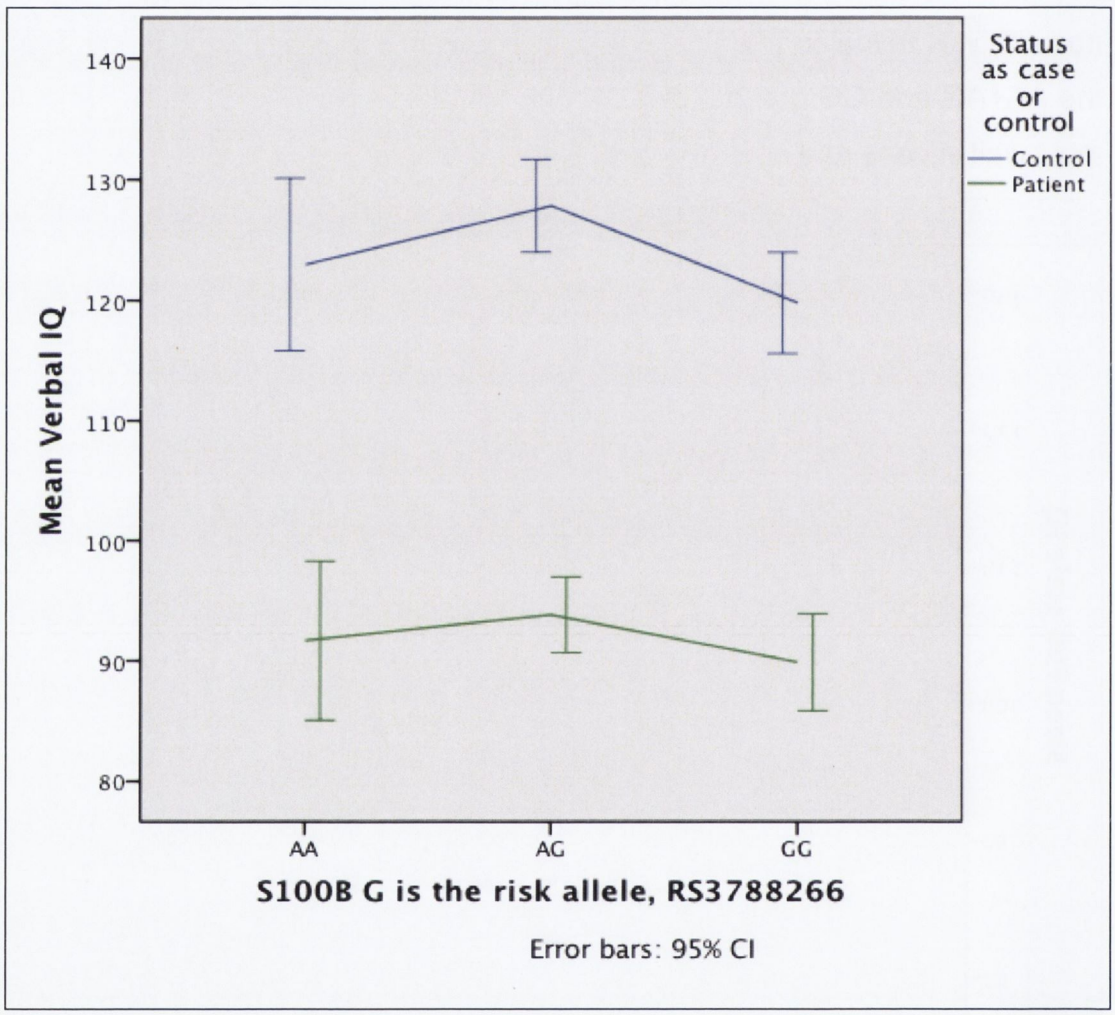


Figure 4.2. Differences in WAIS verbal IQ associated with S100B rs3788266 genotype in cases and controls. The same effect for the rs3788266 genotypes was seen in cases and controls.

The analysis of Performance IQ revealed a significant overall association with rs3788266 genotype ($F_{simple\ effect}$: 4.67, p=0.01, Table 4.4.). This indicates that the association exists to a greater extent in cases compared to controls. The Tukey post hoc analysis revealed that this association was driven by differences between the AA and GG groups (p=0.016), with homozygous G carriers performing least well in the patient group (Figure 4.4.). The Figure represents only the patients as the association is seen in patients only.

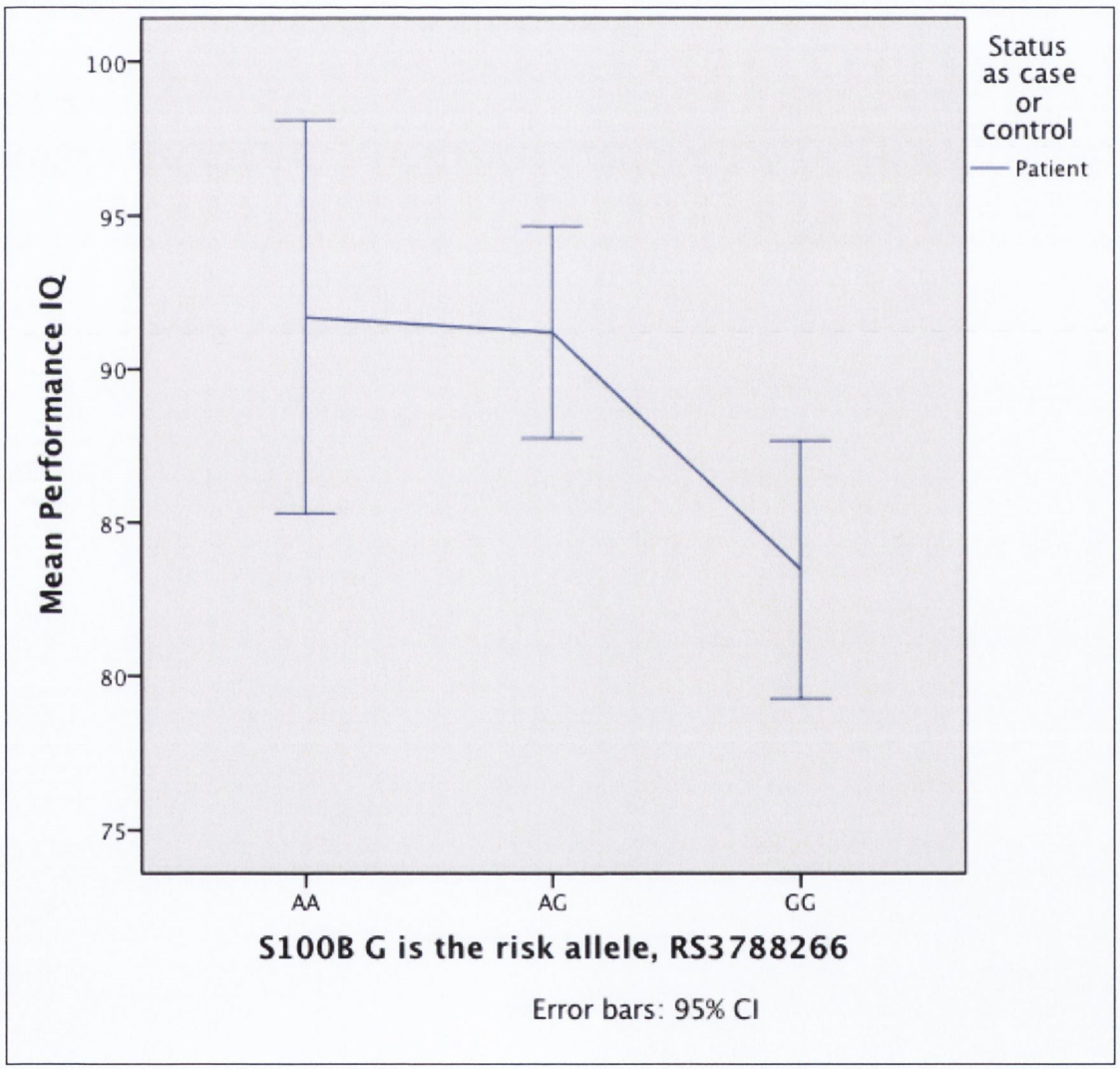


Figure 4.3. Differences in WAIS performance IQ associated with S100B rs3788266 genotype in SZP + SZA cases.

4.3.2.2. Working Memory

In all working memory tasks, controls outperformed combined SZP and SZA cases ($p < 0.001$). No genotype X diagnosis group interaction was observed in any of the 3 working memory tasks.

The WMS letter-number sequence task analysis showed a significant association of rs3788266 genotypes in cases only ($F_{simple\ effect}$: 3.65, p=0.027, Table 4.4). The tukey post hoc analysis revealed that this association was driven by differences between the AA and GG rs3788266 genotype groups (p=0.016, Figure 4.4.), and that the association exists to a greater extend in cases compared to controls. No association was observed between rs3788266 genotypes and CANTAB spatial working memory tasks.

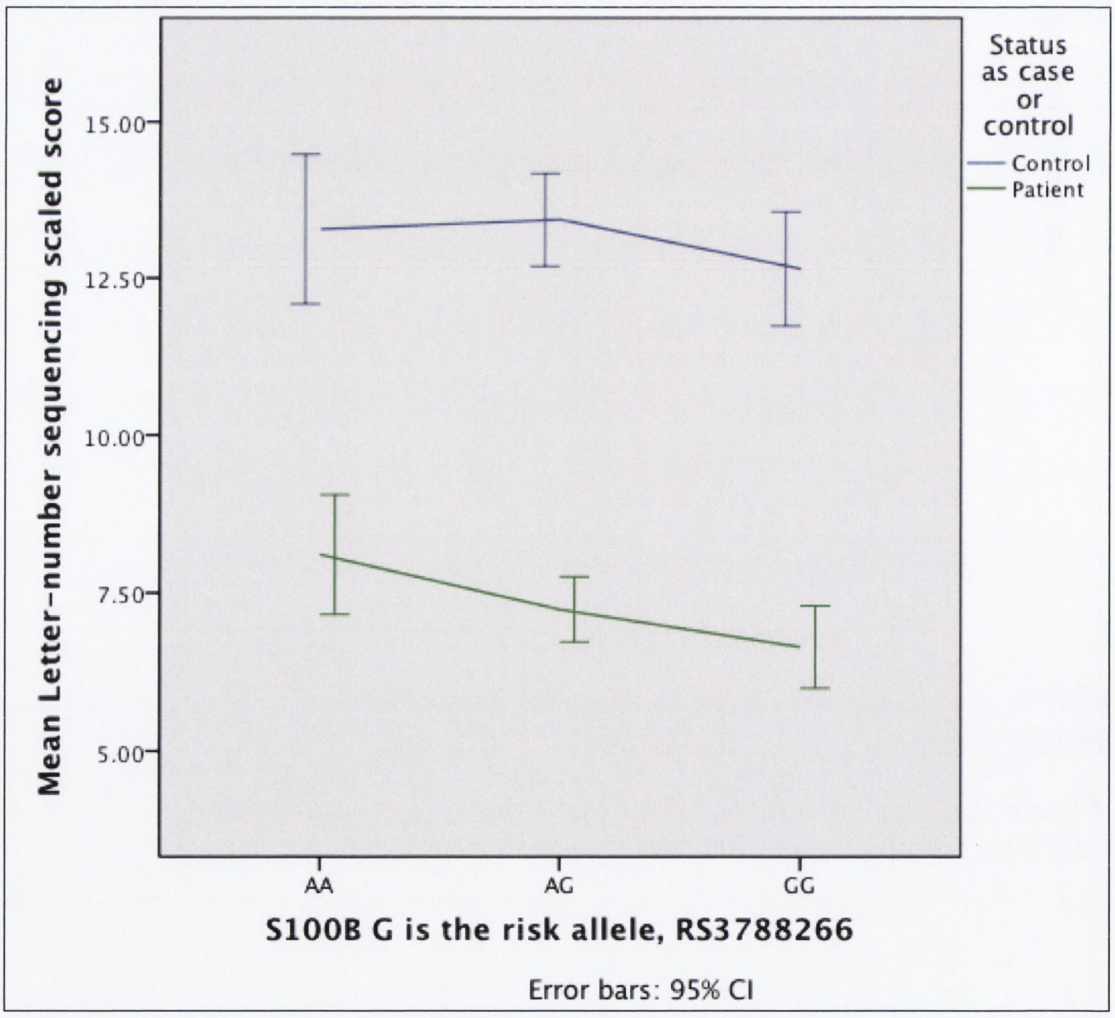


Figure 4.4. Differences in letter-number sequencing task of the Wechsler Memory Scale associated with S100B rs3788266 genotypes.

4.3.2.3. Episodic Memory

Control subjects performed better than combined SZP and SZA cases on all tasks measuring episodic memory ($p < 0.001$). No genotype X diagnosis group interaction was observed in any of the episodic memory tasks.

The WMS logical memory delayed task analysis revealed a significant overall association with rs3788266 genotypes ($F_{main\ effect}$: 3.14, p=0.044, Table 4.4.). Tukey post hoc analysis did not identify any association by differences between the S100B rs3788266 AA+AG vs GG genotype groups for WMS logical memory delayed task (p=0.441).

No association was observed between WMS Faces 1 task and rs3788266 genotypes. The WMS Faces 2 task analysis revealed a significant overall association with rs3788266 genotypes ($F_{main\ effect}$: 3.14, p=0.008, Table 4.4.). Tukey post hoc analysis did not identify any association by differences between the S100B rs3788266 AA+AG vs GG genotype groups for the WMS Faces 2 task (p=0.774).

4.3.2.4. Attention

For attentional control, the control subjects performed better in all given tasks compared with combined SZA and SZP controls, apart from the CPT task for which there was no control data. No genotype X diagnosis interaction was observed. No statistically significant association between the rs3788266 genotype and IDED (adjusted standard score), IDED (6 shapes adjusted) or CPT_IP (2 letters), d' tasks were observed. Analysis of the intradimensional-extradimensional task (IDED score for block 8) revealed a significant association with rs3788266 genotypes in cases only ($F_{simple\ effect}$: 3.39, p=0.035, Table 4.3.). The GG carriers made more mistakes compared to AG and AA carriers. As it is shown in Figure 4.5., the genotypic association is stronger in the patient group.

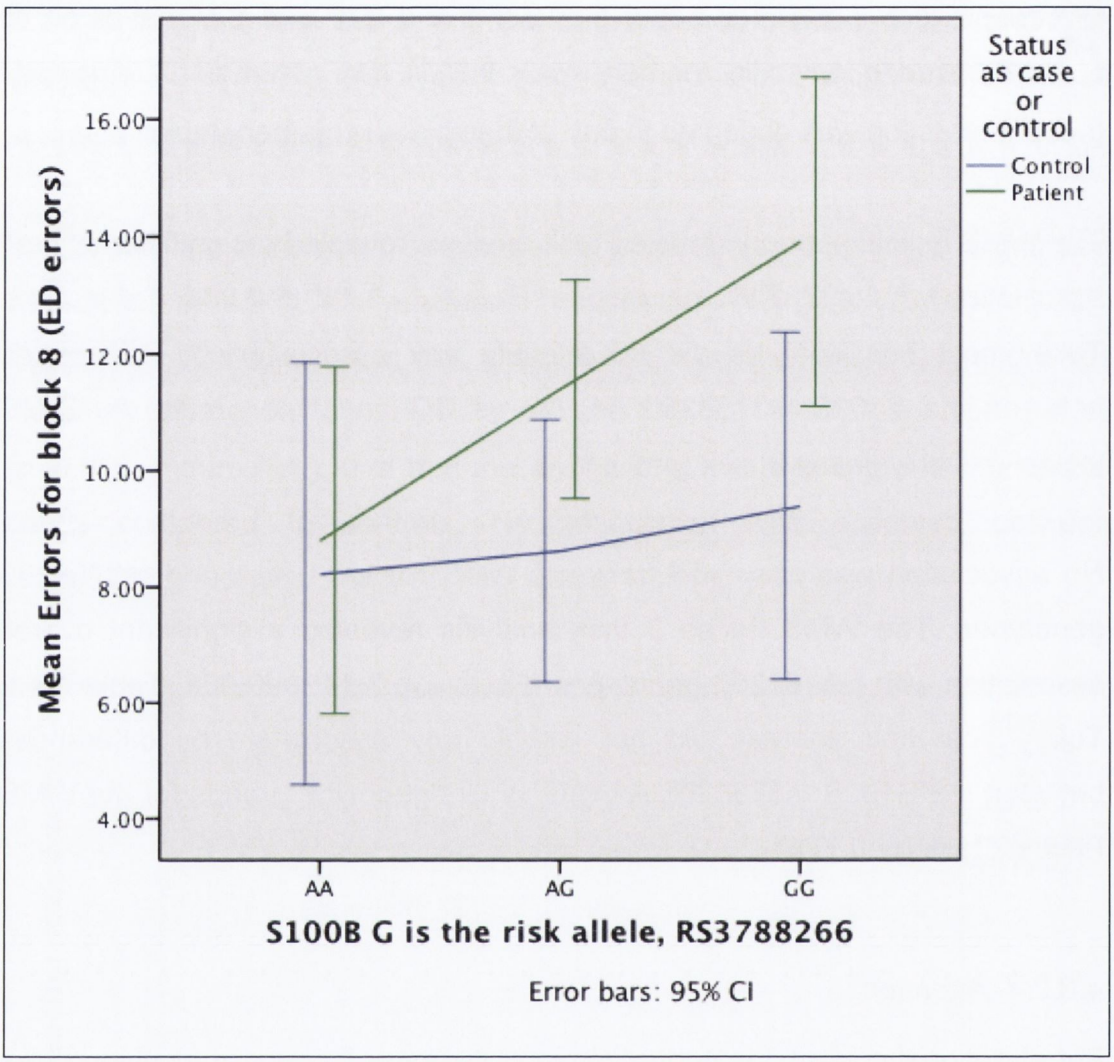


Figure 4.5. Differences in intradimensional-extradimensional task (score for block 8) associated with S100B rs3788266 genotype in cases and controls.

Analysis of the continuous performance test (CPT 3 letters), identical pairs version, revealed a significant overall association of rs3788266 genotypes ($F_{simple\ effect}$=3.46, p=0.033, Table 4.4.) as it is shown Figure 4.6. Tukey post hoc analysis showed that the association was driven by differences between the AA+AG and GG rs3788266 genotype groups (p=0.035).

