INVESTIGATION OF *CIS*-REGULATORY VARIATION IN CANDIDATE GENES FOR PSYCHIATRIC AND NEURODEGENERATIVE DISEASE

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ABBREVIATIONS

3' RACE	3' Rapid amplification of cDNA ends
3' UTR	3' Untranslated region
5' UTR	5' Untranslated region
AD	Alzheimer's disease
AGD	Argyrophilic grain disease
bp	Base pair
CBD	Corticobasal degeneration
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleotide triphosphate
FTD	Frontotemporal dementia
kb	Kilobase
LOD	Logarithm of the odds
Mb	Megabase
mRNA	Messenger ribonucleic acid
OR	Odds ratio
PCR	Polymerase chain reaction
PD	Parkinson's disease
PiD	Pick's disease
PSP	Progressive supranuclear palsy
RDC	Research Diagnostic Criteria
RNA	Ribonucleic acid
RT	Reverse transcription

SNP	Single nucleotide polymorphism
VNTR	Variable number tandem repeat

ABSTRACT

In recent years, molecular genetics research has identified a large number of putative susceptibility genes for a variety of complex psychiatric and neurodegenerative disorders. However, in most instances, the particular functional variants involved have not been identified, and it is typically unclear by what mechanism the pathogenic effect is mediated. Where a genetic association does not appear to be fully explicable by variants that alter the amino acid sequence of a protein, it is a reasonable hypothesis that the association might be mediated by *cis*-acting variants that alter gene expression. This hypothesis was tested in this thesis in relation to 10 putative susceptibility genes for psychiatric and neurodegenerative disorders. The genes were *DISC1*, *RELN*, *GABRA4*, *GABRA5*, *GABRB1*, *GABRB2*, *GABRG2*, *GABRG3*, *NOS1AP* and *MAPT*. Each one of these genes was investigated by assays of relative allelic expression applied to a large number of post-mortem human brain samples. Samples were also genotyped for relevant variants that had previously shown association with disease in order to test those variants for a putative *cis*-regulatory effect. *Cis*-regulatory variation manifested as unequal expression of each parental gene copy at the mRNA level was detected in nearly all of the genes in at least one tissue sample. However, for only two genes (*RELN* and *MAPT*) was evidence obtained that specific variants implicated in disease influenced expression.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1: Genetic Variation in the Human Genome

Genetic variation plays a primary role in phenotypic diversity and is an important determinant of disease susceptibility. In order to affect phenotype, a genetic variant must influence the function of one or more genes. This can happen either by altering the amino acid structure of proteins (protein-coding variation), or by changing the temporal, spatial or quantitative aspects of gene expression (regulatory variation).

The human genome harbours many types of variation in both its structure and sequence. Variants with population frequencies of >1% are referred to as polymorphisms, and by far the most abundant in the human genome are single nucleotide polymorphisms (SNPs), where one of the four nucleotides (adenine, thymine, guanine or cytosine) is substituted with another. The human genome is estimated to harbour ~10 million SNPs (Kruglyak & Nickerson, 2001; Reich, Gabriel & Altshuler, 2003), an average of approximately one every 320 bases. Other types of variant include repeat polymorphisms including microsatellites (repeats of 1–6 nucleotides) and minisatellites (repeats of up to ~60 nucleotides), deletions and insertions (of one or more nucleotides), copy number variants (deletions or duplications of >1 kb), other structural chromosome rearrangements (e.g. inversions, translocations etc.), and abnormalities of whole chromosome number. Only a minority of all genetic variants are expected to have important phenotype effects, most being likely to have no functional consequence.

1.2: Most Genetic Variation Does Not Affect Protein Structure

Protein-coding sequences are, in general, remarkably conserved among higher species. For example, an average orthologous protein in humans and chimpanzees differs by just two

amino acids, and ~29% of proteins are identical (Chimpanzee Sequencing and Analysis Consortium, 2005). Commensurate with this high level of conservation is that protein-coding sequences also show relatively little variation between human individuals. Resequencing efforts have found that human genes contain on average approximately four coding SNPs, of which only half are non-synonymous (i.e. predicted to change the amino acid sequence of the encoded protein) (Cambien *et al.*, 1999; Cargill *et al.*, 1999; Halushka *et al.*, 1999; Stephens *et al.*, 2001; Freudenberg-Hua *et al.*, 2003; Livingston *et al.*, 2004). These same studies have also indicated that coding SNPs are generally less frequent (i.e. have lower minor allele frequencies) than non-coding SNPs, and that non-synonymous SNPs are less frequent than synonymous SNPs (also Tsunoda *et al.*, 2004). Furthermore, only 25–30% of non-synonymous SNPs are predicted to affect protein function (Sunyaev *et al.*, 2001; Ng & Henikoff, 2002; Yue & Moult, 2005), and these SNPs are less frequent than those that are predicted to be functionally neutral (Sunyaev *et al.*, 2001; Freudenberg-Hua *et al.*, 2003; Wong *et al.*, 2003; Livingston *et al.*, 2004). Therefore, it can be estimated that of the ~10 million SNPs in the human genome, only 12,500–15,000 (i.e. <0.002%) are likely to have an important functional effect on protein structure, and most of these are likely to be relatively infrequent at the population level.

The above findings are consistent with the view that non-synonymous SNPs are under strong purifying selective pressure. For example, the finding that only half of observed coding SNPs are non-synonymous is at odds with the expected rate of two thirds if mutations in coding regions were random. Although such variants, are more likely to have significant effects on phenotype than random variants, their apparently low number and frequency suggest that their combined role in determining phenotypic differences may be limited.

It has long been hypothesised that the clear phenotypic differences between humans and chimpanzees are primarily due to alterations in gene regulation rather than protein structure (King & Wilson, 1975). As will be mentioned in later sections, support for this view is growing. More pertinent to the present thesis, however, is whether this hypothesis also applies to differences between human individuals. It has been estimated that humans are more polymorphic at functional regulatory sequences than they are in protein-coding exons (Rockman & Wray, 2002). As will be described in section 1.9, this view has now received empirical support.

1.3: Gene Expression

Gene expression is the cellular process by which DNA sequences of genes are transcribed into RNA and, typically, then translated into proteins. During embryonic development, the particular complement of genes expressed in each cell is integral to the determination of each cell's fate. Throughout life, changes in gene expression are directed by signal transduction cascades to allow cells to alter their function in response to physiological and environmental influences. Hence, gene expression is a highly dynamic process that can be regulated at several levels and in many different ways.

The process of gene expression is illustrated in Figure 1.1. The first stage of gene expression is transcription, during which the DNA sequence of a gene is used as a template for RNA synthesis. The resultant pre-mRNA then undergoes three modifications to form mature mRNA. These modifications include splicing (the excision of introns and joining of exons), 5' capping (the linkage of a 7-methylguanosine nucleoside to the 5' end), and 3' polyadenylation (the linkage of ~200 adenylate residues to the 3' end to form a poly(A) tail). The mature mRNA is then transported out of the nucleus for translation, whereby it is

used to instruct the manufacture of a protein. The end-product of gene expression is not necessarily a protein, however, since many genes do not code for proteins. Such non-coding genes are expressed primarily by transcription, the end product being non-coding RNA (ncRNA). There is growing realisation that ncRNAs may play important roles in coordinating the expression of protein-coding genes (Mattick, 2001; Mattick & Makunin, 2006).

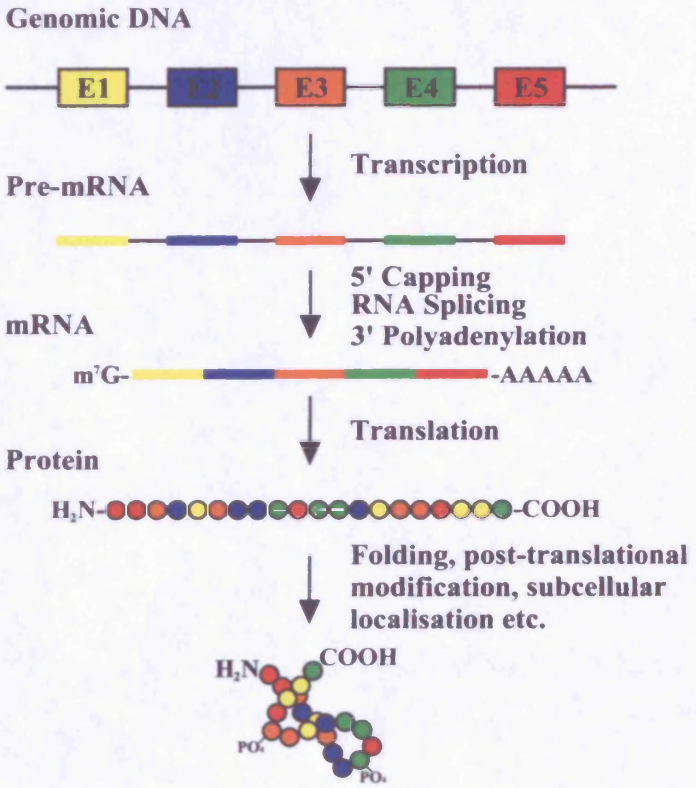

Figure 1.1: The process of gene expression. Genomic DNA is transcribed into pre-mRNA. The pre-mRNA is then modified such that the 5' end is capped, intronic sequences are spliced out, and a poly(A) tail is added to the 3' end. The mature mRNA is then translated into a protein, which adopts a three-dimensional structure and may be subject to further modifications to alter function.

1.4: Gene Regulation by *Cis*-Regulatory Elements and *Trans*-Regulatory Factors

Regulation of gene expression, whether at the level of transcription, pre-mRNA modification, or translation, is controlled by the binding of protein or often RNA factors to

regulatory sequences in the genome. A prime example is the core promoter, normally located at the 5' end of a gene, which includes binding sites (i.e. recognition sequences) for a number of proteins necessary for initiation of transcription. Regulatory elements are also commonly present in introns, as well as in exons, but can also be located many kilobases from the gene(s) they influence, as will be described later. Such sequences on the same DNA or RNA molecule as the gene or transcript being regulated are described as *cis*-regulatory elements.

In contrast to *cis*-regulatory elements, the protein or RNA factors that bind them are usually encoded by distantly located genes, often on different chromosomes. Since they are required to migrate to their site of action, where they influence expression of both chromosomal copies of a gene, they are described as *trans*-regulatory. Examples of *trans*-regulatory factors include RNA polymerases, transcription factors, hormones, various species of regulatory ncRNAs, and even *trans*-acting DNA-DNA interactions (Göndör & Ohlsson, 2009). In terms of their regulation, genes encoding *trans*-regulatory factors (or subunits thereof) are no different from any other genes; hence they too are also regulated by *cis*-regulatory elements and *trans*-regulatory factors.

1.5: Gene Regulation at the Level of Transcription

The expression of a gene can be regulated at any of the stages indicated in Figure 1.1, and even following translation, a protein can be modified to alter its location, activity, and abundance (e.g. Fortini, 2009). However, it is at the first stage, at the level of transcription, that the fundamental aspects of gene expression are controlled. For example, the types of cell in which a gene is expressed, the times in development that it is expressed, and the

degree to which it is expressed in adults, both basally and in response to physiological and environmental signals, are largely determined at this level (Ooi & Wood, 2008).

As already mentioned, the transcription of protein-coding genes is initiated at the core promoter, typically at the 5' end of a gene, and is dependent on the binding and recruitment of multiple proteins including transcription factors and the RNA-synthesising enzyme, RNA polymerase II (Butler & Kadonaga, 2002; Levine & Tjian, 2003). Transcription factors may directly bind to recognition sequences in the promoter, or, like RNA polymerase II, associate with other transcription factors that do. The ensemble of *trans*-regulatory proteins at the core promoter forms the basal transcription complex, which is sufficient to direct only low levels of transcription. A generalised example of a typical core promoter is shown in Figure 1.2.

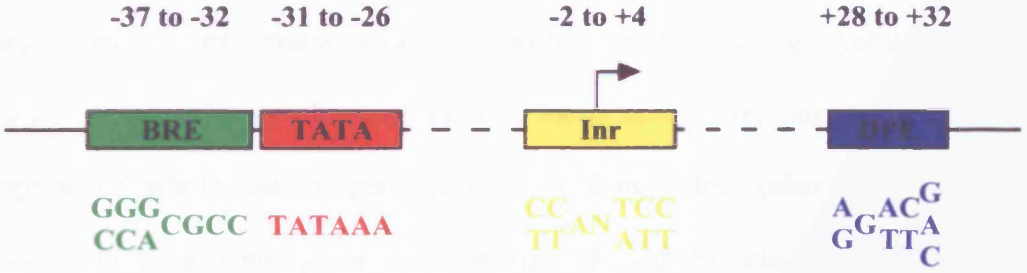

Figure 1.2: A generalised core promoter containing a TFIIB recognition element (BRE), *TATA* box, initiator (Inr), and downstream promoter element (DPE). Approximate nucleotide positions of each element relative to the transcription start site (indicated by an arrow) are shown at the top. Consensus recognition sequences for protein factors that bind each element are shown at the bottom. Figure is adapted from Butler & Kadonaga (2002).

The basal transcription complex is not adequate to initiate more than low levels of transcription. Most genes therefore have a variety of additional *cis*-regulatory elements that, when bound to by specific transcription factors, can interact with the basal

transcription complex to modulate transcriptional rate (Levine & Tjian, 2003; Maston, Evans & Green, 2006). Examples, shown in Figure 1.3, include the proximal promoter, located just 5' of the core promoter, and a number of more distant elements such as enhancers and silencers, which can increase or decrease transcription respectively.

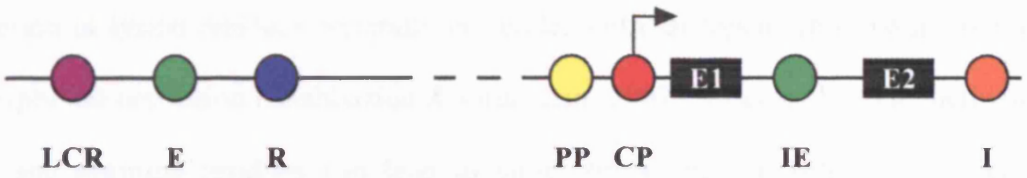

Figure 1.3: *Cis*-regulatory elements involved in transcriptional regulation. LCR = locus control region; E = enhancer; R = repressor; PP = proximal promoter; CP = core promoter; IE = intronic enhancer; I = insulator. E1 and E2 represent exons. The arrow indicates the transcription start site.

These additional elements typically contain recognition sequences for tissue-specific transcription factors, and may mediate responses to signalling molecules such as hormones (e.g. glucocorticoids or retinoic acid) and second messengers (e.g. cyclic AMP). Further examples of *cis*-regulatory elements include locus control regions, which can regulate transcription of whole loci or gene clusters, and insulators (also known as boundary elements), which prevent genes from being affected by *cis*-regulatory elements of neighbouring genes. As indicated in Figure 1.3, transcriptional *cis*-regulatory elements can be located throughout the gene as well as in distal regions. At present, the *cis*-regulatory elements that control expression of most genes have not been characterised, hence, their number, position and functional effect are largely unknown.

Thus far, transcription and its regulation have been described in terms of their reliance on the capacity of various transcription factors to recognise and bind to specific nucleotide sequences within and around a gene. However, transcription is also reliant on a number of

epigenetic factors. Chromosomal DNA is wrapped around specialised proteins known as histones to form chromatin. Chromatin structure can be altered by covalent modifications to the histones, and in turn altered structure can determine ease of access of transcription factors to their target DNA sequences. Consequently, the regulation of chromatin by modification can have profound effects on gene expression. For example, histone acetylation at lysine residues generally correlates with an 'open' chromatin structure and transcriptional activation (Shahbazian & Grunstein, 2007), whereas histone methylation at lysine and arginine residues can lead to either transcriptional activation or repression depending on the particular amino acids targeted (Zhang & Reinberg, 2001). As well as histones, DNA can also be methylated at cytosine bases of CpG dinucleotides. Many *cis*-regulatory elements of genes, particularly promoters, are embedded within CpG islands, sequences of typically 300–3,000 nucleotides with a high (e.g. >50%) CpG dinucleotide content. CpG methylation is generally correlated with transcriptional silencing (Ballestar & Wolffe, 2001; Scarano *et al.*, 2005).

The structure of chromatin and the modification state of histones and DNA play an important role in cell differentiation and the establishment of cell type- and tissue-specific patterns of gene expression (Reik, 2007; Vasanthi & Mishra, 2008). Furthermore, these epigenetic factors, particularly DNA methylation, are central to the maintenance of mono-allelic transcriptional phenomena. These include X inactivation in females, where one of the two X chromosomes is randomly selected for permanent transcriptional silencing (Mohandas *et al.*, 1981), and genomic imprinting, a phenomenon that is estimated to affect 100–200 genes (Falls *et al.*, 1999) whereby one copy of the gene is permanently silenced dependent on parent-of-origin, in some cases in a tissue-specific manner.

1.6: *Cis*- and *Trans*-Regulatory Variants

Variants that occur in *cis*-regulatory elements of genes (*cis*-regulatory variants), or in any functional (i.e. protein-coding or regulatory) sequence of genes that encode *trans*-regulatory factors (*trans*-regulatory variants) have the potential to alter gene expression. For example, *cis*-regulatory variants may abolish, create or modify binding sites for transcription factors, whereas variants in genes that encode transcription factors may alter their production or their capacity to bind DNA or interact with other proteins. As shown in Figure 1.4, a defining characteristic of *cis*-regulatory variants is that they act in an allele-specific manner and thus only influence expression of a gene copy on the same chromosome. Copy number variants that encompass genes are also likely to influence gene expression levels by altering gene dosage in *cis*. In contrast, *trans*-regulatory variants influence expression of both gene copies.

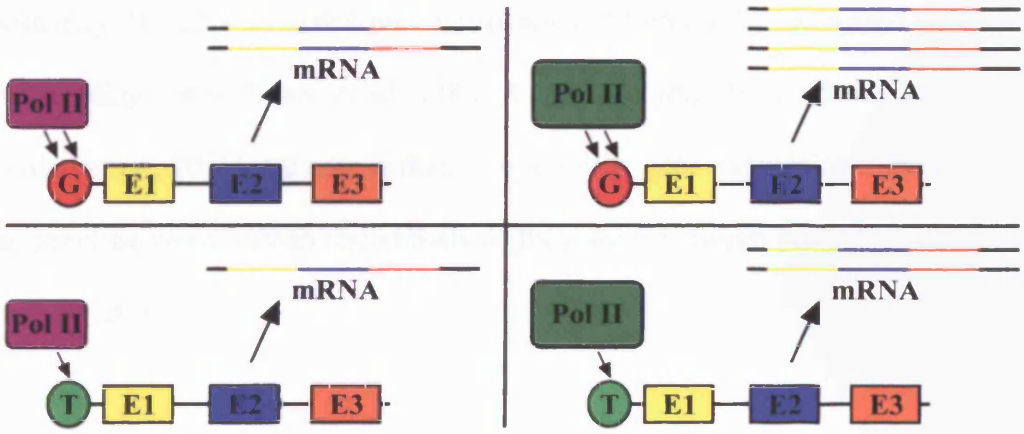

Figure 1.4: *Cis*- and *trans*-regulatory variants. In this hypothetical example, a *cis*-regulatory variant with alleles G (top) and T (bottom) in the core promoter of a gene results in twice as much mRNA being produced from one gene copy than from the other. In contrast, a functional variant in a gene that encodes either a low (left) or high (right) activity *trans*-regulatory protein (e.g. RNA polymerase II) results in a two-fold difference in mRNA from both copies of the target gene.

As regulatory elements that control expression of most genes are largely uncharacterised, the *cis*- or *trans*-regulatory variants that influence expression of any given gene are also largely unknown. Furthermore, unlike variants that affect protein structure, which typically occur in exons and have predictable effects on amino acid sequence, variants that influence expression of a gene can be very difficult to recognise. Although *in silico* tools are under continual development (Wasserman & Sandelin, 2004), variants that have functionally important regulatory effects *in vivo* cannot be reliably predicted based on inspection of DNA sequence alone.

1.7: Natural Variation in Gene Expression

Since the advent of microarray technology, it has become increasingly clear that species, populations, and individuals show substantial variation in their gene expression profiles. For example, comparisons between humans and chimpanzees have found that approximately 10–20% of genes are significantly differentially expressed in a variety of tissues including brain (Enard *et al.*, 2002; Cáceres *et al.*, 2003; Khaitovich *et al.*, 2004, 2005; Gilad *et al.*, 2006). Of note is that, in one study, gene expression differences in brain were as great between human individuals as they were between humans and chimpanzees (Enard *et al.*, 2002).

Human inter-individual differences in gene expression have also been found in peripheral blood. In a study of 75 healthy blood donors, 370 of ~18,000 genes (i.e. ~2%) were found to differ in expression by more than 2 fold relative to the mean in at least five individuals (Whitney *et al.*, 2003). In two subsequent studies (Radich *et al.*, 2004; Eady *et al.*, 2005), the proportion of genes differentially expressed in blood was higher at 5% and 39% respectively. The inconsistent figures between the studies are likely due to differences in

microarray platform, the specific cell types analysed, and in particular, the statistical criteria used to define a significant difference in expression.

Gene expression differences between species and / or strains of several other organisms have also been reported. In a comparison of two strains of the budding yeast *Sacharomyces Cerevisiae*, 1,528 of 6,215 genes (i.e. ~25%) were found to be significantly differentially expressed, whereas at the threshold used, 23 were expected by chance (Brem *et al.*, 2002). In mice, 7,861 of 23,574 genes (i.e. ~33%) were found to be differentially expressed in liver tissue between two parental inbred strains or at least 10% of F_2 progeny (Schadt *et al.*, 2003). Similar proportions of differentially expressed genes have been identified in *Drosophila* (Rifkin, Kim & White, 2003) and *Fundulus* fish (Oleksiak, Churchill & Crawford, 2002). Taken together, these findings suggest that variability in expression of a large number of genes is a natural and common feature of probably all eukaryotic organisms.

1.8: The Heritability of Gene Expression

Although environmental factors are likely to contribute to the above-described variation in gene expression, it is clear from studies of yeast, mice and humans that the expression of many genes is highly heritable (Brem *et al.*, 2002; Schadt *et al.*, 2003; Monks *et al.*, 2004). In humans, heritability of gene expression has been studied using lymphoblastoid cell lines derived from members of several large families that have been made available by the *Centre d'Etudes du Polymorphisme Humain* (CEPH) in Paris, France (Dausset *et al.*, 1990). In one such study, five genes that showed highly variable expression in 35 unrelated individuals were found to be expressed less variably in siblings, and most similarly in monozygotic twins (Cheung *et al.*, 2003), consistent with a genetic component. In a

separate study involving 15 families it was found that of the 2,430 genes that were differentially expressed in children, expression levels of 762 (i.e. ~31%) were significantly heritable (Monks *et al.*, 2004). Median heritability was estimated to be 34%; however, it is of note that, for some genes, genetic factors were predicted to account for 100% of the observed variation in expression. In a smaller study, which measured expression of 25 genes in 10 CEPH families, median heritability was estimated at 42% (Deutsch *et al.*, 2005). Evidence of heritable variation in gene expression in native human tissues (i.e. blood) has also been reported (Sharma *et al.*, 2005; Tan *et al.*, 2005).

1.9: Genetic Mapping of Gene Expression

Genetic variants that contribute to heritable variation in gene expression can be mapped by 'genetical genomic' studies in which gene expression profiling is combined with classical genetic methods such as linkage and association (Jansen & Nap, 2001). In these studies, expression levels of individual genes are treated as quantitative traits, which, just like any other heritable phenotype, are then genetically mapped to one or more expression quantitative trait loci (eQTLs). Given that these studies are based on measures of each gene's absolute expression, effects arising from *cis* or *trans* genetic variation cannot be readily distinguished. These studies have generally therefore categorized eQTLs as either *cis*- or *trans*-acting based on their genetic distance from the regulated gene (e.g. in one study [Morley *et al.*, 2004], eQTLs were defined as *cis*-acting if they were observed within 5 Mb of the regulated gene). This approach has been successfully applied to several organisms, including yeast (Brem *et al.*, 2002; Yvert *et al.*, 2003; Brem & Kruglyak, 2005), fruit flies (Wayne & McIntyre, 2002), fish (Oleksiak, Churchill & Crawford, 2002), mice (Schadt *et al.*, 2003; Chesler *et al.*, 2005), rats (Hubner *et al.*, 2005; Petretto *et al.*, 2006), as well as humans (Schadt *et al.*, 2003; Monks *et al.*, 2004; Morley *et al.*, 2004).

In humans, the genetical genomics approach has been employed in several studies of CEPH family cell lines (Monks *et al.*, 2004; Morley *et al.*, 2004; Deutsch *et al.*, 2005), and, together, these studies have identified significant eQTLs for hundreds of genes. Although these studies differed in scale, design and the number of eQTLs found, a consistent finding is that most of the eQTLs they identified were *trans*-acting. For instance, in the study that mapped the largest number of eQTLs, 110 (77.5%) of the 142 gene expression traits that had the strongest evidence for linkage mapped to a single *trans* eQTL, whereas 27 (19%) mapped to a single *cis* eQTL (Morley *et al.*, 2004). As in other studies (e.g. Monks *et al.*, 2004, Deutsch *et al.*, 2005), this study also found that expression of a smaller proportion of genes was linked to more than one locus. Of the 142 most strongly linked traits, two (1.5%) mapped to a *cis* and a *trans* eQTL, and three (2%) mapped to two trans eQTLs. When the analysis was widened to the 984 expression traits with linkage at a less stringent level of significance, 164 (16%) mapped to more than one eQTL, 152 of which mapped to *trans* eQTLs, and 12 to *cis* and *trans* eQTLs, suggesting that where more than one regulatory locus influences an expression trait, each contributes a smaller effect. Interestingly, consistent with findings in yeast (Brem *et al.*, 2002; Yvert *et al.*, 2003), mice (Schadt *et al.*, 2003; Chesler *et al.*, 2005) and rats (Hubner *et al.*, 2005), this study also identified a number of loci to which expression of a large number of genes was linked. For instance, expression of 31 genes mapped to chromosome 14q32, and expression of 25 mapped to chromosome 20q13, whereas if eQTLs mapped randomly across the genome, no more than four hits was expected to occur at each locus. The authors termed these eQTL hotspots 'master regulators', as they presumably contain polymorphism in genes encoding *trans*-regulatory factors that influence expression of a large number of genes.

The genetics of gene expression has also been studied by genetic association. In one study, 374 expression traits that previously showed evidence of *cis* linkage were followed up by testing SNPs within 50 kb 5' or 3' of the relevant gene for association with the gene's expression level in cell lines from 57 unrelated CEPH individuals (Cheung *et al.*, 2005). It was found that 65 (17%) of these traits were associated with at least one *cis* SNP. In a further analysis, the 27 expression traits that showed the strongest *cis* linkages were tested for association with ~770,000 SNPs genome-wide. Of these 27 traits, 14 (52%) showed genome-wide significant evidence for association with at least one SNP: 12 associated only with *cis* variants, one with a *cis* and a *trans* variant, and one with a *trans* variant only. A notable finding was that although *cis* associations were generally only found at one region of each gene, the associations were found to be approximately equally likely to occur at the 5' end, within, or at the 3' end of the gene.

A genome-wide association analysis of global gene expression in lymphoblastoid cells, assessed by microarray, has more recently been carried out using the 270 individuals genotyped through the HapMap Consortium (Stranger *et al.*, 2007). Analysis of over 2.2 million common (minor allele frequency of >5%) SNPs in each population provided evidence for at least 1,348 genes with association signals in *cis*, and at least 180 in *trans*. Thirty-seven percent of *cis* signals and 15% of *trans* signals were replicated in at least one separate population.

Large-scale association analysis of genetic variation and gene expression has also been performed in native human tissues (Göring *et al.*, 2007; Myers *et al.*, 2007a; Schadt *et al.*, 2008; Emilsson *et al.*, 2008). For example, in a recent report, inter-individual variability in global gene expression in post-mortem human brain was assessed in cerebral cortex

samples from 193 control individuals using genome-wide genotyping and gene expression microarrays (Myers *et al.*, 2007). In this study, 58% of the ~24,000 transcripts on the expression array were found to be cortically expressed in at least 5% of the samples. Furthermore, significant associations were found between 99 transcripts and 433 SNPs acting in *cis* (i.e. within 1 Mb 5' or 3' of the gene), and between 2,876 transcripts and 16,701 SNPs acting in *trans*.

1.10: Functional Assays of Putative Regulatory Variation

Functional assays of genetic variation can be used for two purposes. The first is to identify functional regulatory variants, which can subsequently be used as rational candidates for disease association testing. The aforementioned 'genetical genomics' studies represent good examples of the type of hypothesis-free approach that can be used for this purpose. The second is to test whether or not specific variants, which may have already been found to show association with disease, have effects on gene expression.

A variety of methods are available to assess the wide range of potential regulatory effects that variants can have (Knight, 2003; Prokunina & Alarcón-Riquelme, 2004). A few examples include northern blotting and reverse transcription PCR, which can be used to determine effects on splicing; so called 'DNA footprinting' and 'gel-shift' assays to investigate DNA-protein interactions (e.g. transcription factor binding sites); and bisulphite sequencing to evaluate effects on methylation. In addition to these methods, which primarily test for qualitative effects, a number of methods to investigate quantitative effects on gene expression are also available and commonly used.

The most widely used of such methods is the reporter gene assay. In this assay, a putative regulatory sequence containing either allele of a variant is cloned alongside a reporter gene into a vector, which is then transfected into cultured cells (Alam & Cook, 1990). The activity of the reporter gene product is then quantitatively measured as an indicator of transcriptional efficiency, making it possible to compare the regulatory potential of each allele of the variant sequence.

Reporter gene assays can provide valuable information on the potential of genetic variants to influence gene expression (Hoogendorn *et al.*, 2003). However, it must be appreciated that these assays represent an *in vitro* method where a variant DNA sequence is tested in a highly unnatural transcriptional environment. For instance, the DNA sequence is typically naked (i.e. devoid of chromatin proteins), isolated from other *cis*-regulatory elements, and may be subjected to *trans*-regulatory proteins that it would not normally encounter. For these reasons, variants that are found to have a regulatory effect in reporter gene assays cannot always be assumed to have a similar effect *in vivo* (Cirulli & Goldstein, 2007).

An alternative, commonly used approach is to simply compare the total abundance of a gene's mRNA or protein products (e.g. as quantified by real-time PCR or western blot analysis) between tissue samples derived from individuals of different genotypes. If the variant in question has a regulatory effect it is expected that homozygotes for either allele will have significantly different mRNA or protein levels, and that heterozygotes will have levels in an intermediate range.

This approach has the obvious advantage that variants are tested in their normal chromosomal and physiological context. However, unlike reporter gene assays, effects of

putative regulatory variants are inferred by association, making it more difficult to attribute causative status to a particular variant. In addition, this type of experiment is subject to a number of potentially confounding factors, as tissue samples, particularly human tissues obtained post-mortem, may differ in respects other than genotype for the variant in question. For example, they may differ in terms of pre-agonal state, post-mortem interval, or method of preparation, all of which may influence RNA quality. Differences in pre-mortem exposure to environmental factors (e.g. diet or drugs), variability in intrinsic *trans*-acting molecules such as hormones, and variation in genes encoding *trans*-regulatory proteins or RNAs may also influence gene expression levels. The capacity of this approach to detect modest regulatory effects of any variant is therefore limited, especially in comparisons of human post-mortem tissues where most of these confounds cannot be avoided.

1.11: Assays of Relative Allelic Expression

By comparing the relative expression level of the two chromosomal copies of a gene *within* individual tissue samples, it is possible to control most of the above-mentioned variables that can confound comparisons of total expression *between* samples. This, essentially, is the rationale behind assays of relative allelic expression.

Assays of relative allelic expression enable the indirect screening of genes for the presence of functional sequence variants in *cis*-regulatory elements, as well as other *cis*-acting regulatory phenomena such as epigenetic modification. The basic principle of these assays is illustrated in Figure 1.5.

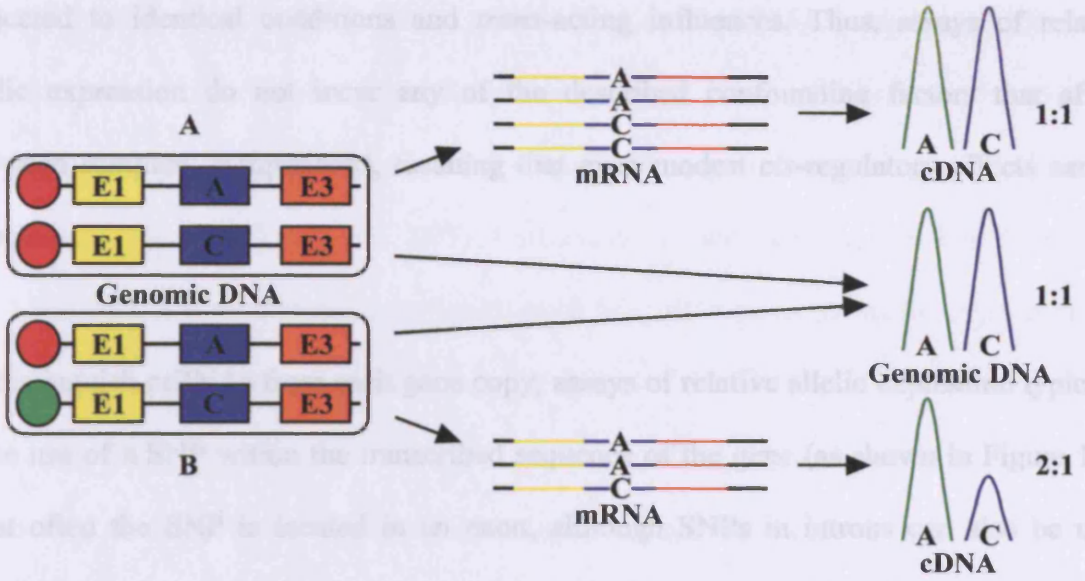

Figure 1.5: Assays of relative allelic expression make use of a SNP within a transcribed exon of a gene (left). In heterozygotes, mRNAs transcribed from each copy of the gene can be distinguished (middle). The relative abundance of mRNAs from each gene copy can therefore be measured in cDNA by a quantitative method of allele discrimination (right). **A:** In the absence of *cis*-regulatory variation (top), each mRNA will be present in a 1:1 ratio. **B:** In heterozygotes for *cis*-regulatory variation (bottom), each mRNA will be present in unequal abundance (e.g. 2:1 ratio). The relative abundance of each gene copy in genomic DNA is also measured as a 1:1 control.

In the absence of *cis*-acting influences that differentially affect expression of each chromosomal copy of a gene, both copies of that gene will be expressed equally (Figure 1.5A). In contrast, if an individual is heterozygous for any *cis*-acting variant that affects a gene's transcription or its mRNA's stability, or if a gene is subject to allele-specific epigenetic modification (e.g. imprinting effects), then each copy of that gene will be expressed at different levels. Accordingly, by measuring the relative expression of each copy of a gene in tissue samples from a series of individuals, the presence of *cis*-regulatory genetic or epigenetic variation can be inferred on the basis of a significant departure from a 1:1 ratio in any one sample (Figure 1.5B). In this 'within samples' approach, the mRNA from each gene copy serves as the perfect internal control for the other, as both are

subjected to identical conditions and *trans*-acting influences. Thus, assays of relative allelic expression do not incur any of the described confounding factors that afflict 'between samples' comparisons, meaning that even modest *cis*-regulatory effects can be detected.

To distinguish mRNAs from each gene copy, assays of relative allelic expression typically make use of a SNP within the transcribed sequence of the gene (as shown in Figure 1.5). Most often the SNP is located in an exon, although SNPs in introns can also be used (Pastinen *et al.*, 2004), in which case pre-mRNA instead of mRNA will be assayed. The first step in any relative allelic expression assay is to genotype the tissue samples for the SNP to identify heterozygotes. The relative abundance of mRNAs from each gene copy is then measured in those samples using a quantitative method of allele discrimination. Examples of such methods used previously include real-time PCR assays (Zhu *et al.*, 2004), polymerase colonies (Butz *et al.*, 2004), microarrays (Lo *et al.*, 2003), and RT-PCR followed by a variety of methods based on single base primer extension (Singer-Sam *et al.*, 1992; Cowles *et al.*, 2002; Yan *et al.*, 2002; Bray *et al.*, 2003a; Pastinen *et al.*, 2004). A primer extension method that uses fluorescent dideoxynucleotides is particularly robust, capable of quantifying very small percentage differences in allelic representation (Norton *et al.*, 2002).

By assessing relative allelic expression in a series of individuals, it is possible to screen a gene for the presence of common *cis*-regulatory variation, at least as this affects gene expression in the assayed tissue. Studies involving a limited number of individuals (typically less than 20 per gene) have found that around 50% of genes analysed show evidence of unequal allelic expression in tissue or cells from at least one individual (Yan *et*

al., 2002; Bray *et al.*, 2003; Lo *et al.*, 2003; Pant *et al.*, 2006), demonstrating that *cis*-regulatory variation is common in the human genome. These studies have revealed that allelic expression differences are typically of modest magnitude (<2 fold), and, for some genes, tissue-specific (Lo *et al.*, 2003). Furthermore, analysis of human lymphoblastoid cell lines derived from families has confirmed that differences in allelic expression are heritable (Yan *et al.*, 2002; Pastinen *et al.*, 2004).

The utility of relative allelic expression assays extends beyond their capacity to simply screen a gene for *cis*-regulatory variation. Since they expose only the variance in expression resulting from *cis*-acting effects, they also provide a particularly powerful method for examining the influence of specific genotypes or haplotypes on a given gene's expression (e.g. Bray *et al.*, 2003b, 2005). This makes them particularly useful for investigating regulatory effects associated with variants implicated in disease.

1.12: Pathogenic Variants in Monogenic Human Disease

Mutations that disrupt gene expression, especially transcription, are known to cause several human genetic disorders (Maston, Evans & Green, 2006). Examples include α- and β-thalassaemia (Antonarakis *et al.*, 1984; Driscoll *et al.*, 1999; Hatton *et al.*, 1990), Gilbert's syndrome (Bosma *et al.*, 1995), and fragile X syndrome (Sutcliffe *et al.*, 1992), each of which involve transcriptional disruption by a different mechanism. However, the majority of identified high penetrance mutations known to cause genetic disease impart their pathogenic effect by altering protein structure. For instance, as of December 2008, the Human Gene Mutation Database (Stenson *et al.*, 2009) lists 35,545 missense and nonsense mutations, but only 817 regulatory mutations. The larger number of mutations in the former class may in part be due to the fact that such mutations are more readily

identifiable. However, the considerably smaller number of regulatory mutations more likely indicates that variants that influence gene expression typically have more subtle phenotypic effects that, independently, are insufficient to cause disease.

1.13: Pathogenic Variants in Complex Human Disease

In contrast to rare dominant Mendelian disorders where the effect of a single mutation is often large enough to cause disease, the genetic architecture of more common disorders such as diabetes, cardiovascular disease, and the major mental illnesses, is generally thought to be considerably more complex. While these disorders often show high heritability (e.g. Hyttinen *et al.*, 2003; Zdravkovic *et al.*, 2002; Cardno *et al.*, 1999; Sullivan, Kendler & Neale, 2003), transmission is not usually consistent with a Mendelian model. Rather, the majority of cases of these conditions are thought by many to be largely attributable to the co-action of variants in multiple genes, as well as environmental factors (Reich & Lander, 2001; Pritchard, 2001; Dean, 2003). According to the common disease / common variant hypothesis (Reich & Lander, 2001) the variants underlying complex disorders are most likely to be common, with each conferring only a small increase in disease risk (e.g. odds ratios [OR] of less than 2). However, this hypothesis has recently been challenged by findings of an increased frequency of rare copy number variants in complex diseases such as autism and schizophrenia (e.g. Sebat *et al.*, 2007; Walsh *et al.*, 2008; Stefansson *et al.*, 2008; International Schizophrenia Consortium, 2008).

There are many examples of variants associated with a complex disease that have been demonstrated to have a *cis*-regulatory effect (Knight, 2005). Among the earliest examples are a variable number tandem repeat (VNTR) polymorphism associated with type 1 diabetes that regulates transcription of the gene encoding insulin (Kennedy *et al.*, 1995), a

SNP in exon 4 of *PTPRC*, which modulates splicing (Lynch & Weiss, 2001) and is associated in some studies with multiple sclerosis, and a SNP in the 5' UTR of *F12* that predisposes to cardiovascular disorders by reducing the translation efficiency of coagulating factor XII (Kanaji *et al.*, 1998). The widespread involvement of variants affecting gene expression in complex diseases is suggested in that many of the most significant genome-wide association hits related to such disorders do not appear explicable in terms non-synonymous changes (e.g. O'Donovan *et al.*, 2008; Stefansson *et al.*, 2009; Newton-Cheh *et al.*, 2009; Song *et al.*, 2009).

1.14: Psychiatric and Neurodegenerative Disease

Psychiatric and certain types of neurodegenerative diseases are prime examples of complex diseases that are currently being tackled by molecular genetics research. Psychiatric diseases have as their defining features disturbances of perception, cognition, mood and behaviour. As their name suggests, neurodegenerative diseases are characterised by the degeneration of neural tissue, and these disorders also frequently have psychiatric as well as neurological manifestations. Pertinent examples of both types of disease, as well as epilepsy, a neurological disorder, are listed in Table 1.1 with brief details of core symptoms, heritability, and lifetime population prevalence.

Microarray studies have found that many psychiatric and neurodegenerative disorders are associated with widespread gene expression changes in brain (Ricciarelli *et al.*, 2004; Hauser *et al.*, 2005; Katsel *et al.*, 2005; Iwamoto & Kato, 2006). However, it is difficult on the basis of gene expression assays alone to distinguish between changes that reflect primary disease mechanisms and secondary manifestations of the disease (Bray, 2008).

Disorder	Core Clinical Features	Heritability	Prevalence (%)
Schizophrenia	Delusions, hallucinations, and cognitive and emotional deficits.	>0.80[a]	1
Bipolar Disorder	Episodes of mania, with or without episodes of depression.	0.85[b]	1
Major Depression	Episodes of clinically significant depression in the absence of mania.	$0.29_{(M)}$, $0.42_{(F)}$[c]	10–20
Generalised Anxiety Disorder	Recurrent, ongoing, prominent and pathological anxiety.	0.32[d]	5
Alcohol / Drug Dependence	Inappropriate or excessive use of alcohol or drugs leading to social, psychological or physical impairment and physical dependence.	$0.50–0.60_{(A)}$[e] $0.45–0.79_{(D)}$[e]	10
Autism	Delayed social-communicative development with restricted patterns of interest or behaviour.	>0.90[f]	0.05
Alzheimer's Disease	Progressive deterioration of memory accompanied by cognitive impairments.	0.60–0.80[g]	5 (over age 65)
Idiopathic Epilepsy	Recurrent seizures and loss of consciousness.	0.80[h]	0.5–1

Table 1.1: The core clinical features, heritability and approximate lifetime population prevalence of several psychiatric and neurodegenerative disorders and the neurological disorder, epilepsy. Heritability estimates for major depression are given separately for males (M) and females (F), and separately for alcohol (A) and other drug (D) dependences. Heritability estimates are from [a]Cardno & Gottesman (2000); [b]McGuffin *et al.* (2003); [c]Kendler *et al.* (2006); [d]Hettema, Neale & Kendler (2001); [e]Dick & Agrawal (2008); [f]Freitag (2007); [g]Gatz *et al.* (2006); [h]Kjeldsen *et al.* (2003).

There is now strong evidence implicating particular susceptibility genes in many psychiatric and neurodegenerative disorders including schizophrenia (International Schizophrenia Consortium, 2008; Stefansson *et al.*, 2009), bipolar disorder (Craddock *et al.*, 2008; Ferreira *et al.*, 2008), autism (Wang *et al.*, 2009), and Alzheimer's disease (Corder *et al.*, 1993; Harold *et al.*, 2009). However, in the vast majority of cases, the true functional variants involved have not been characterised, and the mechanisms by which

they increase susceptibility is unclear. One of the few notable exceptions is the ε4 allele of the *APOE* gene, which is thought to increase risk for Alzheimer's disease through changes in protein structure (Strittmatter *et al.*, 1993a, 1993b) and non-coding variation in *SLC6A4*, which has been found to alter transcription of the serotonin transporter and to be associated with anxiety-related traits (Lesch *et al.*, 1996).

None of the associations between schizophrenia and bipolar disorder and their best supported susceptibility genes that have emerged in the past though positional cloning, for example *NRG1*, *DTNBP1* and *DAOA* (reviewed in Norton, Williams & Owen, 2006) appear to be mediated by variants that affect protein structure. Moreover, unpublished data generated in the Department of Psychological Medicine suggest that the same is also true for the leading candidate genes for these disorders that have been identified more recently by genome-wide association (e.g. *ANK3*, Ferreira *et al.*, 2008; *CACNA1C*, Ferreira *et al.*, 2008; *NRGN*, Stefansson *et al.*, 2009; and *ZNF804A*, O'Donovan *et al.*, 2008). Therefore, if these associations are genuine, it is likely that they are mediated by variants that alter gene expression.

For several of the leading (pre-genome-wide association) schizophrenia susceptibility genes, there is now evidence that this might indeed be the case. For example, using assays of relative allelic expression, it has been found that a haplotype in the *DTNBP1* gene that showed association with schizophrenia in UK and Irish samples (Williams *et al.*, 2004) is associated with reduced expression of that gene's mRNA in human cerebral cortex (Bray *et al.*, 2005). Using real time PCR, a SNP within a haplotype of the *NRG1* gene that shows association with schizophrenia (Stefansson *et al.*, 2002, 2003) was found to be associated with increased expression of a novel *NRG1* mRNA isoform in human hippocampus (Law

et al., 2006). In a subsequent study, a reporter gene assay provided evidence that this SNP in the 5' region of *NRG1* influences transcriptional activity (Tan *et al.*, 2007).

1.15: The Present Series of Studies

There are numerous other examples of genes implicated in psychiatric or neurodegenerative disease where reported associations are not entirely explicable by variants that alter protein structure. A selection of these genes forms the basis for this thesis, which tested the hypothesis that the putative associations between these genes and disease may be mediated by variants that have *cis*-regulatory effects on mRNA abundance.

The genes analysed were as follows:

- *DISC1*: This gene was originally identified as the breakpoint site of a chromosomal translocation in a large family with multiple cases of mental illness. It has since emerged as promising susceptibility candidate for schizophrenia as well as bipolar disorder.
- *RELN*: This gene encodes a large protein that is critical for proper neuronal migration. Reduced *RELN* expression in brain has been reported in schizophrenia and bipolar disorder as well as autism. Genetic association between this gene and autism and schizophrenia has also been reported.
- *NOS1AP*: A positional and plausible functional susceptibility candidate for schizophrenia, with evidence of genetic association and altered expression in schizophrenic and bipolar disorder post-mortem brain.
- *MAPT*: This gene encodes tau, the major constituent of neuronal protein aggregates that are characteristic of several neurodegenerative diseases, including Alzheimer's disease and rare disorders such as progressive supranuclear palsy.

- A selection of six $GABA_A$ receptor subunit genes: The Department of Psychological Medicine, Cardiff University, recently generated strong genetic evidence that implicates several members of this family of genes in the aetiology of a particular subtype of bipolar disorder.

1.16: Aims

The general aims of the present series of studies were as follows:

- To determine whether or not the expression of each of the above genes in human brain is influenced by *cis*-regulatory variation. This was achieved by applying assays of relative allelic expression to a large number of human post-mortem brain tissue samples.
- For genes that showed evidence of unequal allelic expression (and thereby *cis*-regulatory variation), to attempt to link that phenomenon to susceptibility to relevant psychiatric or neurodegenerative diseases. This was achieved by:
    - Genotyping the assayed brain samples for specific variants or haplotypes that have been reported to show association with disease to test whether or not they are also associated with regulation of gene expression in brain.
    - Or, where relative allelic expression assays pointed to an association between specific variants and gene expression, by testing those variants for evidence of association with disease.

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2: GENERAL MATERIALS AND METHODS

This chapter describes in detail the general materials and methods used in the present series of studies. Specific details of materials and methods used for each study are described in relevant chapters.

2.1: Brain Samples

2.1.1: Demographic Data

Post-mortem brain samples used in the present studies were obtained by Dr NJ Bray / Prof MC O'Donovan from three reputable sources with ethical permission to collect and distribute the samples: 1) the MRC London Brain Bank for Neurodegenerative Diseases (London, UK); 2) the Stanley Medical Research Institute (Maryland, USA); and 3) the Karolinska Institute (Stockholm, Sweden). Demographic characteristics of samples from each source are summarised in Table 2.1. All tissue samples were stored in a -80°C freezer prior to extraction.

2.1.2: A Note on DNA and RNA Processing

At the commencement of the present work, brain samples from all subjects had already been processed by Dr Bray. However, as stocks of DNA, RNA and cDNA became depleted, it was necessary to obtain further brain tissue from certain subjects for DNA / RNA extraction and subsequent cDNA synthesis. This replenishment of samples was carried out by the author.

Source	N	Sex		Age at Death		Ethnicity			Diagnosis					Brain Region		
		M	F	M	R	C	A	B	N	S	B	M	A	F	T	P
MRC	67	33	34	73	20–105	67	0	0	44	1	0	0	22	45	22	0
Stanley	71	44	27	45	19–70	66	3	2	23	17	15	16	0	60	0	11
Karolinska	10	6	4	52	35–61	10	0	0	10	0	0	0	0	0	10	0
All	148	83	65	58	19–105	143	3	2	77	18	15	16	22	105	32	11

Table 2.1: Details of brain samples used in the present series of studies. **Sex**: M = male, F = female; **Age at Death**: M = mean average (whole years), R = range (whole years); **Ethnicity**: C = Caucasian, A = Asian, B = Black; **Diagnosis**: N = normal (no known psychiatric or neurological disease), S = schizophrenia, B = bipolar affective disorder, M = major depressive disorder, A = Alzheimer's disease; **Brain Region**: F = frontal lobe, T = temporal lobe, P = parietal lobe. Except for Age at Death, numbers in each cell represent the frequency of individual brain samples obtained from each source that fall in each demographic category.

2.1.3: Genomic DNA Extraction

For each brain sample, up to ~1,000 mg of tissue was taken from a -80°C freezer and freeze-fractured using a hammer and a pre-frozen stainless steel pulveriser. Approximately half of the freeze-fractured tissue was used for DNA extraction, and half for RNA extraction (see later). To obtain DNA, the pulverised sample was loaded into a 2 ml Lysing Matrix D tube (Qbiogene) with 1 ml of STE buffer (Fisher Scientific), and homogenised on a FastPrep® FP120 Cell Disrupter (Qbiogene) for 40 seconds at speed setting 4. The tube was then centrifuged at 13,000 rpm for five minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube to which 100 µl of 10% sodium dodecyl sulphate (Sigma-Aldrich) and 100 µl (2 mg) of proteinase K (Sigma-Aldrich) was added. The tube was then vortexed and incubated at 50°C overnight in a water bath.

The following morning, the digested tissue was divided and transferred in equal volumes (~600 µl) to two microcentrifuge tubes. 1 ml of phenol (Sigma-Aldrich) was added to each

tube. The tubes were then vortexed, incubated at room temperature for five minutes, and centrifuged at 3,300 rpm for four minutes at 10°C. The upper aqueous phases of each tube were transferred to fresh tubes to which 400 µl of phenol (Sigma-Aldrich) and 400 µl of 24:1 chloroform / isoamyl alcohol (Sigma-Aldrich) were added. The tubes were vortexed and centrifuged at 3,300 rpm for four minutes at 10°C. The upper aqueous phases of each tube were again transferred to fresh tubes, this time with 1 ml of 24:1 chloroform / isoamyl alcohol (Sigma-Aldrich). The tubes were vortexed and centrifuged at 3,300 rpm for four minutes at 10°C. To precipitate the DNA, the upper aqueous phases of each tube were transferred to two fresh tubes to which 1 ml of 100% ethanol (Sigma-Aldrich) was added. The precipitated DNA from both tubes was then removed and pooled into a fresh tube and suspended in 1 ml of TE buffer. Extracted DNA samples were left overnight in a fridge to dissolve, and then stored at -20°C.

2.1.4: Total RNA Extraction

Total RNA was originally extracted using the RNAwiz™ isolation reagent (Ambion). Due to the discontinuation of this product, replenishment of RNA samples was instead carried out using Tri Reagent® Solution (Ambion).

For each brain sample, up to ~1,000 mg of tissue was taken from a -80°C freezer and freeze-fractured using a hammer and a pre-frozen stainless steel pulveriser. Approximately half of the freeze-fractured tissue was used for DNA extraction (see above), and half for RNA extraction. To obtain RNA, the pulverised sample was loaded into a 2 ml Lysing Matrix D tube (Qbiogene) with 1 ml of Tri Reagent® Solution (Ambion), and homogenised on a FastPrep® FP120 Cell Disrupter (Qbiogene) for 40 seconds at speed setting 4. Once

homogenised in Tri Reagent, the sample was incubated at room temperature for 10 minutes. The tube was then centrifuged at 13,000 rpm for five minutes at 4°C.

The supernatant was transferred to a fresh microcentrifuge tube to which 200 µl of chloroform (Sigma-Aldrich) was added. The tube was vigorously vortexed, incubated at room temperature for 10 minutes, and centrifuged at 13,000 rpm for 10 minutes at 4°C. The upper aqueous phase was transferred to a second microcentrifuge tube to which 500 µl of isopropanol (Sigma-Aldrich) was added to precipitate the RNA. The tube was vortexed, incubated at room temperature for 10 minutes, and centrifuged at 13,000 rpm for eight minutes at 4°C. The supernatant was discarded and the pellet was washed by adding 1 ml of 75% ethanol (Sigma-Aldrich) and centrifuging at 8,000 rpm for five minutes at 4°C. The ethanol was discarded and the pellet was air-dried for five minutes before being suspended in 40–100 µl of nuclease-free water (Ambion) and stored at -80°C.

2.1.5: DNase Treatment of Extracted RNA Samples

To remove genomic DNA, extracted RNA samples were treated using the DNA-free™ Kit (Ambion). This kit contains a recombinant DNase I (rDNaseI) enzyme in addition to DNase Inactivation Reagent. 40 µl of extracted total RNA was transferred to a 0.6 ml microcentrifuge tube to which 4 µl of 10× DNase I Buffer and 1 µl (2 U) of rDNase I was added. The mixture was gently tapped and then incubated at 37°C for 30 minutes. Following this, 5 µl of DNase Inactivation Reagent was added, and the mixture was incubated at room temperature for two minutes whilst being gently tapped intermittently. To pellet the DNase and Inactivation Reagent, the mixture was centrifuged at 13,000 rpm for two minutes. The supernatant, containing DNA-free RNA, was then transferred to a fresh 0.6 ml tube and stored at -80°C.

1 µl of each DNase-treated RNA sample was diluted in 140 µl of water, which is a dilution factor that is equivalent to that achieved during reverse transcription (described in section 2.1.7) to generate cDNA for the relative allelic expression assay (described in section 2.2.2). This diluted RNA sample was then tested for residual genomic DNA contamination (alongside genomic DNA and water as positive and negative controls) by PCR using primers and conditions that were optimised for the amplification of a genomic DNA sequence. The absence of contaminating genomic DNA was confirmed by the failure of the dilute RNA sample to produce a PCR amplicon detectable by agarose gel electrophoresis.

2.1.6: Spectrophotometry and Dilution of Extracted DNA and RNA Samples

The concentration and purity of extracted DNA and RNA samples was measured by spectrophotometry using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). A 260/280 ratio of $\geq$1.7 was required for DNA and of $\geq$1.8 for RNA. Working aliquots of 1 ml of genomic DNA at a concentration of 8 ng/µl were then prepared and stored at -20°C. Where RNA concentrations were above 1 µg/µl, samples were diluted to 1 µg/µl before being stored at -80°C.

2.1.7: Reverse Transcription

Reverse transcription (RT) involves the use of a reverse transcriptase enzyme to copy RNA into its complementary DNA (cDNA) sequence, which can then be used as a template for PCR Amplification (described in section 2.3.1). For each DNase-treated RNA sample, two separate RT reactions were carried out simultaneously.

Prior to conducting RT reactions, all work surfaces, pipettes and gloves were thoroughly cleaned with RNaseZap® (Ambion) to minimise the potential for RNase contamination. RT

reactions were carried out in a total volume of 20 µl containing 1 µg of total RNA. All reagents were supplied as part of the RETROscript Kit® (Ambion). Table 2.2 shows the volume and concentration of each reagent used in a single reaction, which was carried out in two steps.

Reagent	Supplier	Concentration	Volume (µl)
H_2O (nuclease-free)	Ambion	-	9.0
Random Decamer Primers		50 µM	2.0
RT Buffer		10×	2.0
dNTPs (dATP, dCTP, dGTP, dTTP)		10 mM	4.0
MMLV-RT		100 U/µl	1.0
RNase Inhibitor		10 U/µl	1.0
RNA Template	-	1 µg/µl	1.0
Total	-	-	20

Table 2.2: Details of reagents used in RT reactions.

In the first step, the RNA template was mixed with the random decamer primers and nuclease-free water, heated to 85°C for 3–5 minutes, and then immediately placed on ice. This step was necessary to denature the secondary structure of mRNA to facilitate annealing of primers to the template and was done prior to the second step to prevent the reverse transcriptase and RNase inhibitor enzymes from being denatured. In the second step, all other reagents in Table 2.2 were added to the reaction mix. The reaction was incubated at 44°C for 40 minutes, 55°C for 20 minutes, and then heated to 92°C for 10 minutes to inactivate the enzymes.

2.1.8: Dilution of cDNA Samples

Following completion of RT reactions, 120 µl of sterile water (Phoenix Pharma) was added to each cDNA product (20 µl) to create working samples at what is hereafter referred to as the 'standard cDNA concentration'. These cDNA samples were then stored at -20°C.

2.2: Molecular Analysis Techniques

2.2.1: Genotyping

Samples were primarily genotyped for single nucleotide polymorphisms (SNPs), but other polymorphisms, such as microsatellites, were also genotyped. Genotyping of SNPs was a necessary step in the relative allelic expression assay (described in section 2.2.2) in order to identify informative samples. Additional variants were genotyped to identify carriers of particular alleles or haplotypes of interest.

Genotyping of SNPs

Genotyping of SNPs was performed by single base primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems). This involved the following five steps: 1) PCR Amplification, 2) Post-PCR Cleanup, 3) SNaPshot Primer Extension, 4) Post-SNaPshot Cleanup, and 5) Capillary Gel Electrophoresis. Each of these steps is described in detail in section 2.3. SNP genotype data were processed in GeneScan® Analysis 3.7 (Applied Biosystems) and analysed in Genotyper® 2.5 (Applied Biosystems) as described in section 2.4.1.

Genotyping of Microsatellites

Genotyping of microsatellites was performed by PCR-amplifying the sequence using a 5' fluorescently-labelled forward primer and then measuring the size (in bp) of amplicons by coelectrophoresis with a fluorescent size standard by capillary gel electrophoresis. PCR Amplification and Capillary Gel Electrophoresis are described in detail in section 2.3. Amplicon size data were processed in GeneScan® Analysis 3.7 (Applied Biosystems) and analysed in Genotyper® 2.5 (Applied Biosystems) as described in section 2.4.1.

2.2.2: Relative Allelic Expression Assay

The relative allelic expression assay was the main technique used to address the major questions posed in the present research; hence the bulk of data presented in following chapters was generated by this assay. As described in Chapter 1, the relative allelic expression assay is a method to detect the influence of *cis*-regulatory genetic or epigenetic variation on gene expression at the RNA level. In brief: the relative expression of each parental copy of a gene is measured in brain tissue by quantifying each allele of an exonic SNP in cDNA derived from heterozygous brain samples. If departure from a 1:1 ratio is detected, it is assumed that this reflects the influence of *cis*-acting regulatory variation.

In addition to simply screening a gene for *cis*-regulatory variants, the relative allelic expression assay was also used to investigate potential effects on expression of individual polymorphisms or haplotypes that have been found to be associated with disease. Such effects were tested for by comparing allele ratios from brain samples that are heterozygous for the polymorphism or haplotype with ratios from samples that are homozygous or that do not carry the haplotype.

Three key aspects of the assay are the choice of SNP to analyse, the design of PCR primers, and the application of a correction factor derived from genomic DNA.

SNP Selection

The SNP chosen for analysis was used primarily to distinguish transcripts derived from each of the two parental copies of a gene (i.e. the SNP was not chosen based on hypotheses regarding its functionality). An absolute requirement was that the SNP must be expressed at the RNA level; hence only SNPs within an exon of the gene of interest were selected. It

was preferable to choose a SNP with a high minor allele frequency to maximise the number of informative (i.e. heterozygous) brain samples. In addition, it was important to choose a SNP that is sufficiently distant from the splice junction to allow the design of single-exon primers that are capable of amplifying cDNA and genomic DNA templates (described below). When choosing a SNP for a gene that is alternatively spliced or that has multiple promoters, the exon in which the SNP was located was considered carefully, as this determined the particular transcripts that would be assayed.

Primer Design

PCR primers flanking the SNP were designed to produce the same amplicon from both genomic DNA and cDNA, and were therefore based on sequences from a single exon. The use of single-exon primers meant that genomic DNA could be assayed under identical conditions to cDNA, to serve as a control for potential allelic biases that may arise during PCR amplification or SNaPshot primer extension. More details on primer design are described under PCR Amplification in section 2.3.1.