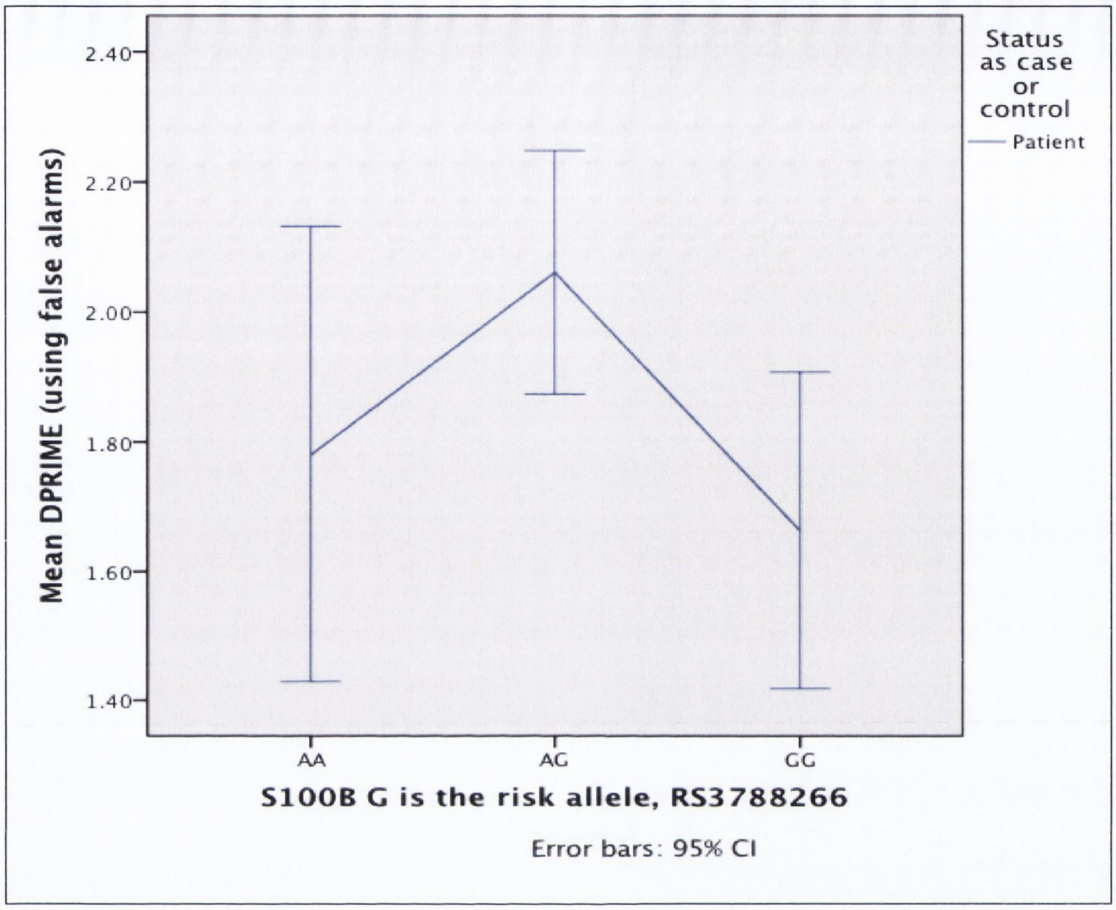


Figure 4.6. Differences in CPT, d' (using false alarm) associated with S100B rs3788266 genotype in patients only. Control subjects were not available for this task.

4.3.2.5. Social Cognition

For all tasks investigating social cognition, there were no differences between controls and combined SZP and SZA cases. There were no statistically significant association between S100B rs3788266 genotypes and all given tasks to investigate the social cognition in cases (simple effect) and combined cases and controls (main effect). A statistical interaction of genotype X diagnosis has been found for IPSAQ externalising bias task ($F_{interaction\ effect}$=3.60, p=0.028, Table 4.4.), this association is driven by differences in the control group.

4.3.3. S100B Serum Concentration

Serum concentrations of S100B protein in the combined set of 220 SZP and SZA cases were measured and analysed for association between clinical symptomatology or measures of cognition and S100B rs3788266 genotypes.

4.3.3.1. S100B Serum Concentration vs Clinical Symptomatology

Correlation analysis of clinical symptomatology has been restricted to BADDS Psychosis Score as this was the only score that was associated with rs3788266 genotypes previously. For this analysis 209 SZP and SZA cases with psychosis phenotypes were used. 11 samples were dropped from this analysis, as they did not present with psychotic symptoms. As shown in Figure 5.7, there is a weak correlation between S100B serum concentration and severity of psychosis (Spearman r = 0.137; p=0.023).

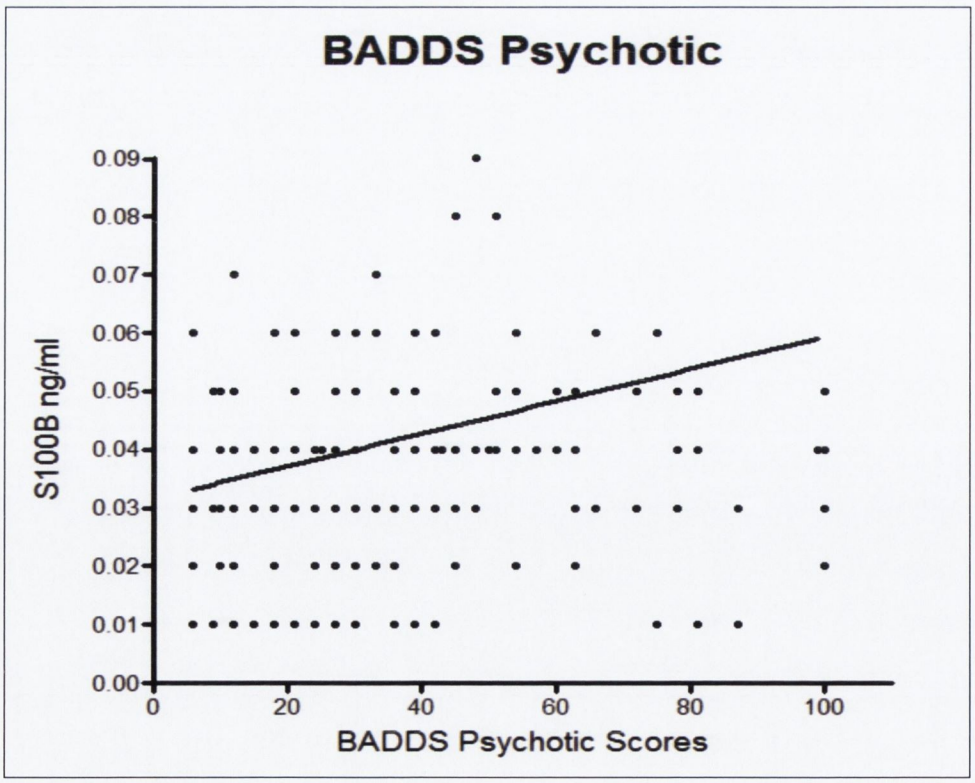


Figure 4.7. Correlation of S100B serum concentration and BADDS Psychosis Score.

4.3.3.2. S100B Serum Concentration vs Cognition

Correlation analysis between S100B serum concentration and cognition was restricted to 3 cognitive domains: IQ (WAIS full-scale IQ, WAIS performance IQ), working memory (WMS letter-number sequence task) and attention (IDED 8 shapes adjusted task) as they have been found in previous analyses to be significantly associated with rs3788266 G Allele (Table 4.4.).

The analysed subjects consisted of 219 SZP and SZA cases, 72.3% male and mean age 39.60 (±11.45). The correlation analysis did not reveal any statistically significant association between serum level of S100B protein and IQ, working memory or attention in SZP and SZA cases (Table 4.5.). However, the level of S100B did correlate with age (r=0.162, *p*=0.011, Figure 4.8.). There were also a positive correlation between the increased level of S100B and poorer WAIS verbal IQ but was statistically not significant (Figure 4.9.).

Table 4.5. Correlation between S100B serum concentration and cognitive performance in all 219 cases

	Spearman r	**p-value**
Age	0.162	0.011*
Age at onset	0.058	0.207
Chlorpromazine (mg)	- 0.043	0.293
WAIS full-scale IQ	- 0.088	0.106
WAIS performance IQ	- 0.018	0.401
WAIS verbal IQ	- 0.133	0.058
WMS letter-number sequence task	- 0.031	0.330
IDED 8 shapes adjusted task	0.065	0.181

* the p-value is statistically significant at < 0.05

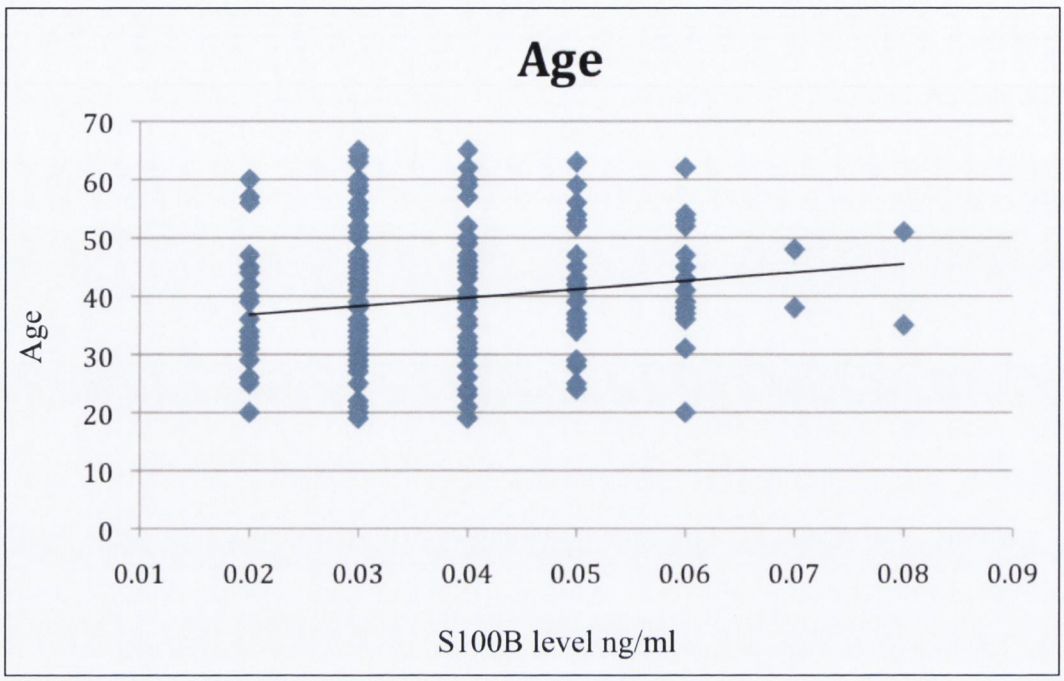


Figure 4.8. Correlation of age with S100B serum concentration. Inceased serum level of S100B protein correlated with increased age in SZP and SZA subjects. The y-axis indicated the age of subjects and the x-axis the concentration of S100B in ng/mL.

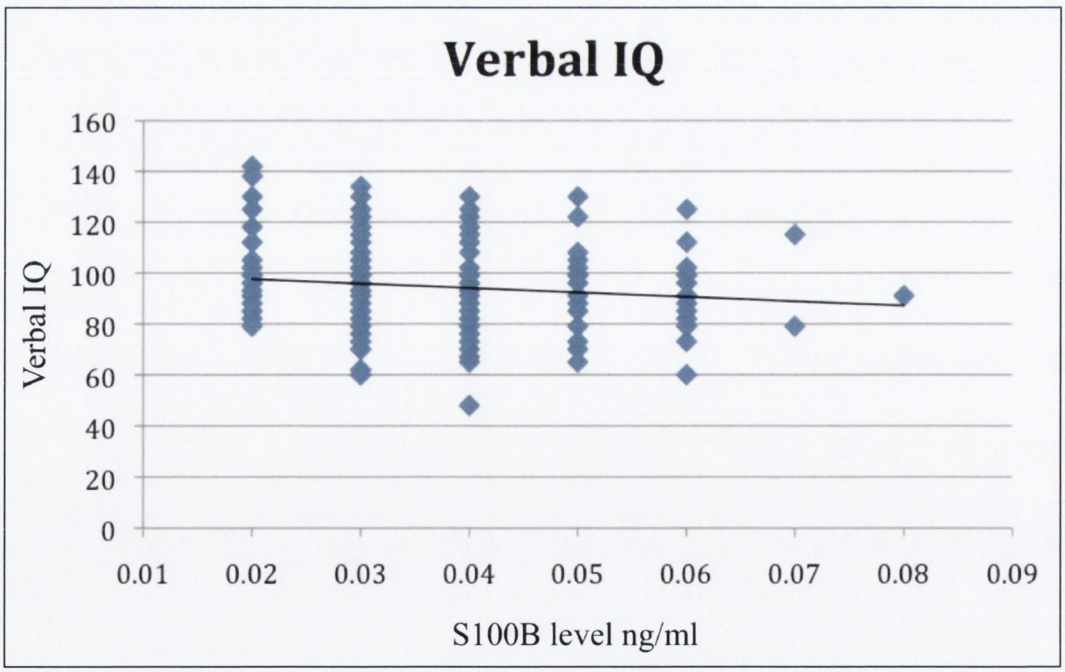


Figure 4.9. Correlation of S100B serum concentration with verbal IQ Score. Increased serum level of S100B protein showed a correlation with poorer level of verbal IQ in SZP and SZA subjects that approached significance. The y-axis indicates the WAIS verbal IQ scores and the x-axis the concentration of S100B in ng/mL.

4.3.3.3. S100B Serum Concentration vs rs3788266

To investigate possible functional effects of rs3788266 on S100B serum levels, an analysis of S100B serum level by rs3788266 genotype was performed in 220 Irish SZP and SZA cases. Four cases could not be genotyped and were therefore dropped from the analysis.

The demographic characteristics of the samples by genotype and the results of the association analysis are summarised in Table 4.6. There was a significant association between S100B serum level and rs3788266 genotype in the SZP and SZA cases (p=0.001). Importantly, the direction of the effect indicates that the putative risk G allele carriers had significantly higher levels of S100B than carriers of the non-risk A allele (Figure 4.10.).

Table 4.6. Association analysis of S100B serum consentration with rs3788266 in the SZP and SZA samples (N=216)

	Genotype	**N (%)**	**S100B (ng/ml)**	***P*-Value**
SZP and SZA cases	AA	39 (18.1)	0.030	**0.001**
	AG	114 (52.8)	0.034	
	GG	63 (29,2)	0.062	

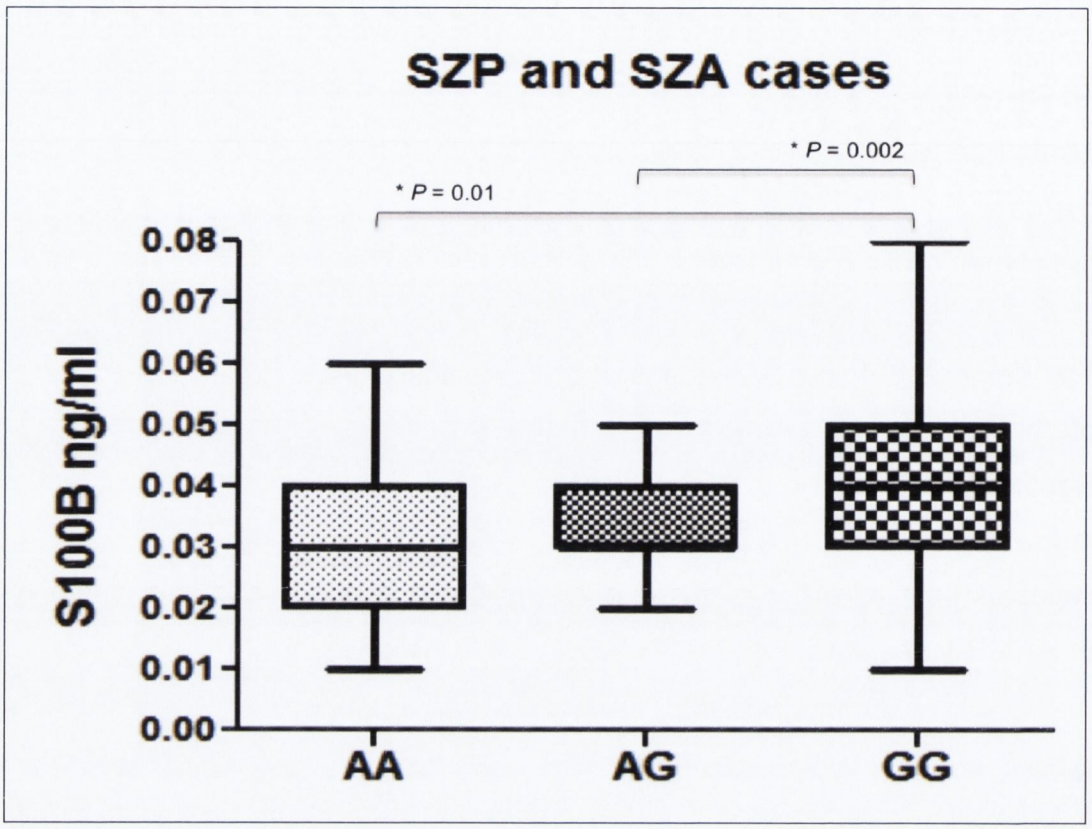


Figure 4.10. Analysis of S100B serum levels by rs3788266 genotype. The S100B protein level in serum was measured with a Electrochemiluminescence Immunoassay. Carriers of the GG genotype show significantly higher levels of S100B than carriers of the AA and AG genotypes.

4.4 Discussion

This study investigated the hypothesis that the risk GG genotype of S100B rs3788266 is associated with poorer cognitive performance and increased clinical symptom severity in SZP, SZA and BPAD, and healthy subjects (cognition analysis only). This study also investigated the hypothesis that higher levels of serum S100B protein would correlate with higher scores for positive symptoms, and lower levels of IQ, deficits in working memory and deficits in attention in SZP and SZA subjects. Irish cases and controls were genotyped for the S100B SNP rs3788266 and divided into 3 genotype groups (AA, AG and GG). Measures of clinical symptomatology (BADDS, SAPS and SANS) and cognition (current IQ, working memory, episodic memory, attentional control and social cognition) were calculated for each group. The serum concentration of S100B protein was measured for a proportion of cases.

For the clinical data, this study indicates a very weak association between the GG genotype of the S100B rs3788266 and severity of psychosis in BPAD subjects. This study also shows a weak correlation between the increased serum S100B protein and severity of psychosis in SZP and SZA subjects as measured by the BADDS psychotic factor. These tests are not corrected for multiple testing and hence must be used with caution. Nevertheless, Those results are in line with the previous studies. Roche S *et al.* (2007) found a significant association between the G-allele of the S100B rs3788266 and psychotic subset of BPAD patients. The G-allele of the S100B rs3788266 was associated with increased S100B protein (Chapter 2). Ling SH *et al.* (2007) have reported that increased S100B protein concentration was associated with severity of psychosis. The concentrations of S100B protein in CSF of SZP cases during an acute psychotic episode were increased compared to matched healthy controls (Steiner J *et al.,* 2006). Falcone T *et al.* (2009) found high level of serum S100B in psychotic children. In a further study, long-term continuous psychotic symptoms in SZP subjects were associated with higher levels of S100B protein (Wiesmann M *et al.*, 1999).