Genomic DNA Correction Factor

In single base primer extension assays that employ SNaPshot chemistry, the two alleles of a SNP are not equally represented – not even in genomic DNA where alleles are usually present in equimolar concentrations. Instead, there is a bias towards specific nucleotides (Moskvina *et al.*, 2005). Given this fact, and that the used assay employed SNaPshot chemistry, it was crucial that raw cDNA allelic ratios were corrected for this bias to obtain an accurate measurement of relative allelic expression. In the absence of copy number variation, null alleles or paralogous sequences, allelic ratios obtained from genomic DNA should reflect a perfect 1:1 ratio of the two alleles. Therefore, where possible, genomic

DNA was always assayed in parallel with cDNA so that allelic ratios obtained from the former could be used to correct those from the latter. How the correction factor was applied is described in section 2.5.1.

Assay Outline

Genomic DNA samples were initially genotyped for the chosen SNP to identify heterozygotes, informative for analysis. Genotyping of SNPs is described in section 2.2.1. For each heterozygote, genomic DNA and two corresponding cDNA samples (i.e. products of two separate RT reactions) were assayed on two separate occasions under identical conditions according to the following five steps: 1) PCR Amplification, 2) Post-PCR Cleanup, 3) SNaPshot Primer Extension, 4) Post-SNaPshot Cleanup, and 5) Capillary Gel Electrophoresis. Each of these steps is described in detail in section 2.3. Raw data were processed in GeneScan® Analysis 3.7 (Applied Biosystems) and analysed in Genotyper® 2.5 (Applied Biosystems) as described in section 2.4.1. Raw allelic ratios were then statistically analysed in Excel 2003 (Microsoft) as described in section 2.5.

Investigation of Effects of Polymorphisms or Haplotypes

When testing for potential *cis*-regulatory effects of disease-associated polymorphisms or haplotypes, the relevant polymorphisms were genotyped in each brain sample. However, since parental DNA samples were not available, unless the individual was homozygous at all other genotyped loci, the precise haplotypes carried on each gene copy (i.e. phased diplotypes) by each assayed brain sample could not be definitively determined. For this reason, the relevant polymorphisms were genotyped in the entire collection of brain samples (i.e. assayed and non-assayed samples) so that the most likely phased diplotype carried by each of the assayed samples with respect to the assayed SNP could be predicted

based on observed patterns of linkage disequilibrium (LD). The analysis of LD and prediction of phased diplotype probabilities are described in section 2.5.

2.2.3: 3' RACE

3' rapid amplification of cDNA ends (3' RACE) is a technique that allows 3' ends (including the poly-A tail) of mRNAs transcribed from a particular gene to be PCR-amplified from cDNA. In the present work it was used to search for novel mRNA isoforms and to verify the 3' exon makeup of known isoforms. The technique involved two steps: 1) reverse transcription using a specially designed primer, and 2) PCR amplification.

Using FirstChoice® Total RNA (Ambion) derived from human brain as template, a RT reaction was carried out as described in section 2.1.7, except that an oligo$(dT)_{17}$ adapter primer, as shown in Table 2.3, was used instead of random decamers. The oligo(dT) portion of the primer is designed to hybridise to the poly(A) tails of mRNAs, thereby facilitating the production of cDNA enriched for 3' ends. Following the RT reaction, a PCR is carried out using a gene-specific forward primer and the adapter primer shown in Table 2.3 as the reverse primer. PCR Amplification is described in detail in section 2.3.1. To increase gene-specificity, PCR products were diluted 1 in 1,000 and then used as template in a nested PCR using a second gene-specific primer. The nested PCR products

Primer	Sequence (5'–3')
Oligo$(dT)_{17}$ Adapter	GAGTCCAGTCGACACAGAGCATTTTTTTTTTTTTTTT
Adapter	GAGTCCAGTCGACACAGAGC

Table 2.3: Details of primers used in 3' RACE.

could then be analysed by agarose gel electrophoresis, excised and extracted from the gel, and then sequenced as described in section 2.3.

2.2.4: Sequence Analysis

If a relative allelic expression assay indicated the presence of *cis*-regulatory variation, sequencing was carried out to search for novel variants in functionally important regulatory domains (e.g. promoters). Sequencing was also employed following 3' RACE to determine the exon makeup of amplified transcripts. Sequencing was performed by the fluorescent dideoxy method using the BigDye® Terminator Kit (Applied Biosystems). This involved the following five steps: 1) PCR Amplification, 2) Post-PCR Cleanup, 3) Sequencing, 4) Post-Sequencing Cleanup, and 5) Capillary Gel Electrophoresis. Each of these steps is described in detail in section 2.3. Sequence chromatograms were analysed in Sequence Navigator™ 1.0.1 (Applied Biosystems) as described in section 2.4.2.

2.3: Core Molecular Methods

2.3.1: PCR Amplification

The first step in all the molecular analyses conducted (e.g. genotyping, relative allelic expression assay, sequencing) was the polymerase chain reaction (PCR). PCR is an *in vitro* method that enables short stretches of DNA sequence (typically 100–500 bp) to be rapidly amplified for further analysis. The method relies on the specific design of two oligonucleotide primers (typically 20–30 bases long) that are complementary to the sequences that flank the stretch of DNA of interest.

PCR Primer Design

All PCR primers were designed by use of the Primer3 program (Rozen & Skaletsky, 2000) available online (http://frodo.wi.mit.edu), unless otherwise specified. Input sequences were obtained from the Ensembl Genome Browser website (http://www.ensembl.org). Care was taken to avoid variants in the primer binding sequence of the template. Primers intended to amplify a SNP for the purpose of relative allelic expression analysis were designed to also avoid, where possible, the inclusion of additional variants in the amplicon. For these primers, the input parameter 'product size ranges' was set at 100–300 bp. For all primers, the 'optimal primer Tm' was set at 58°C. All other input parameters were left at default settings. To ensure that the primer pair suggested by Primer3 would specifically amplify the intended sequence, an *in silico* PCR was carried out using the program on the University of California, Santa Cruz Genome Bioinformatics website (http://genome.ucsc.edu/cgi-bin/hgPcr), which searches for the primer sequences in the latest assembly of the human genome database, and displays the sequence of the predicted amplicon(s).

PCR Protocol

PCRs were carried out in a total volume of 12 µl containing either 6 µl of genomic DNA at a concentration of 8 ng/µl or 6 µl of cDNA at the standard cDNA concentration (see section 2.1.8). The volume and concentration of each reagent used in a single PCR is shown in Table 2.4. Blank PCRs, containing sterile water instead of DNA, were always carried out alongside live PCRs so that any potential bias or inaccuracy that might later result from the presence of contaminating templates (e.g. products of earlier PCRs) could be detected and resolved at an early stage.

Reagent	Supplier	Concentration	Volume (µl)
Sterile H_2O	Phoenix Pharma	-	2.8
PCR Buffer (inc. 15 mM $MgCl_2$)	Qiagen	10×	1.2
Forward Primer	Sigma-Aldrich	10 µM	0.5
Reverse Primer		10 µM	0.5
dNTPs (dATP, dCTP, dGTP, dTTP)	Bioline	9.52 mM	0.9
HotStar Taq™ Polymerase	Qiagen	5 U/µl	0.1
DNA Template	-	*	6
Total	-	-	12

Table 2.4: Details of reagents used in most PCRs. *Genomic DNA was at 8 ng/µl, cDNA was at the standard cDNA concentration (see section 2.1.8).

Thermal cycling was carried out by either a MJ Research PTC-225 DNA Engine Tetrad™ or PTC-220 DNA Engine Dyad™ thermal cycler. A total of 35 cycles were carried out using a thermal cycling program as depicted in Figure 2.1.

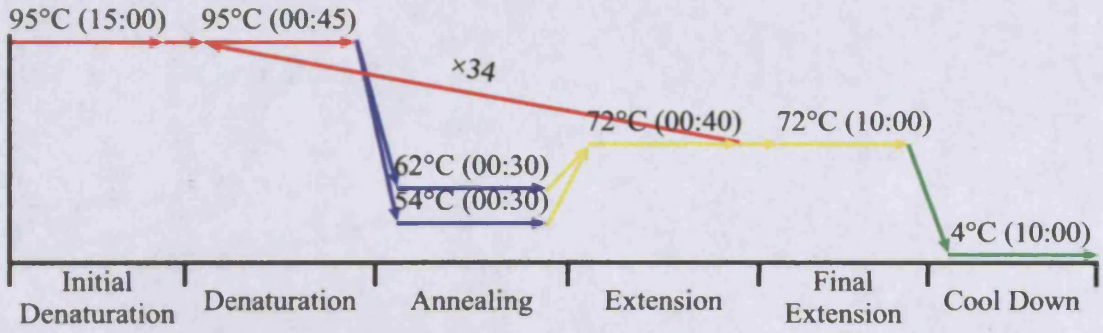

Figure 2.1: Thermal cycling conditions used in PCRs. Incubation time at each temperature is indicated in parentheses in minutes and seconds.

Using the reagent mix shown in Table 2.4 and the thermal cycling program shown in Figure 2.1, the only condition that required optimisation was the annealing temperature. This was achieved by carrying out test PCRs using a thermal cycling program that included an annealing temperature gradient from 54°C to 62°C. Optimal temperatures were then determined by agarose gel electrophoresis and visualisation with an ultraviolet transilluminator. Electrophoresis and transillumination were also used to confirm amplification and absence of contamination whenever PCR was performed.

Variant PCR Protocols

Small variations on the above protocol were sometimes required. When the sequence to be amplified was GC-rich, dimethyl sulfoxide (Sigma-Aldrich) was added to the reaction at 7% volume (i.e. 0.84 µl in a 12 µl reaction) with an adjusted volume of water. For amplification of microsatellites, the volume of genomic DNA template was reduced to 4 µl (i.e. 32 ng) with an adjusted volume of water.

2.3.2: Agarose Gel Electrophoresis

Agarose gel electrophoresis in conjunction with ultraviolet transillumination was mainly used to assess a number of factors related to the quality of a PCR. For instance, it was used

to find the optimal annealing temperature for new PCRs, to confirm that only amplicons of expected sizes were produced, and, most frequently, to simply check that PCRs had worked and that contamination was absent. Less frequently, the method was also employed as part of techniques to identify novel genetic variants or alternatively spliced transcripts (e.g. following 3' RACE PCR). If a PCR produced more than one amplicon, agarose gel electrophoresis was used to separate them by size so that they could be measured and extracted from the gel for further analysis (e.g. sequencing).

Agarose Gels

Agarose gels were made with 2–3% electrophoresis grade agarose (Invitrogen) and either 100 or 200 ml of 1× TBE buffer. Each gel was treated with 3 μl (30 μg) of ethidium bromide (Sigma-Aldrich) per 100 ml of agarose solution.

TBE Buffer

TBE buffer was prepared at 10× concentration. It contained Tris (Fisher Scientific), boric acid (Fisher Scientific), and EDTA (Sigma-Aldrich) at concentrations of 0.97, 0.89, and 0.02 M respectively, in Millipore-purified water. It was diluted 1 in 10 as required.

Size Standard DNA Ladders

Two size standard DNA ladders were used: a 100 bp DNA ladder (Promega) and a 1 Kb Plus DNA ladder (Invitrogen). The 100 bp DNA ladder consists of 11 fragments that range in size from 100–1,000 bp in 100 bp increments, plus an additional fragment at 1,500 bp. The 1 Kb Plus DNA ladder consists of 12 fragments that range in size from 1,000–12,000 bp in 1,000 bp increments, plus eight bands that range in size from 100–1,650 bp (i.e. 100,

200, 300, 400, 500, 650, 850, and 1,650 bp). Each ladder was diluted to ~0.1 μg/μl using the supplied buffer.

Loading Buffer

A stock of loading buffer was prepared at working concentration. It contained 12 ml of 1× TBE buffer, 8 ml of laboratory reagent grade glycerol (Fisher Scientific), and 300 mg of general purpose grade Orange G sodium salt (Fisher Scientific).

Agarose Gel Electrophoresis Protocol

PCR products were mixed with 4 μl of loading buffer per up to 12 μl of product and loaded into pre-formed wells in the agarose gel. When sizing of PCR products was necessary, 5 μl of size standard DNA ladder was loaded into one well. In all electrophoreses, TBE buffer was used. To check that PCRs had worked and that contamination was absent, 4 μl of product was electrophoresed at 120 volts for ~20 minutes. When multiple PCR amplicons were being separated and sized (e.g. following 3' RACE PCR), the entire product was electrophoresed at 80 volts for up to 4 hours as required. Gels were visualised by ultraviolet transillumination using an AutoChemi™ (UVP) imaging system and photographed using the in-built camera.

2.3.3: Extraction of PCR Products from Agarose Gel

Where a PCR reaction gave rise to multiple bands on an agarose gel (e.g. following 3'RACE), specific bands were sometimes excised from the gel following electrophoresis, and the DNA recovered so that it could be sequenced. The extraction procedure was carried out using the QIAquick® Gel Extraction Kit (Qiagen) in conjunction with a microcentrifuge according to the manufacturer's protocol.

Discrete bands were excised from the gel (up to 400 mg) using a clean, sharp scalpel and placed in a microcentrifuge tube. 300 µl of Buffer QG (which contains guanidine thiocyanate) per 100 mg of agarose was then added. The tube was incubated in a water bath at 50°C for 10 minutes whilst being vortexed intermittently. 100 µl of isopropanol (Sigma-Aldrich) per 100 mg of agarose was added, and the tube was vortexed again. The dissolved agarose was then applied to a QIAquick® spin column inside a collection tube and centrifuged at 13,000 rpm for one minute. Following this, 500 µl of Buffer QG was added to the spin column, and the tube was centrifuged again at 13,000 rpm for one minute. 750 µl of ethanol-containing Buffer PE was added to the spin column, which was then allowed to stand for five minutes. The tube was then centrifuged twice at 13,000 rpm for one minute. 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) that had been pre-heated to 70°C was added to the spin column, which was then allowed to stand for one minute. The PCR product was eluted by a final centrifugation at 13,000 rpm for one minute. The eluted product was stored at -20°C.

2.3.4: Post-PCR Cleanup

Some post-PCR molecular analyses (e.g. genotyping, relative allelic expression assay, and sequencing) require unincorporated primer or dNTPs present in the PCR product to be deactivated to avoid potential interference with primer extension reactions. This can be achieved by treatment with shrimp alkaline phosphatase (SAP) and exonuclease I (ExoI). SAP dephosphorylates free nucleotides, rendering them incapable of polymerisation. ExoI degrades unbound primer into free nucleotides which can then be dephosphorylated by SAP.

To each PCR product, 1 µl (1 U) of SAP (GE Healthcare), 0.25 µl (2.5 U) of ExoI (GE Healthcare), and 1.75 µl of sterile water (Phoenix Pharma) was added. The reaction was incubated at 37°C for 60 minutes, and then heated to 80°C for 20 minutes to inactivate the enzymes.

2.3.5: SNaPshot Primer Extension

SNaPshot primer extension involves use of the SNaPshot™ Multiplex Kit (Applied Biosystems) to perform a single base extension reaction. In this reaction, an oligonucleotide primer, specifically designed to anneal immediately adjacent to a known SNP site, is extended by one base with a fluorescently labelled ddNTP (F-ddNTP). Using the kit it is possible to extend multiple primers in a single multiplex reaction. Although originally designed for allele discrimination purposes (e.g. SNP genotyping), SNaPshot chemistry also has excellent quantitative properties (Norton *et al.*, 2002). Hence, SNaPshot was used not just for SNP genotyping, but also for relative allelic expression analysis.

SNaPshot Extension Primer Design

All extension primers were designed by use of the FP Primer program (Ivanov *et al.*, 2004) available online (http://m034.pc.uwcm.ac.uk/FP_Primer.html). If there were known variants in the amplicon in addition to the SNP to be genotyped or analysed, care was taken to avoid them. The input parameter 'minimum length of the primers' was set to 17. All other input parameters were left at default settings. From the list of suggested primer sequences, a primer with low secondary structure and a Tm close to 60°C was used. For multiplex reactions, each primer was designed either a) so that it was at least two bases different in length from the others, or, if the same length as another, b) so that it would be extended with a different option of allelic nucleotides.

SNaPshot Protocol

SNaPshot reactions were carried out in a total volume of 10 µl. Table 2.5 shows the volume and concentration of each reagent used in a singleplex reaction. For multiplex reactions, total extension primer volume remained at 1 µl with the concentration of each primer increased accordingly. Moreover, total PCR product volume increased to a maximum of 4.75 µl with the volume of sterile water reduced accordingly. Multiplex reactions were only carried out for genotyping purposes. All reactions for relative allelic expression analysis were carried out as singleplex.

Reagent	Supplier	Concentration	Volume (µl)
Sterile H_2O	Phoenix Pharma	-	2.75
SNaPshot Buffer	-	3×*	3
Extension Primer	Sigma-Aldrich	1 µM	1
SNaPshot Mix	Applied Biosystems	-	1.25
Clean PCR Product	-	-	2
Total	-	-	10

Table 2.5: Details of reagents used in SNaPshot reactions. *SNaPshot buffer at 3× concentration contained 160 mM Tris-HCl at pH 8 (Fisher Scientific) and 4 mM $MgCl_2$ (Qiagen) in Millipore-purified water.

Thermal cycling was carried out by either a MJ Research PTC-225 DNA Engine Tetrad™ or PTC-220 DNA Engine Dyad™ thermal cycler. A total of 25 cycles were carried out using the thermal cycling program depicted in Figure 2.2.

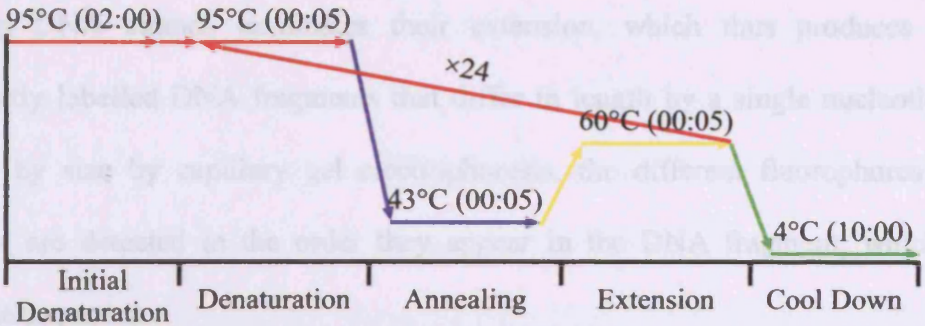

Figure 2.2: Thermal cycling conditions used in SNaPshot primer extension reactions. Incubation time at each temperature is indicated in parentheses in minutes and seconds.

2.3.6: Post-SNaPshot Cleanup

Left untreated, unincorporated F-ddNTPs present in SNaPshot products will comigrate with the fragment(s) of interest during capillary gel electrophoresis and may interfere with base calling. To prevent this, SNaPshot products were treated with shrimp alkaline phosphatase (SAP), which alters the migratory properties of free F-ddNTPs by the removal of 5' phosphoryl groups.

To each SNaPshot product, 0.5 µl (0.5 U) of SAP (GE Healthcare) and 2 µl of dH_2O (Phoenix Pharma) was added. The reaction was incubated at 37°C for 60 minutes, and then heated to 80°C for 20 minutes to inactivate the enzyme.

2.3.7: Sequencing

Sequencing was carried out using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems), which is based on the chain-terminating dideoxy method. In a sequencing reaction using this kit, an oligonucleotide primer, usually the forward or reverse PCR primer, anneals to a template and is extended in the presence of a high concentration of dNTPs and a lower concentration of fluorescently labelled ddNTPs (F-ddNTPs). The incorporation of a F-ddNTP after a random number of dNTPs into

elongating DNA strands terminates their extension, which thus produces a set of fluorescently labelled DNA fragments that differ in length by a single nucleotide. When separated by size by capillary gel electrophoresis, the different fluorophores for each nucleotide are detected in the order they appear in the DNA fragment, which thereby reveals the sequence.

Sequencing Protocol

Sequencing reactions were carried out in a total volume of 10 µl. Table 2.6 shows the volume and concentration of each reagent used in a single reaction. Sequencing of PCR amplicons was carried out in both directions in two separate reactions: one with the forward PCR primer, and one with the reverse PCR primer.

Reagent	Supplier	Concentration	Volume (µl)
BigDye Sequencing Buffer	Applied Biosystems	5×	2
Primer (F or R)	Sigma-Aldrich	3.2 µM	1
BigDye Terminator Mix	Applied Biosystems	2.5×	2
Clean PCR Product	-	-	5
Total	-	-	10

Table 2.6: Details of reagents used in sequencing reactions.

Thermal cycling was carried out by either a MJ Research PTC-225 DNA Engine Tetrad™ or PTC-220 DNA Engine Dyad™ thermal cycler. A total of 26 cycles were carried out using the thermal cycling program depicted in Figure 2.3.

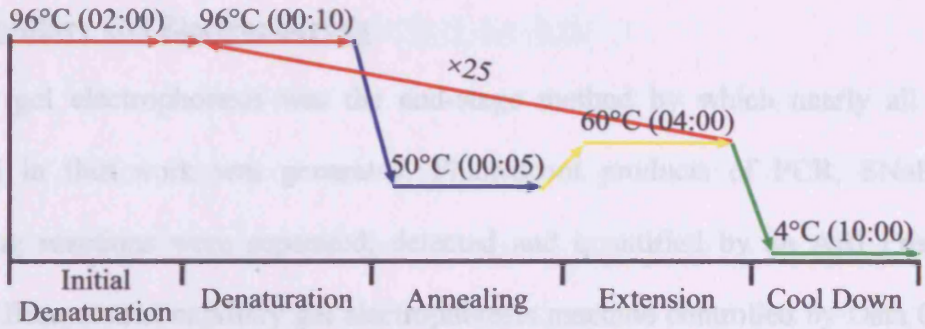

Figure 2.3: Thermal cycling conditions used in sequencing reactions. Incubation time at each temperature is indicated in parentheses in minutes and seconds.

2.3.8: Post-Sequencing Cleanup

Left untreated, unincorporated F-ddNTPs present in sequencing products will comigrate with the fragments of interest during capillary gel electrophoresis and may interfere with base calling. To prevent this, sequencing products were purified using the Agencourt® CleanSEQ® purification system (Beckman Coulter). The CleanSEQ system works by the binding of sequencing products to specially designed paramagnetic beads, which, when exposed to a magnetic field, separates the products from unincorporated dyes, nucleotides, salts and other contaminants that are present in the supernatant. Following removal of the supernatant, the beads are washed in 85% ethanol and eluted into water, which causes the products to be released.

The post-sequencing cleanup procedure was fully automated using a Biomek® NX MC Laboratory Automation Workstation (Beckman Coulter). The Biomek robot was programmed using Biomek® Software 3.2 (Beckman Coulter) to follow the CleanSEQ manufacturer's protocol for dye-terminator removal (Agencourt Bioscience Corp, 2006). Clean sequencing products were eluted into 10 µl of water.

2.3.9: Capillary Gel Electrophoresis

Capillary gel electrophoresis was the end-stage method by which nearly all raw data presented in this work was generated. Fluorescent products of PCR, SNaPshot and sequencing reactions were separated, detected and quantified by an ABI Prism® 3100 (Applied Biosystems) capillary gel electrophoresis machine controlled by Data Collection Software 2.0 (Applied Biosystems). The particular conditions and settings used during electrophoresis varied according to the type of product analysed. The majority of sample products were loaded onto the ABI 3100 by Miss Jennifer Turner.

Electrophoresis of SNaPshot Products

1–2 µl of clean product was mixed with 8–9 µl of Hi-Di™ Formamide (Applied Biosystems) and loaded on to the ABI 3100 machine. The products were electrophoresed on a 36 cm capillary array with POP-4™ Polymer (Applied Biosystems) at 60°C.

Electrophoresis of Fluorescent Microsatellite PCR Products

2 µl of product was mixed with 7.5 µl of Hi-Di™ Formamide (Applied Biosystems) and 0.5 µl of GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) and loaded on to the ABI 3100 machine. The products were electrophoresed on a 36 cm capillary array with POP-4™ Polymer (Applied Biosystems) at 60°C.

Electrophoresis of Sequencing Products

10 µl of clean product was loaded on to the ABI 3100 machine and electrophoresed on a 36 cm capillary array with POP-6™ Polymer (Applied Biosystems) at 55°C.

2.4: Data Analysis

Following capillary gel electrophoresis, data output files for fluorescent PCR assays (i.e. for microsatellite genotyping) and SNaPshot assays (i.e. for SNP genotyping and relative allelic expression) were processed by GeneScan® Analysis 3.7 and analysed in Genotyper® 2.5. Data output files for sequencing assays were analysed in Sequence Navigator™ 1.0.1 (Applied Biosystems).

2.4.1: GeneScan® Analysis 3.7 and Genotyper® 2.5

GeneScan® Analysis 3.7 (Applied Biosystems) is a program that assigns size to detected DNA fragments and quantifies their fluorescent signal. Detected DNA fragments can then be viewed in Genotyper® 2.5 (Applied Biosystems) as peaks in an electropherogram, which can be labelled according to either size (if a size standard was used in the electrophoresis) or height. The height of each peak is proportional to the amount of the DNA fragment that was detected. To ensure only true signals were analysed, a minimum peak height value of 500 was required.

Analysis of Microsatellite Genotype Data

When genotyping microsatellites, peaks were labelled according to size (i.e. length in bp). The size of each peak was proportional to the number of repeats. The number of repeats for each allele was approximately calculated by dividing the size by the number of bases in a single repeat unit after the length of the PCR primers and the non-polymorphic portion of the amplicon was subtracted. The exact numbering of repeats was guided by reported population allele frequencies.

Analysis of SNP Genotype Data

In Genotyper® 2.5, each allele of a SNP is depicted as a peak of a different colour according to the fluorophore attached to each ddNTP in the SNaPshot™ Multiplex Kit (A = green; C = black; G = blue; T = red). Genotypes for each SNP were determined by the presence of peaks in the appropriate colour.

Analysis of Relative Allelic Expression Data

To quantify allelic ratios, peaks were labelled in Genotyper® 2.5 according to height. Peaks heights were required to be in the approximate range of 1,000–6,000 as peak heights outside this range are generally less quantitative. Height data were appended to a table and copied into an Excel 2003 (Microsoft) spreadsheet for statistical analysis.

2.4.2: Analysis of Sequence Data in Sequence Navigator™ 1.0.1

Sequence data output files were analysed in Sequence Navigator™ 1.0.1 (Applied Biosystems). In this program, sequence data is presented as a series of peaks in an electropherogram that differ in colour according to the fluorophore attached to each ddNTP in the BigDye® Terminator Kit (A = green; C = blue; G = black; T = red). The sequence was determined by simply reading from left to right.

2.5: Statistical Analysis

2.5.1: Statistical Correction of Allelic Ratios

Raw peak height data copied from Genotyper 2.5 (Applied Biosystems) were pasted into an Excel 2003 (Microsoft) data sheet that was pre-formulated to calculate a corrected measure of relative allelic expression for each brain sample assayed. The main calculations executed in the data sheet to generate corrected allelic expression ratios are summarised in the equation in Figure 2.4.

$$\text{cDNA ratio}_{cor} = \frac{[Ac/Bc]}{\left[\left(\Sigma\frac{Ag}{Bg}\right)/n\right]}$$

Figure 2.4: Calculation of corrected cDNA allelic ratios. *Ac*, *Bc* = peak height values for alleles *A* and *B* in cDNA. *Ag*, *Bg* = peak height values for alleles *A* and *B* in genomic DNA. *n* = the number of genomic DNA samples assayed.

In essence, raw cDNA ratios were each divided by the mean of all ratios derived from genomic DNA, since, in the absence of any outliers, this was likely to represent the most precise equimolar ratio. Raw genomic DNA ratios were also corrected by the same factor, so that corrected genomic DNA ratios averaged 1:1. Once corrected, replicate cDNA and genomic DNA ratios for each brain sample were averaged, and these average values were subjected to further statistical analyses.

2.5.2: Statistical Power of the Relative Allelic Expression Assay

The power of the relative allelic expression assay to detect effects of unknown *cis*-regulatory variants is based on the binomial distribution, Hardy-Weinberg equilibrium at the regulatory variant, and no LD with the assayed SNP. The probability of a brain sample being homozygous at a putative regulatory variant with alleles in Hardy-Weinberg

equilibrium is $p^2 + q^2$, where p and q are the two allele frequencies. The probability that, of n individuals, all are homozygous (and therefore undetectable by the assay) for the regulatory polymorphism is then $(p^2 + q^2)^n$. This also applies for n individuals selected for heterozygosity at the assayed SNP if there is no LD between the genotypes at each polymorphism. The power to detect at least one heterozygote is then $1 - (p^2 + q^2)^n$. If the assayed and regulatory SNPs are in LD, then a higher proportion of samples selected for heterozygosity at the assayed SNP will also be heterozygous for the regulatory SNP and the power will be increased.

2.5.3: Statistical Comparison of Allele Ratios

Evidence for *cis*-regulatory polymorphism is obtained when cDNA ratios are significantly different from 1:1, as represented by ratios obtained from genomic DNA. To test whether or not cDNA ratios were significantly different from genomic DNA ratios, the independent samples t-test was used. The t-test was also used to test for potential *cis*-regulatory effects of disease-associated polymorphisms or haplotypes by comparing cDNA ratios from brain samples that are heterozygous for the polymorphism or haplotype with ratios from samples that are homozygous, or that do not carry the haplotype. All t-tests were two-tailed, assumed unequal variance, and were performed using Excel 2003 (Microsoft). The power to detect a significant difference in cDNA ratios between heterozygotes and homozygotes for disease-associated polymorphisms or haplotypes was calculated using the Statistical Power Calculator available on the DSS Research website (http://www.dssresearch.com/toolkit/spcalc/power_a2.asp).

2.5.4: Prediction of Phased Diplotypes

To determine the most likely phased diplotype carried by each brain sample, and phase with respect to the assayed SNP, unphased genotype data from all brain samples were first analysed in EH Plus (Zhao, Curtis & Sham, 2000) to predict the frequency of each possible haplotype. The probability that each brain sample carries a specific phased diplotype was then calculated using an in-house program (designed by Dr V Moskvina) by first reconstructing all possible phased diplotypes for each brain sample based on their observed genotypes at each locus. The expected distribution of phased diplotype frequencies, given the predicted haplotype frequencies in the entire brain sample collection, was then used to identify the most probable phased diplotype for each brain sample. The probability that a brain sample carries a particular phased diplotype is then the frequency of that diplotype divided by the sum of the frequencies for all possible diplotypes.

2.5.5: Linkage Disequilibrium Analysis

D' and r^2 measures of linkage disequilibrium (LD) between two polymorphisms (e.g. an assayed SNP and a disease-associated variant) were calculated using Haploview 4.0 (Barrett *et al.*, 2005).