The increased level of S100B protein has been found to be associated in many independent studies with negative symptoms (Rothermundt M *et al.*, 2001b, Schroeter ML *et al.*, 2003; Ling SH *et al.*, 2007). A study by Tan Y *et al.* (2010) found a positive correlation between serum S100B protein levels and negative symptoms during the acute phase of SZP. Rothermundt M *et al.* (2004c) reported that high levels of S100B protein were correlated with negative symptomatology in SZP subjects and that the levels decreased after 6 month of treatment. However, in a subgroup of patients with persistent negative symptoms the S100B level remained high. This has been interpreted as being associated with persistent psychopathology rather than as a result of antipsychotic treatment. It has been reported that patients with high levels of S100B have problems in emotional expression, contact with others, spontaneity and taking initiatives (Rothermundt M *et al.*, 2004c). Negative symptoms have been frequently linked with cognitive impairments (Rothermund M *et al.*, 2004b).

At a cognitive performance level, this study showed a weak association between the GG genotype of rs3788266 and poorer IQ, verbal working memory and attention in SZP, SZA and healthy subjects. Homozygote GG carriers show lower scores of WAIS full-scale IQ, WAIS verbal IQ and WAIS performance IQ compared to AG and AA cariers, with both cases and controls displaying a similar genotype effect. Carriers of the G risk allele show poorer level of verbal working memory than carriers of the non-risk A allele and also both cases and controls displayed a similar genotype effect for verbal working memory. These tests are not corrected for multiple testing and hence must be interpreted with extreme caution.

For attention, the IDED task (score for block 8) analysis showed that the carriers of the GG risk genotype made more mistakes compared to AG and AA genotypes. In addition, the CPT 3 letters task and CPT 4 letters task analysis indicated that the homozygote GG risks genotype carriers show lower scores compared to AG or AA carriers. There were no association between the G allele of S100B rs3788266 and poorer performance in any of the tasks when analysing the episotic memory and social cognition. Previous

functional and animal studies Found corretation between increased level of S100B protein and deficits in attention processes that normalised after treatment in patients with major depression (Hetzel G *et al.*, 2005; Dietrich DE *et al.*, 2003).

This study investigated possible association between the serum level of the S100B protein in SZP and SZA cases and IQ, verbal working memory and attention. This study was restricted to analysis of WAIS full-scale IQ, WAIS verbal IQ and WAIS performance IQ, letter number sequencing and IDED task (score for block 8) as they have shown assococition with cognitive impairments from the genotype analyses using rs3788266. Serum levels of S100B protein correlated with age indicating an association between increased serum level of S100B protein and increased age. This is in line with previous findings; a systematic meta-analysis investigating the level of S100B in mood disorders found a correlation between age and S100B protein levels (Schroeter ML *et al.*, 2010). In the present study, there was no correletion between increased serum level of S100B protein and poor scores of IQ, verbal working memory or attention. Finally, this study found an assciation between increased levels of S100B protein and the G allele of S100B rs3788266 in SZP cases, which is in line with previous findings (Chapter 2).

Those results suggest a weak association between an S100B variant and verbal intelligence and verbal working memory. A study by Pedersen A *et al.* (2008) reported that first episode patients and chronic schizophrenic patients with normal S100B serum concentration showed no cognitive deficits but the chronic patients with impairment in verbal memory had significantly increased S100B protein levels. The lack of significant association in the present study could be explained by differences in sample characteristics. Persistently high S100B protein levels might impair verbal memory only in chronic schizophrenic cases, however the subjects in the present study were not restricted to chronic patients. Antipsychotic medication may affect the serum level of S100B protein as has been shown in previous studies. The serum level of S100B has been shown to both decrease (Rothermundt M *et al.*, 2001b; Sarandol A *et al.*, 2007; Ling SH *et al.*, 2007; Steiner J *et al.*, 2008)

and increase (Schroeter ML *et al.*, 2003) after antipsychotic treatment. Also Steiner J *et al.* (2010) reported reduction of S100B release from astrocytic C6 and oligodendrocytic OLN-93 cell lines after haloperidol and clozapine treatment.

Various animal and functional studies support the role of S100B protein in spatial memory (Nishiyama H *et al.*, 2002; Roder JK *et al.*, 1996; Gerlai R & Roder J 1996; Whitaker-Azmitia PM *et al.*, 1997; Winocur G *et al.*, 2001). Variants within S100B gene (rs9722 and rs11542311) have been associated with visuospatial disabilities in SZP (Zhai J *et al.*, 2010). In the present study no association between spatial memory and S100B rs3788266 was found. Zhai J *et al.* (2010), however, did not investigate the rs3788266 variant, so the results are not directly comparable.

The mechanism by which S100B protein levels might affect cognition is not known but there are several possibilities. It is believed that increased levels of S100B protein in SZP patients might cause a shift in neuronal regeneration-degeneration balance towards degeneration, which could reduce synapses and dendrites (Ling SH *et al.,* 2007). Tan Y *et al.* (2010) hypothesized that increased levels of S100B might affect the interaction of neuron and glial cells, which could lead to elevated neurotransmission release and thus contribute to the cognitive deficits in SZP. Indeed, S100B has been found to be involved in dopaminergic and glutamatergic neurotransmission (Tramontina F *et al.,* 2006; Rothermundt M *et al.*, 2007; Liu Y *et al.*, 2008). Suchankova P *et al.*, 2010 hypothesized that increased levels of S100B up-regulates the NFkB transcription factor, which in turn might result in the transcription of several pro-inflammatory cytokines that could affect the normal brain function.

Overall, these data suggest that the functional variant rs3788266 at S100B very weakly associated with cognitive performance and possibly symptom severity. These results are interesting but need to be interpreted with caution. The analyses have been performed in small samples and the results have not

been corrected for multiple testing. This present study needs to be confirmed by replication in suitably powered independent studies.

Chapter 5

Association and Epistasis Analysis of S100B and SIX-Family Genes with Schizophrenia and Bipolar Disorder

5.1. Introduction

Family, twin, and adoption studies indicate that genetics play a major role in the etiology of SZP and BPAD. The heritability is estimated to account for at least 80% of the disease risk for BPAD and SZP (Carroll LS & Owen MJ, 2009). Complex diseases display a complex pattern of inheritance with variable age-at-onset and incomplete penetrance, and thus they are likely to be caused by many genes in combination with environmental risk factors (Goodwin FK & Jamison KR, 2007).

The two main types of study used to map disease gene are linkage and association. Linkage studies utilize family samples whereas association studies used population-based samples (Goodwin FK & Jamison KR, 2007). Association studies are a set of methods that are used to identify correlations between genetics polymorphisms and the expression of phenotypes such as BPAD or SZP. Two main methods for these tests are the "case-control" and "family-based" paradigms. The association test approach is based on the premise that (a) disease variant alleles will have significantly different frequency distributions in affected versus unaffected individuals (case-control design), or (b) that disease variant alleles will have a transmission pattern that is significantly different from that expected under Mendelian laws of inheritance (family-based design). A positive association can mean either the polymorphism is directly involved in disease pathogenesis or is in linkage disequilibrium (LD) with the pathogenic variant or is a chance finding (Goodwin FK & Jamison KR, 2007).

LD is the non-random association of alleles at two or more loci. It is a correlation between polymorphisms (e.g. SNPs) that is caused by their shared history of mutation and recombination. LD decays as the distance between SNPs increases because there is more opportunity for recombination. LD plays a central role in association analysis. When performing an association study at a candidate gene, the local pattern of LD will determine the number and density of markers that is needed to capture a high proportion of genetic variation (Flint-Garcia SA *et al.*, 2003). The most commonly used measures

for LD are D' and r^2, which are both scaled between 0 and 1. The D'=1.0 represents complete LD (i.e. no recombination between two markers) and D'=0 signifies no LD. The LD coefficient r^2 represents the proportion of observation in which two specific pairs of alleles occur together. If 2 SNPs are perfectly correlated they have an r^2 of 1.0. An r^2 of 0 can be interpreted as no observation of correlation (Manolio TA *et al.,* 2008). The genome consists of haplotype "blocks", which are regions of high LD separated by recombination hotspots. These blocks are characterized as genomic regions with low recombination rates, which means that SNPs within these blocks are often in high LD with each other. For this reason "tagging" SNPs can be selected in order to describe the genetic variation of this block (Figure 5.1.). The HapMap data allow for the identification of tag SNPs specific for a particular population (Hirschhorn JN & Daly MJ, 2005).

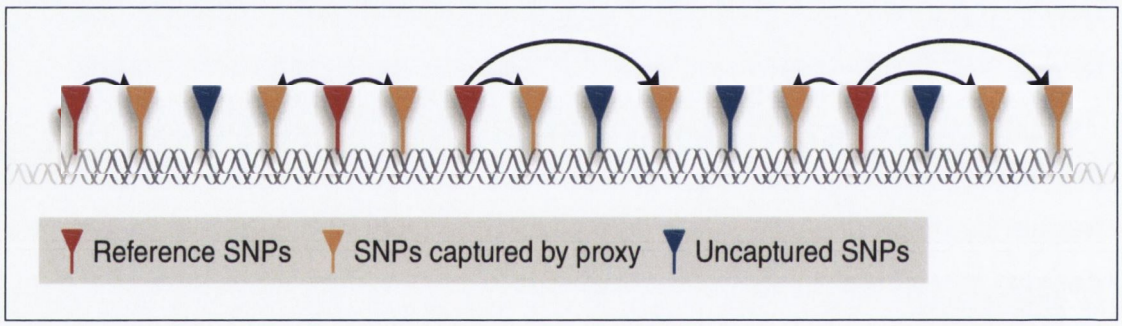


Figure 5.1. Schematic of a genomic region to be tested for association with a phenotype. The region contains 16 SNPs, any one of which could affect the phenotype. The four SNPs in red are genotyped directly (these are the reference or **tag SNPs**). The eight SNPs in orange are captured through correlations (linkage disequilibrium) with the SNPs in red (as denoted by arrows). The four SNPs in blue are neither genotyped nor correlated with genotyped SNPs, and so phenotypic association with any of these uncaptured SNPs would be missed (adopted from Kruglyak L, 2005).

Two major hypotheses have been proposed to explain the underlying genetic cause of common disease. These are common disease-common variant (CDCV) and common disease-rare variant (CDRV) hypotheses (Figure 5.2.). CDCV implies that genetic susceptibility to a common disease is caused by the inheritance of multiple genetic variants with low penetrance, that are common in the population (minor allele frequency (MAF) >5 %), meaning that

the disease is polygenic (Owen MJ *et al.,* 2009). CDRV proposes that the genetic susceptibility to a common disease caused by the inheritance of a number of low frequency variants (MAF <1%) with high penetrance (Psychiatric GWAS Consortium Coordinating Committee *et al.,* 2009). Candidate gene studies and genome-wide association studies (GWAS), described below, are based on the CDCV hypothesis. There is another hypothesis, the two-hit hypothesis, which proposes that there are gene-gene interactions between common SNPs, such that association cannot be detected by testing SNPs or genes individually (Gershon ES *et al.,* 2011).

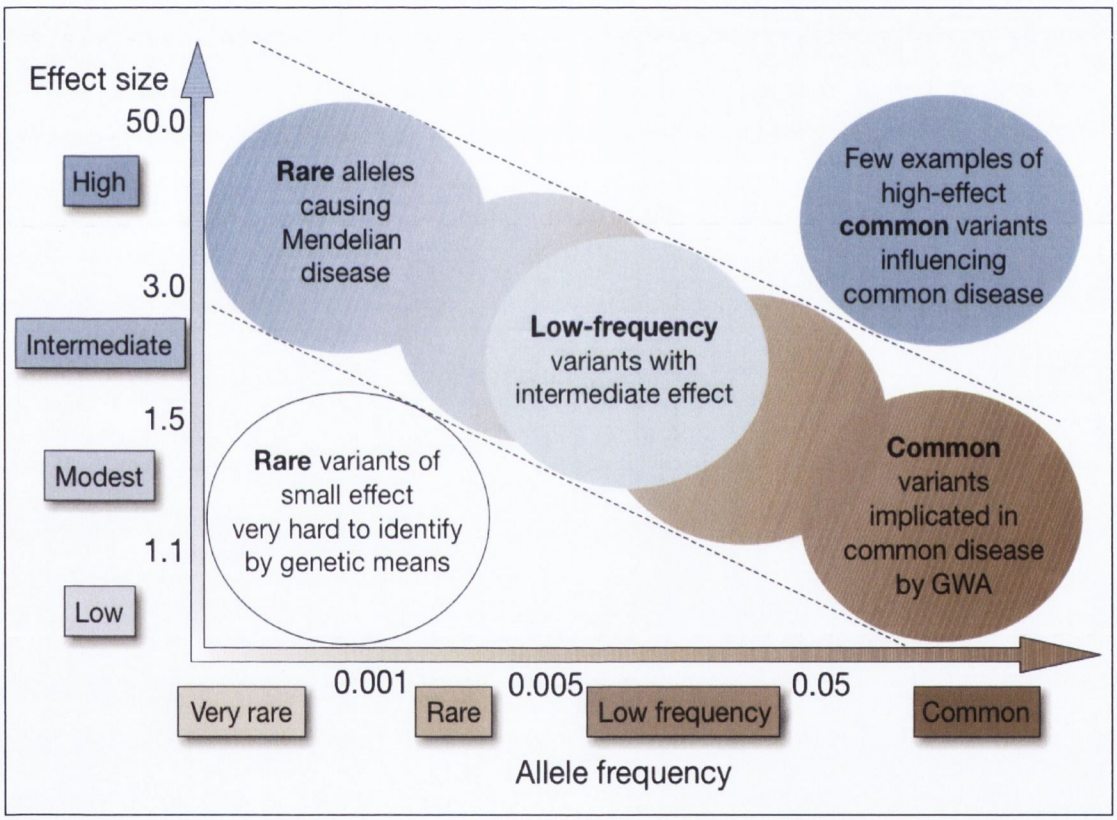


Figure 5.2. Allele frequency and genetic effects of disease associated different type of variants. The Y-axis represents the effect of the variant in terms of odd ratios and the X-axis indicates the minor allele frequencies. To the upper left of the figure are rare variants of large effect. To the lower right of the figure are common variants of small effect (adapted from Manolio TA *et al.*, 2009).

For many years association studies for BPAD or SZP have been at the level of the candidate gene where a small number of SNPs or other common genetic markers are tested in relatively small association samples, usually

case-control samples. Genes were selected on the basis of being functional candidates based on a biological hypothesis or positional candidates based on location under a linkage peak. Despite being numerous, these studies were largely ineffective at identifying definite risk genes. For example in SZP research, 1,727 association studies, 1,008 candidate genes, 8,788 polymorphisms and 287 meta-analysis have been published (http://www.szgene.org/, April 2011).

New advances in genetic technology, the Human Genome Project and the HapMap Project have made genome-wide association studies (GWAS) possible. The international HapMap Project (www.hapmap.org) has developed a haplotype map of the human genome, which can describe the pattern of correlation within common human variation. The HapMap consortium validated 4 million SNPs, including 2.8 million of the estimated 10 million common SNPs in a number of samples called reference panels from different major world populations (Psychiatric GWAS Consortium Coordinating Committee *et al.*, 2009). GWAS can directly study up to 1 million genetic polymorphisms across the whole genome and this number can be increased several-fold using imputation (Marchini J *et al.,* 2007). GWAS is usually performed as a case-control design with studies including many thousands of samples.

The GWAS approach has been applied to many complex diseases since 2007. GWAS identified numerous new susceptibility loci for diseases like type 2 diabetes, Crohn's disease, BPAD, SZP and others (Monalio TA *et al.,* 2008). A list of the GWAS studies can be found at the Catalog of Published Genome Wide Association studies from the National Human Genome Research Institute (http://www.genome.gov/26525384). At the time of writing this thesis, there have been 12 published GWAS that included BPAD subjects (WTCCC, 2007; Baum AE *et al.*, 2008a; Sklar P *et al.*, 2008; Ferreira MA *et al.*, 2008; Hattori E *et al.*, 2009; Scott LJ *et al.*, 2009; Smith EN *et al.*, 2009; Zhang D *et al.*, 2009; Huang J *et al.*, 2010; Djurovic S *et al.*, 2010; Lee MT *et al.*, 2010; Curtis D *et al.*, 2011) plus 2 BPAD GWAS meta-analysis (Wang KS *et al.,* 2010; Liu Y *et al.*, 2011) and 13 GWAS that included subjects with SZP

(Lencz T *et al.*, 2007; O'Donovan M *et al.*, 2008; Shifman S *et al.*, 2008; Sullivan PF *et al.*, 2008; Walsh T *et al.*, 2008; Stefansson H *et al.*, 2009; Kirov G *et al.*, 2009; Need AC *et al.*, 2009; Purcell SM *et al.*, 2009; Shi J *et al.*, 2009; Athanasiu L *et al.*, 2010; Ikeda M *et al.*, 2011; Curtis D *et al.*, 2011) plus 3 SZP GWAS meta-analysis (Stefansson H *et al.*, 2009; Shi J *et al.*, 2009; Wang KS *et al.*, 2010). The best-supported genetic associations from GWAS for BPAD are at the DGKH, NCAN, PALB2, ANK3 and CACNA1C genes and for SZP are at ZNF804A, NRGN, TCF4 and the MHC region on chromosome 6 (for review see chapter 1, section 1.2.3.). A resent meta-analysis has also found an association between ZNF804A and both BPAD and SZP (Williams HJ *et al.*, 2011).

As described in earlier chapters, I have been studying the rs3788266 SNP that was reported by Roche S *et al.* (2007) as being associated with psychotic BPAD in a small family-based study. The location of this SNP within the promoter region of the gene suggested that the rs3788266 might affect gene expression. I was able to show that the SNP did impact on expression of S100B using *in vitro* and *in vivo* methods (chapter 2). Bioinformatics analysis revealed that the disease-associated G allele of rs3788266 disrupts Trex/MEF3 consensus recognition site, which is located 1.4Kb upstream of the transcription start site. The Trex/MEF3 site is bound by the Six-family of transcriptional activator proteins, suggesting that this SNP might affect S100B expression. Six-family proteins are expressed in the brain and regulate brain development and possibly differentiation/maturation of neuronal cells. Interesting, there is a cluster of Six-family genes at chromosomal location 14q23, which was identified as a potential BPAD susceptibility locus (Cassidy F *et al.*, 2007), suggesting that Six-family genes could also confer susceptibility to BPAD. Indeed, SIX5 has been implicated in the pathophysiology of myotonic dystrophy, a neuromuscular disorder that also includes neuropsychiatric impairment (Hanson IM, 2001; Sato S *et al.*, 2002). Members of this highly conserved gene family are organised into three phylogenetic groups: SIX1/2, SIX3/6 and SIX4/5. The SIX1, SIX2, SIX4 and SIX5 transcription factors have been reported to bind the Trex/MEF3 consensus recognition site. I investigated the binding of Six-family

transcription factors using EMSA and found that SIX1 and SIX4 binds to the site of rs3788266 at S100B and there was an allele-specific effect on binding (Chapter 3).