2.5.6: Other Statistical Analyses

Other statistical analyses, such as linear regression analysis, were performed using SPSS for Windows 14.0 (SPSS Inc.).

CHAPTER 3

DISRUPTED IN SCHIZOPHRENIA 1 (*DISC1*)

CHAPTER 3: DISRUPTED IN SCHIZOPHRENIA 1 (*DISC1*)

3.1: Introduction

3.1.1: The *DISC1* Gene and Protein

The *DISC1* gene (OMIM: 605210) was originally identified in a large Scottish family in which a balanced chromosomal translocation t(1;11)(q42.1;q14.3) cosegregated with schizophrenia, bipolar disorder and other major mental illnesses (St Clair *et al.*, 1990). The breakpoint on chromosome 1 directly interrupted two novel genes, which were named Disrupted in Schizophrenia 1 (*DISC1*) and 2 (*DISC2*) (Millar *et al.*, 2000a). The *DISC1* gene spans ~414 kb of chromosome 1q42, includes 13 exons, and encodes a full length protein of 854 amino acids. The translocation interrupted the coding sequence, potentially resulting in a truncated protein that lacks the C-terminal 257 amino acids (Millar *et al.*, 2000a). *DISC2* appears to specify a non-coding RNA that is transcribed from the antisense strand, and which may regulate *DISC1* expression (Millar *et al.*, 2000a).

DISC1 is expressed in both the embryonic and adult brain, and is particularly prominent in the hippocampus throughout all stages of life (Austin *et al.*, 2003, 2004; Schurov *et al.*, 2004; Lipska *et al.*, 2006). In mice, significant peaks in protein expression are detectable during embryonic neurogenesis and at puberty (Schurov *et al.*, 2004), suggesting that *DISC1* plays an important role in brain development and maturation. The amino acid sequence of *DISC1* predicts a scaffold protein, and protein-protein interaction assays have identified >50 potential binding partners (Millar *et al.*, 2003, 2005; Miyoshi *et al.*, 2003, 2004; Morris *et al.*, 2003; Ozeki *et al.*, 2003; Brandon *et al.*, 2004; Ogawa *et al.*, 2005) several of which are indeed involved in neurodevelopmental processes. Two of the most studied are fasciculation and elongation protein ζ 1 (*FEZ1*), linking *DISC1* with axon

guidance and outgrowth (Miyoshi *et al.*, 2003), and nuclear distribution gene E homologue-like 1 (*NUDEL*), which is known to form a complex with lissencephaly 1 (*LIS1*) (Sasaki *et al.*, 2000; Niethammer *et al.*, 2000), mutations in which cause severe disorders of neuronal migration (Pilz *et al.*, 1998; Cardoso *et al.*, 2002). More recently *DISC1* was also found to interact with, and thereby inhibit, phosphodiesterase 4B (*PDE4B*) (Millar *et al.*, 2005). This is a potentially important finding since *PDE4B* inactivates cAMP, a second messenger molecule that plays a key role in learning, memory and mood (Davis *et al.*, 1995; Lamprecht, 1999; Bauman, Goehring & Scott, 2004).

3.1.2: The *DISC1* Gene in Psychiatric Populations

Following the initial report on the Scottish family, a number of genetic linkage and association studies have suggested that *DISC1* may operate more generally as a susceptibility gene for mental illness. Chromosome 1q42 has been reported to show linkage to schizophrenia (Ekelund *et al.*, 2001; Ekelund *et al.*, 2003; Hwu *et al.*, 2003), schizoaffective disorder (Hamshere *et al.*, 2005), bipolar disorder (Curtis *et al.*, 2003; Macgregor *et al.*, 2004) and autism (Buxbaum *et al.*, 2004). Furthermore, SNPs and haplotypes in *DISC1* have shown association with schizophrenia, schizoaffective disorder and bipolar disorder in several independent populations (Hennah *et al.*, 2003; Hodgkinson *et al.*, 2004; Callicott *et al.*, 2005; Cannon *et al.*, 2005; Thomson *et al.*, 2005a; Chen *et al.*, 2007; Qu *et al.*, 2007). Association with major depressive disorder (Hashimoto *et al.*, 2006) and autistic spectrum disorders (Kilpinen *et al.*, 2008) has also been reported. There have, however, been many negative association reports in schizophrenia and bipolar disorder (e.g. Devon *et al.*, 2001; Kockelkorn *et al.*, 2004; Zhang *et al.*, 2005b, Sanders *et al.*, 2008). However, even large negative studies do not exclude the involvement of a gene given the presumed genetic complexity of these disorders and the expected small effect

sizes involved for many risk alleles (International Schizophrenia Consortium, 2009). It should also be noted that across the diverse populations, not all of the associated haplotypes overlap, indeed when put together they span the entire gene (Hennah *et al.*, 2006; Chubb, 2008). This may reflect a mixture of true and false associations, population differences in linkage disequilibrium (LD) structure, or, more likely given the distance across which the signals span, the presence of multiple susceptibility variants.

In order for *DISC1* to increase susceptibility to mental illness in the general population, it must contain sequence variants that either alter the structure of the encoded protein or influence the temporal, spatial or quantitative aspects of its expression. A common non-synonymous base substitution, which encodes Ser704Cys, has been reported to be associated with mental illness, and has also been shown to influence hippocampal and cingulate grey matter volume (Callicott *et al.*, 2005; Hashimoto *et al.*, 2006) and various measures of cognitive function (Callicott *et al.*, 2005; Thomson *et al.*, 2005b). Two other non-synonymous polymorphisms in *DISC1*, which encode Arg264Gln and Phe607Leu, also form parts of haplotypes that have been reported to show altered representation in schizophrenia (Hennah *et al.*, 2003; Cannon *et al.*, 2005). However, these and other associated haplotypes include non-coding variants that do not appear to tag polymorphism with obvious effects on *DISC1* protein structure. Although screening of *DISC1* has identified a small number of rare frameshift and missense mutations (Devon *et al.*, 2001; Sachs *et al.*, 2005), in every case the variant either did not segregate with illness, or has been found in similar frequency in healthy control individuals (Devon *et al.*, 2001; Green *et al.*, 2006). Taken as a whole, these findings raise the possibility that the reported associations between *DISC1* and mental illness are mediated, at least in part, by variants that affect *DISC1* expression.

One prominent example of a haplotype that might influence *DISC1* expression is HEP3, a common two SNP haplotype (comprising alleles T and A at SNPs rs751229 and rs3738401 respectively) spanning ~62 kb from intron 1 to exon 2 of *DISC1*. In a sample from Finland, this haplotype was originally reported to be under-transmitted to schizophrenic females (Hennah *et al.*, 2003), but the finding was later interpreted to reflect over-transmission to schizophrenic males when population frequency was taken into account (Hennah *et al.*, 2005). It was also reported to be associated with cognitive endophenotypes related to schizophrenia such as deficits in visual working memory and visual attention (Hennah *et al.*, 2005). The association between this haplotype and males with psychotic disorder has also been observed in a second Finnish sample (Palo *et al.*, 2007). Moreover, an overlapping haplotype (which also includes the A allele at SNP rs3738401) was found to be under-represented in schizoaffective disorder patients in a study involving a North American white population (Hodgkinson *et al.*, 2004).

3.1.3: Altered *DISC1* Expression as a Potential Pathogenic Mechanism in Psychiatric Disease

The potential pathogenic importance of altered *DISC1* expression is suggested by the observation that lymphoblastoid cell lines derived from family members carrying the t(1;11) translocation show ~50% lower *DISC1* expression than cell lines from family members without the translocation (Millar *et al.*, 2005). Since no truncated protein could be detected, the authors concluded that increased susceptibility of translocation carriers to mental illness was probably due to haploinsufficiency of *DISC1*.

Consistent with this view are findings that down-regulation of *DISC1 in vivo* and *in vitro* give rise to developmental anomalies that are of potential relevance to schizophrenia. For

example, down-regulation of *Disc1* by RNA interference (RNAi) has been found, in embryonic mice, to impair neuronal migration and dendritic arborisation (Kamiya *et al.*, 2005), and in adult mice, to lead to cellular positioning defects in the hippocampus (Duan *et al.*, 2007). Moreover, in cell culture, down-regulation of *DISC1* by RNAi has been reported to inhibit neurite outgrowth (Kamiya *et al.*, 2005; Shinoda *et al.*, 2007; Taya *et al.*, 2007), and promote premature neuronal differentiation accompanied by reduced neural progenitor proliferation (Mao *et al.*, 2009). Although these results all support decreased expression of *DISC1* as the pathogenic mechanism, it should, however, be noted that similar and additional phenotypes are also reported to result from expression of the putative truncated *DISC1* protein product acting in a dominant-negative manner (Kamiya *et al.*, 2005; Hikida *et al.*, 2007; Taya *et al.*, 2007).

Whether or not brain *DISC1* expression is altered in psychiatric patients is not clear. One study did not find a difference in *DISC1* mRNA abundance in prefrontal cortex between subjects with schizophrenia or bipolar disorder and control subjects (Dean *et al.*, 2007). However, in the hippocampus, a modest (~20%) *increase* in *DISC1* protein was observed in schizophrenic subjects compared with controls (Lipska *et al.*, 2006). The hypothesis that *DISC1* contains common regulatory variation, and that this contributes to susceptibility to mental illness, is suggested by a recent study in which a putative risk haplotype for bipolar disorder was associated with reduced *DISC1* expression in lymphoblastoid cell lines (Maeda *et al.*, 2006). However, a further study did not find a correlation between SNPs that showed association with schizophrenia and *DISC1* mRNA abundance in human brain (Lipska *et al.*, 2006). The existence of regulatory variation in *DISC1*, and its relevance to psychiatric illness, therefore remains open to question.

3.1.4: The Present Study

The reports of association between *DISC1* and various psychiatric disorders are currently not explicable by variants that alter protein structure, and there is evidence compatible with the hypothesis that alterations in *DISC1* expression could be relevant to disease. The primary aim of the present study was to investigate the possibility that *DISC1* contains common regulatory polymorphism. To this end, 65 informative individuals were screened for altered allelic expression. Samples were also genotyped for the HEP3 haplotype to test for a potential *cis* effect of this haplotype on *DISC1* expression. In selected individuals, the proximal *DISC1* promoter region was also sequenced in an effort to identify potential regulatory variants.

3.2: Materials and Methods

Brain samples and the general methods used for DNA and RNA processing, genotyping, and the relative allelic expression assay are described in more detail in Chapter 2.

3.2.1: Samples

Post-mortem brain tissue derived from frontal, temporal or parietal cortex of 148 unrelated anonymised individuals obtained from three sources (the MRC London Brain Bank for Neurodegenerative Diseases, the Stanley Medical Research Institute, and the Karolinska Institute) were used in this study. For each tissue sample, genomic DNA and total RNA were extracted by standard procedures. RNA was then treated with DNAse and reverse transcribed to cDNA.

3.2.2: Genotyping

PCR primers for the primary SNP used for relative allelic expression analysis (rs3738401), and for SNPs rs821616 and rs751229, were designed by use of the Primer3 program (Rozen & Skaletsky, 2000). PCR was carried out in a total reaction volume of 12 μl containing 48 ng of genomic DNA, and consisted of 35 cycles. Genotyping was carried out by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and primers designed by use of the FP Primer program (Ivanov *et al.*, 2004). All primer sequences are shown in Table 3.1, which, where relevant, also shows the annealing temperature used during PCR cycling.

Polymorphism	Primer	Sequence (5'–3')	Ta (°C)
rs3738401*	F:	GAACGTGGAGAAGCAGAAGG	58
	R:	AGAATGCATGTCACGCTCTG	
	EXT:	ACCCGCGATGTCTCTCTC	-
rs821616*	F:	ACTTGGAAGCTTGTCGATTG	58
	R:	CATCCATCTGCCTCTCATCT	
	EXT:	CTTCCTGGAGCTGTAGGC	-
rs751229	F:	TGAATTGAACTGAATCCCTTTTT	60
	R:	GAGATGGGTTTCTGCCATGT	
	EXT:	CTTAAAAACTTAGATTTTCAGCC	-

Table 3.1: Details of primers used in the study of the *DISC1* gene. Asterisks indicate SNPs used for relative allelic expression analysis. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

3.2.3: Linkage Disequilibrium Analysis and Phased Diplotype Prediction

Linkage disequilibrium (LD) analyses were performed using Haploview 4.00 (Barrett *et al.*, 2005) and were based on genotype data for the entire collection of 148 brain samples. The probability that each brain sample carried the HEP3 haplotype comprised of SNPs rs751229 and rs3738401 was calculated using EH Plus (Zhao, Curtis & Sham, 2000) and an in-house program, as described in Chapter 2 (section 2.5.4).

3.2.4: Relative Allelic Expression Assay

The SNP chosen for the primary relative allelic expression analysis was rs3738401. This SNP has a minor allele frequency of 0.30 in the HapMap CEU sample, is located in exon 2 of the *DISC1* gene, and is present on all four principal *DISC1* mRNAs known to be transcribed in human brain (see Figure 3.1). The SNP is also present on low abundance

hybrid transcripts formed through intergenic splicing of *DISC1* and the adjacent gene translin-associated factor X (*TSNAX*) (Millar *et al.*, 2000b).

Genomic DNA from all brain samples was initially genotyped, as described, for the SNP to identify heterozygotes. For each heterozygous sample, cDNA produced from two separate reverse transcription reactions, alongside corresponding genomic DNA, was assayed on two separate occasions (i.e. 2 × {2 cDNA + 1 genomic DNA}). Genomic DNA and cDNA were assayed under identical conditions. This allowed the average allelic ratio obtained from genomic DNA (representing a 1:1 ratio) to be used to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to the assay.

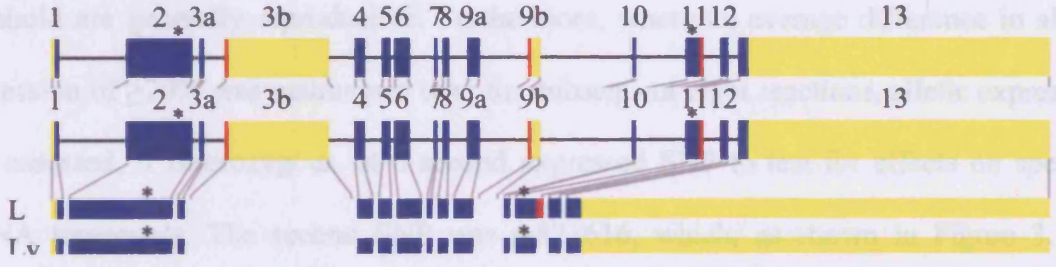

Figure 3.1: The organisation of the *DISC1* gene and its four major mRNA transcripts. L = Long isoform (NM_018662), Lv = Long variant isoform (NM_001012957), S = Short isoform (NM_001012959), Es = Extremely short isoform (NM_001012958). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons, and the red rectangle represents the alternatively spliced 66 bp portion of exon 11. Asterisks indicate the positions of assayed SNPs rs3738401 in exon 2 and rs821616 in exon 11.

PCR consisted of 35 cycles and was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA or 6 µl of cDNA at the standard cDNA concentration (see Chapter 2, section 2.1.8). Primers, shown in Table 3.1, were located in a single exon and therefore amplified an identical sequence from both templates. Allelic representation was measured by primer extension using the SNaPshot™ Multiplex Kit (Applied

Biosystems) and the primer shown in Table 3.1. Peak heights of allele-specific extended primers were determined using Genotyper 2.5 (Applied Biosystems). The ratio of cDNA peak heights, corrected using the average ratio of genomic DNA peak heights, was then used to calculate the relative expression of mRNAs transcribed from each parental chromosome in each individual sample.

To confirm altered allelic expression, individuals showing expression of one allele at a level 20% or more than the other were assayed on four further occasions (i.e. 4 × {2 cDNA + 1 genomic DNA}). In line with previous studies (Yan *et al.*, 2002; Bray *et al.*, 2003), an allelic expression difference ≥20% for any individual sample was chosen as indicative of the influence of *cis*-acting regulatory polymorphism since measurements surpassing this threshold are generally reproducible. Furthermore, where an average difference in allelic expression of ≥20% was maintained over the subsequent eight reactions, allelic expression was assessed, if heterozygous, at a second expressed SNP to test for effects on specific mRNA transcripts. The second SNP was rs821616, which, as shown in Figure 3.1, is located on exon 11 and present on the L and Lv transcripts only. The procedure for this SNP was identical to that used for rs3738401, but the primers used were those shown in Table 3.1.

3.2.5: Sequencing

In selected individuals, approximately 1 kb 5' to the *DISC1* transcription start site was sequenced to determine the presence or absence of putative regulatory variants in the proximal promoter region. Primers used for PCR and sequencing are shown in Table 3.2. PCR was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA, and consisted of 35 cycles. Sequencing was performed in both directions by the fluorescent

dideoxy method using the BigDye® Terminator Kit (Applied Biosystems). Where PCR generated two products of different sizes, the products were electrophoresed, excised from the gel, purified, and then sequenced.

Target Sequence	Primer	Sequence (5'–3')	Ta (°C)
$^{-}$1045–$^{-}$795	F:	GAACTCCTGGACTCAAGCAA	60
	R:	TCAGGGATAGGAGGGAGAAC	
$^{-}$970–$^{-}$612	F:	GGGCAAACACACTGATTTTA	60
	R:	GCACCCCCTAGGTAGCAC	
$^{-}$668–$^{-}$348	F:	CATGTGTAGCCCTTCCTTGT	60
	R:	GTGACCACACAGTGATGGAG	
$^{-}$451–$^{-}$103	F:	TCTCTTTCCAGGGTCATCAG	60
	R:	CTGTTTCCTTTCCAGCATTG	
$^{-}$233–$^{+}$146	F:	AGCAGCGTGTGTGTGTATGT	60
	R:	CTAGGACTCTGTGGCAGCTC	

Table 3.2: Details of primers used to PCR amplify and sequence the *DISC1* proximal promoter region. F = forward primer, R = reverse primer, Ta = annealing temperature used during PCR cycling. Target sequence positions are relative to the transcription start site.

3.3: Results

3.3.1: Assayed Samples

A total of 65 genotyped individuals were heterozygous for the expressed SNP rs3738401 and therefore informative for allelic expression analysis. Their mean age at death was 57 years (SD = 19.69), 36 were male, and 29 were female. Over half (n = 33) had no known history of psychiatric or neurological disease, 10 had schizophrenia, eight had bipolar disorder, four had major depression, and 10 had Alzheimer's disease. Samples were derived from frontal (n = 45), temporal (n = 14), and parietal (n = 6) brain tissue.

3.3.2: Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.09. No correlation was observed with sex, age, diagnostic status or brain region assayed. In the primary analysis, transcripts tagged by the G allele at SNP rs3738401 were on average 8.8% more abundant than those tagged by the A allele (Figure 3.2). The difference between allelic ratios obtained with cDNA and genomic DNA was significant ($P = 6.3 \times 10^{-8}$). Four samples displayed an allelic expression difference ≥20% (Figure 3.2). However, when these samples were assayed a further four times, this level of allelic expression difference was maintained by just one individual (Figure 3.3). The individual (Individual A, Figure 3.3) was a 57-year-old anonymous male with no known history of psychiatric or neurological disorder. For this individual, the average G:A ratio over eight replicate cDNA measurements was 1.53:1 (95% C.I. = 1.37–1.68:1). Thus, in this individual, expression of the G allele was increased by 53% (using the A allele as an arbitrary reference point), or expression of the A allele reduced by 34% (using the G allele

as an arbitrary reference point). Observed cDNA ratios from this individual were significantly different from ratios derived from genomic DNA ($P = 9.86 \times 10^{-5}$).

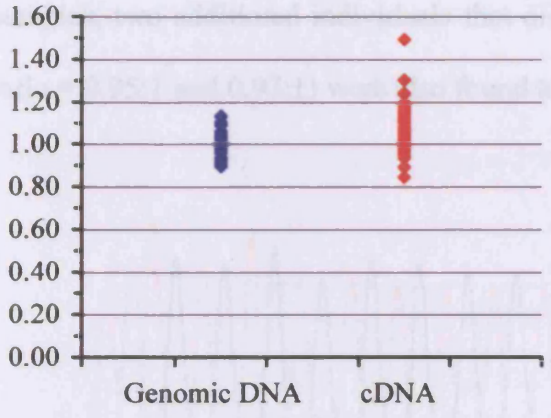

Figure 3.2: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 65 individuals heterozygous for SNP rs3738401. Data are presented as the G allele relative to the A allele (G:A). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

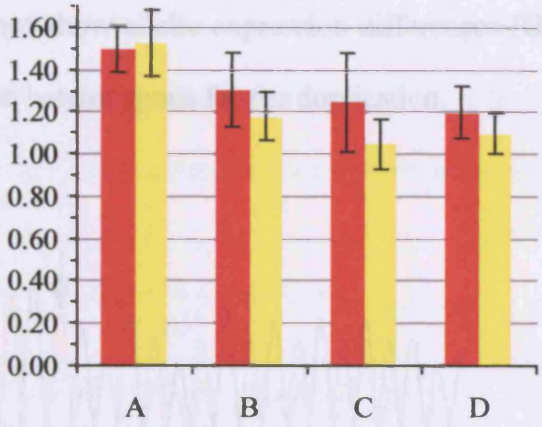

Figure 3.3: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for four individuals (A, B, C, D) that showed an initial allelic expression difference ≥20%. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

Relative allelic expression was assessed in this individual at a second exonic SNP, rs821616, for which the individual was also heterozygous. When assayed at this SNP, the average T:A ratio in cDNA from this individual was 1.60:1 (95% C.I. = 1.34–1.87:1).

3.3.3: Sequencing of the *DISC1* Promoter Region

Sequencing of approximately 1 kb immediately upstream of the *DISC1* transcription start site in the individual that showed a robust allelic expression difference of ≥20% (Individual A) and in three individuals that showed close to equal allelic expression identified a novel insertion polymorphism (Figure 3.4). The insertion was a duplication of the 22 nucleotides at positions ¯168 to ¯147 relative to the *DISC1* transcription start site

(nucleotides 675–696 in reference sequence AF222982). The duplication was inserted in tandem with the reference sequence. The individual that showed a robust allelic expression difference was heterozygous for this duplication. However, after screening a further eight samples, two additional individuals that did not show allelic expression differences (G:A ratio = 0.95:1 and 0.97:1) were also found to be heterozygous for the duplication.

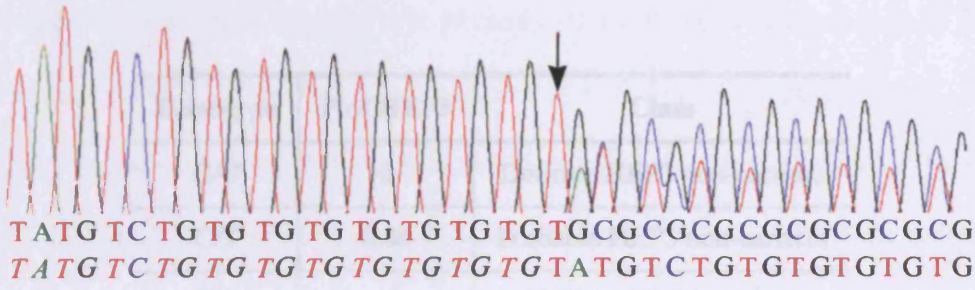

Figure 3.4: Sequencing trace showing the heterozygous duplication of the 22 nucleotides at positions ¯168–¯147 relative to the *DISC1* transcription start site in the individual that showed a consistent allelic expression difference. The sequence on the chromosome without the duplication is shown on the upper line; the duplicated sequence on the other chromosome is shown on the lower line. The arrow indicates the start of the duplicated sequence. The reference sequence is shown in italics.

The duplicated sequence includes a TG repeat that has previously been reported to be polymorphic (Devon *et al.*, 2001; Kockelkorn *et al.*, 2004). In the present study, all chromosomes carrying the duplicated sequence carried one $(TG)_8$ repeat unit and one $(TG)_{10}$ unit. On the chromosome without the insertion, the individual that showed a consistent allelic expression difference carried a $(TG)_9$ unit. Some individuals that showed no allelic expression differences were heterozygous for two repeat units that differed in length to a greater extent (e.g. $(TG)_9$ and $(TG)_{14}$).

3.3.4: Effect of SNP rs751229 and the HEP3 haplotype

The HEP3 haplotype is comprised of alleles A and T respectively of SNPs rs3738401 (the assayed SNP) and rs751229, which were found to be in moderate LD ($D' = 0.70$, $r^2 = 0.32$). In the 148 brain samples genotyped in this study, the estimated frequency of this haplotype is 0.06. The probability that the 65 assayed brain samples (all of which were heterozygous at SNP rs3738401) carried the HEP3 haplotype based on their genotype at

Genotype	*P* of HEP3	Class
C/C	0	Definite HEP3 non-carriers
C/T	0.06	Probable HEP3 non-carriers
T/T	1	Definite HEP3 carriers

Table 3.3: The estimated probability that the 65 assayed brain samples carry the HEP3 haplotype based on their genotype at SNP rs751229.

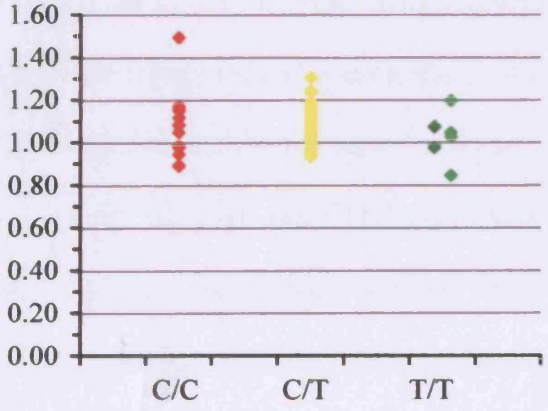

Figure 3.5: Scatter plot showing allelic ratios for cDNA derived from 65 individuals heterozygous for SNP rs3738401 stratified by genotype for SNP rs751229. Data are presented as the G allele relative to the A allele (G:A). Individuals with genotype T/T (i.e. definite HEP3 carriers) are separated according to sex (dark green = males, light green = females). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

SNP rs751229 is shown in Table 3.3. As indicated in Table 3.3, assayed brain samples that were heterozygous for SNP rs751229 most likely do not carry the HEP3 haplotype (*P* of not carrying HEP3 = 0.94).

In the 65 brain samples assayed, cDNA ratios did not significantly differ between homozygotes and heterozygotes for SNP rs751229 (*P* = 0.49; Figure 3.5). Similarly, cDNA ratios did not significantly differ between definite HEP3 carriers (i.e. T/T at SNP rs751229) and definite or probably non-carriers (i.e. C/C or C/T at SNP rs751229; *P* = 0.20). There was also no significant difference between male and female HEP3 carriers (*P* = 0.89). These analyses had 80% power to detect a ratio difference of 0.08 between SNP rs751229 homozygotes and heterozygotes, 0.11 between HEP3 carriers and non-carriers, and 0.20 between male and female HEP3 carriers (*P* = 0.05).

3.4: Discussion

The present study sought to test the hypothesis that *DISC1* contains common regulatory polymorphism, which might underlie reported genetic associations between *DISC1* and psychiatric disorders in general populations. An assay of relative allelic expression applied to a total of 65 post-mortem brain samples revealed just one that showed a robust allelic expression difference of ≥20%. In that sample, derived from a 57-year-old male with no known psychiatric or neurological diagnosis, expression of one gene copy was ~50% greater than expression of the other. The identified *cis* effect on expression was found to apply to the L and Lv *DISC1* transcripts, although an effect on the S and Es transcripts could not be excluded.

Overall, the data suggest that polymorphisms or haplotypes that have moderately large (i.e. ≥20%) effects on *DISC1* expression in brain are, at least in Caucasian populations, rare with a frequency of <0.01 in the samples screened. This frequency is much lower than that of most haplotypes that have been reported to be associated with psychiatric illness in such populations. Thus, while the present study supports the existence in rare cases of *cis*-acting variation with a moderately large influence on *DISC1* expression in adult brain, it does not support the hypothesis that the relatively common haplotypes associated with schizophrenia and bipolar disorder tag functional variants that act through this mechanism. It is, however, possible that the rare observation of allelic expression difference is relevant to one of the many rare haplotypes that have been reported to be associated with illness (e.g. Thomson *et al.*, 2005), although this hypothesis cannot be tested using inferred haplotypes from a single subject. Furthermore, it is difficult to speculate as to whether the as yet unknown functional variant is a risk or protective factor, as it is not known if its

effect is to suppress or enhance *DISC1* expression. A decrease in *DISC1* expression has been found to impair neurite outgrowth and cerebral cortex development (Kamiya *et al.*, 2005; Shinoda *et al.*, 2007; Taya *et al.*, 2007) and, consistent with the likely effect of the *DISC1* translocation, would presumably confer risk. An increase in *DISC1* expression in response to atypical antipsychotic drug treatment has been observed (Chiba *et al.*, 2006), and, therefore, might be protective.

The search in the *DISC1* proximal promoter region for variation that may have given rise to the single observation of substantial allelic expression difference uncovered two potential candidates: a novel 22 bp duplication variant and a TG repeat polymorphism, which was also included in the duplicated sequence. The individual that showed an allelic expression difference was heterozygous for the duplication. However, two further individuals that did not show allelic expression differences were also heterozygous for it, suggesting the variant is a frequent polymorphism which, at least alone, does not affect *DISC1* expression. The individual was also heterozygous for the TG repeat polymorphism but this is also probably not a functional variant, as the difference in repeat length between the duplicated and non-duplicated chromosomes was very small, and a number of individuals that did not show allelic expression differences were heterozygous for two alleles of more different lengths. To identify the true functional variant, further mutation screening of *DISC1* would be required. It is also possible, however, that the allelic expression difference might be due to heterozygosity at multiple common variants (including the duplication) comprising a haplotype, none of which alone are unique to the individual screened.

In addition to the single observation of a moderately large allelic expression difference, mRNAs tagged by the G allele at assayed SNP rs3738401 were generally slightly (8.8% on average) more abundant than mRNAs tagged by the A allele. This finding is consistent with the presence of a *cis*-acting regulatory variant of small effect that is in high LD with the assayed SNP. Although this has the potential to promote susceptibility to psychiatric disease, high quality imputed data (average maximum posterior call probability >0.99; missing data proportion <3%) generated in the Department of Psychological Medicine as part of recent genome-wide association studies (Wellcome Trust Case Control Consortium, 2007; Craddock *et al.*, 2008; O'Donovan *et al.*, 2008) – with which the author was not involved – did not indicate an association with schizophrenia ($P = 0.85$) or bipolar disorder ($P = 0.99$).

The comparison of cDNA ratios between brain samples with different genotypes at SNP rs751229 did not reveal any significant differences. The T allele of this SNP along with the A allele of the assayed SNP rs3738401 comprise the HEP3 haplotype that has previously been found to be over-transmitted to Finnish males with psychotic disorder (Hennah *et al.*, 2003, 2005; Palo *et al.*, 2007). That cDNA ratios from definite HEP3 carriers were not significantly different from definite or probable HEP3 non-carriers suggests that if the association of this haplotype with psychosis is genuine, it unlikely to be mediated by effects on *DISC1* mRNA abundance in brain. However, given the power of this comparison (80% to detect a difference of 11%), small effects of this haplotype on mRNA abundance cannot be excluded.

It is important to highlight that *DISC1* plays a major a role in brain development and its expression is developmentally regulated. For instance, in mice, *DISC1* expression peaks

during periods of active neurogenesis (Schurov *et al.*, 2004), peaks again at puberty (Schurov *et al.*, 2004), and is expressed in some regions of the developing brain that do not express *DISC1* in adulthood (Austin *et al.*, 2004). Similarly, in humans, *DISC1* is more prominently expressed during prenatal, neonatal and pubertal stages of life than in adulthood (Lipska *et al.*, 2006). It is therefore possible that risk alleles or haplotypes may exert effects on *DISC1* expression exclusively in a developmental context; in which case, their existence would not have been detected in the present study as only adult brain tissue was assayed. Similarly, the present study cannot rule out variants that might exert effects in specific brain regions not studied. A noteworthy example is the dentate gyrus, the region of highest *DISC1* expression in the adult brain (Austin *et al.*, 2003, 2004), and where recent evidence suggests the gene plays an important role in adult neurogenesis (Duan *et al.*, 2007). Finally, the present study does not address quantitative or qualitative post-transcriptional effects, for example in the abundance of the protein or its subcellular distribution. Both may be of importance as, in schizophrenic subjects, a 20% increase in *DISC1* protein in the hippocampus (Lipska *et al.*, 2006), and an altered subcellular distribution of the protein in the orbitofrontal cortex (Sawamura *et al.*, 2005) has been found.

3.4.1: Conclusions

The results of the present study do not provide evidence that reports of genetic association between *DISC1* and psychiatric disorder can be explained by variants that result in changes in *DISC1* mRNA abundance in adult brain. Future investigations of the functional variants underlying disease association with *DISC1* should focus on post-transcriptional mechanisms and / or earlier developmental stages.

CHAPTER 4

REELIN (*RELN*)

CHAPTER 4: REELIN (*RELN*)

4.1: Introduction

4.1.1: The *RELN* Gene and Reelin

The human *RELN* gene (OMIM: 600514) spans ~518 kb of chromosome 7q22, includes 65 exons and encodes the large (3,460 amino acids) extracellular matrix protein reelin. During brain development, reelin is secreted by Cajal-Retzius cells located in the marginal zone, and plays a key role in the determining the final position of neurons radially migrating from the subventricular zone (Jossin, 2004). It was originally identified as the defective gene product in reeler mice, which exhibit severe cerebellar and cortical malformations and widespread neuronal ectopia (D'Arcangelo *et al.*, 1995). It binds two low density lipoprotein receptors, apolipoprotein E receptor 2 (*APOER2*) and very low density lipoprotein receptor (*VLDLR*) (D'Arcangelo *et al.*, 1999), and initiates a signal cascade that involves phosphorylation of disabled 1 (*DAB1*) (Howell, Herrick, & Cooper, 1999) and activation of Src family protein tyrosine kinases (Arnaud *et al.*, 2003). In the adult brain, it is largely synthesised by a subset of GABAergic interneurons in the neocortex and hippocampus, and glutamatergic neurons in the cerebellum (Pesold *et al.*, 1998). In addition to its role in brain development, recent evidence suggests that reelin signalling is also important for synaptic plasticity and cognitive functions such as learning and memory (Herz & Chen, 2006).

4.1.2: *RELN* Expression in Human Disease

RELN and its protein product have been implicated in the aetiology and pathology of several psychiatric and neurodegenerative diseases. While null mutations in *RELN* cause an autosomal recessive form of lissencephaly (Hong *et al.*, 2000; Chang *et al.*, 2007; Zaki

et al., 2007), altered reelin expression has been reported in several other psychiatric and neurodegenerative disorders, including schizophrenia, bipolar disorder, autism and Alzheimer's disease.

In schizophrenia and bipolar disorder, *RELN* mRNA and reelin protein have been reported to be reduced by ~50% in prefrontal cortex, temporal cortex, hippocampus, caudate nuclei and cerebellum (Impagnatiello *et al.*, 1998; Guidotti *et al.*, 2000; Fatemi, Earle & McMenomy, 2000; Fatemi *et al.*, 2005b). The origin of this reduction, at least in schizophrenia, has been localised to interstitial white matter neurons in hippocampus, and in prefrontal cortex, to these same neuron types as well as layer I neurons and interneurons (Eastwood & Harrison, 2003, 2006). Furthermore, in a recent meta-analysis of ~100 post-mortem neurochemical markers analysed in the Stanley Consortium brain sample (including tissue from 15 each of individuals with schizophrenia, bipolar disorder and major depression, and 15 healthy controls), a decreased level of reelin mRNA and protein was found to be among the most striking neuropathological findings (Torrey *et al.*, 2005). Importantly, the decrease in brain reelin levels was not related to post-mortem interval, the use of medication, nor a reduction in neuronal number (Impagnatiello *et al.*, 1998; Guidotti *et al.*, 2000; Fatemi, Earle & McMenomy, 2000; Eastwood & Harrison, 2003, 2006; Torrey *et al.*, 2005), suggesting that it constitutes part of the disease process.

Reelin protein has been found to be significantly decreased by ~70% in superior frontal cortex, by 40% in the cerebellum, and non-significantly by ~70% in the parietal cortex of adults with autism, compared with tissue from healthy controls (Fatemi *et al.*, 2005a). In the superior frontal cortex and cerebellum, reductions in reelin protein were accompanied by 4.7 and 3.9 fold reductions in *RELN* mRNA and 14.2 and 2.8 fold increases in *VLDLR*

mRNA respectively. Significant reductions in *DAB1* mRNA were also observed. In a study that measured blood levels of reelin in 28 monozygotic autistic twins, their parents and six normal siblings, reelin levels were found to be 60–70% decreased in all family members when compared with levels in eight unrelated controls (Fatemi, Stary & Egan, 2002).

Reelin has also been implicated in the neuropathology of Alzheimer's and other neurodegenerative diseases. There is evidence to suggest that the reelin signal pathway regulates phosphorylation of the microtubule associated protein tau (*MAPT*) (Hiesberger *et al.*, 1999), and hyperphosphorylated tau is the major constituent of neurofibrillary tangles (a pathologic hallmark of Alzheimer's and other neurodegenerative diseases). Increased levels of reelin have been found in cerebrospinal fluid of patients with Alzheimer's disease, frontotemporal dementia, progressive supranuclear palsy and Parkinson's disease (Botella-López *et al.*, 2006). *RELN* mRNA and reelin protein are also reported to be 40% increased in frontal cortex of Alzheimer's patients compared with controls (Botella-López *et al.*, 2006).

4.1.3: Epigenetic Regulation of the *RELN* Promoter

The *RELN* promoter is embedded within a CpG island, which extends from ~1200 bp upstream ($^{-}$1200) to ~200 bp downstream ($^{+}$200) of the transcription start site (Chen *et al.*, 2002b). This CpG island is controlled by several regulatory proteins (Grayson *et al.*, 2005). Most notable is DNA methyltransferase 1 (*DNMT1*), which, by methylation of CpG dinucleotides and subsequent recruitment of further proteins, has the effect of silencing *RELN* transcription (Noh *et al.*, 2005; Dong *et al.*, 2005; Kundakovic *et al.*, 2007).

In post-mortem brains of individuals with schizophrenia and bipolar disorder, over-expression of *DNMT1* and increased levels of the methyl donor S-adenosyl methionine (SAM) have been observed in the same neurons that show reduced expression of reelin (Veldic *et al.*, 2004, 2005, 2007; Guidotti *et al.*, 2007). Furthermore, particular CpG dinucleotides between positions $^{-}$444 and $^{-}$259 and non-standard CpNpG trinucleotides at positions $^{-}$139 and $^{-}$134 have been found to be hypermethylated in schizophrenia (Abdolmaleky *et al.*, 2005; Grayson *et al.*, 2005). However, a subsequent report did not confirm these findings and presented additional data to highlight possible methodological errors in the previous two studies (Tochigi *et al.*, 2008). Aberrant epigenetic regulation of the *RELN* 5' region in schizophrenia and bipolar disorder is still suggested, however, by a study that employed a different methodology. Although no significant difference in methylation was observed at positions $^{+}$131, $^{+}$227 and $^{+}$229 between schizophrenia, bipolar and control subjects, a correlation between age and methylation status was detected in the control subjects but not in the psychiatric subjects (Tamura *et al.*, 2007).

4.1.4: ***RELN* in the Genetics of Psychiatric Disease**

Genome-wide linkage scans and meta-analysis have consistently identified chromosome 7q21–q36 as a region likely to harbour susceptibility variants for autism (Badner & Gershon, 2002; Trikalinos *et al.*, 2006; Freitag, 2007; Yang & Gill, 2007). The q22 region in particular, to which the *RELN* gene maps, has shown significant linkage not only with autism (IMGSAC, 2001a, 2001b; Lamb *et al.*, 2005; Schellenberg *et al.*, 2006; Trikalinos *et al.*, 2006; AGPC, 2007), but also with schizophrenia (Ekelund *et al.*, 2000; Wedenoja *et al.*, 2008). *RELN* has therefore been the subject of several candidate gene association studies for both disorders.

Most association studies of *RELN* have focussed on a variable length CGG repeat located in the 5' UTR of the gene immediately upstream of the start codon. In the first analysis of this polymorphism in a Caucasian population, eight alleles were identified, ranging from 4 to 23 repeats, with 8 and 10 repeats being the most frequent (Persico *et al.*, 2001). In both case-control and family-based analyses, long alleles (≥11 repeats) were associated with increased risk for autism (Persico *et al.*, 2001). In a second study of autism, cases and controls did not differ in allelic or genotypic frequencies, but a more powerful family-based association test showed that long alleles were transmitted more often than expected to autistic children (Zhang *et al.* 2002). The authors also noted that they found no evidence for instability of the repeat during transmission. A further seven studies have analysed the repeat with respect to autism. One found association with the 10 repeat allele (Skaar *et al.*, 2005), one showed distorted paternal transmission of the 10 and 11 repeat alleles in an Indian population (Dutta *et al.*, 2007), and the remainder were negative (Krebs *et al.*, 2002; Bonora *et al.*, 2003; Devlin *et al.*, 2004; Li *et al.*, 2004; Ashley-Koch *et al.*, 2007).

The 5'UTR repeat has been less thoroughly assessed in the context of schizophrenia. In two small studies of Japanese and Chinese Han populations, no association was found (Akahane *et al.* 2002; Huang & Chen, 2006). In a Caucasian sample, a higher frequency of the homozygous 8 repeat genotype was observed in controls compared with cases and the 10 repeat allele was significantly more frequent in patients that did not respond to antipsychotic drug treatment than in patients that did or normal controls (Goldberger *et al.*, 2005).

There is evidence to suggest that the CGG repeat may be functional. In a comparison between 10 pairs of age-matched autistic male individuals it was found that those carrying

at least one long (≥12 repeats) allele had on average 25% less reelin in their plasma than those carrying two short (≤10 repeats) alleles (Lugli *et al* 2003). Furthermore, in neuronal and non-neuronal cell lines transfected with constructs encompassing the *RELN* 5' UTR upstream of the luciferase gene it was found that luciferase activity declined as the number of repeats increased (Persico *et al.* 2006). This finding was explained in terms of predicted mRNA folding and translational efficiency. However, given the location of the repeat within the same CpG island as the *RELN* promoter, an effect of the repeat on transcription by a methylation-related mechanism is an equally plausible explanation.

Several polymorphisms within *RELN* other than the CGG repeat have been reported to show association with autism (Skaar *et al.*, 2005; Serajee *et al.*, 2006; Ashley-Koch *et al.*, 2007; Li *et al.*, 2008). With the possible exception of one SNP which may affect splicing of exon 6 (Persico *et al.*, 2001), none of the associated polymorphisms are predicted to have a significant effect on protein structure. Although a small number of rare non-conservative missense mutations in *RELN* that cosegregate with autism have been found (Bonora *et al.*, 2003), these variants were too infrequent to account for the strong linkage signal observed at the 7q22 locus (IMGSAC, 2001a, 2001b). Furthermore, despite extensive mutation screening, only one relatively frequent non-conservative SNP (encoding Ser630Arg) in *RELN* has been identified, and both alleles were found to have similar frequencies in autistic individuals and controls (Bonora *et al.*, 2003). Taken together, these findings strongly suggest that if the reported associations are genuine, they are most likely mediated by sequence variants that alter *RELN* expression.

A mutation screen of ~1 kb of the *RELN* promoter region in 100 Chinese Han schizophrenic patients identified a SNP at position $^{-}$728, which was predicted to alter a

transcription factor binding site (Chen *et al.*, 2002a). In a small case-control study this SNP showed a non-significant ($P = 0.08$) trend for association with schizophrenia (Chen *et al.*, 2002). In a Finnish population, a number of non-coding variants showed significant associations with various neuropsychological endophenotypes related to memory and executive functions (Wedenoja *et al.*, 2007). In an Ashkenazi Jewish population, a genome-wide association scan identified a female-specific association of SNP rs7341475 in intron 4 of *RELN* with schizophrenia (Shifman *et al.*, 2008). The SNP was then tested in a further four case-control sample sets: three of European ancestry (UK, Ireland and USA) and one from China. The UK sample set was that collected by the Department of Psychological Medicine. In total there were 2,274 cases (768 females, 1,506 males) and 4,401 controls (2,194 females, 2,207 males). The female-specific association of the same allele was replicated in the UK sample set, and was replicated again when data from the other three populations were combined. When data from all five populations was combined, the estimated female-specific odds ratio (OR) for carrying the common G/G genotype was 1.58 ($P = 8.8 \times 10^{-7}$) (Shifman *et al.*, 2008). The replicated association of the same SNP in the same allelic direction with the same disorder in several populations provides strong, though not genome-wide significant, evidence that the SNP is or tags a true pathogenic variant. How the sex-specificity of the risk allele can be explained is, however, unclear.

4.1.5: The Present Study

The altered expression of reelin in psychiatric and neurodegenerative disease may be due, at least in part, to *cis*-regulatory variation within or nearby the *RELN* gene itself. This hypothesis is supported by the reviewed genetic evidence, the putative functional effect of the 5' UTR repeat, and the equivocal findings relating to the methylation status of the

promoter in psychosis. The present study formally tested this hypothesis by employment of a relative allelic expression assay. Putative effects of the 5' UTR repeat and SNP rs7341475 polymorphisms on *RELN* expression were also investigated. Moreover, the repeat polymorphism was also tested for association with schizophrenia and bipolar disorder in a case-control analysis.

4.2: Materials and Methods

Brain samples and the general methods used for DNA and RNA processing, genotyping, and the relative allelic expression assay are described in more detail in Chapter 2.

4.2.1: Samples

Post-mortem brain tissue derived from frontal, temporal or parietal cortex of 148 unrelated anonymised individuals obtained from three sources (the MRC London Brain Bank for Neurodegenerative Diseases, the Stanley Medical Research Institute, and the Karolinska Institute) were used in this study. For each tissue sample, genomic DNA and total RNA were extracted by standard procedures. RNA was then treated with DNAse and reverse transcribed to cDNA.

4.2.2: Genotyping

PCR primers for the SNP used for relative allelic expression analysis (rs2229864), and for SNP rs7341475, were designed by use of the Primer3 program (Rozen & Skaletsky, 2000). PCR was carried out in a total reaction volume of 12 μl containing 48 ng of genomic DNA, and consisted of 35 cycles. Genotyping was carried out by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and primers designed by use of the FP Primer program (Ivanov *et al.*, 2004). For the 5' UTR repeat polymorphism, primers were as described previously (Persico *et al.*, 2001), with a 5' FAM label on the forward primer. PCR was performed as it was for the SNPs, but with a 12 μl reaction volume that included 7% DMSO. Amplicons were then sized by coelectrophoresis with a size standard on a

capillary electrophoresis machine. All primer sequences are shown in Table 4.1, which, where relevant, also shows the annealing temperature used during PCR cycling.

Polymorphism	Primer	5' Label	Sequence (5'–3')	Ta (°C)
rs2229864*	F:	-	AGGACCGTTATGCTGGACAC	58
	R:	-	AAACATGTCAAAGGCGATCC	
	EXT:	-	CCCCAGTACCCCAGCA	-
rs7341475	F:	-	CTGCTCTGGCACTTTCTAGC	58
	R:	-	TCTTGGGAATGGTATGCAGT	
	EXT:	-	GGCACTTTCTAGCCATGAAAT	-
5' UTR CGG	F:	FAM	CGGCGTCTCCAAAACTGAAT	58
	R:	-	AACAGCGCTAGGAGGAAAGT	

Table 4.1: Details of primers used in the study of the *RELN* gene. The asterisk indicates the SNP used for relative allelic expression analysis. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

4.2.3: Relative Allelic Expression Assay

The SNP chosen for relative allelic expression analysis was rs2229864. This SNP has a minor allele frequency of 0.30 in the HapMap CEU sample, is located in exon 50 of the *RELN* gene, and is present on both principal *RELN* mRNAs known to be transcribed in human brain (see Figure 4.1). The SNP may also be present on more minor transcripts that are generated by alternative polyadenylation signals in an extension of exon 63 (Rouvroit *et al.*, 1999).

Genomic DNA from all brain samples was initially genotyped, as described, for the SNP to identify heterozygotes. For each heterozygous sample, cDNA produced from two separate reverse transcription reactions, alongside corresponding genomic DNA, was assayed on

two separate occasions (i.e. 2 × {2 cDNA + 1 genomic DNA}). Genomic DNA and cDNA were assayed under identical conditions. This allowed the average allelic ratio obtained from genomic DNA (representing a 1:1 ratio) to be used to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to the assay.

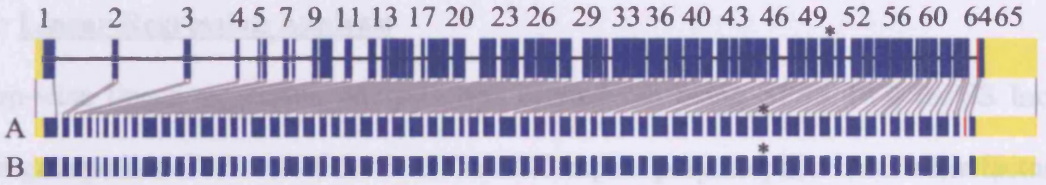

Figure 4.1: The organisation of the *RELN* gene and its two major mRNA transcripts. A = full length mRNA (NM_005045), B = alternatively spliced mRNA (NM_173054). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons, and the red rectangle represents the alternatively spliced microexon 64. The asterisk indicates the position of assayed SNP rs2229864 in exon 50.

PCR consisted of 35 cycles and was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA or 6 µl of cDNA at the standard cDNA concentration (see Chapter 2, section 2.1.8). Primers, shown in Table 4.1, were based on a single exon and therefore amplified an identical sequence from both templates. Allelic representation was measured by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and the primer shown in Table 4.1. Peak heights of allele-specific extended primers were determined using Genotyper 2.5 (Applied Biosystems). The ratio of cDNA peak heights, corrected using the average ratio of genomic DNA peak heights, was then used to calculate the relative expression of mRNAs transcribed from each parental chromosome in each individual sample.

To confirm altered allelic expression, select individuals showing expression of one allele at a level 20% or more than the other were assayed on four further occasions (i.e. 4 × {2

cDNA + 1 genomic DNA}). In line with previous studies (Yan *et al.*, 2002; Bray *et al.*, 2003), an allelic expression difference ≥20% for any individual sample was chosen as indicative of the influence of *cis*-acting regulatory polymorphism since measurements surpassing this threshold are generally reproducible.

4.2.4: Linear Regression Analysis

A step-wise linear regression analysis was carried out using SPSS 14.0 (SPSS Inc.) to investigate potential effects of the 5' UTR CGG repeat polymorphism and other factors on *RELN* expression. Predictor variables included the difference in repeat number between the two alleles carried by an individual (i.e. CGG difference), rs7341475 genotype, age, sex, diagnosis, and brain region assayed. The dependent variable was the expression ratio of the highest relative to the lowest expressed allele (i.e. H:L cDNA ratio) for each individual.

4.2.5: Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) analyses were performed using Haploview 4.00 (Barrett *et al.*, 2005) and were based on genotype data for the entire collection of 148 brain samples.

4.2.6: Case-Control Association Analysis of the 5' UTR CGG Polymorphism

The 5' UTR CGG repeat polymorphism was indirectly tested for association with schizophrenia and bipolar disorder by a case-control association analysis. The Department of Psychological Medicine recently contributed to genome-wide case-control association studies of schizophrenia and bipolar disorder, which were comprised of 479 individuals with schizophrenia, 1,868 individuals with bipolar disorder, and 2,939 control individuals (Wellcome Trust Case-Control Consortium, 2007; O'Donovan *et al.*, 2008). Imputed genotype data were generated for these cases and controls by Dr J Marchini (Oxford

University) using IMPUTE (Marchini *et al.*, 2007). It should be noted that imputed genotype data for the schizophrenia and bipolar disorder case-control datasets were generated using slightly different versions of IMPUTE, resulting in minor differences in the estimated allele frequencies. The samples analysed and the genesis of the datasets (with which the author was not involved) are described in detail in the source publications.

Rather than genotyping the repeat polymorphism in these individuals, it was instead genotyped in the 90 European individuals (consisting of 30 trios) that comprise the HapMap CEU samples. Patterns of LD in these samples were found to be representative of that in the individuals analysed in the case-control studies (WTCCC, 2007). Based on previous association findings (Persico *et al.*, 2001; Goldberger *et al.*, 2005), genotypes for the repeat polymorphism were biallelically coded as short (≤10 repeats) versus long (≥11 repeats) and 8 repeats versus ≥10 repeats and merged with HapMap SNP data. Using Haploview 4.00 (Barrett *et al.*, 2005), SNPs in high LD (i.e. r^2) with the polymorphism were then identified and examined for evidence of association with disease in the imputed data for those SNPs, which were not present on the genotyping arrays used in the genome-wide association studies.

4.2.7: Calculation of Statistical Power for Case-Control Association Analyses

The statistical power of the case-control analyses to detect association of the 5' UTR repeat polymorphism with schizophrenia and bipolar disorder was calculated by use of Epi Info™ 3.3 (Centers for Disease Control and Prevention). Since SNPs in LD with the polymorphism were tested for association as a surrogate for the polymorphism itself, the calculation was based on case and control samples sizes that had been adjusted by multiplication by the value of r^2.

4.3: Results

4.3.1: Assayed Samples

A total of 66 genotyped individuals were heterozygous for the expressed SNP rs2229864 and therefore informative for allelic expression analysis. Their mean age at death was 60 years (SD = 19.67), 34 were male, and 32 were female. More than half (n = 39) had no known history of psychiatric or neurological disease, eight had schizophrenia, five had bipolar disorder, three had major depression, and 11 had Alzheimer's disease. Samples were derived from frontal (n = 45), temporal (n = 15), and parietal (n = 6) brain tissue.

4.3.2: Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.07. No correlation was observed with sex, age, diagnostic status or brain region assayed. Three samples displayed an allelic expression difference of ≥20%, two of which were clear outliers (Figure 4.2). One was an 85-year-old anonymous male with no known history of neurological or psychiatric disorder. For this individual (individual A), the average C:T ratio over four replicate cDNA measurements was 1.30:1. The other outlier was an 83-year-old anonymous male with a history of Alzheimer's disease. For this individual (individual B), the average C:T ratio over four replicate cDNA measurements was 0.60:1. When these individuals' samples were assayed a further four times, the average C:T ratios over eight replicate cDNA measurements were 1.44:1 (95% C.I. = 1.31–1.57:1) and 0.68:1 (95% C.I. = 0.64–0.73:1) respectively (Figure 4.3). Thus, expression of the C allele was increased by 44% in individual A and reduced by 32% in individual B (using the T allele as an arbitrary reference point), or expression of the T allele was reduced by 31% in individual A or increased by 46% in individual B (using the

C allele as an arbitrary reference point). Observed cDNA ratios from both individuals were significantly different from ratios obtained with genomic DNA ($P = 6.89 \times 10^{-5}$ and 3.85×10^{-5} respectively).

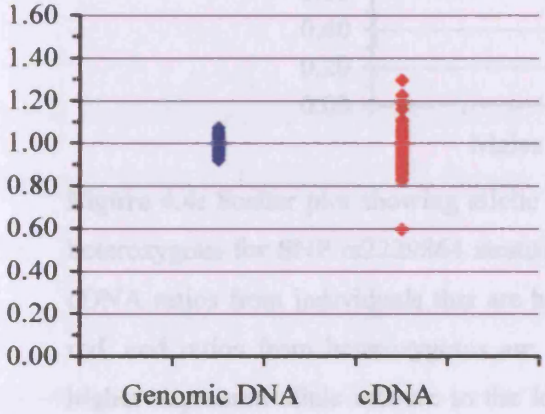

Figure 4.2: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 66 individuals heterozygous for SNP rs2229864. Data are presented as the C allele relative to the T allele (C:T). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

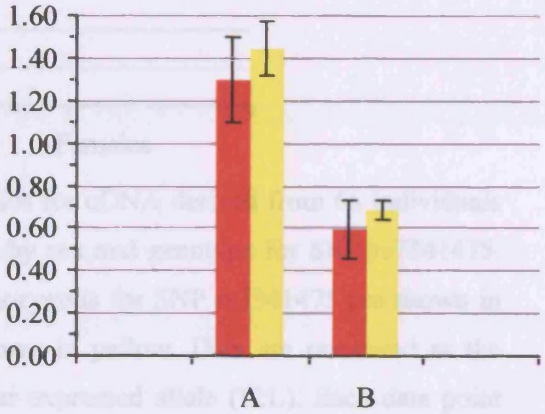

Figure 4.3: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for two individuals (A, B) that showed an initial allelic expression difference ≥20%. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

4.3.3: Effect of SNP rs7341475 in Males and Females

LD analyses showed that there was no LD between the assayed SNP rs2229864 and the putative schizophrenia risk SNP rs7341475 ($D' = 0.01$, $r^2 = 0.00$). cDNA ratios denoted as the more highly expressed allele relative to the lower expressed allele (i.e. H:L) did not significantly differ between homozygotes and heterozygotes for SNP rs7341475 ($P = 0.96$; Figure 4.4). This was also apparent when males and females were analysed separately ($P =$ 0.80 and 0.45 respectively). These analyses had 80% power to detect a ratio difference of 0.08 in males and females combined, 0.11 in males, and 0.07 in females ($P = 0.05$).

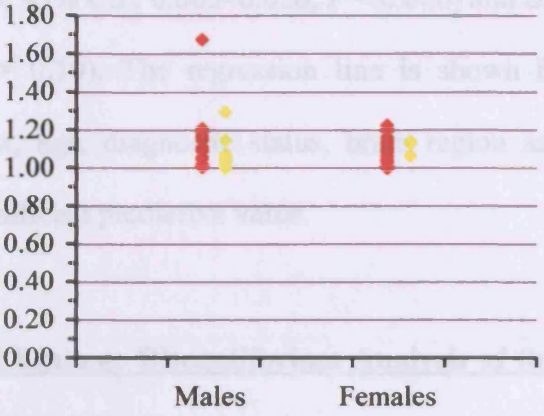

Figure 4.4: Scatter plot showing allelic ratios for cDNA derived from 66 individuals heterozygous for SNP rs2229864 stratified by sex and genotype for SNP rs7341475. cDNA ratios from individuals that are homozygous for SNP rs7341475 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the higher expressed allele relative to the lower expressed allele (H:L). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

4.3.4: Effect of the 5' UTR CGG Repeat

In a stepwise linear regression analysis, the difference in length of the two CGG repeat alleles carried by each individual (i.e. CGG difference) was a significant predictor of H:L

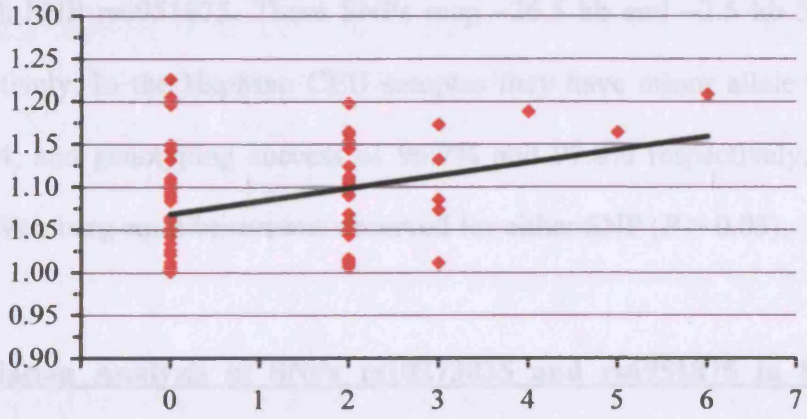

Figure 4.5: Scatter plot showing the regression of CGG difference (i.e. difference in length of the two CGG repeat alleles carried by each individual) with H:L cDNA ratios (adjusted $r^2 = {}^{+}0.10$, $P = 0.006$, $n = 64$). The regression equation is H:L cDNA ratio = 1.068 + (0.015 × CGG Difference). Outlying individuals (individuals A and B) were excluded.

cDNA ratios (B = 0.015, 95% C.I.: 0.005–0.026, $P = 0.006$) and accounted for ~10% of the variance (adjusted $r^2 = 0.10$). The regression line is shown in Figure 4.5. No other variables, including sex, age, diagnostic status, brain region assayed, or coefficient of variation, displayed significant predictive value.

4.3.5: Genotyping and Linkage Disequilibrium Analysis of the 5' UTR CGG Repeat Polymorphism in HapMap CEU Samples

The 5' UTR CGG repeat polymorphism was successfully genotyped in 89 of the 90 (98.89%) HapMap CEU samples. Samples genotyped in duplicate yielded concordant genotypes and all genotypes were confirmed by a second, more experienced, researcher. Six alleles were observed, ranging from 8 to 15 repeats, with 8 and 10 repeat alleles being the most frequent. All genotypes were consistent with Mendelian expectations.

LD analysis revealed that when biallelically coded as short (≤10 repeats) versus long (≥11 repeats), the CGG repeat polymorphism exhibited highest LD ($r^2 = 0.80$) with SNP rs10273035. When coded as 8 repeats versus ≥10 repeats, highest LD ($r^2 = 0.89$) was observed with SNP rs6951875. These SNPs map ~26.5 kb and ~2.5 kb 5' of the CGG repeat respectively. In the HapMap CEU samples they have minor allele frequencies of 0.08 and 0.34, and genotyping success of 96.7% and 97.8% respectively. No deviation from Hardy-Weinberg equilibrium was observed for either SNP ($P > 0.05$).