In general, gene-gene interactions are critical for gene regulation, signal transduction, biochemical network and various other physiological and developmental pathways (Moore JH, 2003). The S100B gene produces a protein with many biological functions. Its potential as a susceptibility factor for BPAD and SZ may be related to a number of these functions that are disturbed by an increased level of S100B. Given the functional link between S100B and the Six-family proteins, and the location of the Six-family genes in a BPAD linkage region, I was interested to investigate possible epistasis between S100B and these genes contributing to psychosis risk.

Several genetic studies have also shown a relationship between SNPs in the S100B gene and SZP, BPAD and major depressive disorder (MDD), which is further support for a role for S100B in the aetiology of psychiatric disorders (Liu J *et al.,* 2005, Roche S *et al.*, 2007, Yang K *et al.,* 2009). Also, Lambert JC *et al.* (2007) has shown association of SNPs within the S100B gene with low cognitive performance and dementia in the elderly. Liu J *et al.* (2005) reported association between SZP and rs11542311, particularly a haplotype spanning rs11542311 and rs9722, and hypothesised a genetic predisposition for increased S100B expression as a relevant pathophysiological mechanism.

In this chapter I will pull together all available data on genetic variation at S100B and its putative role in susceptibility to neuropsychiatric phenotypes. Firstly, this involves reviewing all published candidate gene studies. The Neuropsychiatric Genetics Research Group at TCD has a large Irish psychosis case-control sample and is centrally involved in two SZP GWAS. The S100B rs3788266 SNP will be investigated for association with psychosis using available Irish samples. The GWAS datasets will also be mined to investigate the possibility of associated variants in the Six-family genes and test for interaction with rs3788266 at S100B.

5.2. Material and Methods

5.2.1. MAP of Genetic Studies of S100B

I investigated all studies of the S100B gene published to date using PUBMED to find out if there are any other reported associations between rs3788266 or other SNPs at S100B with psychiatric or related phenotypes. A SNP map of the gene S100B was created and the location of SNPs was determined according to the UCSC genome browser (March 2006 assembly; www.genome.ucsc.edu). I have also investigated the LD structure of S100B SNPs (50kb up or downstream) using CEU samples with Haploview (Barrett JC *et al.*, 2005)

5.2.2. Association Analysis at rs3788266

5.2.2.1. Sample Preparation

The following case and control samples were available to me at the beginning of this study. All study participants had provided written informed consent in line with ethics committee guidelines. The GASP (Genetic Association study of Schizophrenia and related Psychoses) case-control samples, the RPGI (Resource for Psychiatric Genetics in Ireland) samples were used for this study. RPGI and GASP consisted of 696 SZP, 248 BPAD and 151 schizoaffective disorder (SZA) cases.

GASP cases consisted of 538 subjects, which have been interviewed with Psychiatrist or trained psychiatric nurse for Structured Clinical Interview DSM (SCID-P). All cases were over the age of 18 and provided written informed consent. The diagnosis was made using the consensus lifetime best estimate method with DMS-IV criteria. The RPGI samples used for this study consist of 557 cases that were independent of the GASP samples. Cases were diagnosed using DSM-IV criteria and were recruited from five sites across the Republic of Ireland and Northern Ireland using the same inclusion/exclusion criteria as for GASP samples.

The 1,858 Trinity Biobank controls were drawn from the blood donors in Ireland who are unlikely to have SZP as subjects taking medication are prohibited from donating blood. All participating individuals in this control collection gave also a written informed consent and met the same ethical criteria as GASP and RPGI cases. The Neuropsychology control sample consisted of 157 subjects that were prepared and assessed as described in chapter 4 (section 4.2.1).

Genomic DNA from whole blood samples were extracted using the phenol-chloroform method (Sambrook J *et al.*, 1989), QIAamp DNA Blood Maxi Kit (Qiagen, Crawley, UK) or semi-automated 'Gentra' method (Qiagen) at the Trinity Biobank. Stock DNA was dissolved in standard Tris-EDTA buffer and stored at -20°C. Nanodropper (Nanodrop, Wilmington, DE, USA) was used to create working stock of 10ng/μL from stock DNA.

5.2.2.2. Genotyping

All samples were genotyped for rs3788266 A/G using the predesigned TaqMan® Assay (ID: C_25800807_10) from Applied Biosystems (Warrington, UK). The TaqMan® genotyping method is dependent on allele-specific flourophore release, in this case FAM for allele A and VIC for allele G, from a complementary probe during a PCR reaction. Each reaction consisted of about 20ng of genomic DNA, 0.125μl TaqMan assay mix (containing probe and primers) and 2.5μl universal MasterMix (containing Taq Polymerase, dNTPs and reaction buffer from Applied Biosystems, Warrington, UK) made-up to a final volume of 5μl using double-processed tissue-culture grade water. To minimise the risk of systematic errors biasing the overall outcome, a number of quality control measures were implemented at this stage. Case and controls DNA samples were mixed across each 96-well plate. Non-template (negative) controls, HapMap CEU samples of known genotype (positive controls) and duplicate controls were used to measure genotyping accuracy. HapMap CEU genomic DNA samples were obtained from the Coriell Institute for Medical Research, USA. 384-well plates are used for this genotyping experiment. Amplification and allelic discrimination for the assay were

performed using Applied Biosystems 7900HT Sequence Detection System starting 15 min at 95°C (for polymerase activation) flowed by 40 cycles of 95°C for 15 sec and 65°C for 60 sec. Following amplification, individual genotypes were determined automatically based on the wavelength of fluorescent signal emitted from each reaction. All reactions that were at low intensities or ambiguous genotypes were omitted. Failed reactions were repeated.

5.2.2.3. Analysis of Association using PLINK

To investigate whether there is an association at rs3788266 and SZP or BPAD each subsample was tested individually for association with a standard X^2 test for association using PLINK (Purcell S *et al.*, 2007). The Hardy-Weinberg equilibrium was also tested for each subsample using this software.

5.2.3. Bioinformatics Analysis of SIX-Family Genes

The ISC, WTCCC and WTCCC2 (narrow and broad) GWAS datasets (described below) were used to investigate if there is any association between SNPs within SIX1, SIX2, SIX4 and SIX5 genes and SZP and/or BPAD. Gene co-ordinates were downloaded from UCSC Genome Browser table section (www.genome.ucsc.edu) using hg18 March 2006 assembly and dbSNP 130.

To investigate LD, SNP data were downloaded from HapMap release #28 (Phases 1, 2 & 3 - merged genotypes & frequencies; www.hapmap.org) using the CEU population and Haploview v4.2 (http://www.broad.mit.edu/mpg/haploview; Barrett JC *et al.*, 2005) was used to generate LD measurements. Haploview was also used to investigate how well SNPs within the 50 kb regions up and downstream of SIX1, SIX2, SIX4 and SIX5 genes were captured by GWAS SNPs. The tagger algorithm was used in Haploview with settings of r^2 >0.8 and MAF >0.01.

5.2.3.1. ISC Dataset

The International Schizophrenia Consortium (ISC) samples consist of 3,332 of DSM-IV schizophrenia cases and 3,587 controls from eight samples collected in five European countries (ISC *et al.*, 2009). All subjects were genotyped using the Affymetrix Genome-Wide Human SNP array, version 5.0 and 6.0 (Affymetrix, Santa Clara, California, USA). My group in TCD are members of the ISC and contributed the Irish sample of 275 SZP cases (all from GASP above), 866 controls (all from Trinity Biobank above) to this study. Genotype and phenotype files are obtained from the ISC web-site (http://pngu.mgh.harvard.edu/isc/).

5.2.3.2. WTCCC Dataset

The Welcome Trust Case Control Consortium (WTCCC) data consist of 1,868 BPAD cases and 2,938 control subjects typed with the Affymetrix Gene Chip 500K mapping array set (WTCCC, 2007). All subjects were self-identified as white European and living in the UK. All control subjects were from the 1958 British Birth Cohort or were from UK Blood Donors. This data was downloaded from the WTCCC website following a submission by Dr. Derek Morris.

5.2.3.3. WTCCC2 Dataset

The Welcome Trust Case Control Consortium 2 (WTCCC2) GWAS is an all-Ireland study of psychosis (TCD PI = Prof. Aiden Corvin). Samples consisted of a narrow psychosis case sample (SZP (n=1 418), schizoaffective disorder (n=182) or schizophreniform disorder (n=6); total = 1,606) and a broad psychosis case sample (narrow plus 442 BPAD cases; total = 2,048) and 1,794 controls sampled in Ireland. The sample set is predominantly made up of GASP and RPGI cases and Trinity Biobank controls from TCD and an Irish case sample collected by Virginia Commonwealth University (VCU; PI = Prof. Kenneth Kendler). The subjects were genotyped on the Affymetrix 6.0 SNP genotyping array. At time of writing a manuscript describing this study was under review. There was an overlap between the samples in the WTCCC2

study and the Irish subset of the ISC study (GASP cases and approximately half of the Trinity Biobank controls).

5.2.4. Epistasis

Given the prior evidence of interaction of SIX-family transcription factors and S100B gene (chapter 3), I tested for SNPxSNP epistasis using the PLINK --*epistasis* option (http://pngu.mgh.harvard.edu/purcell/plink/; Purcell S *et al.*, 2007). The full WTCCC2 narrow and broad samples were used in this analysis.

5.3. Results

5.3.1. Bioinformatics Analysis of S100B gene

The S100B gene is located at chromosome 21q22.3 and composed of 3 exons. The first one represents the 5'-untranslated region. The second exon encodes a single EF-hand and the third exon encodes a Ca^{2+}-binding domain (Allore RJ *et al.*, 1988). The promoter contains several regulatory transcription elements (Castets F *et al.*, 1997).

I searched for studies that have investigated SNPs at S100B for an association with BPAD, SZP, MDD, Alzheimer's Disease (AD) and behaviour, or with gene expression and found 10 studies. At the time of generating this table, data from the WTCCC2 GWAS of psychosis was not available. The summaries of the results from 10 studies are listed in Table 5.1. I also created a table that lists all investigated SNP within or close to the S100B gene from those 10 studies (Table 5.2.) Figure 5.3 shows the genomic organization of the S100B gene and the LD structure between the associated SNPs from those 10 studies. Twelve SNPs within or close to S100B gene show some level of significant association in at least one study; 4 of those SNPs were located upstream of the gene (rs12152107, rs13047696, rs9978658, rs3788266), 5 SNPs are intronic (rs11911834, rs2186358, rs34722617, rs9984765, rs2300403), 1 SNP is exonic (rs11542311), 1 SNP in the 3'UTR (rs9722) and 1 SNP is downstream of the gene S100B (rs2839350). Only 3 SNPs (rs3788266, rs9722, rs11542311) reached statistically significance (*p*-value < 0.05) in more than one study.

I also examined the haplotype block pattern at the S100B gene at chr21:46,842,959-46,849,463 (hg18) including 50 kb region up and downstream of the gene to consider potential regulatory polymorphisms. As is shown in Figure 5.4., this locus does not contain well-defined haplotype blocks.

Table 5.1. Association Studies of S100B gene.

#	Reference	Subjects, Phenotype and Ethnicity	Associated SNPs	Results
1	**Liu J** *et al.*, 2005	384 SZP cases 401 controls (Han Chinese)	rs11542311 rs9722	No association was found when 4 SNPs were investigated individually. A haplotype rs11542311 - rs9722 (G-C) was found associated with SZP. The SNPs V1 (-960 C>G) and V2 (rs8128872; -111 C>T) are found in S100B promoter: the SNP V2 is on a Sp1 transcription factor binding site (Sp1 CCCCG**C**C, bold is the C allele of V2).
2	**Roche S** *et al.*, 2007	125 BPAD type1 trios (Irish)	rs2839350 rs3788266 rs2186358	The SNPs rs2839350 (P=0.022) and rs3788266 (P=0.031) were significantly associated with BPAD. Both SNPs (rs2339350 (P=0.016) and rs3788266 (P=0.009)) were more significantly associated in the psychotic subset. When the sample was extended to 151 BPAD Type I trios, it was found that rs2186358 (intron) now had an association with BPAD (P= 0.0019). Associations were also observed at the haplotype level. The rs2839351 and rs9722 (C-C) haplotype was associated with BPAD and the rs2839363 and rs3788266 (G-A) haplotype was associated in the psychotic subgroup.
3	**Lambert JC** *et al.*, 2007	2,517 Dementia, AD cases 2,131 controls (6 different EU populations)	rs2300403	rs2300403 has been associated with lower cognitive performance in 3 different populations (P=0.0005) and was associated with developing dementia or AD in 6 populations with the effect stronger in women and older subjects (P=0.0006).
4	**Yang K** *et al.*, 2009	152 MDD cases 150 controls (Han Chinese)	rs9722 rs11911834	No association was found between major depressive disorder (MDD) and 2 investigated SNPs (rs9722 and rs11542311). However, rs9722 was associated with age of onset of disease (P=0.03) in MDD cases. Also an association between the haplotype constructed from the 2 SNPs and MDD was observed.
#	**Reference**	**Subjects, Phenotype and Ethnicity**	**Associated SNPs**	**Results**

5	**Hohoff C** *et al.*, 2010	196 mRNA and serum samples (healthy German)	rs9722 rs11542311 rs9984765	The SNP rs9722 (P=0.001), and S100B haplotype T-G-G-A (rs2186358-rs11542311-rs2300403-rs9722, P=0.0004) were associated with elevated S100B serum level. rs11542311 (P = 0.01), rs9984765 (P=0.046) and the haplotypes G/C-A-T-C (rs11542311-rs2839356-rs9984765-rs881827, P=0.004) were associated with S100B mRNA expression.
6	**WTCCC, 2007**	1,868 BPAD cases 2,938 controls (British)	none	11 SNPs were genotyped across the 50kb regions up and downstream of the S100B gene but none of the SNPs were found to be associated with BPAD.
7	**ISC, 2009**	3,322 SZP cases 3,587 controls (8 European samples)	rs34722617 rs9978658 rs13047696 rs12152197	16 SNPs were genotyped across the 50kb regions up and downstream of the S100B gene and 4 of the SNPs were found to be associated at p-value < 0.05.
8	**Zhai J** *et al.*, 2010	304 SZP cases 196 controls (Chinese)	rs9722 rs11542311	Two SNPs (A allele of rs9722, the G allele of rs1051169, and the AG haplotype) were associated with the poorer performance of spatial ability in schizophrenia patients.
9	**Suchankova P** *et al.*, 2010	270 Women 247 Men (healthy Swedish)	rs9722 rs11542311	Two SNPs were analyzed with respect to personality traits assessed using the Temperament and Character Inventory (TCI). In men, the SNPs rs11542311 and rs9722 were found to significantly influence the TCI dimension of self-directedness (P=0.042 and P=0.0021, respectively). Low scores of dimension of self-directedness are seen in depression and SZP.
10	**Dagdan E** *et al.*, 2011	87 BPAD Irish cases, 67 healthy relatives, and196 healthy	rs3788266	G allele of rs3766266 SNP was associated with increased level of S100B serum in 3 sample groups.

Table 5.2. Map of S100B gene Association Studies.

Position	SNP	Alleles[a]	Function	1[b] SZP	2 BPAD	3 Dementia+ AD	4 MDD	5 healthy	6 BPAD	7 SZP	8 SZP	9 healthy	10 BPAD
46839749	rs3804040	C/G	unknown		ns								
46841624	rs2839349	A/G	unknown								ns		
46841647	rs2839350	A/G	unknown		.016				ns	ns			
46841713	rs2839351	C/T	unknown		ns								
46842970	rs9983698	C/T	untranslated-3						ns				
46843666	rs9722	C/T	untranslated-3	.008[c]	ns		.03	.001		ns	.023	.001	
46844238	rs2239574	C/T	intron					ns	ns	ns			
46844295	rs881827	C/T	intron		ns	ns		ns	ns	ns	ns		
46844364	rs2239575	C/T	intron			ns			ns	ns			
46845481	rs2300403	C/T	intron			.0005		ns					
46845507	rs2300404	A/G	intron			ns							
46845861	rs2300405	C/T	intron			ns							
46845932	rs6518303	C/T	intron			ns							
46846082	rs9984765	C/T	intron					.046	ns	ns			
46846394	rs34722617	A/T	intron							.009			
46846460	rs2839355	C/T	intron		ns	<.05							
46846571	rs2839356	C/T	intron			ns		ns	ns	ns			
46846657	rs1051169[d]	C/G	coding-synon	.008[c]		<.05		.01	ns	ns	<.05[c]	.004	
46846802	rs2186358	A/C	intron		.0019	ns		ns					
46846948	rs11911834	G/T	intron				<.05[c]						
46847314	rs2839357	A/G	intron								ns		
46847806	rs2839359	C/T	intron		ns								
46849173	rs2839363	A/G	intron		ns								
46850221	rs2839364	C/T	near-gene-3			ns		ns					
46850373	rs2839365	C/G	near-gene-3			ns							
46850782	rs3788266	A/G	near-gene-3		.009	ns					ns		.0001
46850870	rs2839366	A/T	near-gene-3							ns			
46851511	rs9978658	G/T	unknown							.0009			
46851604	rs13047696	A/G	unknown							.006			
46852307	rs12152107	A/C	unknown							.003			
46853887	rs8133385	A/G	unknown						ns				
46854892	rs9982863	A/G	unknown						ns	ns			

[a] associated; [b] see table 5.1; [c] SNP associated in haplotype; [d] rs11542311 was replaced by this SNP ID in dbSNP129 onwards; ns = tested but not significant

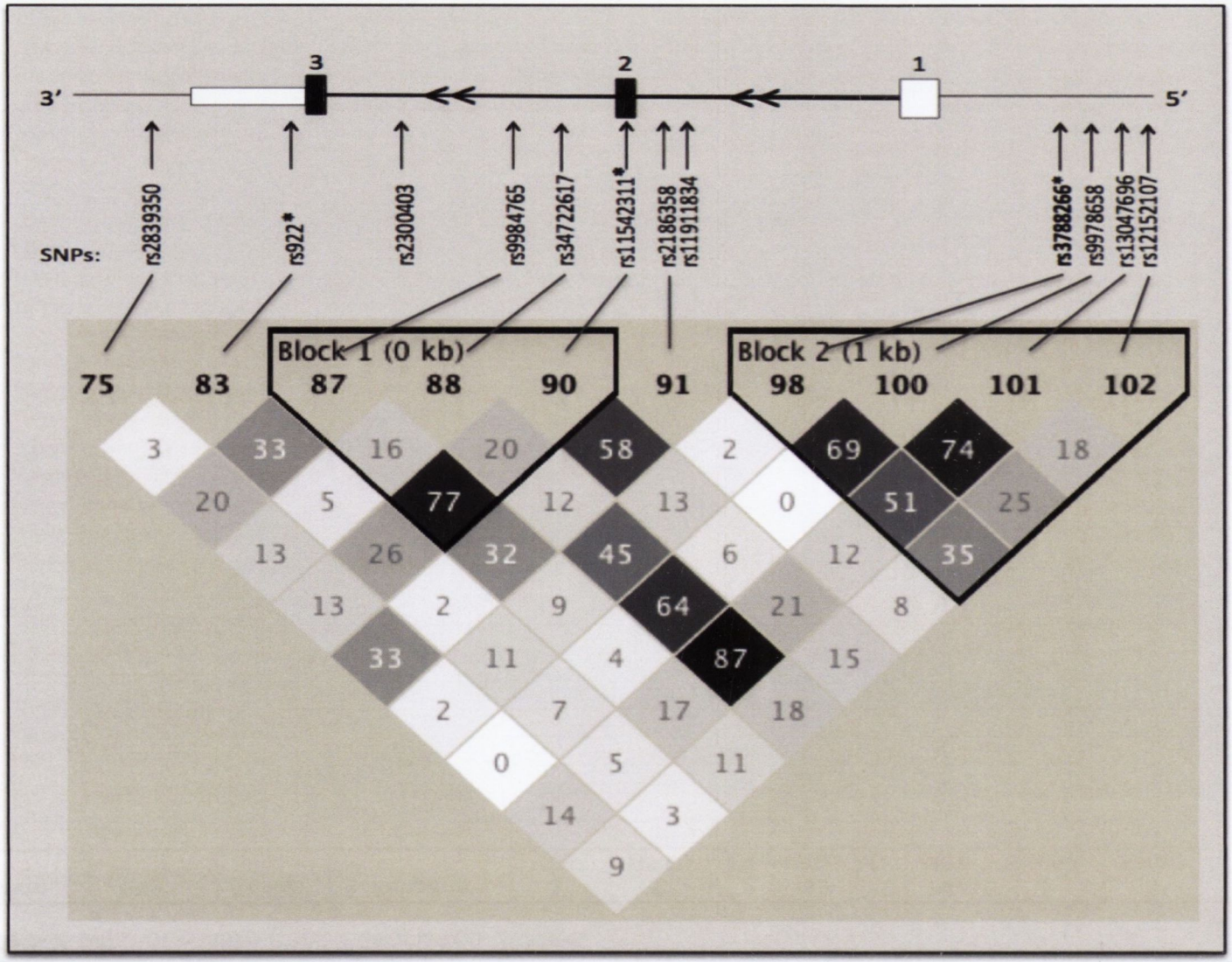


Figure 5.3. Genomic organization of the 6.5-kb S100B gene with translated region spanning exons 2 and 3 (given as black bars) according to the UCSC genome browser (March 2006 assembly). The SNP positions relative to the gene are indicated by arrows (*associated in more than one study). Below is the LD structure (generated by Haploview) of the significantly associated S100B gene variants. The r^2 values are based upon CEU genotype data and numbers below the SNPs identifiers refer to HapMap. This figure does not include 2 associated SNPs (rs2300403 and rs11911834) as data was not available in HapMap.