4.3.6: Association Analysis of SNPs rs10273035 and rs6951875 in Schizophrenia Cases and Controls

Imputed genotype and allele frequency data for SNPs rs10273035 and rs6951875 were obtained for 479 schizophrenia cases and 2,939 controls (Table 4.2). Missing data

proportions were 1.84% (8 cases, 55 controls) and 27.94% (131 cases, 824 controls) for each SNP respectively. The average maximum posterior genotype call probabilities were 0.99 and 0.91 respectively. No deviation from Hardy-Weinberg equilibrium was observed for either SNP in cases or controls ($P > 0.05$).

SNP	Allele or Genotype	Frequency		*P*
		Cases	Controls	
rs10273035	C	898 (0.95)	5,550 (0.96)	0.19
	T	44 (0.05)	218 (0.04)	
	C/C	428 (0.91)	2,671 (0.93)	
	C/T	42 (0.09)	208 (0.07)	0.42
	T/T	1 (0.00)	5 (0.00)	
rs6951875	A	257 (0.37)	1,630 (0.39)	0.42
	G	439 (0.63)	2,600 (0.61)	
	A/A	44 (0.13)	328 (0.16)	
	A/G	169 (0.49)	974 (0.46)	0.36
	G/G	135 (0.39)	813 (0.38)	

Table 4.2: Summary of schizophrenia case-control data for SNPs rs10273035 and rs6951875. SNP alleles associated with longer CGG repeat alleles (i.e. ≥11 repeats for SNP rs1027303 or ≥10 repeats for rs6951875) in HapMap CEU samples are underlined. Genotype and allele frequencies are based on imputed data. Frequencies expressed as fractions of total number of cases or controls are shown in parentheses. The significance *P* value for allelic and genotypic tests of association is indicated.

Imputation did not reveal any allelic or genotypic association between SNP rs10273035 and schizophrenia (Table 4.2). Similarly, no association was observed with SNP rs6951875 (Table 4.2). Based on the frequency of longer alleles of the CGG repeat (i.e. ≥11 repeats = 11.9% or ≥10 repeats = 63.6%) and allowing for its LD with the tested SNPs (i.e. $r^2 = 0.80$

or 0.89) in the HapMap CEU samples, it was calculated that these analyses had 80% power to detect association of the CGG repeat polymorphism with schizophrenia with ORs of ≥1.38 or ≥1.37 respectively.

4.3.7: Association Analysis of SNPs rs10273035 and rs6951875 in Bipolar Disorder Cases and Controls

Imputed genotype and allele frequency data for SNPs rs10273035 and rs6951875 were obtained for 1,868 bipolar disorder cases and 2,938 controls (Table 4.3). Missing data proportions were 4.02% (85 cases, 108 controls) and 24.34% (452 cases, 718 controls) for each SNP respectively. The average maximum posterior genotype call probabilities were 0.99 and 0.92 respectively. No deviation from Hardy-Weinberg equilibrium was observed for SNP rs10273035 in cases or controls ($P > 0.05$), or for SNP rs6951875 in controls. In cases, genotypes for the latter SNP deviated significantly from Hardy-Weinberg equilibrium ($P < 0.001$).

Imputation did not reveal any allelic or genotypic association between SNP rs10273035 and bipolar disorder (Table 4.3). Similarly, no association was observed with SNP rs6951875 (Table 4.3). Based on the frequency of longer alleles of the CGG repeat (i.e. ≥11 repeats = 11.9% or ≥10 repeats = 63.6%) and allowing for its LD with the tested SNPs (i.e. r^2 = 0.80 or 0.89) in the HapMap CEU samples, it was calculated that these analyses had 80% power to detect association of the CGG repeat polymorphism with bipolar disorder with ORs of ≥1.22 or ≥1.16 respectively.

SNP	Allele or Genotype	Frequency		*P*
		Cases	Controls	
rs10273035	C	3,451 (0.97)	5,469 (0.97)	0.70
	T	115 (0.03)	191 (0.03)	
	C/C	1,669 (0.94)	2,644 (0.94)	
	C/T	113 (0.06)	181 (0.06)	0.54
	T/T	1 (0.00)	5 (0.00)	
rs6951875	A	1,158 (0.41)	1,774 (0.40)	0.44
	G	1,674 (0.59)	2,666 (0.60)	
	A/A	267 (0.19)	372 (0.17)	
	A/G	624 (0.44)	1,030 (0.46)	0.20
	G/G	525 (0.37)	818 (0.37)	

Table 4.3: Summary of bipolar disorder case-control data for SNPs rs10273035 and rs6951875. SNP alleles associated with longer CGG repeat alleles (i.e. ≥11 repeats for SNP rs1027303 or ≥10 repeats for rs6951875) in HapMap CEU samples are underlined. Genotype and allele frequencies are based on imputed data. Frequencies expressed as fractions of total number of cases or controls are shown in parentheses. The significance *P* value for allelic and genotypic tests of association is indicated.

4.4: Discussion

The present study had four main objectives: 1) to determine whether or not the *RELN* gene contains common regulatory polymorphism, which could potentially influence risk for several psychiatric disorders; 2) to test whether or not SNP rs7341475, identified as a risk factor for schizophrenia in women, is associated with altered *RELN* expression; 3) to test whether or not a 5' UTR CGG repeat polymorphism, previously reported to be associated with autism, influences *RELN* expression at the mRNA level; and 4) if the CGG repeat was indeed found to influence *RELN* expression, to identify SNPs in high LD with it and to test those SNPs for association with schizophrenia and bipolar disorder using imputed genotype data obtained in a large genome-wide case-control study. To achieve these objectives, an assay of relative allelic expression was applied to a total of 66 post-mortem brain samples, which were also genotyped for SNP rs7341475 and the CGG repeat polymorphism.

Of the 66 heterozygotes assayed, three showed an allelic expression difference of ≥20%, two of which were clear outliers. Repeat assays of these individuals showed a robust allelic expression difference of ~45% in both individuals. The higher expressed allele was different in each individual. Hence, if the two individuals carried the same putative regulatory variant, which is suggested by their similar allelic difference, then the lower expressed allele is in phase with different alleles at the assayed SNP. The putative variant is, therefore, unlikely to be in high LD (i.e. *D'*) with the assayed SNP.

These data suggest that polymorphisms or haplotypes that have moderately large effects (i.e. ≥20%) on *RELN* expression in brain exist but are, at least in Caucasian populations,

relatively rare with a frequency of <0.02 in the samples screened. This frequency is much lower than that of most SNPs that have been reported to be associated with autism in such populations. Thus, while the present study supports the existence in rare cases of *cis*-acting variation that has a moderately large influence on *RELN* expression in adult brain, it does not support the hypothesis that the relatively common SNPs associated with autism tag functional variants that act through this mechanism. Nonetheless, it remains a distinct possibility that the putative regulatory variant(s) detected in the present study may influence risk of psychiatric disorders in which expression of *RELN* in brain is altered. Indeed, for autism, it is perhaps even likely, given the previous (Lugli *et al.*, 2003; Persico, Levitt & Pimenta, 2006) and present work (discussed later) that suggests that the 5' UTR repeat polymorphism reported to be associated with the disorder has functional effects on *RELN* expression.

The comparison of H:L cDNA ratios between homozygotes and heterozygotes for SNP rs7341475 did not reveal any significant differences in males and females combined, nor in each sex separately. This finding suggests that the reported association of the SNP with schizophrenia in women is not mediated by an alteration in *RELN* mRNA abundance. It is of note, however, that these analyses did not have sufficient power to detect a significant ratio difference of <0.08 in both sexes combined, or <0.07 in females, hence the present study cannot exclude the possibility that SNP rs7341475 influences risk by very small effects on expression. Furthermore, it is possible that SNP rs7341475 is not a functional variant, but merely serves as proxy for a separate variant that is in high LD with it. In this case, and if the variant indeed has effects on mRNA abundance, only a fraction of SNP rs7341475 heterozygotes, and even some homozygotes for the risk allele, would be expected to show a significant allelic expression difference. Consistent with this was that

one of the outlying male individuals with a H:L cDNA ratio of 1.44:1 was a SNP rs7341475 heterozygote. However, one other male individual with a similar ratio was homozygous for the non-risk allele, which suggests that *cis*-regulatory variants with effects on mRNA abundance are not exclusive to haplotypes tagged by the risk allele.

In the test of whether or not the CGG repeat polymorphism has functional effects on *RELN* mRNA abundance, it was assumed that if it did, then the difference in repeat number of the two alleles carried by each individual (i.e. CGG difference) should account for some of the inter-individual variation in allelic expression imbalance. Genotyping of the repeat polymorphism in the assayed individuals identified genotypes with CGG differences ranging from zero (i.e. homozygotes) to six repeats (i.e. 8/14 repeat genotype). In a stepwise linear regression analysis it was found that CGG Difference was the only significant predictor of H:L cDNA ratio, and accounted for ~10% of the measured inter-individual variation in that variable. The regression equation was H:L cDNA ratio = 1.068 + (0.015 × CGG Difference), which indicates that as repeat number difference increases by one, allelic expression imbalance increases by 1.5%.

These data are in agreement with two previous studies, which also provided evidence that the CGG repeat polymorphism has functional effects on reelin expression (Lugli *et al.*, 2003; Persico, Levitt & Pimenta, 2006). In both studies, which only measured expression at the protein level, higher repeat numbers were associated with lower expression. The data from the present study do not distinguish the direction of the relationship between repeat number and mRNA expression level. While direct assay of the repeat is theoretically possible given its location within mRNA sequence, this was not performed because of unknown effects of repeat size on the efficiency of reverse transcription, which is not

necessarily controlled for by assay of genomic DNA. However, given that the *RELN* promoter is known to be regulated by methylation, it is tempting to assume that higher repeat numbers are also associated with lower mRNA expression due to the extra potential methylation sites that the CGG repeats provide. It is of note that in comparison with reported effects on protein abundance (Lugli *et al.*, 2003; Persico, Levitt & Pimenta, 2006), the effect of the repeat polymorphism on mRNA abundance is relatively small. This difference in effect size on expression at the two levels suggests that the effect previously observed on protein expression cannot be explained merely by an effect on mRNA abundance (e.g. transcriptional effects). Hence, while the present study provides the first evidence that the repeat polymorphism has functional effects on mRNA expression, it is also consistent with previous evidence, which suggests that it additionally influences protein levels by separate effects on mRNA folding and translational efficiency (Persico, Levitt & Pimenta, 2006).

If the repeat is a true susceptibility variant for autism, then its pathogenicity may be mediated by a combination of all the above-described effects. The same can be proposed in relation to Alzheimer's disease with which the polymorphism was also found to be associated after the present work was conducted (Seripa *et al.*, 2008). Interestingly, in that study, genotypes containing shorter alleles (i.e. 8/8 and 8/10 repeat genotypes) were more frequent in female but not male patients with Alzheimer's disease than controls, consistent with increased levels of reelin detected in affected brains (Botella-López *et al.*, 2006). However, there is no obvious explanation for the sex-specific effect, which might be a chance observation resulting from multiple post-hoc testing and reduced power in the smaller subgroups.

The longer alleles (≥11 repeats) of the repeat are reported to confer susceptibility to autism (Persico *et al.*, 2001; Zhang *et al.*, 2002), which, given previous and present findings, is consistent with reduced levels of reelin found in brains of autistic individuals (Fatemi *et al.*, 2005a). In view of this, it was reasoned that the polymorphism might similarly be a good candidate susceptibility variant for schizophrenia and bipolar disorder, in which reduced brain expression of reelin has proved to be a replicable finding (Torrey *et al.*, 2005). For the sake of efficiency, the polymorphism was genotyped in HapMap CEU samples to identify SNPs in high LD with it, which could then be tested for association with the disorders using imputed genotype data already generated as part of genome-wide association studies in the Department of Psychological Medicine. When biallelically coded as short (≤10 repeats) versus long (≥11 repeats) and 8 repeats versus ≥10 repeats, the CGG repeat polymorphism was found to exhibit highest LD with SNPs rs10273035 (r^2 = 0.80) and rs6951875 (r^2 = 0.89) respectively. Imputation analyses of these SNPs did not reveal any significant allelic or genotypic association with schizophrenia or bipolar disorder.

The present association analyses represent, to the authors knowledge, the largest association study of the *RELN* 5' UTR CGG polymorphism with schizophrenia to date, having 80% power to detect association with ORs of ≥1.37. That no significant association was detected is consistent with two previous, less powerful, studies of the same polymorphism that also did not detect any association with schizophrenia in Asian populations (Akahane *et al.* 2002; Huang & Chen, 2006). Patient data on responsiveness to antipsychotic drug treatment was not available in the present study, hence whether or not alleles of 10 repeats or longer were more frequent in non-responders than in responders, as previously observed in a Caucasian population (Goldberger *et al.*, 2005), could not be determined. The present association analyses also represent the first time the

polymorphism has been studied in relation to bipolar disorder, and had 80% power to detect association with ORs of ≥1.16. That no significant association was detected suggests that the polymorphism does not confer susceptibility to bipolar disorder either.

The above suggestion that the CGG repeat polymorphism does not confer susceptibility to schizophrenia or bipolar disorder is only valid given the described power of the analyses to detect effects of the stated sizes. It is also noteworthy that the calculated power levels did not take into consideration the LD relationship between the rare 4 repeat allele and the tested SNPs, as this allele was not detected in the HapMap CEU samples. Nonetheless, the present study strongly suggests that if the CGG repeat polymorphism does confer susceptibility to schizophrenia and bipolar disorder, then its effect is likely to be very small. Given that most common allele effect sizes are now expected to be substantially smaller than many previously expected (International Schizophrenia Consortium, 2009), this is an important caveat; hence, the present study certainly does not exclude the involvement of this polymorphism in these disorders.

The replicated finding that *RELN* expression is down-regulated in brains of patients with schizophrenia and bipolar disorder has led to the hypothesis that reduced levels of the protein increases vulnerability to development of those disorders (Guidotti *et al.*, 2000; Fatemi, 2001, 2005; Costa *et al.*, 2002). However, the previous and present evidence that the CGG repeat polymorphism has a functional effect on *RELN* expression and is not associated with either disorder does not support that hypothesis. Also arguing against it, is that of all individuals reported to date to be heterozygous for null alleles of *RELN* (and that express 50% normal levels of reelin protein), none have demonstrated any clinical signs of psychosis (Hong *et al.*, 2000; Chang *et al.*, 2007; Zaki *et al.*, 2007). Given that such null

alleles have a larger effect on expression compared with the repeat polymorphism, these findings could collectively be interpreted to suggest that reduced brain levels of reelin in patients with schizophrenia and bipolar disorder may represent a benign consequence, or side-effect, of a true pathogenic process or of the disorders themselves. For example, according to one hypothesis, reduced expression of *RELN* is a consequence of the same pathogenic process that leads to down-regulation of the gene that encodes the 67 kDa form of glutamic acid decarboxylase (GAD_{67}) in brains of the same patients (Noh *et al.*, 2005; Veldic *et al.*, 2004, 2005). Multiple studies have found that variants in the promoter region of that gene, *GAD1*, are associated with schizophrenia and bipolar disorder (Addington *et al.*, 2005; Lundorf *et al.*, 2005; Straub *et al.*, 2007; Zhao *et al.*, 2007; Du *et al.*, 2008), although the data are not yet conclusive in favour of a true association.

An alternative hypothesis is that more important than the level of reelin in brain *per se* in determining vulnerability to psychosis is the capacity for reelin levels to change or be maintained as the brain matures. Under this hypothesis, the decreased brain level of reelin observed in patients with schizophrenia and bipolar disorder may be pathogenic not because it is lower than in healthy individuals, but either because it decreased with time or at a certain point, or failed to increase as might normally occur at appropriate stages of development. Possibly relevant to this hypothesis is the recent finding that the degree of methylation of the *RELN* promoter correlates with age in healthy individuals, but not in individuals with schizophrenia or bipolar disorder (Tamura *et al.*, 2007).

4.4.1: Conclusions

The present study adds to previous evidence that the CGG repeat polymorphism in the 5' UTR of the *RELN* gene has a functional effect on *RELN* expression in brain. Accordingly,

altered brain levels of reelin in disorders with which the polymorphism is reportedly associated (e.g. autism, Alzheimer's disease) potentially represents a primary pathogenic defect. However, SNPs tagging the repeat polymorphism were not found to be associated with schizophrenia or bipolar disorder, suggesting that the previously observed decrease in brain level of reelin may represent a secondary feature of those disorders. Also consistent with this view is that no effect on mRNA abundance of a SNP (rs7341745) associated with schizophrenia in women was detected. The pathogenic mechanism of this SNP requires further investigation. The present study also detected a low frequency variant with a larger *cis*-regulatory effect on *RELN* expression. The contribution of this as yet unknown variant to psychiatric and neurodegenerative disease may also deserve further investigation.

CHAPTER 5

$GABA_A$ RECEPTOR SUBUNIT GENES

(*GABRA4, GABRA5, GABRB1,*

GABRB3, GABRG2, GABRG3)

CHAPTER 5: $GABA_A$ RECEPTOR SUBUNIT GENES

5.1: Introduction

5.1.1: GABA

The amino acid, γ-aminobutyric acid (GABA) is the most widely distributed and abundant inhibitory neurotransmitter in the adult brain (Sivilotti & Nistri, 1991). An estimated 30–40% of all brain neurons and synapses utilise GABA (Bloom & Iversen, 1971; Roberts, 1986). The majority of GABAergic neurons are interneurons that modulate excitability of local neuronal circuits (Roberts, 1986). In addition, GABAergic neurons play a central role in nearly all stages of brain development including neuronal proliferation, migration, differentiation, and synapse formation and refinement (Owens & Kriegstein, 2002). Hence, given its widespread presence and utilisation, one could say that GABA is involved in all brain functions and all brain diseases (Olsen, 2002).

5.1.2: $GABA_A$ Receptors

GABA acts via two classes of ionotropic and metabotropic receptors, $GABA_A$ and $GABA_B$ respectively (Barnard *et al.*, 1998). Most of its neurobiological actions are mediated by the $GABA_A$ class (Sieghart & Sperk, 2002). $GABA_A$ receptors are pentameric ligand-gated chloride ion (Cl^-) channels (Sieghart *et al.*, 1999; Olsen, 2002) that, in adult brain, mediate fast synaptic inhibition (Connors, Malenka & Silva, 1988). They are made up of various combinations of α (α_1 to α_6), β (β_1 to β_3), γ (γ_1 to γ_3), δ, ε, θ, π, and ρ (ρ_1 to ρ_3) subunits (Barnard *et al.*, 1998; Bonnert *et al.* 1999), each encoded by separate genes. In the brain, the most abundant and widespread subtype assemblies are composed of two α, two β and one γ subunits (Sieghart *et al.*, 1999; Möhler, 2006). In less abundant and more localised receptors, the γ subunit is substituted with a δ, ε, or π subunit, whereas the β subunit can be

replaced with the θ subunit (Barnard *et al.*, 1998; Whiting, 2003). The ρ subunits are believed to form pentamers only with other ρ subunits and are preferentially expressed in the retina (Barnard *et al.*, 1998).

$GABA_A$ receptors can be modulated by a variety of pharmacologically and clinically important drugs including alcohol, anaesthetics, barbiturates, benzodiazepines, convulsants, and steroids (Sieghart, 1995, 2006, Olsen, 2002). Each class of drug acts at different sites of the channel; hence, each has varying affinities for receptors with different subunits (Sieghart, 1995; Korpi, Gründer & Lüddens, 2002). In addition to regulating brain excitability, different $GABA_A$ receptor assemblies play significant roles in the control of anxiety, sleep, and cognition including learning, memory and sensorimotor processing (Möhler *et al.*, 2004; Möhler, 2006). This has led to the use and ongoing development of $GABA_A$ receptor subtype-selective drugs in the treatment of a wide range of psychiatric and neurological conditions (Whiting, 2003; Guidotti *et al.*, 2005; Korpi & Sinkkonen, 2006). Epilepsy, pain, sleep disorders, affective disorders, psychoses, and alcohol and substance abuse are a few examples.

5.1.3: GABAergic Dysfunction in Psychiatric Disease

Abnormalities in GABA neurotransmission, both pre- and post-synaptic, have been described in relation to several psychiatric disorders, particularly schizophrenia and bipolar disorder (Blum & Mann, 2002; Guidotti *et al.*, 2005). In relation to these particular disorders, several studies have found a significant reduction (up to 70%), relative to controls, in cortical or cerebellar levels of glutamic acid decarboxylase (GAD), particularly the 67 kDa form (GAD_{67}) (Akbarian *et al.*, 1995b; Impagnatiello *et al.*, 1998; Guidotti *et al.*, 2000; Volk *et al.*, 2000; Vawter *et al.*, 2002; Hashimoto *et al.*, 2003; Woo, Walsh &

Benes, 2004; Fatemi *et al.*, 2005b; Veldic *et al.*, 2005), which is the main rate-limiting enzyme for GABA synthesis in the brain (Asada *et al.*, 1997). According to a recent meta-analysis of ~100 post-mortem neurochemical markers analysed in the Stanley Consortium brain sample (including tissue from 15 each of individuals with schizophrenia, bipolar disorder and major depression, and 15 healthy controls), the reduction in this enzyme was among the most striking neuropathological findings (Torrey *et al.*, 2005). Similar decreases in brain levels of GAD have also been described in relation to major depression (Fatemi *et al.*, 2005b) and autism (Fatemi *et al.*, 2002; Yip, Soghomonian & Blatt, 2007). In addition to reduced GAD, schizophrenia and bipolar disorder are also associated with other deficiencies in GABAergic function. These include decreases in reelin (see Chapter 4), the GABA membrane transporter (Woo *et al.*, 1998), and parvalbumin (Lewis, Volk & Hashimoto, 2004).

5.1.4: Altered Expression of $GABA_A$ Receptor Subunits as a Pathophysiological Feature of Schizophrenia and Bipolar Disorder

A number of studies have suggested that expression of $GABA_A$ subunits, and hence the composition of $GABA_A$ receptors, is responsive to changes in intensity of GABAergic stimulation (Steiger & Russek, 2004; Guidotti *et al.*, 2005). Consistent with this, changes in $GABA_A$ subunit expression have been observed in the same disorders that are associated with reductions in GAD. In schizophrenia, binding assays using [^{3}H]muscimol (a potent $GABA_A$ receptor agonist that binds to the same site as GABA) on post-mortem brain tissue have shown increased binding in prefrontal cortex, cingulate cortex, hippocampus, and other brain regions when compared with normal controls (Hanada *et al.*, 1987; Benes *et al.*, 1992, 1996a, 1996b; Dean *et al.*, 1999; Deng & Huang, 2006; Newell *et al.*, 2007). In bipolar disorder, increased binding of [^{3}H]flumazenil (a benzodiazepine antagonist), but

not [^{3}H]muscimol was observed in prefrontal cortex (Dean *et al.*, 2001), and alterations in [^{3}H]flumazenil binding were also observed in hippocampus (Dean *et al.*, 2005). These latter findings suggest that in bipolar disorder, the up-regulation in receptor expression may be specific to subtypes that are sensitive to benzodiazepines.

Alterations in $GABA_A$ subunit expression associated with schizophrenia and bipolar disorder have also been examined at mRNA and individual protein levels. In an initial study, which utilised *in situ* hybridisation, expression levels of six subunits (α_1, α_2, α_5, β_1, β_2 and γ_2) were not significantly different in prefrontal cortex from individuals with schizophrenia and controls (Akbarian *et al.*, 1995a). In a number of subsequent reports, however, consistent with the binding assay data, α_1 and α_5 subunit mRNAs, particularly the latter, were found by quantitative RT-PCR and *in situ* hybridisation to be significantly increased in prefrontal cortex of schizophrenic brains relative to controls (Impagnatiello *et al.*, 1998; Ohnuma *et al.* 1999). Increased expression of these receptors in schizophrenia has also been detected by immunohistochemistry at the protein level (Ishikawa *et al.*, 2004b; Guidotti *et al.*, 2005). Furthermore, according to one report, the density of postsynaptic neurons with α_2-containing receptors is significantly increased in the brains of schizophrenic individuals, but not individuals with major depression (Volk *et al.*, 2002). In a further study, expression of α_6 subunit mRNA was found to be 1.37 fold lower in peripheral blood leukocytes of schizophrenic patients, as compared with their unaffected siblings (Petryshen *et al.*, 2005).

By immunohistochemistry, the β_2/β_3 subunits were found to be increased in both schizophrenia and bipolar disorder prefrontal cortex samples, although the antibody used could not distinguish between the two subunits (Ishikawa *et al.*, 2004a). It was later found

by real-time PCR that expression of the β_2 subunit was reduced in schizophrenia (Zhao *et al.*, 2006), which, if confirmed, suggests that the increased protein levels detected in the previous study may be specific to the β_3 subunit.

The γ_1/γ_3 subunits have also been examined by immunohistochemistry and found to be significantly increased in bipolar disorder, but not schizophrenia (Ishikawa *et al.*, 2004a). With regards to the γ_2 subunit, one study that employed *in situ* hybridisation and semi-quantitative RT-PCR found that although overall expression levels were only modestly and non-significantly reduced in schizophrenia, the ratio of long and short splice forms of this subunit was significantly altered in patient samples when compared with controls (Huntsman *et al.*, 1998).

The above described studies together suggest that changes in expression of $GABA_A$ subunits, particularly in prefrontal cortical areas, may play an important role in the pathophysiology of schizophrenia and bipolar disorder. However, alterations in expression of $GABA_A$ subunits may represent a compensatory response to diminished GABAergic neurotransmission. This is suggested by the observation that subunit expression is altered in the same brain samples that show reduced GAD levels (Impagnatiello *et al.*, 1998) and GABA content (Ohnuma *et al.*, 1999). That mRNA for the GABA transporter, which generally functions to remove GABA from the synapse, has been found at reduced levels concomitant with a reduction in GAD (Ohnuma *et al.*, 1999; Volk *et al.*, 2001) and increased receptor subunit expression (Volk *et al.*, 2002) is also consistent with this view. Furthermore, although increased [^{3}H]muscimol binding has been observed even in schizophrenic patients with little or no exposure to antipsychotic drugs (Benes *et al.*, 1992), it is possible that some reports of altered subunit gene expression reflect effects of

medication. However, it is also possible that altered $GABA_A$ receptor expression plays a direct role in susceptibility to schizophrenia, bipolar disorder, and other psychiatric disorders. Thus, the genes encoding these receptors are plausible functional candidates for these conditions.

5.1.5: Genomic Organisation of $GABA_A$ Subunit Genes

The genes encoding $GABA_A$ subunits are genomically organised into clusters on different chromosomes. In most instances, these clusters include genes coding for α, β and γ subunits consistent with the composition of most receptor subtypes. Phylogenetic tree analysis suggests that the different gene clusters arose from a single ancestral cluster following a series of duplication events (Russek, 1999; Darlison, Pahal & Thode, 2005). In the human genome, the major subunit gene clusters are located on chromosome 4p12 (α_2, α_4, β_1 and γ_1 subunits), 5q31–q35 (α_1, α_6, β_2, γ_2 and π subunits), 15q11–q13 (α_5, β_3 and γ_3 subunits) and Xq28 (α_3, ε and θ subunits). The remaining subunits are located on chromosome 6q13–q16 (ρ_1 and ρ_2 subunits), 1p36 (δ subunit) and 3q11 (ρ_3 subunit).

5.1.6: $GABA_A$ Subunit Genes in Psychiatric and Neurological Populations

$GABA_A$ subunit genes have been tested for association with a variety of psychiatric and neurological disorders. Previous evidence supports their possible involvement in alcohol dependence (Parsian & Cloninger, 1997; Noble *et al.*, 1998; Loh *et al.*, 1999; Parsian & Zhang, 1999; Schuckit *et al.*, 1999; Song *et al.*, 2003; Covault *et al.*, 2004; Dick *et al.*, 2004; Edenberg *et al.*, 2004; Radel *et al.*, 2005), schizophrenia (Lo *et al.*, 2004; Liu *et al.*, 2005; Petryshen *et al.*, 2005; Yu *et al.*, 2006; Lo *et al.*, 2007; Zhao *et al.*, 2007), bipolar disorder (Papadimitriou *et al.*, 1998; Massat *et al.*, 2002; Yamada *et al.*, 2003; Horiuchi *et al.*, 2004), major depression (Yamada *et al.*, 2003), autism (Cook *et al.*, 1998; Martin *et*

al., 2000; Menold *et al.*, 2001; Buxbaum *et al.*, 2002; Shao *et al.*, 2003; McCauley *et al.*, 2004; Ma *et al.*, 2005; Ashley-Koch *et al.*, 2006), and epilepsy (Mulley *et al.*, 2003; Chou *et al.*, 2003, 2007; Audenaert *et al.*, 2006; Maljevic *et al.*, 2006).

Recently, the Department of Psychological Medicine contributed towards a large genome-wide association study of seven common diseases, one of which was bipolar disorder (Wellcome Trust Case Control Consortium, 2007). The study examined ~2,000 cases for each disorder (1,868 for bipolar disorder) and a shared set of 2,938 controls, all of which were genotyped for ~500,000 SNPs across the genome. In the bipolar data set, one of the higher ranked association signals was from SNP rs7680321 in the *GABRB1* gene (encoding the $GABA_A$ β_1 subunit), with the C allele being more frequent in cases than controls (OR = 1.36, $P = 6.2 \times 10^{-5}$).

In an effort to increase biological homogeneity, a follow-up study sought to identify the phenotypic subset of cases that showed the strongest association signal at SNP rs7680321 (Craddock *et al.*, 2008). The association signal was found to be significantly enriched in the 279 cases that met Research Diagnostic Criteria (RDC; Spitzer *et al.*, 1978) for schizoaffective disorder, bipolar type (OR = 1.80, $P = 3.8 \times 10^{-6}$). When individuals with this diagnosis were compared with the original set of controls, SNPs rs6414684 in *GABRB1*, rs3934674 in *GABRA4* (α_4 subunit), rs17561681 in *GABRA5* (α_5 subunit), rs890319 in *GABRB3* (β_3 subunit), and rs854579 in *GABRR3* (ρ_3 subunit) showed gene-wide significant evidence for association, independent of the signal at SNP rs7680321. Moreover, although not included in the final report, early statistical analyses also identified SNPs in *GABRG3* (γ_3 subunit) as showing association, albeit at more nominal levels of significance.

A notable outcome of the study is that none of the most strongly associated SNPs in each gene are predicted to alter the amino acid sequence of an encoded protein. Indeed, according to online SNP databases and several published mutation screening efforts (Madia *et al.*, 2003; Ma *et al.*, 2006; Urak *et al.*, 2006), such polymorphisms have not been identified in *GABRA5*, *GABRB1*, and *GABRB3*, at least not in Caucasian populations. Furthermore, although non-synonymous SNPs are known to exist in *GABRA4*, *GABRG3*, and *GABRR3*, those SNPs either showed no evidence of association or showed much weaker evidence than the top hit. Thus it may be hypothesised that the associations are mediated by sequence variants that alter gene expression, which in turn may modify the subunit composition of $GABA_A$ receptors, and thereby their physiological and pharmacological properties.