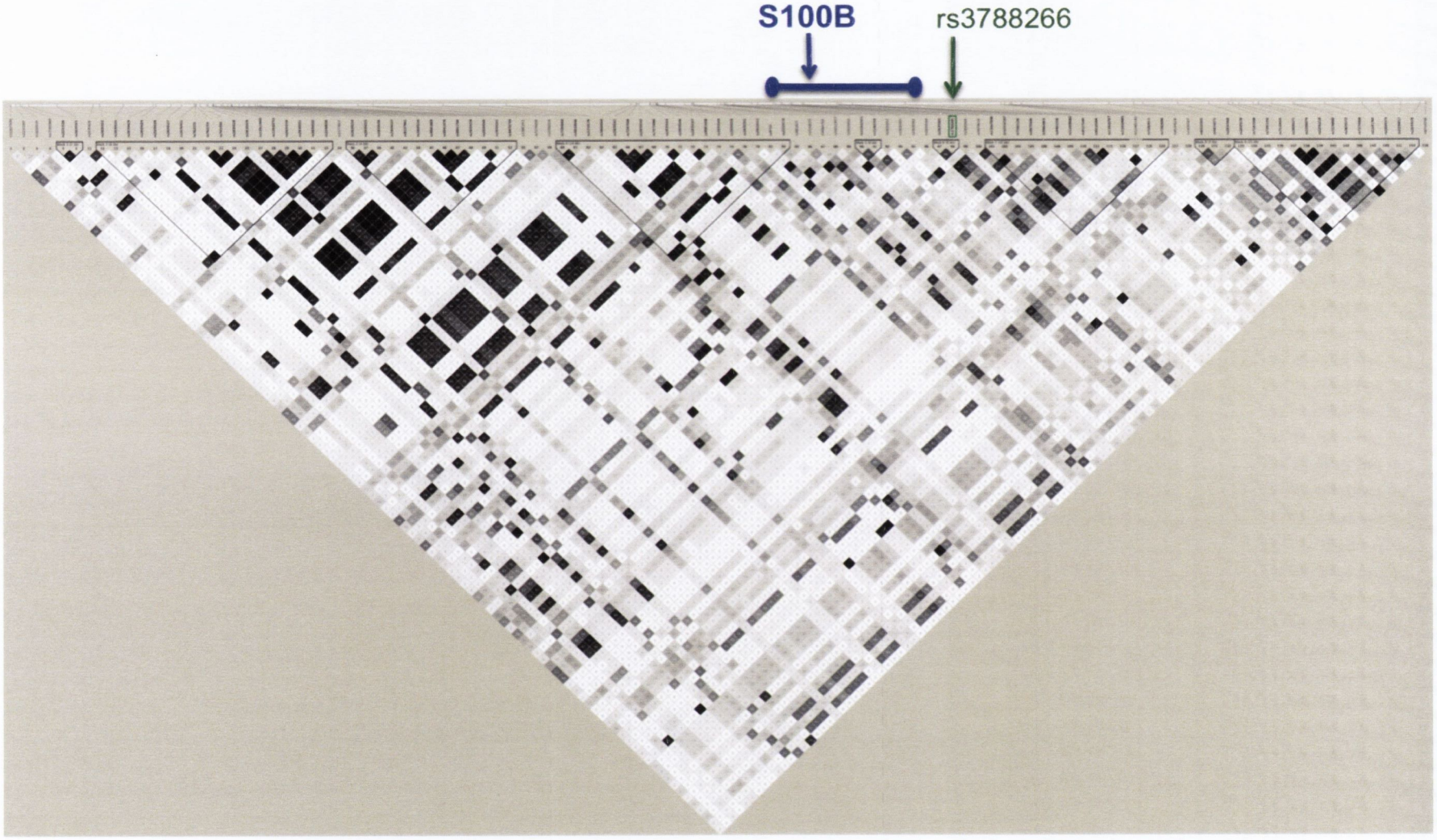


Figure 5.4. LD (r^2) structure from common HapMap CEU SNPs at +/- 50 kb of the S100B gene. Blue arrow indicates the location of S100B gene and the location of the rs3788266 SNP is marked in green. Image generated using Haploview.

The association study with biggest sample size was the ISC (almost 7,000 subjects) and the statistically most significant disease associated SNP is rs9978658 (G allele is risk allele). Interestingly, this SNP is in very high LD with rs3788266 (D'=1.0, r^2=0.70; risk G allele of rs3788266 is on same haplotype as G allele of rs9978658). The SNP rs3788266 was not directly genotyped in the ISC study. Therefore, I decided to genotype rs3788266 in available Irish samples in my lab and perform an association study.

5.3.2. Association Study

I investigated the association of rs3788266 with SZP and BPAD using the GASP + RPGI cases and Biobank + Neuropsychology controls. The samples were in Hardy-Weinberg equilibrium p-value < 0.05 (Table 5.3). As it is shown in Table 5.4, there was no evidence of association at rs3788266 with SZP or BPAD.

Table 5.3. List of samples used for the association study of rs3788266 with BPAD and SZP.

Sample	MAF	X2	HWE P	H1[a]	NH1[b]	HET	NHET	H2	NH2
GASP all cases	0.448	0.352	0.552	A/A	105	A/G	273	G/G	160
RPGI all cases	0.468	1.149	0.283	A/A	116	A/G	290	G/G	151
Biobank controls	0.471	5.654	0.017	A/A	387	A/G	977	G/G	494
Neuropsy. controls	0.452	4.929	0.026	A/A	39	A/G	64	G/G	54
[a] Homozygous genotype 1; [b] number of Homozygous genotype 1 in sample									

Table 5.4. Association analysis results for rs3788266.

Samples	Sample size		MAF[a]		χ^2	p-value	OR	95% C.I.
	Cases	Controls	Cases	Controls				
SZP	696	2023	0.4569	0.4691	0.6204	0.4309	0.9521	0.842 - 1.076
BPAD	248	2023	0.4718	0.4691	0.01264	0.9105	1.011	0.838 - 1.218
BPAD+SZP+SZA Combined	1095	2023	0.4589	0.4691	0.5943	0.4408	0.9598	0.864 - 1.065

[a] Frequency of minor A-Allele is presented.

5.3.3. SIX-Family Genes

I investigated SNPs at the six-family genes (SIX1, SIX2, SIX4 and SIX5) for a possible association with SZP or BPAD using the ISC (SZP), WTCCC (BPAD) and WTCCC2 (psychosis) data. The SIX1 and SIX4 genes are located on chromosome 14q23.1 where linkage with BPAD was previously reported (Cassidy F *et al.*, 2007). SIX2 is located on chromosome 2p21 and SIX5 is on chromosome 19q13.32.

5.3.3.1. SIX1 Gene

The SIX1 gene is located on chr14:60,181,170-60,185,908 (hg18). Table 5.5 shows all the SNPs that are located within the gene SIX1. There are 22 SNPs with the SIX1 gene. One is a frameshift mutation (rs34585587) and there is one other missense SNP (rs17850414). None of the SNPs has a MAF > 10% in HapMap CEU population.

Table 5.5. Variants located within the SIX1 gene.

SNP	Location	Alleles	Class	Function
rs61993831	60181257	A/G	single	untranslated-3
rs3832952	60181482	-/AT	deletion	untranslated-3
rs3742638	60181633	A/G	single	untranslated-3
rs3742637	60181709	G/T	single	untranslated-3
rs33943216	60181987	-/A	deletion	untranslated-3
rs63554860	60181987	A/T	single	untranslated-3
rs59016233	60181998	-/A	deletion	untranslated-3
rs57517528	60182264	C/T	single	untranslated-3
rs10144415	60182419	C/G	single	untranslated-3
rs35069179	60182503	C/T	single	untranslated-3
rs60585662	60182504	-/T	deletion	untranslated-3
rs61991660	60182507	C/T	single	untranslated-3
rs12101095	60182773	A/G	single	coding-synon
rs61515165	60183008	C/T	single	coding-synon
rs34899649	60183078	-/A	deletion	intron
rs72534789	60183092	-/A	deletion	intron
rs71114127	60183100	-/A	deletion	intron
rs58483512	60183721	A/G	single	intron
rs35969503	60184728	-/C	deletion	intron
rs34585587	**60185168**	**-/G**	**insertion**	**frameshift**
rs73309461	60185330	C/T	single	coding-synon
rs17850414	**60185365**	**A/G**	**single**	**missense**

I investigated the SIX1 gene polymorphisms for a possible association with SZP and BPAD using the ISC, WTCCC and WTCCC2 data. 50kb up and downstream of the gene was considered to include potential regulatory polymorphisms. All SNPs genotyped by ISC, WTCCC and WTCCC2 studies are listed in Table 5.6. Only two SNPs (rs10483727 and rs11158297) were associated at *p*-value < 0.05, both in the ISC data.

Table 5.6. SNP results from GWAS studies in the region of the SIX1 gene.

SNP	Location	ISC *p*-value (SZP)	WTCCC *p*-value (BPAD)	WTCCC2 narrow *p*-value (SZP)	WTCCC2 broad *p*-value (SZP+BPAD)
rs10483727	60142628	**0.0233**	n/a	0.9393	0.9056
rs6573317	60145460	n/a	0.1380	n/a	n/a
rs761557	60147237	0.6204	n/a	0.7792	0.6845
rs10142842	60154958	0.6509	0.0561	0.7519	0.6222
rs4901995	60160382	0.8758	0.1700	0.5404	0.5299
rs6573320	60161775	0.7759	0.2070	0.5392	0.5331
rs10143202	60194693	0.4611	0.0718	0.1662	0.1773
rs4899015	60195789	0.1176	n/a	0.4541	0.7009
rs11158297	60217834	**0.0311**	0.1457	0.456	0.7528
rs964412	60224521	0.6336	0.3283	0.7256	0.5493
rs1018456	60228546	0.1929	0.1507	0.4702	0.7813
rs1018457	60228623	0.6209	0.3612	0.7515	0.5687
rs1955689	60233783	0.7509	0.3591	0.6537	0.4822

red = significant at *p*-value < 0.05; n/a = not analyzed

Coverage of common SNPs in this region in the GWAS datasets were good but not complete. The results of a coverage analysis of the GWAS SNPs are shown at Table 5.7. For this study the number and percentage of the common SNPs captured by GWAS was determined Haploview 4.2 using pairwise tagging only (r^2>0.8). Common HapMap CEU SNPs in this instance have a MAF>0.1, with <1 Mendelian error, greater than 80% call rate and Hardy-Weinberg p-value >0.001.

5.3.3. SIX-Family Genes

I investigated SNPs at the six-family genes (SIX1, SIX2, SIX4 and SIX5) for a possible association with SZP or BPAD using the ISC (SZP), WTCCC (BPAD) and WTCCC2 (psychosis) data. The SIX1 and SIX4 genes are located on chromosome 14q23.1 where linkage with BPAD was previously reported (Cassidy F *et al.*, 2007). SIX2 is located on chromosome 2p21 and SIX5 is on chromosome 19q13.32.

5.3.3.1. SIX1 Gene

The SIX1 gene is located on chr14:60,181,170-60,185,908 (hg18). Table 5.5 shows all the SNPs that are located within the gene SIX1. There are 22 SNPs with the SIX1 gene. One is a frameshift mutation (rs34585587) and there is one other missense SNP (rs17850414). None of the SNPs has a MAF > 10% in HapMap CEU population.

Table 5.5. Variants located within the SIX1 gene.

SNP	Location	Alleles	Class	Function
rs61993831	60181257	A/G	single	untranslated-3
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rs3742637	60181709	G/T	single	untranslated-3
rs33943216	60181987	-/A	deletion	untranslated-3
rs63554860	60181987	A/T	single	untranslated-3
rs59016233	60181998	-/A	deletion	untranslated-3
rs57517528	60182264	C/T	single	untranslated-3
rs10144415	60182419	C/G	single	untranslated-3
rs35069179	60182503	C/T	single	untranslated-3
rs60585662	60182504	-/T	deletion	untranslated-3
rs61991660	60182507	C/T	single	untranslated-3
rs12101095	60182773	A/G	single	coding-synon
rs61515165	60183008	C/T	single	coding-synon
rs34899649	60183078	-/A	deletion	intron
rs72534789	60183092	-/A	deletion	intron
rs71114127	60183100	-/A	deletion	intron
rs58483512	60183721	A/G	single	intron
rs35969503	60184728	-/C	deletion	intron
rs34585587	**60185168**	**-/G**	**insertion**	**frameshift**
rs73309461	60185330	C/T	single	coding-synon
rs17850414	**60185365**	**A/G**	**single**	**missense**

I investigated the SIX1 gene polymorphisms for a possible association with SZP and BPAD using the ISC, WTCCC and WTCCC2 data. 50kb up and downstream of the gene was considered to include potential regulatory polymorphisms. All SNPs genotyped by ISC, WTCCC and WTCCC2 studies are listed in Table 5.6. Only two SNPs (rs10483727 and rs11158297) were associated at *p*-value < 0.05, both in the ISC data.

Table 5.6. SNP results from GWAS studies in the region of the SIX1 gene.

SNP	Location	ISC *p*-value (SZP)	WTCCC *p*-value (BPAD)	WTCCC2 narrow *p*-value (SZP)	WTCCC2 broad *p*-value (SZP+BPAD)
rs10483727	60142628	**0.0233**	n/a	0.9393	0.9056
rs6573317	60145460	n/a	0.1380	n/a	n/a
rs761557	60147237	0.6204	n/a	0.7792	0.6845
rs10142842	60154958	0.6509	0.0561	0.7519	0.6222
rs4901995	60160382	0.8758	0.1700	0.5404	0.5299
rs6573320	60161775	0.7759	0.2070	0.5392	0.5331
rs10143202	60194693	0.4611	0.0718	0.1662	0.1773
rs4899015	60195789	0.1176	n/a	0.4541	0.7009
rs11158297	60217834	**0.0311**	0.1457	0.456	0.7528
rs964412	60224521	0.6336	0.3283	0.7256	0.5493
rs1018456	60228546	0.1929	0.1507	0.4702	0.7813
rs1018457	60228623	0.6209	0.3612	0.7515	0.5687
rs1955689	60233783	0.7509	0.3591	0.6537	0.4822

red = significant at *p*-value < 0.05; n/a = not analyzed

Coverage of common SNPs in this region in the GWAS datasets were good but not complete. The results of a coverage analysis of the GWAS SNPs are shown at Table 5.7. For this study the number and percentage of the common SNPs captured by GWAS was determined Haploview 4.2 using pairwise tagging only (r^2>0.8). Common HapMap CEU SNPs in this instance have a MAF>0.1, with <1 Mendelian error, greater than 80% call rate and Hardy-Weinberg p-value >0.001.

Table 5.7. Coverage of SIX1 gene with WTCCC, WTCCC2 and ISC studies (analysed with Haploview tagger algorithm).

Affymetrix platform	Study	Assayed SNPs	Common CEU SNPs	Captured at $r^2>0.8$
5.0	WTCCC	12	50	43 (86%)
6.0	ISC/WTCCC2	12	50	43 (86%)

The ISC and WTCCC2 data tagged 12 SNPs within the target region. The tagged 12 SNPs captured 43 of 50 common SNPs at this locus. The seven SNPs that were not captured are marked in red at Figure 5.5. The SNPs within the SIX1 gene, which are listed in Table 5.5 are not captured or tagged in the GWAS studies.

The SNP rs10483727 was found to be significantly associated with SZP in ISC data (p = 0.023). This SNP is located upstream of the SIX1 gene and is not in LD with any other SNP. The SNP rs11158297 was also found to be significantly associated with in ISC data (P=0.031) and is only in LD with SNPs further downstream of the SIX1 gene (Figure 5.5). As none of the SNPs within the SIX1 gene are tagged by the GWAS studies, primarily because none are known to have a MAF >10%, further analysis is needed to determine if any of these SNPs are possibly associated with SZP or BPAD.