5.1.7: The Present Study

The association of $GABA_A$ subunit genes with bipolar disorder, especially RDC-defined schizoaffective disorder, bipolar type, does not appear to be explicable by variants that affect protein structure. It is likely, therefore, that the associations are mediated by *cis*-regulatory effects on gene expression, which may influence the brain's capacity to compensate for a presynaptic deficit in GABAergic transmission. To test this hypothesis, assays of relative allelic expression targeted at several of the associated genes were applied to varying numbers of brain samples from heterozygous individuals. The genes assayed were *GABRA4*, *GABRA5*, *GABRB1*, *GABRB3*, and *GABRG3*. For a number of these genes, samples were also genotyped for the SNPs that showed association with RDC-defined schizoaffective disorder, bipolar type to test for putative *cis* effects on expression. Furthermore, given the widespread expression of the γ_2 subunit in the brain and its

requirement for high-affinity benzodiazepine binding, which is altered in bipolar disorder, the *GABRG2* gene was also assayed.

5.2: Materials and Methods

Brain samples and the general methods used for DNA and RNA processing, genotyping, and the relative allelic expression assay are described in more detail in Chapter 2.

5.2.1: Samples

Post-mortem brain tissue derived from frontal, temporal or parietal cortex of 66 unrelated anonymised individuals obtained from three sources (the MRC London Brain Bank for Neurodegenerative Diseases, the Stanley Medical Research Institute, and the Karolinska Institute) were used in this study. For each tissue sample, genomic DNA and total RNA were extracted by standard procedures. RNA was then treated with DNAse and reverse transcribed to cDNA.

5.2.2: Genotyping

PCR primers for the SNPs used for relative allelic expression analysis of each gene and for SNPs rs7680321 and rs6414684 in *GABRB1* and rs17561681 in *GABRA5* were designed by use of the Primer3 program (Rozen & Skaletsky, 2000). PCR was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA, and consisted of 35 cycles. Genotyping was carried out by fluorescent primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and primers designed by use of the FP Primer program (Ivanov *et al.*, 2004). Primer sequences for SNPs used for relative allelic expression analysis and for SNPs rs17561681, rs7680321 and rs6414684 are shown in Tables 5.1 and 5.2 respectively, which, where relevant, also show the annealing temperature used during PCR cycling.

Gene	Polymorphism	Primer	Sequence (5'–3')	Ta (°C)
GABRA4	rs7660336	F:	TGGCAAAAGGAAATGGAATA	56
		R:	GGCTCAGAGAACAAAATTTCA	
		EXT:	AGCCAACATAAGAAAGGGAA	-
GABRA5	rs140682	F:	TGCGTACCCTAATTCTGAAGTC	58
		R:	CCATCAGGTGGTACTGGTTC	
		EXT:	CGTACCCTAATTCTGAAGTCGT	-
GABRB1	rs10016388	F:	AAAGGGGAGAGAAGCAAGAA	58
		R:	AGAGCCACCACTTTACATATCC	
		EXT:	GGTGTCATATACTTATGAAGGTCTATTAC	-
	rs10028945	F:	CAGCCATCCAATTGGTTTTA	58
		R:	CTGCTAAAGATCTACCTGTCTAACTTC	
		EXT:	GCTAAAGATCTACCTGTCTAACTTCT	-
GABRB3	rs11637141	F:	CATCCCCAAATGTGTCTTGT	58
		R:	GATGAAAACAAACCCCATCA	
		EXT:	AATGAGTCACTTACTTCATATCCTCTC	-
GABRG2	rs418210	F:	AAGAAAATTTCCTCCACATTCA	57
		R:	GCCCAAGGACACAAAATAAA	
		EXT:	TCTCTTTAGATCCAAAATAAATGGA	-
GABRG3	rs140679	F:	ACGCGTGTCCTACGTGAC	60
		R:	CGTGGTGGTTGGTTTTCTAC	
		EXT:	CCATGGACCTTTTTGTGAC	-

Table 5.1: Details of primers used for relative allelic expression analysis of $GABA_A$ receptor subunit genes. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

Gene	Polymorphism	Primer	Sequence (5'–3')	Ta (°C)
GABRA5	rs17561681	F:	CAAAGACAGCCACTCCATTT	58
		R:	GTCACTCTCCCAGCAGAAAA	
		EXT:	CACTCCATTTTAAGACCAGATGA	-
GABRB1	rs7680321	F:	ACGAAACTTAAAATATGCTGTGAA	58
		R:	CAGGTGGAGGAAGCATTTTA	
		EXT:	GAAACTTAAAATATGCTGTGAACTACA	-
	rs6414684	F:	TGTTGTGATTTCCACAATGG	58
		R:	GAAGTGCAGCCTGAAAATGT	
		EXT:	AGATGGAATTTATGCCAAATGAAAA	-

Table 5.2: Details of primers used for genotyping SNPs rs17561681, rs7680321, and rs6414684. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

5.2.3: Relative Allelic Expression Assay

Details of SNPs chosen for relative allelic expression analysis of each gene are described in Table 5.3. Their location on known mRNA transcripts of each gene is illustrated in Figures 5.1–5.6.

Genomic DNA from all brain samples was initially genotyped, as described, for all SNPs to identify heterozygotes. For each heterozygous sample, cDNA produced from two separate reverse transcription reactions, alongside corresponding genomic DNA, was assayed on two separate occasions (i.e. 2 × {2 cDNA + 1 genomic DNA}). Genomic DNA and cDNA were assayed under identical conditions. This allowed the average allelic ratio obtained from genomic DNA (representing a 1:1 ratio) to be used to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to the assay.

Gene	Polymorphism	Exon	MAF
GABRB1	rs10016388	3' UTR*	0.08
	rs10028945	3' UTR*	0.28
GABRA4	rs7660336	3' UTR	0.48
GABRA5	rs140682	8	0.07
GABRB3	rs11637141	3' UTR	0.23
GABRG2	rs418210	3' UTR	0.20
GABRG3	rs140679	8	0.49

Table 5.3: Details of SNPs chosen for relative allelic expression analysis of $GABA_A$ receptor subunit genes. Exon = the exon in which the SNP is located as shown in each gene figure (Figures 5.1–5.6). MAF = minor allele frequency in HapMap CEU samples. *SNPs rs10016388 and rs10028945 are present in UTRs of different *GABRB1* mRNA transcripts (Figure 5.1).

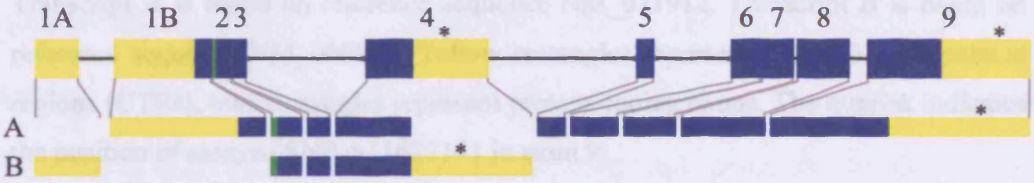

Figure 5.1: The organisation of the *GABRB1* gene and its two mRNA transcripts. Transcript A is based on reference sequence NM_000812. Transcript B is based on cDNA clones DB172039 and AW301170 as indicated in AceView. Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. The green rectangle represents protein-coding sequence in transcript A, and UTR sequence in transcript B. Asterisks indicate the positions of assayed SNPs rs10016388 in exon 4 and rs10028945 in exon 9.

Figure 5.2: The organisation of the *GABRA4* gene and its one reference mRNA transcript (NM_00809). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. The asterisk indicates the position of assayed SNP rs7660336 in exon 9.

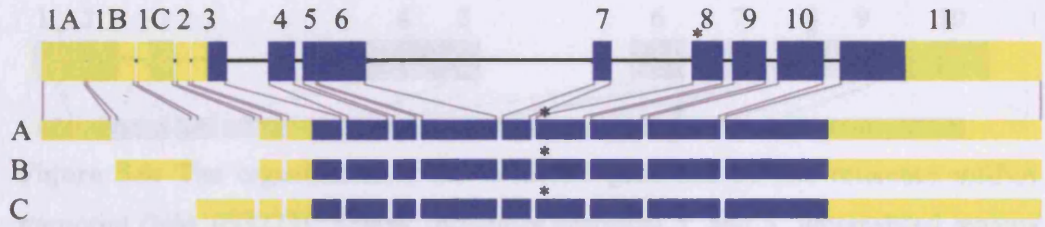

Figure 5.3: The organisation of the *GABRA5* gene and its three mRNA transcripts. Transcript A is based on reference sequence NM_000810. Transcript B and C are based on descriptions provided by their discoverers (Kim *et al.*, 1997). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. The asterisk indicates the position of assayed SNP rs140682 in exon 8.

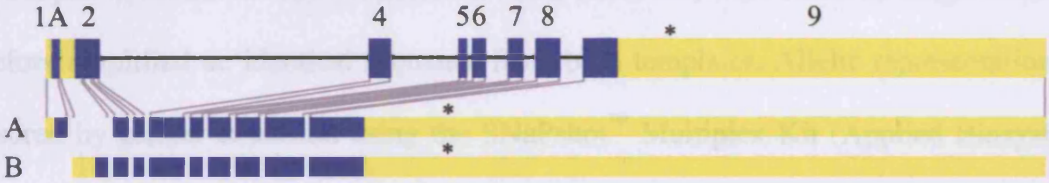

Figure 5.4: The organisation of the *GABRB3* gene and its two mRNA transcripts. Transcript A is based on reference sequence NM_021912. Transcript B is based on reference sequence NM_000814. Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. The asterisk indicates the position of assayed SNP rs11637141 in exon 9.

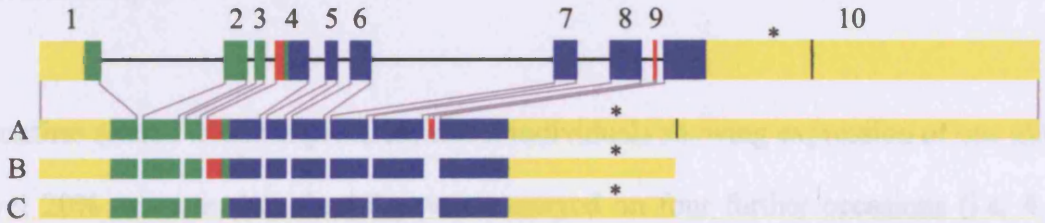

Figure 5.5: The organisation of the *GABRG2* gene and its three mRNA transcripts. Transcript A is based on reference sequence NM_198904. Transcript B is based on reference sequence NM_000816. Transcript C is based on reference sequence NM_198903. Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. Green rectangles represent protein-coding sequence / exons in transcripts A and C, and UTR sequence in transcript B. Red rectangles represent an alternatively spliced portion of exon 4 and the alternatively spliced exon 9. The asterisk indicates the position of assayed SNP rs418210 in exon 10.

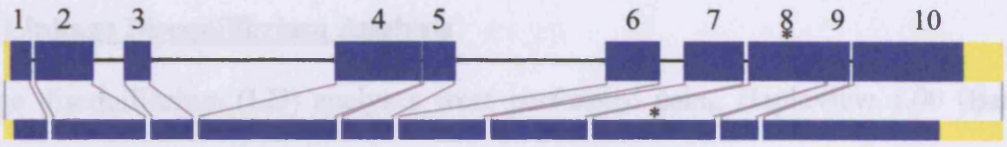

Figure 5.6: The organisation of the *GABRG3* gene and its one reference mRNA transcript (NM_033223). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons. The asterisk indicates the position of assayed SNP rs140679 in exon 8.

PCR consisted of 35 cycles and was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA or 6 µl of cDNA at the standard cDNA concentration (see Chapter 2, section 2.1.8). Primers, shown in Table 5.1, were based on single exons and therefore amplified an identical sequence from both templates. Allelic representation was measured by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and the primers shown in Table 5.1. Peak heights of allele-specific extended primers were determined using Genotyper 2.5 (Applied Biosystems). The ratio of cDNA peak heights, corrected using the average ratio of genomic DNA peak heights, was then used to calculate the relative expression of mRNAs transcribed from each parental chromosome in each individual sample.

To confirm altered allelic expression, select individuals showing expression of one allele at a level 20% or more than the other were assayed on four further occasions (i.e. 4 × {2 cDNA + 1 genomic DNA}). In line with previous studies (Yan *et al.*, 2002; Bray *et al.*, 2003), an allelic expression difference ≥20% for any individual sample was chosen as indicative of the influence of *cis*-acting regulatory polymorphism since measurements surpassing this threshold are generally reproducible.

5.2.4: Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) analyses were performed using Haploview 4.00 (Barrett *et al.*, 2005) and were based on genotype data for the entire collection of 66 brain samples.

5.2.5: Checking for Genetic Association

Where evidence for *cis*-regulatory variation in high LD with the assayed SNP was obtained in any gene assay, that SNP was checked for evidence for association with schizophrenia, bipolar disorder and RDC-defined schizoaffective disorder, bipolar type in imputed datasets generated in the Department of Psychological Medicine. These were the datasets generated as part of the WTCCC genome-wide association study (WTCCC, 2007) and the bipolar disorder study that followed it (Craddock *et al.*, 2008). The author was in no way involved in the genesis of either of these datasets.

5.3: Results

5.3.1: *GABRB1*

Assayed Samples

Individuals informative for allelic expression analysis included 18 heterozygotes for SNP rs10028945, which is located within the 3' UTR of the reference mRNA transcript (NM_000812; Figure 5.1), and 17 heterozygotes for SNP rs10016388, which is located within the 3' UTR of an alternative mRNA predicted by AceView based on two cDNA clones (DB172039 and AW301170; Figure 5.1). Five individuals were heterozygous at both SNPs. The mean age at death for the total of 30 informative individuals was 63 years (SD = 19.50), 19 were male, and 11 were female. A large proportion (n = 28) had no known history of psychiatric or neurological disease, one had schizophrenia, and one had major depression. Samples were derived from frontal (n = 19), temporal (n = 5), and parietal (n = 6) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility for the assay at SNP rs10028945 with an average coefficient of variation (SD / mean) of 0.06. The average coefficient of variation for the assay at SNP rs10016388, located within the alternative mRNA, was higher at 0.19. However, this was largely due to a few samples showing very large within-sample variation. Samples with a coefficient of variation of >2 SDs from the mean (n = 4) were therefore excluded, resulting in a coefficient of variation 0.08. In neither assay was significant correlation observed with sex, age, diagnostic status or brain region assayed.

Reference mRNA Transcript

Analysis of the reference transcript (NM_000812) at SNP rs10028945 did not reveal any samples that showed allelic expression differences of ≥20% and cDNA ratios were not significantly different from genomic DNA ratios (P = 0.57; Figure 5.7). LD analyses showed that SNP rs10028945 was in low LD with putative risk SNP rs7680321 (D' = 0.28, r^2 = 0.00) and not in LD with putative risk SNP rs6414684 (D' = 0.03, r^2 = 0.00). cDNA ratios for the two samples derived from heterozygotes for SNP rs7680321 were close to 1:1 (Figure 5.7) and cDNA ratios denoted as the more highly expressed allele relative to the lower expressed allele (i.e. H:L) did not significantly differ between homozygotes and heterozygotes for SNPs rs7680321 (P = 0.13) or rs6414684 (P = 0.36) (Figure 5.8). These analyses had 80% power to detect a ratio difference of 0.06 and 0.09 respectively (P = 0.05).

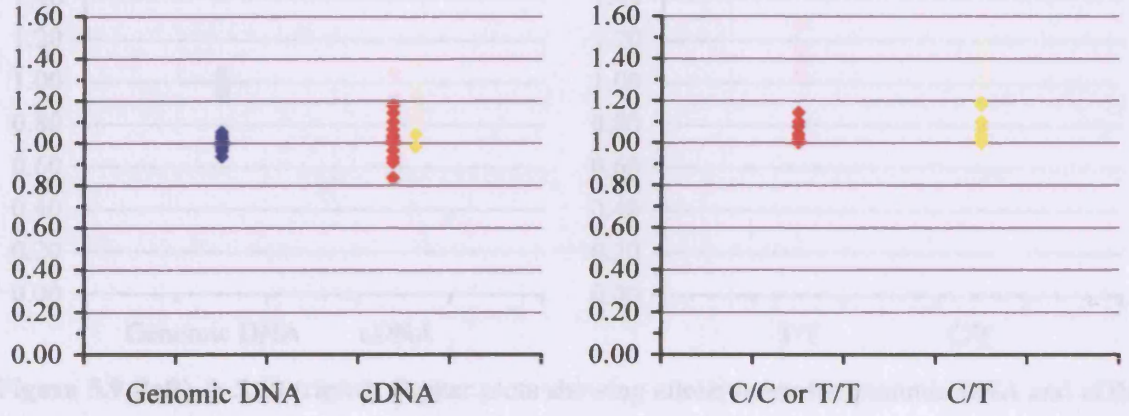

Figure 5.7 (left) & 5.8 (right): Scatter plots showing allelic ratios for genomic DNA and cDNA derived from 18 individuals heterozygous for SNP rs10028945. In Figure 5.7, cDNA ratios from individuals that are homozygous for SNP rs7680321 are shown in red, and ratios from heterozygotes are shown in yellow. In Figure 5.8, cDNA ratios from individuals that are homozygous for SNP rs6414684 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the C allele relative to the T allele (C:T) in Figure 5.7 and as the higher expressed allele relative to the lower expressed allele (H:L) in Figure 5.8. Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

Alternative mRNA Transcript

Analysis of the alternative mRNA transcript (DB172039 and AW301170) at SNP rs10016388 showed that transcripts tagged by the A allele were on average ~9% less abundant than those tagged by the T allele (Figure 5.9). The difference between allelic ratios obtained with cDNA and genomic DNA was significant (P = 0.005). LD analyses showed that SNP rs10016388 was in high LD with putative risk SNP rs7680321 (D' = 1.00, r^2 = 0.43) such that the C allele of the latter was predicted to always be in phase with the A allele of the former. LD between SNP rs10016388 and putative risk SNP rs6414684 was low (D' = 0.13, r^2 = 0.00). cDNA ratios did not significantly differ according to homozygosity and heterozygosity for SNP rs7680321 (P = 0.67; Figure 5.9), and when denoted as the more highly expressed allele relative to the lower expressed allele (i.e. H:L)

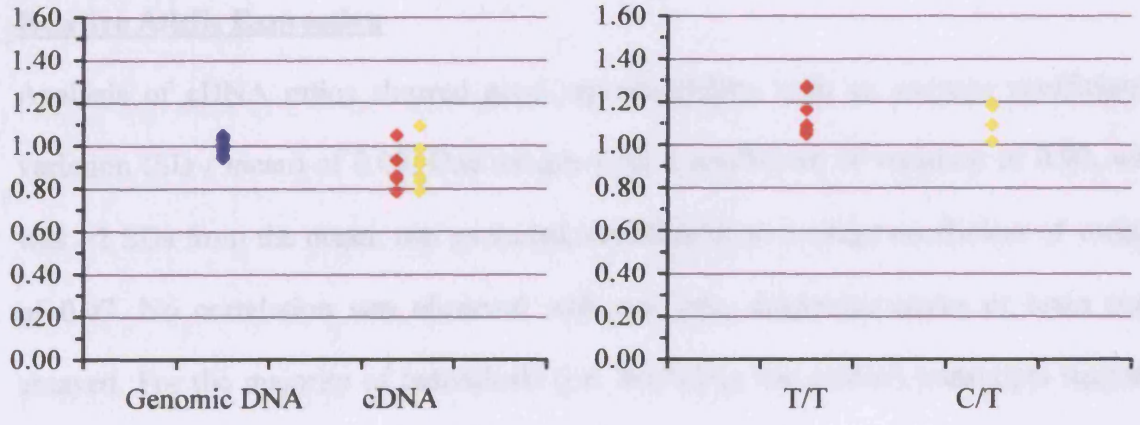

Figure 5.9 (left) & 5.10 (right): Scatter plots showing allelic ratios for genomic DNA and cDNA derived from 13 individuals heterozygous for SNP rs10016388. In Figure 5.9, cDNA ratios from individuals that are homozygous for SNP rs7680321 are shown in red, and ratios from heterozygotes are shown in yellow. In Figure 5.10, cDNA ratios from individuals that are homozygous for SNP rs6414684 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the A allele relative to the T allele (A:T) in Figure 5.9, and as the higher expressed allele relative to the lower expressed allele (H:L) in Figure 5.10. Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

they did not significantly differ between homozygotes and heterozygotes for SNP rs6414684 either (P = 0.80; Figure 5.10). These analyses had 80% power to detect a ratio difference of 0.14 and 0.11 respectively (P = 0.05).

5.3.2: *GABRA4*

Assayed Samples

A total of 28 genotyped individuals were heterozygous for the expressed SNP rs7660336 and therefore informative for allelic expression analysis. Their mean age at death was 63 years (SD = 20.98), 15 were male, and 13 were female. None had a known history of psychiatric or neurological disease. Samples were derived from frontal (n = 20), temporal (n = 3), and parietal (n = 5) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.09. One sample with a coefficient of variation of 0.90, which was >2 SDs from the mean, was excluded, resulting in an average coefficient of variation of 0.07. No correlation was observed with sex, age, diagnostic status or brain region assayed. For the majority of individuals (i.e. excluding one outlier) transcripts tagged by the C allele were on average ~4% less abundant than those tagged by the G allele (Figure 5.11). The difference between allelic ratios obtained with cDNA and genomic DNA was significant (P = 0.003). Only one sample displayed an allelic expression difference of ≥20% (Figure 5.11). This sample was derived from a 54-year-old anonymous female with no known history of psychiatric or neurological disorder. For this individual, the average C:G ratio over four replicate cDNA measurements was 1.43:1. When this individual's

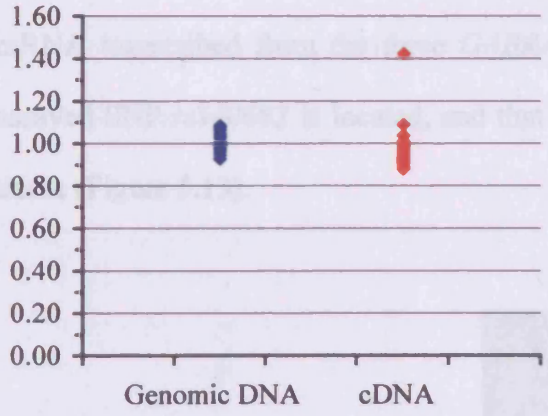

Figure 5.11: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 27 individuals heterozygous for SNP rs7660336. Data are presented as the C allele relative to the G allele (C:G). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

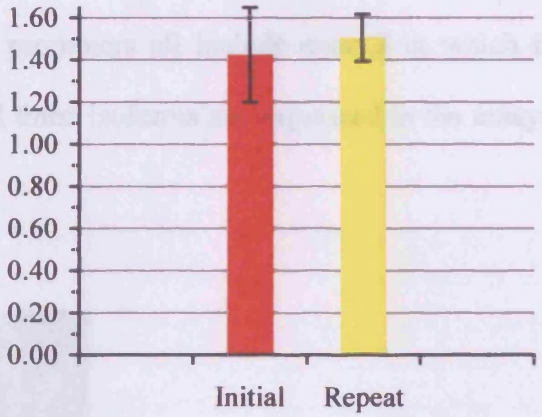

Figure 5.12: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for the individual that showed an initial allelic expression difference ≥20%. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

sample was assayed a further four times, the average C:G ratio over eight replicate cDNA measurements was 1.50:1 (95% C.I. = 1.39–1.62:1; Figure 5.12). Thus, in this individual, expression of the C allele was increased by 50% (using the A allele as an arbitrary reference point), or expression of the G allele reduced by 34% (using the G allele as an arbitrary reference point). Observed cDNA ratios from this individual were significantly different from ratios obtained with genomic DNA ($P = 5.29 \times 10^{-6}$).

5.3.3: *GABRA5*

Transcript Characterisation

GABRA5 has one reference mRNA transcript (NM_000810), but three isoforms of this mRNA transcribed from different promoters and differing in their first exon (Figure 5.3) have previously been reported (Kim *et al.*, 1997). RT-PCR using primers to amplify from

exon 1A, 1B and 1C to exon 8 of *GABRA5* using brain cDNA as template showed that mRNA transcribed from the three *GABRA5* promoters all include exon 8 in which the assayed SNP rs140682 is located, and that all three isoforms are expressed in the assayed tissue (Figure 5.13).

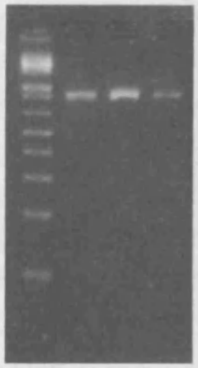

Figure 5.13: Gel photograph showing a 1 Kb Plus DNA ladder (lane 1), and amplicons produced by PCR with forward primers complementary to exon 1A (lane 2), 1B (lane 3), and 1C (lane 4) and a reverse primer complementary to exon 8.

Assayed Samples

A total of 28 genotyped individuals were heterozygous for the expressed SNP rs140682 and therefore informative for allelic expression analysis. Their mean age at death was 66 years (SD = 19.22), 14 were male, and 14 were female. A large proportion (n = 27) had no known history of psychiatric or neurological disease, and one had major depression. Samples were derived from frontal (n = 20), temporal (n = 6), and parietal (n = 2) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed very good reproducibility, with an average coefficient of variation (SD / mean) of 0.03. No correlation was observed with sex, age, diagnostic status

or brain region assayed. A small number of samples (n = 4) displayed allelic expression differences of ≥20%, however there were no major outliers, and ratios ranged from 0.79:1 to 1.25:1 (Figure 5.14).

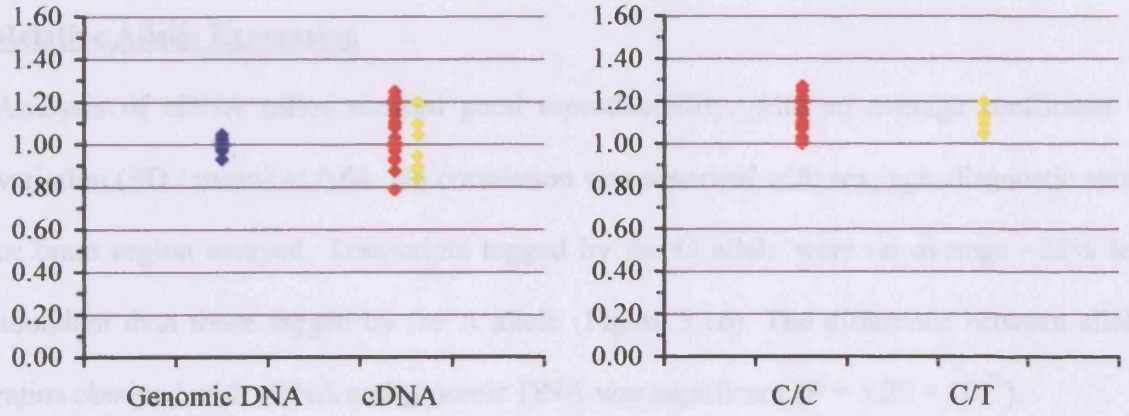

Figure 5.14 (left) & 5.15 (right): Scatter plots showing allelic ratios for genomic DNA and cDNA derived from 28 individuals heterozygous for SNP rs140682. cDNA ratios from individuals that are homozygous for SNP rs17561681 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the C allele relative to the T allele (C:T) in Figure 5.14, and as the higher expressed allele relative to the lower expressed allele (H:L) in Figure 5.15. Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

LD analyses showed that there was moderate LD between the assayed SNP rs140682 and putative risk SNP rs17561681 (D' = 0.62, r^2 = 0.04). cDNA ratios denoted as the more highly expressed allele relative to the lower expressed allele (i.e. H:L) did not significantly differ between homozygotes and heterozygotes for SNP rs17561681 (P = 0.60; Figure 5.15). This analysis had 80% power to detect a ratio difference of 0.08 (P = 0.05).

5.3.4: *GABRB3*

Assayed Samples

A total of 25 genotyped individuals were heterozygous for the expressed SNP rs11637141 and therefore informative for allelic expression analysis. Their age at death was 65 years

(SD = 19.03), 13 were male, and 12 were female. A large proportion (n = 22) had no known history of psychiatric or neurological disease, and three had schizophrenia. Samples were derived from frontal (n = 17), temporal (n = 3), and parietal (n = 5) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.04. No correlation was observed with sex, age, diagnostic status or brain region assayed. Transcripts tagged by the G allele were on average ~23% less abundant than those tagged by the A allele (Figure 5.16). The difference between allelic ratios obtained with cDNA and genomic DNA was significant ($P = 5.29 \times 10^{-19}$).

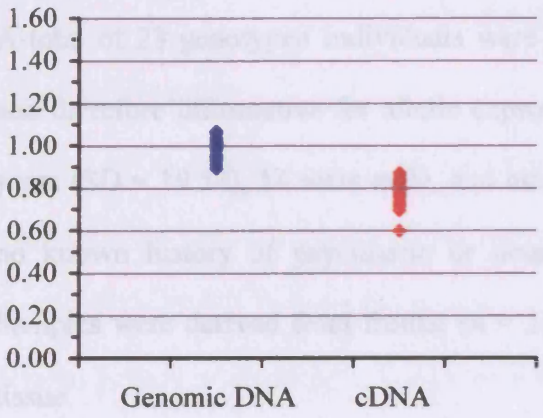

Figure 5.16: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 25 individuals heterozygous for SNP rs11637141. Data are presented as the G allele relative to the A allele (G:A). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

Figure 5.17: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for the individual that showed the largest allelic expression difference. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

One sample, derived from an 89-year-old anonymous female with no known history of neurological or psychiatric disorder, showed even more pronounced allelic expression

difference. For this individual the average G:A ratio over four replicate cDNA measurements was 0.60:1. When this individual's sample was assayed a further four times, the average G:A ratio over eight replicate cDNA measurements was 0.62:1 (95% C.I. = 0.60–0.65:1; Figure 5.17). Thus, in this individual, expression of the G allele was reduced by 38% (using the A allele as an arbitrary reference point), or expression of the A allele increased by 60% (using the G allele as an arbitrary reference point). Observed cDNA ratios from this individual were significantly different from ratios obtained with genomic DNA ($P = 1.66 \times 10^{-12}$).