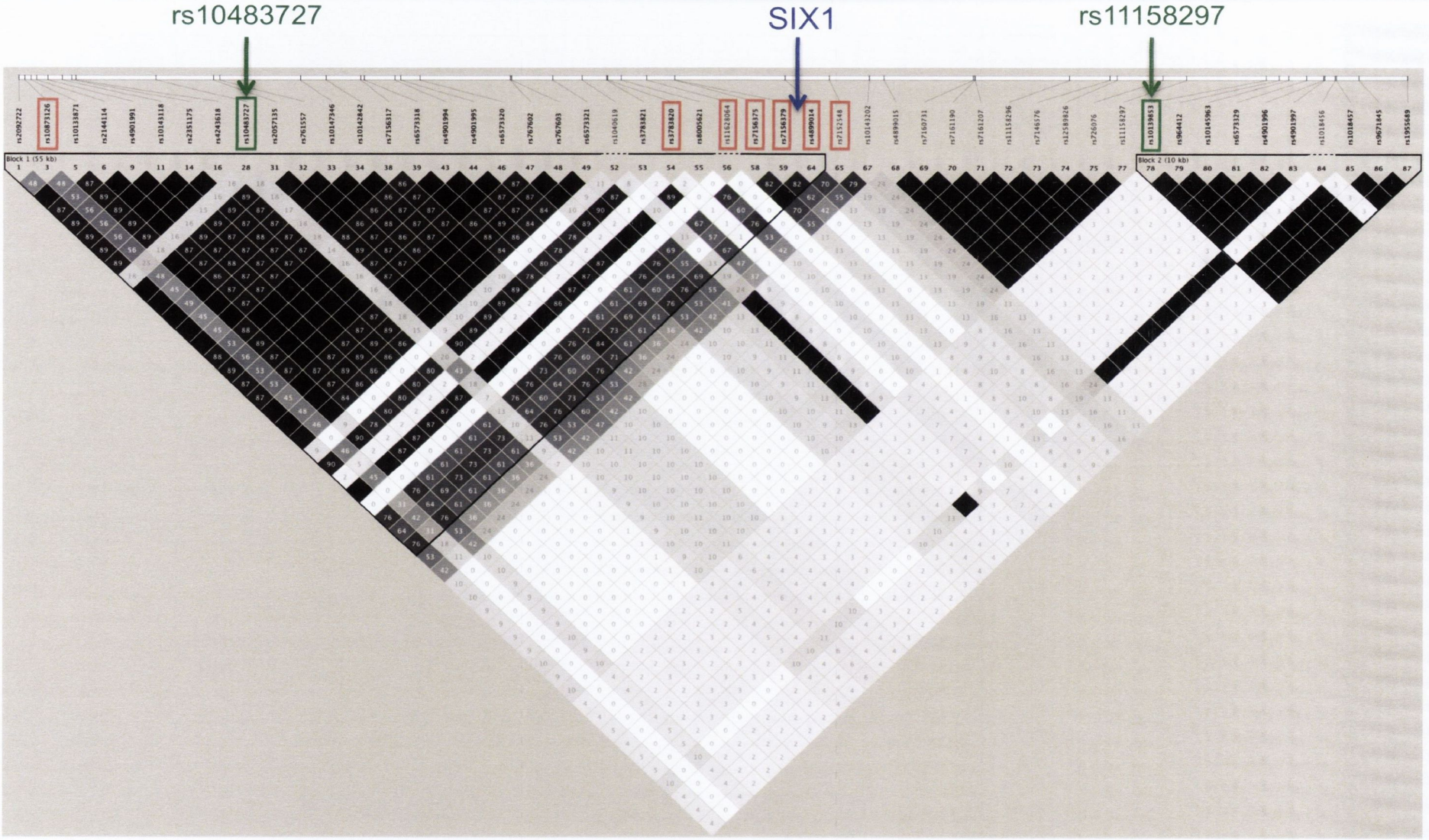

Figure 5.5. Common CEU SNPs at +/- 50 kb of SIX1 gene. Pair-wise LD values (r^2) are shown. The SNPs marked in green are associated in GWA studies and the SNPs marked in red are not captured by GWAS data. Blue arrow indicates the location of SIX1 gene. Image generated using Haploview.

5.3.3.2. SIX2 Gene

The SIX2 gene is located on chr2:45085847-45090026 (hg18). Table 5.8 shows all SNPs that are located within the SIX2 gene. There are 20 SNPs with the SIX2 gene and two of them are missense SNPs (rs4143307 and rs17856709).

Table 5.8. SNPs located within the gene SIX2.

SNP	Location	observed	Class	Function
rs2290030*	45086189	G/T	single	untranslated-3
rs2290029*	45086331	C/T	single	untranslated-3
rs61203820	45086543	A/C	single	untranslated-3
rs3738975*	45087279	C/G	single	intron
rs3738974*	45087305	A/C	single	intron
rs12992612	45087493	G/T	single	intron
rs1529973*	45087564	C/G	single	intron
rs2164705	45087586	C/G	single	intron
rs2959230*	45087616	G/T	single	intron
rs2959229*	45087866	A/G	single	intron
rs3768917*	45088438	C/G	single	intron
rs895631*	45088513	A/G	single	intron
rs3754641	45088658	A/G	single	intron
rs4434041	45088682	C/G	single	intron
rs72877603	45088847	A/T	single	intron
rs71422122	45089040	C/T	single	intron
rs1118180	45089105	A/C	single	intron
rs4143307	**45089275**	**C/G**	**single**	**missense**
rs17856709	**45089281**	**C/T**	**single**	**missense**
rs11540435	45089500	C/T	single	nonsense

*MAF > 10% in HapMap CEU population.

I investigated the SIX2 gene polymorphisms for a possible association with SZP and BPAD using the ISC, WTCCC and WTCCC2 data. 50 kb region up and downstream of the gene was considered in my analysis to include sites of potential regulatory polymorphisms. All SNPs from the GWAS data are listed in Table 5.9. I found several SNPs (marked in red), which were had a *p*-value < 0.05 in at least one of the GWAS.

Table 5.9. SNP results from GWAS studies in the region of the SIX2 gene.

SNP	Location	ISC *p*-value (SZP)	WTCCC *p*-value (BPAD)	WTCCC2 narrow *p*-value (SZP)	WTCCC2 broad *p*-value (SZP+BPAD)
rs163513	45038728	0.2687	0.1082	0.3499	0.4509
rs895636	45041857	0.4066	0.4381	0.9302	0.8591
rs340514	45042515	**0.0269**	0.4478	0.915	0.9139
rs4953155	45043000	0.2934	n/a	0.9498	0.7439
rs748948	45104183	n/a	0.4806	n/a	n/a
rs873745	45046677	0.6477	0.7113	0.4436	0.6985
rs881309	45047238	0.6723	n/a	0.5871	0.3922
rs163503	45050643	0.5044	0.7993	0.1643	0.1944
rs163502	45051161	0.7057	n/a	0.4286	0.1984
rs908279	45053513	0.6536	0.2937	0.4992	0.4786
rs11692566	45054404	0.5346	n/a	0.06906	0.08001
rs485348	45056571	0.5298	n/a	0.3587	0.2307
rs7584817	45057690	0.8596	n/a	0.3288	0.345
rs478918	45062457	0.4377	0.9143	0.05946	**0.04032**
rs565217	45062481	0.3465	0.1926	0.8619	0.7132
rs566941	45062642	0.4571	n/a	0.1448	0.0857
rs569875	45062982	0.7698	n/a	0.4786	0.3461
rs486043	45063584	0.682	n/a	0.466	0.3367
rs2917760	45064733	0.5729	0.5456	0.1239	0.07374
rs2917761	45064788	0.9814	n/a	0.1002	0.07772
rs2959242	45065596	0.9999	0.8006	0.09928	0.07227
rs2917767	45069783	0.253	0.6380	0.07908	0.05823
rs6705604	45070089	0.1914	0.2391	0.8025	0.6053
rs2922011	45070210	0.3852	0.4035	0.09349	0.06771
rs10179001	45071567	0.5673	n/a	0.2896	0.3466
rs2917769	45073852	0.6613	n/a	0.1126	0.1248
rs767491	45074720	0.5407	n/a	0.1217	0.1009
rs2922008	45075451	0.8018	n/a	0.1245	0.1244
rs2922007	45077013	0.5882	n/a	0.4029	0.3431
rs17032926	45077075	0.706	0.1851	0.5237	0.3522
rs2290030	45086190	**0.0408**	n/a	0.9261	0.6809
rs2959229	45087867	0.4189	n/a	0.0684	0.05189
rs3768917	45088439	0.8591	n/a	0.4349	0.5876
rs964992	45092214	0.3157	0.4103	0.1347	0.1164
rs964991	45092225	0.2561	n/a	0.1654	0.1312
rs921115	45095120	0.3738	0.5518	0.2177	0.1963
rs2921996	45101274	0.104	n/a	0.2267	0.141
rs12469303	45102346	0.4981	n/a	0.2982	0.4191
rs2921993	45102522	0.1719	0.4154	0.1808	0.09906

rs2959264	45103341	0.2114	0.5699	0.1491	0.08574
rs4953173	45103903	**0.0346**	n/a	0.3585	0.2169
rs4952731	45106219	0.2528	0.8658	0.824	0.6127
rs2921988	45106904	**0.0115**	n/a	0.4044	0.1886
rs17032936	45107082	0.2267	n/a	0.7158	0.8744
rs2921987	45108031	**0.0122**	0.8146	0.2067	0.1021
rs17032946	45108226	0.1857	0.3108	0.3617	0.5688
rs13004637	45111656	**0.0102**	n/a	0.3669	0.4926
rs4952732	45113997	0.0356	n/a	0.3207	0.1375
rs6544780	45123326	**0.0014**	n/a	0.5921	0.7446
rs1868023	45126291	0.3669	n/a	0.6842	0.5878
rs921119	45126316	**0.0164**	0.6801	0.5149	0.6174
rs921120	45126545	**0.0101**	0.9722	0.7638	0.8455
rs4952736	45128862	0.1115	n/a	0.5045	0.2754
rs10171794	45129207	0.9864	n/a	0.5645	0.6637
rs4952738	45131104	0.0551	0.619	0.7922	0.9386
rs4953185	45131257	**0.0439**	0.6646	0.7456	0.963
rs6544781	45132298	0.0538	0.8068	0.8376	0.6689
rs4953187	45132854	**0.0346**	0.8156	0.9484	0.7476
rs10191680	45135045	0.7289	n/a	0.2688	0.2661
rs2922017	45135096	0.621	0.0994	0.3209	0.506

red = significant at *p*-value < 0.05; n/a = not analyzed

The WTCCC data genotyped 30 SNPs within -/+50kb SIX2 gene and captured 65 of 101 SNPs at this locus. The WTCCC2 and ISC studies genotyped 62 SNPs with the target region and these SNPs captured 90 of 100 known common SNPs at this locus (Table 5.10). The 10 SNPs that were not captured by WTCCC2 and ISC studies are marked in red in Figure 5.6. There is only one SNP (rs2290030) within the boundaries of the SIX2 gene that was associated at *p*-value < 0.05 in GWAS and this was only in the ISC data. There is no strong LD between any other SNP within the SIX2 gene and SNPs that have a *p*-value < 0.05 in at least one of the GWAS (Figure 5.6.).

Table 5.10. Coverage of SIX2 gene with WTCCC, WTCCC2 and ISC studies.

Affymetrix platform	**Study**	**Assayed SNPs**	**Common CEU SNPs**	**Captured at r^2>0.8**
5.0	WTCCC	30	100	65 (65%)
6.0	ISC/WTCCC2	62	100	90 (90%)

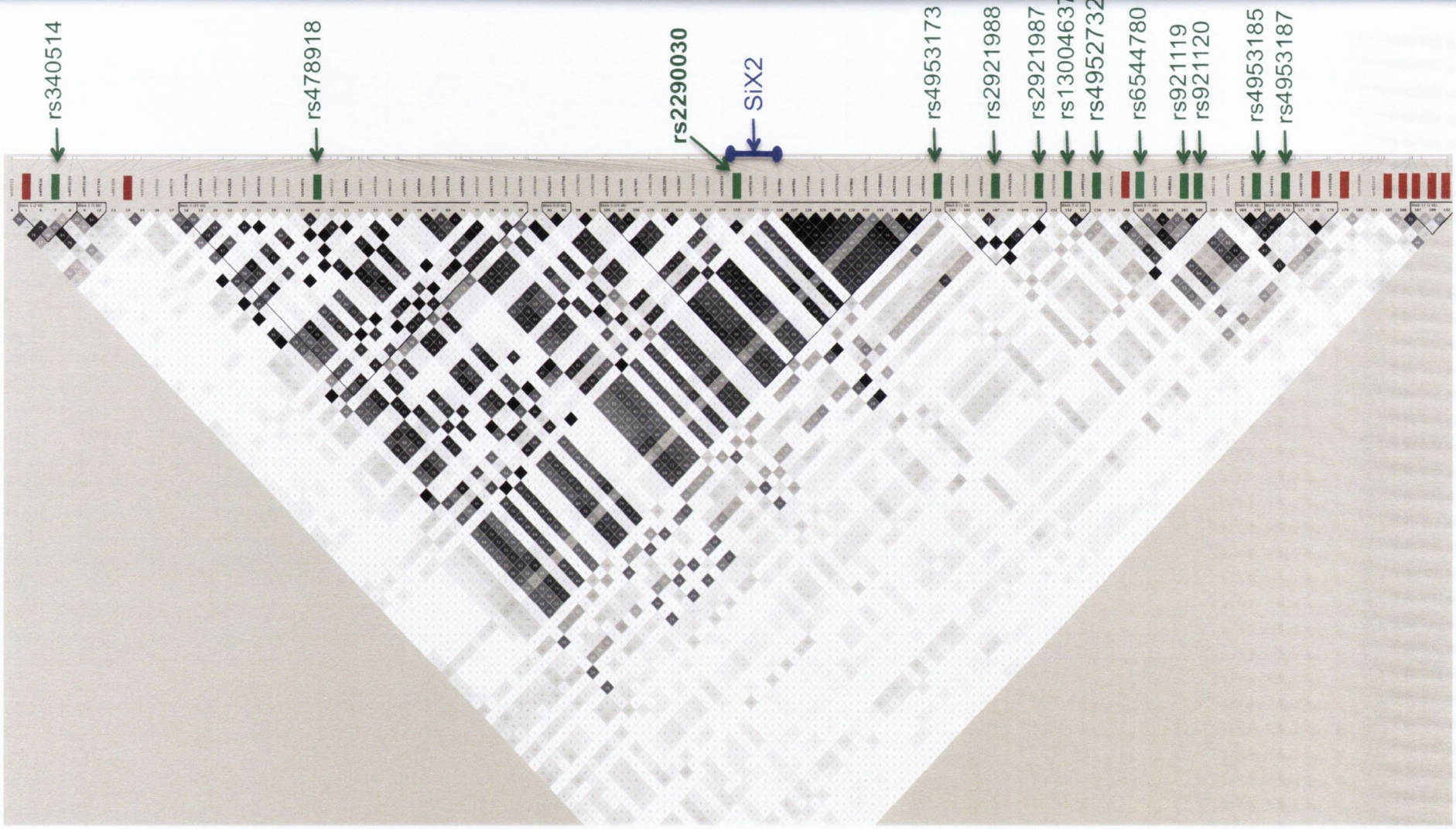

Figure 5.6. Common CEU SNPs at +/- 50 kb of SIX2 gene. Pair-wise LD values (r^2) are shown. The SNPs marked in green are associated in GWAS studies and the SNPs marked in red are not captured by GWAS data. Blue arrow indicates the location of SIX2 gene. Image generated using Haploview.

5.3.3.3. SIX4 Gene

The SIX4 gene is located on chr14:60,246,009-60,260,545 (hg18) and variants in this region are listed in Table 5.11. There are 61 SNPs with the SIX4 gene and 4 of them are missense SNPs.

Table 5.11. Variants located with the gene SIX4.

SNP	Location	observed	Class	Function
rs35262827	60246174	-/G	insertion	untranslated-3
rs10133777	60246212	A/G	single	untranslated-3
rs2180830*	60246238	A/G	single	untranslated-3
rs72722230	60246639	A/T	single	untranslated-3
rs8017327*	60247427	A/G	single	untranslated-3
rs72118294	60247488	-/ATATAT	insertion	untranslated-3
rs10641711	60247493	-/ATATAT	insertion	untranslated-3
rs33950200	60247494	-/ATATAT	insertion	untranslated-3
rs58793115	60247498	-/TATATA	insertion	untranslated-3
rs8017918*	60247532	A/G	single	untranslated-3
rs11847871*	60247780	A/C	single	untranslated-3
rs35674269	60248016	A/G	single	untranslated-3
rs11158303	60248032	A/G	single	untranslated-3
rs10873128	60248126	A/G	single	untranslated-3
rs72123147	60248256	-/A	insertion	untranslated-3
rs72147821	60248262	-/A	insertion	untranslated-3
rs67222444	60248264	-/A	insertion	untranslated-3
rs72369008	60248266	-/A	insertion	untranslated-3
rs71114154	60248270	-/A	insertion	untranslated-3
rs66647808	60248271	-/A	insertion	untranslated-3
rs4899016*	60248662	G/T	single	untranslated-3
rs12882994	60249167	C/G	single	untranslated-3
rs12884102	60249169	C/T	single	untranslated-3
rs7146756	60249187	G/T	single	untranslated-3
rs7145317	60249190	A/T	single	untranslated-3
rs61741142	**60249946**	**A/G**	**single**	**missense**
rs3742636*	**60250410**	**A/C**	**single**	**missense**
rs35020088	60250677	-/A	deletion	intron
rs17097753	60251004	C/T	single	intron
rs11352616	60251431	-/G	deletion	intron
rs33995433	60251433	-/G	deletion	intron
rs71423429	60251434	C/G	single	intron
rs71423430	60251437	C/T	single	intron

SNP	Location	observed	Class	Function
rs71432538	60251440	CT/TC	mnp	intron
rs11449594	60251443	-/A	insertion	intron
rs68148452	60251444	-/A	insertion	intron
rs35634945	60251452	-/A	insertion	intron
rs34651135	60251500	-/G	insertion	intron
rs17097755*	60251647	G/T	single	intron
rs71416074	60251838	A/C	single	intron
rs17097756	60252131	C/T	single	intron
rs7146305	60252429	C/T	single	intron
rs7146721	60252701	C/T	single	intron
rs61991694	60252720	A/T	single	intron
rs35893105	60253783	-/A	insertion	intron
rs35697752	60254747	-/G	insertion	intron
rs17097757*	60254880	C/T	single	intron
rs34744237	60254991	-/C	insertion	intron
rs34310967	60255054	-/C	insertion	intron
rs55805390	60255062	A/G	single	intron
rs58160480	60255293	G/T	single	intron
rs7141965	60255401	A/G	single	intron
rs2351272	60255498	A/T	single	intron
rs7142026	60255627	A/G	single	intron
rs17097760	60255748	C/T	single	intron
rs17834412*	60256016	A/G	single	intron
rs61743413	**60256312**	**A/G**	**single**	**missense**
rs61740042	**60256486**	**A/G**	**single**	**missense**
rs35696046	60257866	-/A	insertion	intron
rs12896047	60258075	A/C	single	intron
rs7160166	60258261	A/G	single	intron

*MAF > 10% in HapMap CEU population.

I investigated the SIX4 gene SNPs for a possible association with SZP and BPAD using the ISC, WTCCC and WTCCC2 data. 50 kb regions up and downstream of the SIX4 gene were included in the analysis (chr14: 60,196,009-60,310,545; hg18). All SNPs genotyped in the GWAS are listed in Table 5.12. I found only one SNP rs11158297 (marked in red) that was associated at *p*-value < 0.05, but only in the ISC data.

Table 5.12. All GWAS SNPs in the region of +/- 50kb of the SIX4 gene.

SNP	Location	ISC *p*-value (SZP)	WTCCC *p*-value (BPAD)	WTCCC2 narrow *p*-value (SZP)	WTCCC2 broad *p*-value (SZP+BPAD)
rs11158297	60217834	**0.0311**	0.1457	0.456	0.7528
rs964412	60224521	0.6336	0.3283	0.7256	0.5493
rs1018456	60228546	0.1929	0.1507	0.4702	0.7813
rs1018457	60228623	0.6209	0.3612	0.7515	0.5687
rs1955689	60233783	0.7509	0.3591	0.6537	0.4822
rs1956869	60301147	n/a	0.7519	n/a	n/a

red = significant at *p*-value < 0.05; n/a = not analyzed

The WTCCC included just 6 SNPs +/-50kb of SIX4 gene and tagged 38 of 43 common SNPs. The ISC and WTCCC2 data included just 5 SNPs within the SIX4 region, which tagged 37 of 43 common SNPs at r^2>0.8 (Table 5.13). The 6 SNPs that were not captured are marked in red in Figure 5.7. The SNP rs11158297, which was associated in the ISC data, is in LD (D'=1 and r^2=1) with rs2180830, rs8017327 and rs11847871 within the 3'UTR of the SIX4 gene (Figure 5.7).

Table 5.13. Coverage of SIX4 gene with WTCCC, WTCCC2 and ISC studies.

Affymetrix platform	Study	Assayed SNPs	Common CEU SNPs	Captured at r^2>0.8
5.0	WTCCC	6	43	38 (88%)
6.0	ISC/WTCCC2	5	43	37 (86%)

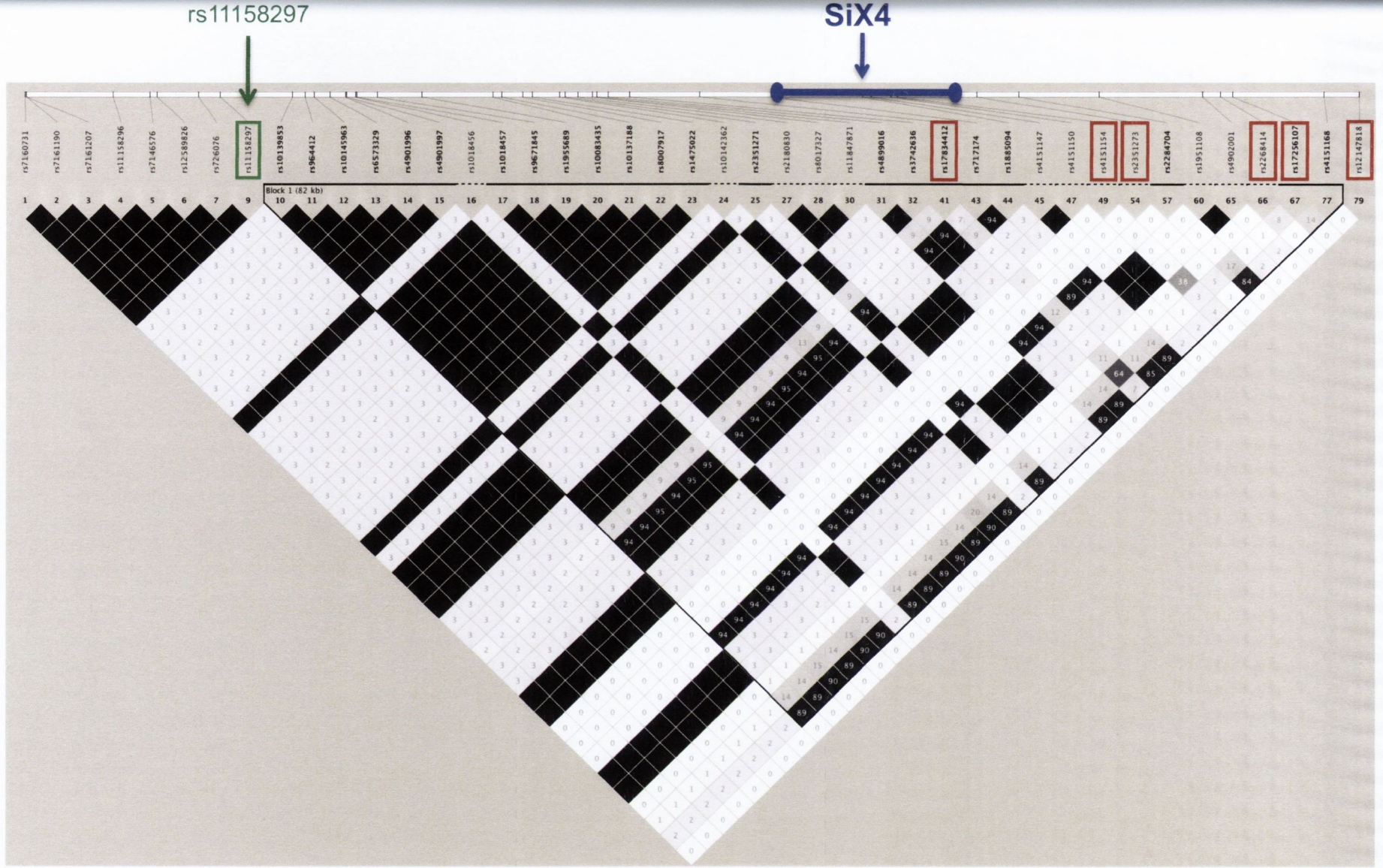

Figure 5.7. Common CEU SNPs at +/- 50 kb of SIX4 gene. Pair-wise LD values (r^2) are shown. The SNP marked in green is associated in GWAS studies and the SNPs marked in red are not captured by GWAS SNPs. Blue arrow indicates the location of SIX4 gene. Image generated using Haploview.

5.3.3.4. SIX5 Gene

The SIX5 gene is located on chr19:50,959,884-50,964,152 (hg18). A list of variants that are located within the SIX5 gene is shown at Table 5.14. There are 9 SNPs with the SIX5 gene and 4 of them are missense and one of them is frameshift mutation.

Table 5.14. Variants located with the SIX5 gene.

SNP	Location	observed	Class	Function
rs60009313	50960267	A/G	single	untranslated-3
rs60934577	50960292	C/T	single	untranslated-3
rs7257515	50960553	C/T	single	untranslated-3
rs7257701	50960554	A/G	single	untranslated-3
rs35750338	**50960713**	**-/T**	**insertion**	**frameshift**
rs2341097*	**50960741**	**C/T**	**single**	**missense**
rs2014576*	**50960915**	**C/T**	**single**	**missense**
rs2014377*	**50961152**	**C/G**	**single**	**missense**
rs34132142	**50961660**	**G/T**	**single**	**missense**
rs10410349	50962479	C/T	single	intron
rs3745802*	50963016	G/T	single	intron
rs4803853	50963076	A/G	single	intron
rs12981796	50963642	C/T	single	coding-synon

*MAF > 10% in HapMap CEU population.

I investigated the SIX5 gene for a possible association with SZP and BPAD using the ISC, WTCCC and WTCCC2 data. All SNPs in the 50 kb regions up and downstream of the SIX5 gene that were genotyped in the GWAS data are listed in Table 5.15 (chr19:50,559,884-51,464,152; hg18). I found only one SNP rs8111071 (marked in red) that was associated at *p*-value < 0.05, but only in the WTCCC data.

Table 5.15. All GWAS SNPs in the region of +/- 50kb of the SIX5 gene.

SNP	Location	ISC *p*-value (SZP)	WTCCC *p*-value (BPAD)	WTCCC2 narrow *p*-value (SZP)	WTCCC2 broad *p*-value (SZP+BPAD)
rs9807819	50931438	0.2293	n/a	0.5174	0.4699
rs16979989	50936008	n/a	0.0543916	n/a	n/a
rs10418603	50942065	0.969	0.7613	0.9972	0.9677
rs16980013	50959293	0.6361	0.9916	0.6224	0.5221
rs3760843	50980546	0.1514	0.9990	0.6499	0.7000
rs8111071	50999246	0.6843	**0.0083**	0.6754	0.4753
rs10402263	51005598	0.3829	n/a	0.5061	0.5191

red = significant at *p*-value < 0.05; n/a = not analyzed

The WTCCC data included only 5 SNP within the target region and tagged only 15 of 50 common SNPs at r^2>0.8. The ISC and WTCCC2 data included 6 SNPs within the 50kb region up and downstream of SIX5, which tagged 23 of 50 common SNPs (Table 5.16). Those SNPs that were not captured are marked in red in Figure 5.8.

Table 5.16. Coverage of SIX5 gene with WTCCC, WTCCC2 and ISC studies.

Affymetrix platform	Study	Assayed SNPs	Common CEU SNPs	Captured at r^2>0.8
5.0	WTCCC	5	50	15 (30%)
6.0	ISC/WTCCC2	6	50	23 (46%)

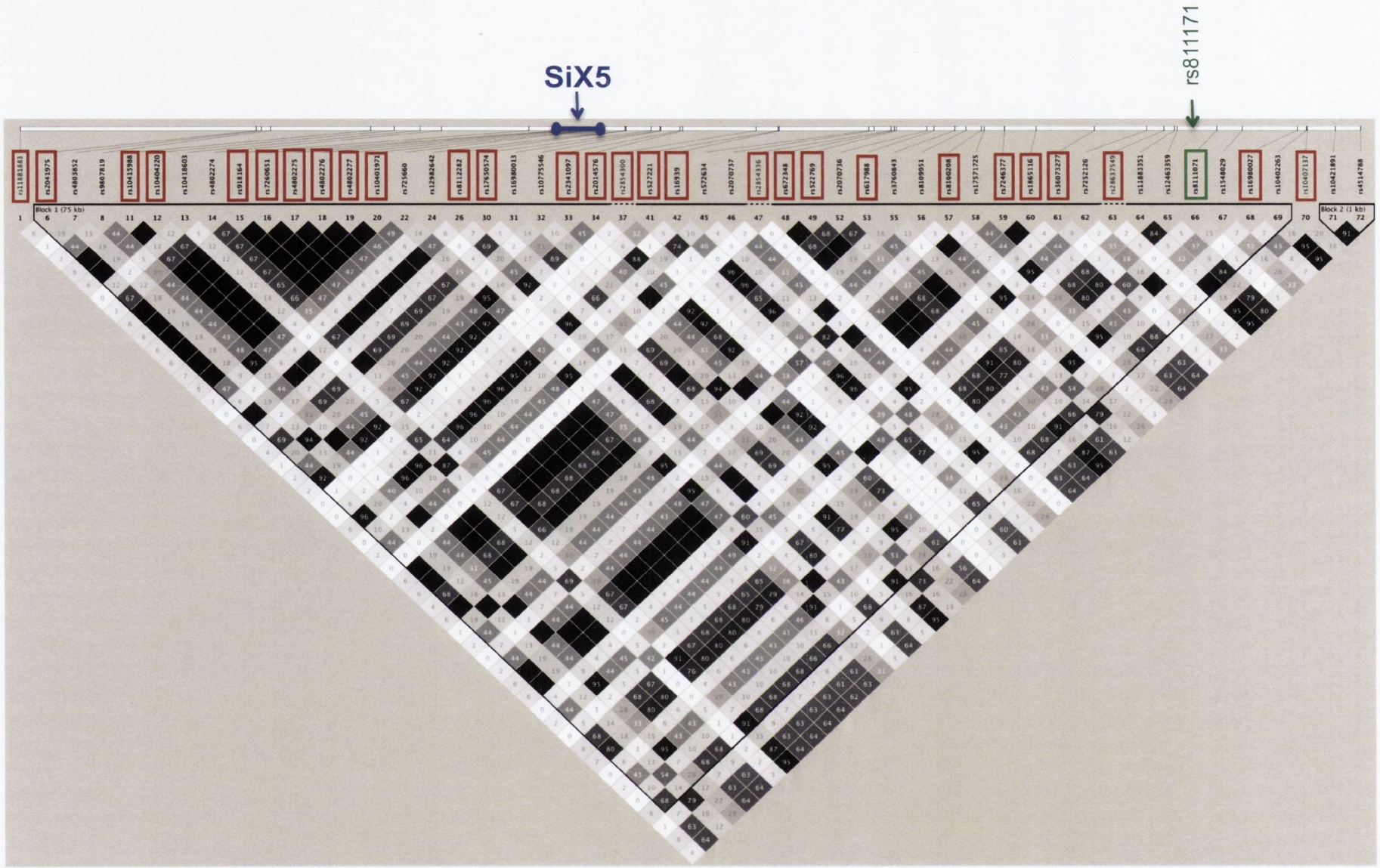

Figure 5.8. Common CEU SNPs at +/- 50 kb of SIX5 gene. Pair-wise LD values (r^2) were shown. The SNPs marked in red were not captured by GWAS SNPs. Blue arrow indicates the location of SIX5 gene. Image generated using Haploview.

5.3.4. Epistasis

I investigated for epistasis between selected SNPs in the SIX1, SIX2, SIX4 and SIX5 and the S100B gene promoter SNP rs3788266 in the GWAS data. I did not have access to raw genotype data from the WTCCC BPAD GWAS and thus could not test for epistasis in that sample. I did have genotype data from the ISC and WTCCC2 GWAS. There is substantial overlap between these samples. Although the WTCCC2 dataset is smaller, I decided to use it for the epistasis analysis because it is a large all-Ireland homogeneous sample unlike the multi-site ISC dataset. In addition, it gave me the opportunity to test for epistasis with both a narrow and broad psychosis phenotype.

Multiple testing is a major problem for epistasis analysis, therefore I restricted my analysis to a small number of SNPs from the Six-family genes. The SNPs for epistasis analysis were selected if they were associated in the ISC or WTCCC2 GWAS or are in high LD with a SNP located within the selected gene. Two SNPs (rs10483727 and rs11158297) at SIX1, 1 SNP (rs2290030) at SIX2, 1 SNP at SIX4 (rs1955689) and 2 SNPs at SIX5 (rs16980013 and rs3760843) were selected.

The results for epistasis analysis are shown in Table 5.17 for the WTCCC2 narrow phenotype and Table 5.18 for the WTCCC2 broad phenotype. Only one SNP (rs1955689) in WTCCC2 narrow dataset reached a p-value < 0.05. Two SNPs (rs1955689 and rs3760843) in WTCCC2 broad dataset reached a p-value < 0.05.

Table 5.17. Epistasis analysis of SIX1, SIX2, SIX4 and SIX5 SNPs with S100B (rs3788266) using WTCCC2 narrow samples.

Interaction	SNPs	OR	χ^2	p-value
SIX1x S100B	rs10483727x rs3788266	1.093	1.393	0.2378
SIX1x S100B	rs11158297x rs3788266	0.7353	1.803	0.1794
SIX2x S100B	rs2290030x rs3788266	1.053	0.2514	0.6161
SIX4x S100B	rs1955689x rs3788266	0.8461	4.567	**0.0326**
SIX5x S100B	rs16980013x rs3788266	1.108	1.767	0.1838
SIX5x S100B	rs3760843x rs3788266	0.8584	2.616	0.1058

Table 5.18. Epistasis analysis of SIX1, SIX2, SIX4 and SIX5 SNPs with S100B (rs3788266) using WTCCC2 broad samples.

Interaction	SNPs	OR	χ^2	p-value
SIX1 x S100B	rs10483727 x rs3788266	1.106	2.039	0.1533
SIX1 x S100B	rs11158297 x rs3788266	0.7865	1.254	0.2629
SIX2 x S100B	rs2290030 x rs3788266	1.024	0.06216	0.8031
SIX4 x S100B	rs1955689 x rs3788266	0.8556	4.525	**0.0334**
SIX5 x S100B	rs16980013 x rs3788266	1.073	0.9311	0.3346
SIX5 x S100B	rs3760843 x rs3788266	0.8371	3.967	**0.0464**

5.4. Discussion

I investigated all association studies published to date that analysed S100B gene variants and psychiatric or related phenotypes, and gene expression, and found 10 studies. Those studies have reported genetic association between S100B SNPs and SZP, major depression and BPAD (Liu J *et al.,* 2005; Roche S *et al.,* 2007; Yang K *et al.,* 2009). Lambert JC *et al.* (2007) has shown association of SNPs within the S100B gene with low cognitive performance and dementia in the elderly. Suchankova P *et al.* (2010) reported that gene variants within the S100B gene influencing the personality trait of self-directedness and low scores of self-directedness has been reported in several psychiatric disorders like depression (Smith DJ *et al.,* 2005) and SZP (Hori H *et al.,* 2008). Zhai J *et al.* (2010) also reported genetic association between the S100B gene variants and spatial disability in SZP subjects.

S100B is similar to many other candidate genes for psychiatric phenotypes. It has been analyzed in many studies and there are various reported results for SNPs and haplotypes but few strong results that are consistently replicated in multiple independent samples. GWAS studies have the advantage of assaying a large number of SNPs in very large samples. I accessed large GWAS datasets from WTCCC and ISC and looked for a genetic association between S100B gene variants and BPAD and SZP. I investigated 50 kb region up and downstream of the gene in order to include potential regulatory polymorphisms. My SNP of interest rs3788266 was not directly genotyped or well tagged (r^2>0.8) by both datasets but interestingly the strongest association signal at the S100B gene in the ISC dataset identifies SNP rs9978658 (p=0.0009). This SNP was in high LD with rs3788266 (D'=1.0 and r^2=0.70). The risk allele of rs9978658 is on the same haplotype as the risk allele of rs3788266 from the original small Irish BPAD family-based association study. rs9978658 is not on the Affymetrix 5.0 SNP array, hence it was not genotyped in the WTCCC BPAD GWAS.

I investigated the possible genetic association between my rs3788266 SNP and SZP and BPAD further by directly genotyping it in 1,095 Irish GASP and

RPGI cases and 2,023 Irish Biobank and Neuropsychology controls. I found no association between the rs3788266 and susceptibility to SZP and/or BPAD in these samples. At the completion of the project work for this chapter, I was able to access imputed data from the full all-Ireland WTCCC2 GWAS of psychosis. This sample largely consists of the Irish cases and controls mentioned above plus an additional ~1,000 cases from VCU. The association result for rs3788266 remains negative in both the narrow (p=0.8817) and broad (p=0.6895) phenotype samples.

These data suggest that the initial association study by Roche S *et al.* (2007) detected a false positive association at rs3788266. There are possible reasons why it may in fact be a true positive association that does not replicate in these GWAS data. The initial association signal in the family-based sample was stronger when only BPAD probands that had psychosis were considered. Data on rs3788266 was not available from the WTCCC BPAD GWAS and the association data presented here is predominantly based on SZP samples. Although the sample size of the GWAS is large, they may not be big enough to detect an association with SZP and BPAD. O'Donovan M *et al.* (2008) reported that to achieve 80% power, more than 10,000 cases and comparable controls are needed to detect small effect ORs of less than 1.12-1.16. Results from previous GWAS suggest that we need more samples to detect loci of small effect sizes and therefore multiple large datasets greatly would enhance power (McCarthy MI *et al.,* 2008). Further studies using larger sample sizes and containing cases with a psychotic phenotype would be required to definitely rule out an association with rs3788266 and psychotic illness.

I also examined large GWAS datasets from the WTCCC and ISC for a genetic association between the SIX-family transcription factor genes SIX1, SIX2, SIX4 and SIX5 variants and SZP and BPAD. Six-family transcription factors are reported to be expressed in the brain and regulate brain development and possibly differentiation and maturation of neuronal cells (Ohto H *et al.,* 1998; Kawakami K *et al.,* 2000). My investigations of these genes are detailed in chapter 3 and my important finding from the EMSA studies was that SIX1 and

SIX4 transcription factor binding to the S100B promoter at rs3788266 is allele-specific.

My analysis revealed that the SIX-family transcription factor genes SIX1, SIX2, SIX4 and SIX5 were not all well tagged in the GWAS studies. A number of different SNPs were associated in different GWAS but there was no evidence of a strong association for any SNP with either BPAD or SZP that was present in more than one sample. Similar to S100B, until very large samples are tested for all variants present, a possible association between the SIX-family genes and psychosis cannot be ruled out but to date neither the GWAS data available to me or any studies in the literature support a role for genetic variation at these genes in BPAD or SZP.

I finally tested the hypothesis that gene x gene interaction of SIX-family transcription factors x S100B would be significantly associated with SZP and BPAD. The problem with epistasis analysis is the potential for false positives due to the large number of tests that are possible, even when considering just interaction between two genes. Therefore, I restricted my analysis to just a small number of SNPs at the SIX-family genes and rs3788266 at S100B. This analysis only revealed one significant result in the WTCCC2 narrow psychosis sample for SNP rs1955689 close to the SIX4 gene. This SNP was selected for epistasis analysis because it is in complete LD with rs3742636 (D'=1.0 and r^2=1.0), which is located in SIX4 gene and leads to a missense mutation. The functional impact of this SNP has not been experimentally tested but the PolyPhen-2 database (http://genetics.bwh.harvard.edu/pph2/dbsnp/rs3742636.html) predicts that it is probably damaging and might, e.g., change the affinity of this transcription factor binding to S100B promoter. This significant epistasis result was also present in the WTCCC2 broad psychosis sample along with a second significant result between rs3760843 at SIX5 and S100B. The SNP rs3760843 was selected because it was closest SNP available to SIX5 gene. This SNP is located in intron of the proximal gene. These results are only borderline significant and have not been corrected for multiple tests but would be interesting to test again in independent sample sets.

General Discussion

A modest linkage signal for BPAD was previously identified at chr21q22, close to the S100B gene (Cassidy F *et al.*, 2007). In a follow-up study by the same group, an association with psychotic BPAD was found at rs3788266 (Roche S *et al.*, 2007). This SNP is located within the S100B promoter and thus raises the possibility of altered expression in disease susceptibility. Indeed, elevated serum S100B protein concentration has previously been implicated in the pathology of both BPAD and SZP (Schroeter ML *et al.*, 2009; Schroeter ML *et al.*, 2010), perhaps mediated through psychosis, a shared phenotype between BPAD and SZP (Goodwin FK & Jamison K, 2007). The initial aim of my thesis was to investigate if this common genetic variant in the S100B promoter causes altered expression of the protein and thus contributes to biology of the illness and is associated with SZP and BPAD.

In this thesis, the functional effect of variation at rs3788266 was established by both *in vivo* and *in vitro* methods. The risk allele for BPAD was associated with increased serum S100B concentration in BPAD cases, unaffected family members, and in unrelated controls. This effect was later reproduced in a sample of SZP cases. In the reporter gene assay, the risk allele conferred higher expression than the protective allele. Finally, using an electrophoretic mobility shift assay, transcription factor binding affinity was found to be altered in the presence of the BPAD risk allele.

Several transcription factor databases predicted an allelic effect of rs3788266 on transcription factor binding. Binding of Six-family transcription factors to the site of rs3788266 was investigated experimentally. Transcription factor SIX1, which is known to suppress neuronal gene expression (Bricaud O & Collazo A, 2011), bound only to the 'protective' A allele. SIX4 bound to both alleles but preferentially to the risk allele. This finding is in agreement with the other experimental results suggesting that increased binding of SIX4 transcription factor to the risk/G allele at rs3788266 is likely to increase the level of S100B expression.

In my endophenotype analysis, individuals homozygous for the risk allele performed worse in general IQ, performance IQ, verbal IQ, verbal working

memory and attention tasks. Furthermore, the risk allele was correlated with severity of psychosis in SZP and SZA. Increased serum S100B protein concentration in SZP and SZA subjects correlated with severity of psychosis and age, which was in agreement with published findings (Schroether ML *et al.*, 2010; Ling SH *et al.*, 2007). Finally, a trend between increased S100B concentration and verbal IQ was observed.

It is worth mentioning that this study had limitations. Clinical status has been reported several times to relate to the level of S100B. Increased level of S100B has been predominantly found in acute phase of SZP (Rothermundt M *et al.*, 2009). Antipsychotic medication has been reported to both decrease (Rothermundt M *et al.*, 2001b; Sarandol A *et al.*, 2007; Ling SH *et al.*, 2007; Steiner J *et al.*, 2008) and increase (Schroeter ML *et al.*, 2003) the level of S100B. The clinical and medication status of the BPAD patients was not known and therefore could not be included in the analyses. The effect of rs3788266 on serum concentration in drug free SZP and BPAD probands with comparable healthy controls should be investigated.

There are several studies implicating genetic variation at S100B in psychiatric disorders. In particular, S100B rs3788266 has been found to be associated with psychotic BPAD. Nevertheless, the data from this thesis does not support S100B rs3788266 as a risk variant for BPAD or SZP. None of the other common variants within the S100B gene investigated in GWAS provided an association signal that replicated across the GWAS datasets. Epistasis between SNPs at SIX-family genes and rs3788266 was also tested for contribution to disease risk but only a weak interaction was observed between both rs3788266 and SIX4, and rs3788266 and SIX5.

GWAS of BPAD and SZP have now been performed on up to 50,000 individuals and there are no associations at S100B that surpass genome-wide significance (The Schizophrenia Psychiatric GWAS Consortium *et al.*, 2011; Psychiatric GWAS Consortium Bipolar Disorder Working Group *et al.*, 2011). The rs3788266 SNP would have been imputed and therefore captured in

those resent GWAS studies. However, the present study found that the SNP rs3788266 may be associated with endophenotypes (symptom severity or cognitive performance), but these have not been investigated by GWAS. Although the GWAS studies do not directly implicate S100B variants, some of the strongest findings relate to S100B biology. GWAS studies indicated involvement of ion channels in the pathology of BPAD (Ferreira MA *et al.*, 2008). S100B is a Ca^{2+} binding protein and its major intracellular role is to regulate calcium homeostasis. S100B also binds to Cu^{2+} (Nishikawa T *et al.*, 1997) and Zn^{2+} (Donato R, 1991; Heizmann CW, 1999; Zimmer DB *et al.*, 1995), and such binding influences Ca^{2+} binding capacity (Heinzmann CW & Cox JA, 1998). Neurogranin has been found as a risk gene for SZP in GWAS reaching genome-wide significance (Stefansson H *et al.,* 2009). Interestingly, S100B inhibits the phosphorylation of neurogranin (Sheu FS *et al.,* 1995).

Although GWAS studies did not identify association between S100B gene and SZP or BPAD, it may still play a role in these disorders. S100B gene copy number variation has been identified in schizophrenic cases. Saus E *et al.* (2010) reported two duplications and one deletion in a sample of 191 SZP cases and none in 341 controls. The CNV study by the ISC (2008) identified six duplications in 3,391 SZP cases and none in 3,181 controls. All six CNV carriers were Portuguese samples. These results are very interesting as they could support the hypothesis of genetic mutation at S100B causing illness by increasing S100B protein concentration by increasing expression of the gene due to increased copy number. Additional rare variants, either coding or in regulatory regions such as the promoter region of S100B, may also contribute to disease risk. Future studies using next generation sequencing might contribute more knowledge about the association of rare variants within the S100B promoter or gene with SZP and BPAD.

SZP and BPAD have strong genetic component but a full understanding of the genetic contribution to these disorders is still elusive. The common variants studied in GWAS do not explain a large proportion of the heritability. Other studies now provide evidence that rare variants, specifically CNVs, are important risk factors for these disorders, especially SZP (Stefansson H *et al.,*

2008; Walsh T *et al.*, 2008; Xu B *et al.,* 2008; Zhang D *et al.*, 2008; McCarthy SE *et al.*, 2009; Kirov G *et al.*, 2009b; Rujescu D *et al.*, 2009; Mulle JG *et al.*, 2010; Vacic V *et al.*, 2011; Levinson DF *et al.*, 2011). The availability of the next-generation whole exome and whole genome sequencing now allows analysis of genetic variation much smaller than large structural variants, down single nucleotide variants. One of the first applications of this technology to psychiatric genetics is the study of *de novo* mutations as a genetic cause of SZP. There are 2 published studies that have investigated the exome of the SZP patients for *de novo* mutations (Girard SL *et al.*, 2011; Xu B *et al.*, 2011). Girard SL *et al.* (2011) reported increased exonic *de novo* mutation rate in individuals with SZP by investigating 15 SZP trios. Xu B *et al.* (2011) examined 53 sporadic SZP cases and 22 controls and identified 40 *de novo* mutations in 27 cases. Studies will scale up in terms of sample numbers and move from whole exome to whole genome studies to continue the study of rare variation. Major challenges lie ahead for large sequencing studies such as functional annotation of variants, determining best study design (family-based versus case-control) and developing methods for extending the unit of investigation beyond the individual variant to genes and pathways.

There are several hypotheses as to how increased S100B concentration might contribute to the pathology of BPAD and SZP. The function of S100B protein is summarized in Figure 6.1(A) and mechanisms of how increased S100B protein might contribute to the pathology of both BPAD and SZP are outlined in Figure 6.1(B). The S100B protein is a multi-functional protein and it affects the functions of microglia, neurons and myogenic cells with different outcomes depending on its concentration (Rothermundt M *et al.,* 2004b). It has previously been suggested that increased S100B serum concentration might impair the regeneration-degeneration balance towards neurodegeneration, which may be part of the pathology of SZP (Rothermundt M *et al.*, 2004c). The present study has shown elevation of S100B concentration at the level of nanomoles. Thus because apoptosis is induced only at the micromolar level, this makes S100B-induced neurodegeneration an unlikely cause of pathogenesis for SZP and BPAD.

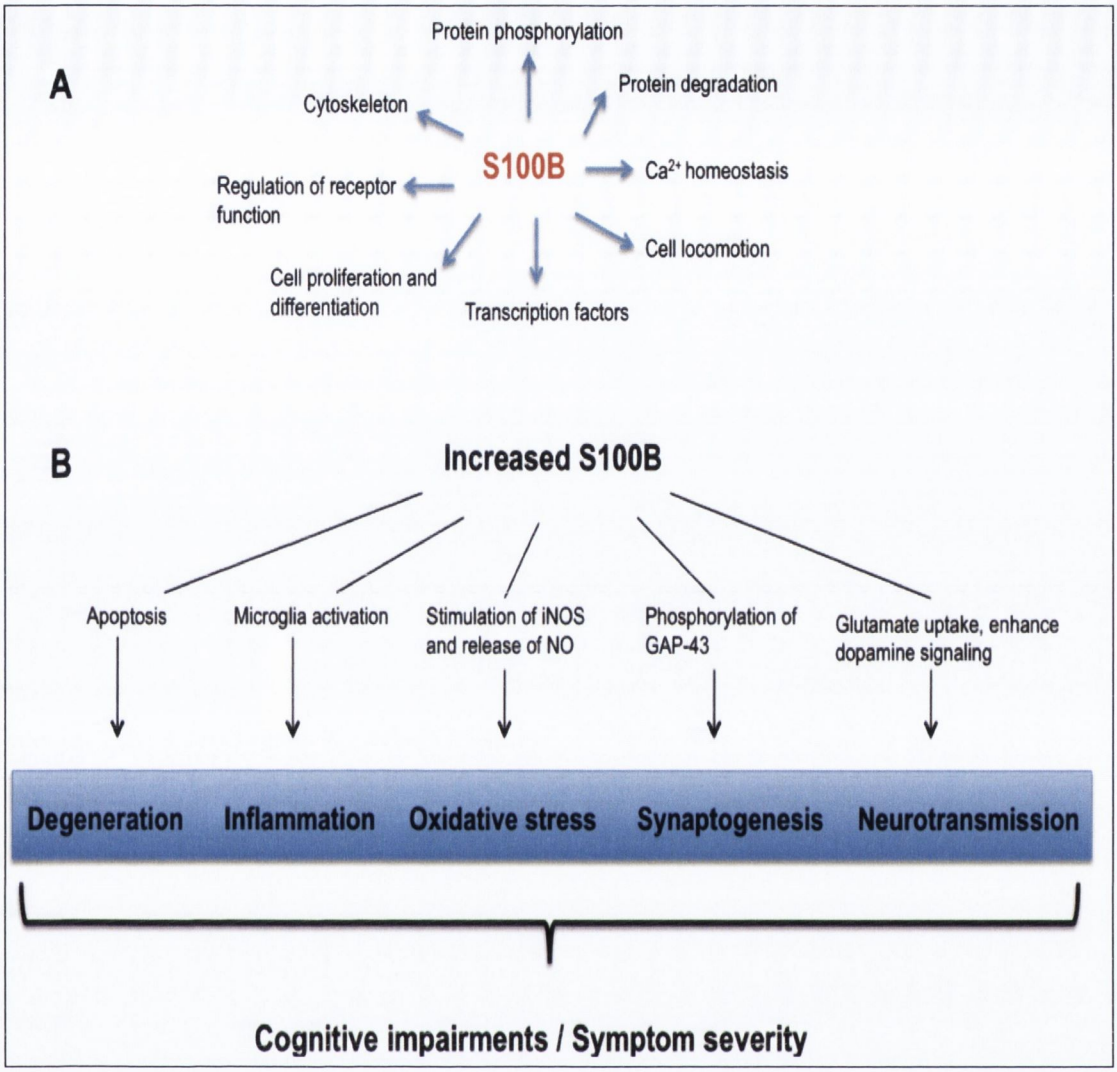

Figure 6.1. (A) Schematic representation of intracellular regulatory effects of S100B (modified from Donato R & Heinzmann CW, 2010). (B) Mechanism how increased S100B could be involved in cognitive impairments and symptom severity.

Neuroinflammation has been implicated in pathology of SZP (Song XQ *et al.,* 2009; Anisman H *et al.,* 2008) and may also be implicated in BPAD. S100B plays a role in neuroinflammation by activating the transcription factor NFkB and microglia (Adami C *et al.*, 2001; Donato R *et al.*, 2009). One potential result of this is increased production of inflammatory cytokines that, if expressed strongly enough, would be damaging to normal neuronal activity. Increased level of S100B involved in the nitric oxide synthase activation and a subsequent generation of nitric oxide (Adami C *et al.*, 2004; Hu J *et al.*, 1996).

Oxidative stress has been linked to SZP and cognitive impairments in SZP (Bitanihirwe B *et al.,* 2011; Berk M *et al.,* 2011).

Disturbances in neurotransmitter release have been hypothesized in the pathology of SZP and BPAD (Goodwin FK & Jamison K, 2007; Keshavan MS *et al.,* 2011). Increased levels of S100B might affect the interaction of neuron and glial cells, which could lead to elevated neurotransmission release and thus contribute to the cognitive deficits in SZP (Tan Y *et al.,* 2010). S100B is involved in the regulation and maintenance of the serotonergic nervous system (Eriksen JL *et al.,* 2002; Rothermundt M *et al.*, 2004b; Shapiro LA *et al.*, 2010) and is also involved in dopaminergic and glutamatergic neurotransmission (Tramontina F *et al.,* 2006; Rothermundt M *et al.*, 2007; Liu Y *et al.*, 2008). Inhibitory and stimulatory effects of glutamate on the secretion of S100B and on the stimulation of glutamate uptake into astrocytes by S100B have been reported (Tramontina F *et al.,* 2006; Ciccarelli R *et al.*, 1999; Nardin P *et al.*, 2007). Liu Y *et al.* (2008) reported that S100B interacts with dopamine receptor 2 and enhances dopamine signalling. Any alteration within the S100B protein structure affecting the binding to the dopamine receptor might contribute to the pathology of the SZP or BPAD by altering dopamine signalling.

Dysfunction of the neuronal synapse is strongly implicated in the pathogenesis of both schizophrenia and BPAD (Goodwin FK & Jamison K, 2007; Keshavan MS *et al.,* 2011). S100B has an important role in regulating the protein kinase C phosphorylation of GAP-43, which is a growth-associated protein that is involved in axonal growth and synaptogenesis during the development, synaptic remodelling and long-term potentiation (Lin LH *et al.*, 1994; Rothermundt M *et al.,* 2004b). Long-term potentiation is the physiological correlate of long-term memory (Swanson LW *et al.,* 1982).

It remains unknown whether increased serum concentration of S100B in SZP and BPAD is a contributing factor to disease progression or whether it is an effect of the disease. The results from this thesis do not give a clear answer if the increased level of S100B is causing illness or just an epiphenomenon of

illness. The present study specifically asked if genetic variation at S100B contributes to cause of illness. Although the SNP was found to be functional, the large association datasets did not support a causal role for the variant in either disorder. The analysis of a relatively small patient and control sample suggests that this SNP might impair cognitive performance and has impact on symptom severity in SZP and BPAD subjects. These endophenotypes have not been studied in sample sizes equivalent to the GWAS studies. Evidence for neuropsychological effects of S100B concentration has been identified in SZP where increased levels are associated with poor cognitive performance, negative symptoms and poor therapeutic response (Rothermundt M *et al.,* 2009). This is supported by animal studies, which showed a negative effect of increased S100B level on behaviour, learning and memory (Rothermundt M *et al.,* 2004b).

The genetic variation at S100B that was studied could contribute to risk by interacting with or modifying a genetic effect elsewhere. Further studies on transcription factors and other regulatory proteins that bind the S100B promoter might answer this question. Variants in the genes that encode the transcription factors that bind to rs3788266 might lead to altered expression level of those transcription factors or altered binding affinity for the S100B promoter. In combination, these genetic factors might lead to increased levels of S100B in SZP and BPAD.

In conclusion, although I have established that the rs3788266 variant in the S100B promoter is functional, I have not been able to prove that it is responsible for increasing risk of illness. Regardless, S100B remains a very interesting gene and protein to study because it offers a rare and important insight into the biology of psychosis, regardless of it being cause or effect.

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