5.3.5: *GABRG2*

Assayed Samples

A total of 23 genotyped individuals were heterozygous for the expressed SNP rs418210 and therefore informative for allelic expression analysis. Their mean age at death was 60 years (SD = 19.54), 14 were male, and nine were female. A large proportion ($n = 22$) had no known history of psychiatric or neurological disease, and one had schizophrenia. Samples were derived from frontal ($n = 15$), temporal ($n = 5$), and parietal ($n = 3$) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.07. No correlation was observed with sex, age, diagnostic status or brain region assayed. Transcripts tagged by the C allele were on average 10% more abundant than those tagged by the T allele (Figure 5.18). The difference between allelic ratios obtained with cDNA and genomic DNA was significant ($P = 2.17 \times 10^{-5}$).

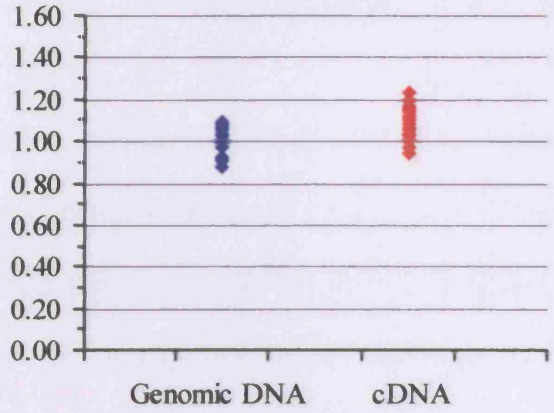

Figure 5.18: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 23 individuals heterozygous for SNP rs418210. Data are presented as the C allele relative to the T allele (C:T). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

5.3.6: *GABRG3*

Assayed Samples

A total of 30 genotyped individuals were heterozygous for the expressed SNP rs140679 and therefore informative for allelic expression analysis. Their mean age at death was 59 years (SD = 20.53), 16 were male, and 14 were female. A large proportion (n = 29) had no known history of psychiatric or neurological disease, and one had schizophrenia. Samples were derived from frontal (n = 20), temporal (n = 5), and parietal (n = 5) brain tissue.

Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with an average coefficient of variation (SD / mean) of 0.08. One sample with a coefficient of variation of 0.70, which was >2 SDs from the mean, was excluded, resulting in an average coefficient of variation of 0.06. No correlation was observed with sex, age, diagnostic status or brain region assayed. No samples displayed allelic expression differences of ≥20%, and cDNA ratios were not significantly different from genomic DNA ratios (P = 0.81; Figure 5.19).

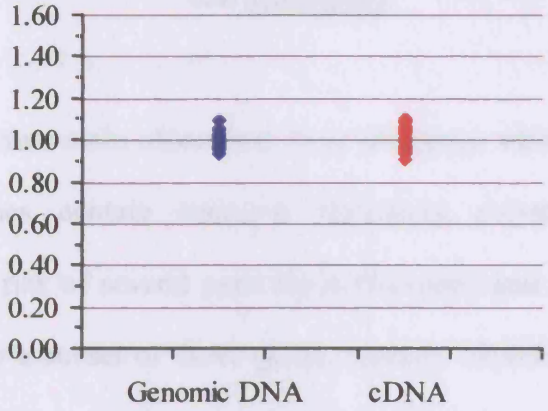

Figure 5.19: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 29 individuals heterozygous for SNP rs140679. Data are presented as the T allele relative to the C allele (T:C). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

5.4: Discussion

The present study had two main objectives: 1) to determine whether or not a selection of $GABA_A$ subunit genes contain common regulatory polymorphism, which might conceivably influence risk of several psychiatric disorders; and 2) to test whether or not particular SNPs within a subset of those genes, recently identified in the Department of Psychological Medicine as risk factors for bipolar disorder, are associated with altered gene expression. To achieve these objectives, assays of relative allelic expression were applied to a series of brain samples, which were also genotyped for the SNPs in question. The data for each assayed gene are discussed in turn.

5.4.1: *GABRB1*

GABRB1 was the index $GABA_A$ subunit gene that through its association with bipolar disorder in a genome-wide study (WTCCC, 2007), and more strongly with RDC-defined schizoaffective disorder, bipolar type in a follow-up study (Craddock *et al.*, 2008), pointed to the possible involvement of the $GABA_A$ receptors in these disorders. Two independent association signals were detected in this gene: the index signal was with SNP rs7680321, and the second was with SNP rs6414684. In the present study, potential *cis* effects on *GABRB1* expression in brain were investigated using assays of relative allelic expression targeted at two of this gene's mRNAs, including the reference transcript (NM_000812) and an alternative transcript that was indicated in AceView (DB172039 and AW301170).

None of 18 heterozygotes assayed for the reference transcript at SNP rs10028945 showed an allelic expression difference of $\geq$20%, suggesting that polymorphisms or haplotypes that have large effects on expression of this mRNA in brain are not likely to be common, at

least in Caucasian populations. Only two of the assayed samples were heterozygous for the risk SNP rs7680321. That both had cDNA ratios close to 1:1 suggests that the association of this variant with bipolar disorder is not mediated by a direct effect of this SNP on expression of the main *GABRB1* transcript. Furthermore, that H:L cDNA ratios did not significantly differ between homozygotes and heterozygotes for SNP rs6414684 suggests that the association of that variant with RDC-defined schizoaffective disorder, bipolar type is also not mediated by a pathogenic alteration in expression of the main *GABRB1* transcript.

In the assay of the alternative *GABRB1* transcript, mRNAs tagged by the A allele at SNP rs10016388 were generally slightly (~9% on average) less abundant than mRNAs tagged by the T allele, with two samples showing an A allelic decrease of $\geq$20%. These findings are consistent with the presence of a *cis*-acting regulatory variant of small effect that is in high LD with the assayed SNP. The assayed SNP rs10016388 is not included in the dataset used in the WTCCC genome-wide association study (WTCCC, 2007). Given the association between this SNP and allelic expression in the present study, testing it for association with bipolar disorder may be warranted.

Although the assayed SNP was found to be in high LD with SNP rs7680321, cDNA ratios did not significantly differ between homozygotes and heterozygotes for this SNP, and neither did they for SNP rs6414684. These findings suggest that the association of those SNPs with bipolar disorder and RDC-defined schizoaffective disorder, bipolar type is not mediated by a pathogenic alteration in mRNA abundance. However, since mRNAs tagged by the risk C allele of SNP rs7680321 were generally under-expressed relative to those tagged by the non-risk T allele, the possibility that reduced expression of the alternative

mRNA isoform is relevant to but not sufficient for the pathogenic effect of the SNP cannot be ruled out.

5.4.2: *GABRA4*

In the assay of the only known *GABRA4* mRNA transcript at SNP rs7660336, only one of 27 heterozygotes showed an allelic expression difference of ≥20%. In that sample, derived from a 54-year-old female with no known psychiatric or neurological diagnosis, expression of one gene copy was ~50% greater than the other. Aside from that one sample, the present study also found that mRNAs tagged by the C allele at SNP rs7660336 were generally a little less (~4% on average) abundant than mRNAs tagged by the G allele.

The first of these findings suggests that polymorphisms or haplotypes that have large effects on *GABRA4* expression in brain are, at least in Caucasian populations, rare with a frequency of <0.02. This frequency is much lower than that of the minor allele of SNP rs3934674, which was the variant in *GABRA4* that was associated with RDC-defined schizoaffective disorder, bipolar type (Craddock *et al.*, 2008). Although this SNP was not genotyped in any of the brain samples assayed, given its minor allele frequency of 0.22 in HapMap CEU samples, it is expected that almost half the assayed samples would have been heterozygous for it. Hence, that only one sample showed an allelic expression difference of ≥20% suggests that SNP rs3934674 does not have a large *cis*-regulatory effect on *GABRA4* mRNA abundance.

In contrast, the present study cannot rule out the possibility that SNP rs3934674 has a small effect on *GABRA4* mRNA abundance, as samples with a C:G cDNA ratio of <1:1 may have been heterozygous for that SNP. However, LD between the assayed SNP

rs7660336 and SNP rs3934674 in Caucasians is relatively low (D' = 0.261, r^2 = 0.02 in HapMap CEU samples), whereas the allelic expression data are more suggestive of a *cis*-regulatory variant that is in high LD with the assayed SNP. In the datasets used in the WTCCC follow-up study (Craddock *et al.*, 200), high quality imputed data (average maximum posterior call probability >0.99; missing data proportion <1%) for assayed SNP rs7660336 did not indicate an association with schizophrenia (P = 0.65), bipolar disorder (P = 0.36) or RDC-defined schizoaffective disorder, bipolar type (P = 0.79). Therefore, it is unlikely that the association between *GABRA4* and RDC-defined schizoaffective disorder, bipolar type is mediated by a pathogenic alteration in *GABRA4* mRNA abundance.

5.4.3: ***GABRA5***

In the assay of *GABRA5* at SNP rs140682, cDNA ratios ranged from 0.79 to 1.25. A notable feature of the data is that, despite this large range of values, cDNA ratios for each sample were very highly replicable, with an unusually low average coefficient of variation of <0.03. A low coefficient of variation narrows the confidence intervals within which the 'true' allelic expression ratio for each brain sample is likely to fall, thereby increasing confidence in the accuracy of the measurements. In view of this, the data, taken as whole, are suggestive of either multiple *cis*-regulatory variants of small to moderate effect or a single variant of variable size effect in low LD with the assayed SNP.

cDNA ratios did not significantly differ between homozygotes and heterozygotes for SNP rs17561681, which was the variant in *GABRA5* that was associated with RDC-defined schizoaffective disorder, bipolar type (Craddock *et al.*, 2008). Although this may imply that the SNP does not influence mRNA abundance, in light of the suggestive evidence that

GABRA5 may contain multiple *cis*-regulatory variants, the possibility remains that any regulatory effect of the SNP might be masked by regulatory effects of other variants.

GABRA5 has three mRNAs that are transcribed from separate promoters, and that differ only in their 5' UTR sequence (Kim *et al.*, 1997). The assayed SNP rs140682 is located in exon 8, which is included in all three mRNAs. RT-PCR using brain cDNA as template showed that all three promoters were active in the assayed brain tissue. Therefore, if *GABRA5* has multiple *cis*-regulatory variants, it is plausible that their effects, including any putative effect of SNP rs17561681, might be specific to mRNAs transcribed from a particular promoter. Hence, the possibility that the association of SNP rs17561681 with RDC-defined schizoaffective disorder, bipolar type is mediated by an isoform-specific pathogenic alteration in mRNA abundance cannot be ruled out. Transcript-specific assays of allelic expression based on primers that span alternate exons and the assayed SNP would allow this possibility to be investigated.

5.4.4: ***GABRB3***

In the assay of *GABRB3* at SNP rs11637141, all brain samples exhibited an allelic expression difference in the same direction, such that mRNAs tagged by the G allele were on average 23% less abundant than mRNAs tagged by the A allele. In addition, one sample showed an even more pronounced difference of 40%. These findings are consistent with the presence of a *cis*-acting regulatory variant of moderate effect that is in high LD with the assayed SNP, and plausibly with a second, less frequent variant of similar or larger effect. The data are also consistent with SNP rs11637141 having a *cis*-regulatory effect itself.

The SNP in *GABRB3* associated with RDC-defined schizoaffective disorder, bipolar type was SNP rs890319 (Craddock *et al.*, 2008). This SNP was not genotyped in any of the brain samples assayed. However, in Caucasians, LD between the assayed SNP rs11637141 and SNP rs890319 is very low (D' = 0.085, r^2 = 0.004 in HapMap CEU samples). Therefore, although not conclusive, the data from the present study do not support the hypothesis that the association of SNP rs890319 with RDC-defined schizoaffective disorder, bipolar type is mediated by a pathogenic alteration in *GABRB3* mRNA abundance. It is important to note, however, that *GABRB3* has two known mRNAs (Kirkness & Fraser, 1993), and that the assayed SNP rs11637141 is located in the 3' UTR of both. Thus, the present study cannot rule out the possibility that SNP rs890319 has a *cis*-regulatory effect that is mRNA-specific. However, if SNP rs11637141 is functional, as the data may suggest, then, given its location in the 3' UTR, it is likely that the detected allelic expression difference reflects an effect on mRNA degradation that influences the abundance of both isoforms.

Given the strong association between the assayed SNP and allelic expression in the present study, the potential importance of *GABRB3* expression in bipolar disorder could be assessed by testing this SNP for genetic association with the disorder. However, in the datasets used in the WTCCC follow-up study (Craddock *et al.*, 200), high quality imputed data (average maximum posterior call probability >0.98; missing data proportion <3.5%) for SNP rs11637141 did not indicate an association with bipolar disorder (P = 0.45) or RDC-defined schizoaffective disorder, bipolar type (P = 0.21). Similarly, the data did not indicate an association with schizophrenia either (P = 0.78). This suggests that variation in *GABRB3* mRNA abundance in brain probably does not influence susceptibility to any of these disorders.

5.4.5: *GABRG2*

In the assay of *GABRG2* at SNP rs418210, mRNAs tagged by the C allele were generally slightly (~10% on average) more abundant than mRNAs tagged by the T allele, with two samples showing a C allelic increase of ≥20%. These findings are consistent with the presence of a *cis*-acting regulatory variant of small effect that is in high LD with the assayed SNP. However, *GABRG2* is known to have three mRNA isoforms, and SNP rs140682 is present on all of them. Thus which of these mRNAs the putative regulatory variant affects is unclear.

Variants in *GABRG2* did not previously show any evidence for association with bipolar disorder or RDC-defined schizoaffective disorder, bipolar type (WTCCC, 2007, Craddock *et al.*, 2008). In the datasets used in the WTCCC follow-up study (Craddock *et al.*, 200), high quality imputed data (average maximum posterior call probability >0.98; missing data proportion <2%) for assayed SNP rs418210 did not indicate an association with schizophrenia (P = 0.55), bipolar disorder (P = 0.54) or RDC-defined schizoaffective disorder, bipolar type (P = 0.87). Therefore, it can be inferred that whatever small change (i.e. increase or decrease of one, two or all three mRNAs) in *GABRG2* mRNA abundance in brain was detected in this study, it probably does not influence susceptibility to these disorders.

5.4.6: *GABRG3*

The assay of *GABRG3* at SNP rs140679 did not reveal any evidence of *cis*-regulatory variation in the 29 brain samples analysed. This finding suggests that if polymorphisms or haplotypes with detectable effects on expression of this gene's only known mRNA in brain exist, they are likely to be rare, at least in Caucasian populations. Hence, any association

between common variants in this gene and psychiatric illness, including RDC-defined schizoaffective disorder, bipolar type, is unlikely to be mediated by a pathogenic alteration in *GABRG3* mRNA abundance.

5.4.7: Further Discussion

The present studies do not support the hypothesis that the reported associations of SNPs in $GABA_A$ subunit genes with bipolar disorder and RDC-defined schizoaffective disorder, bipolar type are mediated by alterations in expression levels of those genes in brain. In fact, the results of each gene assay argue against this hypothesis in three different ways. Firstly, the *GABRB1* and *GABRA5* data clearly demonstrate that the risk SNPs have no more influence on mRNA abundance in the tissue assayed than any other variant within or nearby those genes. Secondly, the *GABRA4*, *GABRB3*, and *GABRG2* data clearly suggest that *cis*-acting variants that influence abundance of those genes' mRNA do operate in brain; however, these variants do not appear to be associated with disease. And thirdly, the *GABRG3* data suggest that the gene does not contain variants that influence mRNA abundance.

It is important to note that it is possible that the risk SNPs in each gene may each be serving as proxies for separate less frequent *cis*-regulatory variants in high LD (as defined by D') with them. In this case, only a fraction of heterozygotes for the risk SNPs, and even some homozygotes for the risk alleles, would be expected to show a significant allelic expression difference. If such variants were of sufficiently low frequency, it is possible that too few brain samples were assayed in the present studies to detect them. However, the brain sample with the outlying cDNA ratio observed in the *GABRA4* assay could potentially be one example.

The assays of *GABRB1* (alternative transcript), *GABRA4*, *GABRA5*, and particularly *GABRB3* each provided evidence of *cis*-regulatory variation that was, or was very likely to be, unrelated to the risk SNPs identified in each gene. If a variant within a gene has a known functional effect, but does not increase risk for a disorder, it is a reasonable assumption that any susceptibility variants within the same gene likely act via a different mechanism. In view of this, the effects of risk SNPs identified in each of these genes may be more complex, or more specific, than simply altering expression levels in brain. For example, their effects may be specific to certain mRNA isoforms, brain regions or cell types, or may only occur at critical periods of development, or be dependent on one or more *trans* factors that are disease-specific.

The variants detected in the present studies, including the assay of *GABRG2*, may be of potential relevance to the aetiology or pathophysiology of several other psychiatric or neurological disorders. Their potential influence is likely dependent not only on whether they increase or decrease mRNA abundance, but also on the basal expression level of the gene in individual cells relative to other subunit genes that make up functional $GABA_A$ receptor channels. The assembly of multimeric channels such as $GABA_A$ receptors may, in theory, be constrained by whichever of the required subunits of the channel is least expressed (and therefore rate-limiting). Hence, in theory, the detected variants might only influence susceptibility if they affect or determine the least expressed subunit of at least one receptor subtype. With this and the logical potential for gene × gene interaction effects in mind, the variants underlying the allelic expression differences observed in these studies, if identified, could be reasonable functional candidates for case-control association studies of relevant psychiatric or neurological disorders.

The SNP assayed for *GABRG2* (rs418210) is part of a haplotype that was previously found to be associated with schizophrenia in a Finnish family sample (Turunen *et al.*, 2003). The detection of a *cis*-regulatory variant in high LD with this SNP is, therefore, particularly intriguing and warrants further work to identify it. The present study also suggests that the SNP assayed for *GABRB3* (SNP rs11637141) is, or is in perfect LD with, a functional *cis*-regulatory variant of moderate effect. In the two studies that have analysed this SNP in relation to autism (Tochigi *et al.*, 2007; Kim *et al.*, 2008), no evidence for association was found. This suggests that whatever influence this SNP has on *GABRB3* mRNA expression in brain (i.e. increase or decrease in expression), it and other variants with a similar effect probably do not influence susceptibility to that disorder. In contrast, promoter variants in *GABRB3* have previously been implicated in the aetiology of childhood absence epilepsy (Urak *et al.*, 2006). Hence, analysis of SNP rs11637141 in relation to that disorder is warranted. The SNP is also a reasonable functional candidate for any other psychiatric or neurological disorder that *GABRB3* might be implicated in.

GABRB3, *GABRA5* and *GABRG3* map to within the imprinted 15q11-q13 region that is associated with Prader-Willi and Angelman syndromes. It is therefore noteworthy that the present studies demonstrate conclusively that none of these genes are imprinted in human brain, at least not in the regions assayed. This is in agreement with a recent study (Hogart *et al.*, 2007), which also detected biallelic expression of these genes in frontal cortex of human control brain samples. Interestingly, this study also found highly skewed or monoallelic expression of these genes in brain samples from individuals with Rett syndrome or autistic spectrum disorders. The authors attributed these findings to dysregulation of epigenetic mechanisms in those disorders.

5.4.8: Conclusions

The present studies do not support the hypothesis that the association of SNPs in $GABA_A$ subunit genes with bipolar disorder and RDC-defined schizoaffective disorder, bipolar type is mediated by *cis*-regulatory influences on mRNA abundance. *Cis*-regulatory variants in a number of these genes do, however, exist and may be relevant to other psychiatric or neurological disorders.

CHAPTER 6

NITRIC OXIDE SYNTHASE 1 ADAPTER PROTEIN (*NOS1AP*)

CHAPTER 6: NITRIC OXIDE SYNTHASE 1 ADAPTER PROTEIN (*NOS1AP*)

6.1: Introduction

6.1.1: The *NOS1AP* Gene, the NOS1 Adapter Protein, and Nitric Oxide

The human *NOS1AP* gene (formerly *CAPON*; OMIM: 605551) encodes the neuronal nitric oxide synthase 1 (NOS1) adapter protein. This protein was originally identified in rat as a binding partner of neuronal nitric oxide synthase (nNOS) (Jaffrey *et al.*, 1998), an enzyme that synthesises the gaseous transmitter, nitric oxide, in the brain (Huang *et al.*, 1993). When bound to nNOS, the NOS1 adapter protein prevents the enzyme from associating with postsynaptic density proteins, PSD-93 and PSD-95 (Jaffrey *et al.*, 1998). The interaction between nNOS and these proteins is important for targeting nNOS to the postsynaptic N-methyl-D-aspartate (NMDA) receptor complex and for subsequent NMDA receptor-mediated nitric oxide release (Brenman *et al.*, 1996a, 1996b). Hence, the principal function of the NOS1 adapter protein is thought to be to negatively regulate the level of nitric oxide in the brain.

The primary function of nitric oxide is to stimulate guanylyl cyclase to catalyze the production of the second messenger, cyclic guanosine monophosphate (cGMP) (Friebe & Koesling, 2003). Through pathways dependent on and independent of this molecule, nitric oxide is known to play a wide range of roles in brain function. For example, it has been shown to regulate the release of numerous neurotransmitters (including glutamate, GABA, dopamine, and others), it modulates neurotransmitter receptor function, plays a key role in synaptic plasticity, and is involved in neuronal development and control of gene expression (Yun, Dawson & Dawson, 1996, 1997; Prast & Philippu, 2001). Since nitric oxide is an oxygen radical, at higher concentrations it is neurotoxic and thus may be involved in the

pathophysiology of various neurological and neurodegenerative disorders (Dawson & Dawson, 1998; Boje, 2004). A role for nitric oxide in the pathophysiology of schizophrenia and other psychiatric disorders has also been proposed, although it is unclear whether an increase or decrease in nitric oxide levels is involved (Akyol *et al.*, 2004; Bernstein, Bogerts & Keilhoff, 2005). Support for a role of nitric oxide in psychiatric disease is also provided by findings that non-coding variants in the *NOS1* gene have been reported to be associated with schizophrenia in some samples (Shinkai *et al.*, 2002; Fallin *et al.*, 2005; Reif *et al.*, 2006; O'Donovan *et al*, 2008, Tang *et al.*, 2008), although these studies have reported association to different alleles, making meta-analysis impossible.

6.1.2: The *NOS1AP* Gene in Psychiatric Populations

Given the diverse roles attributed to nitric oxide in the brain, its possible neuropathophysiological involvement, and the fact that the NOS1 adapter protein negatively regulates the activity of nNOS, the *NOS1AP* gene is an attractive functional candidate for psychiatric disease susceptibility. Moreover, the gene, which contains 10 exons and spans ~300 kb of chromosome 1q23, is a good positional candidate, as linkage studies and meta-analysis have identified this region as a likely susceptibility locus for schizophrenia (Shaw *et al.*, 1998; Brzustowicz *et al.*, 2000, 2002; Gurling *et al.*, 2001; Hwu *et al.*, 2003; Lewis *et al.*, 2003, Ng *et al.*, 2009) and autistic spectrum disorders (Auranen *et al.*, 2002; Ylisaukko-oja *et al.*, 2004, 2006).

In a study of 22 European-Canadian families multiply affected by schizophrenia, the maximum LOD score achieved at 1q23 was 6.5 (Brzustowicz *et al.*, 2000). The susceptibility locus was then fine-mapped to a 1 Mb region (Brzustowicz *et al.*, 2002), in which several polymorphisms, all located within *NOS1AP*, were subsequently shown to be

in significant linkage disequilibrium (LD) with the disease (Brzustowicz *et al.*, 2004). Prior to the work presented here, association between polymorphisms within or nearby *NOS1AP* and schizophrenia had also been reported in Spanish (Rosa *et al.*, 2002), Chinese Han (Zheng *et al.*, 2005) and Colombian (Miranda *et al.*, 2006) populations, although in a British sample no association was found (Puri *et al.*, 2006). No meta-analyses of the association of *NOS1AP* with schizophrenia have, as of September 2008, been reported.

6.1.3: Altered *NOS1AP* Expression as a Potential Pathogenic Mechanism in Psychiatric Disease

Sequencing of the coding region of *NOS1AP* in individuals from the Canadian linkage sample did not identify any non-synonymous coding variants (Brzustowicz *et al.*, 2004), suggesting that the association, if genuine, is mediated by variants that affect expression. Consistent with a pathological role for altered *NOS1AP* expression, a novel 'short' NOS1 adapter protein isoform was recently reported to show ~50% increased expression at the mRNA level in post-mortem brain samples from individuals with schizophrenia and bipolar disorder when compared with normal controls (Xu *et al.*, 2005). The observed increase was associated with alleles of *NOS1AP* SNPs (rs1415263, rs4145621, rs2661818) that had previously been found to show association with schizophrenia (Brzustowicz *et al.*, 2004). No significant differences in expression of the full length isoform, which is controlled by a different promoter, were observed.

6.1.4: The Present Study

The *NOS1AP* gene does not contain common variants that affect protein structure, hence the association between polymorphisms in this gene and schizophrenia, if genuine, may be mediated by *cis*-acting variants that alter *NOS1AP* expression. This hypothesis is supported

by one study, described above, which found an increase in expression associated with putative risk SNP alleles. In the present study, the hypothesis was tested again, this time using a relative allelic expression assay, which, whilst controlling for potential *trans*-acting confounds, can detect genuine effects of *cis*-acting regulatory variation. As in the study described above, short and full length isoforms were assayed separately in order to detect transcript-specific effects. Samples were genotyped for the three SNPs that were reported to show genetic association with schizophrenia in the Canadian families to test for their proposed *cis* effect on expression.

6.2: Materials and Methods

Brain samples and the general methods used for DNA and RNA processing, genotyping, and the relative allelic expression assay are described in more detail in Chapter 2.

6.2.1: Samples

Post-mortem brain tissue derived from frontal, temporal or parietal cortex of 148 unrelated anonymised individuals obtained from three sources (MRC London Brain Bank for Neurodegenerative Diseases; Stanley Medical Research Institute; and Karolinska Institute) were used in this study. For each tissue sample, genomic DNA and total RNA were extracted by standard procedures. RNA was then treated with DNAse and reverse transcribed to cDNA.

6.2.2: Genotyping

PCR primers for the SNPs used for relative allelic expression analysis (rs3751284 and rs348624), and for SNPs rs1415263, rs4145621 and rs2661818, were designed by use of the Primer3 program (Rozen & Skaletsky, 2000). PCR was carried out in a total reaction volume of 12 μl containing 48 ng of genomic DNA, and consisted of 35 cycles. Genotyping was carried out by fluorescent primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and primers designed by use of the FP Primer program (Ivanov *et al.*, 2004). All primer sequences are shown in Table 6.1, which, where relevant, also shows the annealing temperature used during PCR cycling.

Polymorphism	Primer	Sequence (5'–3')	Ta (°C)
rs3751284*	F:	CCTTTGAGGTCTGCCACAAG	58
	R:	CCTGAGCTGTTGCTGTTCCT	
	EXT:	GAGCTGTTGCTGTTCCTCTC	-
rs348624*	F:	CATTCATGTCCCTCTCTTCTCTC	58
	R:	TGGAGCATGTCCTTGTTCTG	
	EXT:	AGCAAAAGCTGATGCAC	-
rs1415263	F:	TGTTTGGAGGAAAATGTGGT	58
	R:	GAGCGTGGTATCAACCAAAC	
	EXT:	ATTAGGCATTCCCAATTCCTTTATC	-
rs4145621	F:	TTTTATTCACCCCAACTCCA	58
	R:	CTATTGTCCATCACCCCTTG	
	EXT:	TGACTTAAATCCAACTCTTCTTGTTTC	-
rs2661818	F:	GATCCTTGTCTCCTTGAGCA	58
	R:	AGATGGGATTGGAAGGAAAC	
	EXT:	TTCAACCTCCTTCCACTATGA	-

Table 6.1: Details of primers used in the study of the *NOS1AP* gene. Asterisks indicate SNPs used for relative allelic expression analysis. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

6.2.3: Relative Allelic Expression Assay

The SNPs chosen for relative allelic expression analysis were rs3751284 and rs348624. In the HapMap CEU sample, frequency information for SNP rs3751284 is not available but the minor allele frequency for SNP rs348624 is 0.10. The two SNPs are located in exons 6 and 9 of the *NOS1AP* gene respectively. Both SNPS are present on the full length brain-expressed mRNA transcript, but only the latter is present on the short transcript (see Figure 6.1).

Figure 6.1: The organisation of the *NOS1AP* gene and its two mRNA transcripts. The full length (FL) transcript is based on reference sequence NM_014697. The short (Sh) transcript is based on a description provided by its discoverers (Xu *et al.*, 2005). Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons, and the red rectangle represents the 5' 54 bp coding portion of exon 9 that is unique to the short isoform. The black vertical line indicates a shorter 3' UTR present in some transcripts. Asterisks indicate the positions of assayed SNPs rs3751284 in exon 6 and rs348624 in exon 9.

Genomic DNA from all brain samples was initially genotyped, as described, for both SNPs to identify heterozygotes. For each heterozygous sample, cDNA produced from two separate reverse transcription reactions, alongside corresponding genomic DNA, was assayed on two separate occasions (i.e. 2 × {2 cDNA + 1 genomic DNA}). Genomic DNA and cDNA were assayed under identical conditions. This allowed the average allelic ratio obtained from genomic DNA (representing a 1:1 ratio) to be used to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to the assay.

PCR consisted of 35 cycles and was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA or 6 µl of cDNA at the standard cDNA concentration (see Chapter 2, section 2.1.8). Primers, shown in Table 6.1, were based on single exons and therefore amplified an identical sequence from both templates. The forward primer for SNP rs348624 was based on a 5' portion of exon 9 that was unique to the short transcript. Allelic representation was measured by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and the primers shown in Table 6.1. Peak heights of allele-specific extended primers were determined using Genotyper 2.5 (Applied Biosystems).

The ratio of cDNA peak heights, corrected using the average ratio of genomic DNA peak heights, was then used to calculate the relative expression of mRNAs transcribed from each parental chromosome in each individual sample.

To confirm altered allelic expression, select individuals showing expression of one allele at a level 20% or more than the other were assayed on four further occasions (i.e. 4 × {2 cDNA + 1 genomic DNA}). In line with previous studies (Yan *et al.*, 2002; Bray *et al.*, 2003), an allelic expression difference ≥20% for any individual sample was chosen as indicative of the influence of *cis*-acting regulatory polymorphism since measurements surpassing this threshold are generally reproducible.

6.2.4: Linkage Disequilibrium Analysis and Diplotype Prediction

Linkage disequilibrium (LD) analyses were performed using Haploview 4.00 (Barrett *et al.*, 2005) and were based on genotype data for the entire collection of 148 brain samples. The probability that each brain sample carried a particular phased diplotype comprised of assayed SNPs rs3751284 and rs348624 was calculated using EH Plus (Zhao, Curtis & Sham, 2000) and an in-house program, as described in Chapter 2 (section 2.5.4).

6.3: Results

6.3.1: Assayed Samples

Fifty-seven and 28 genotyped individuals were heterozygous for the expressed SNPs rs3751284 and rs348624 respectively, and therefore informative for allelic expression analysis. Twelve individuals were heterozygous at both SNPs. The mean age at death for the total of 73 informative individuals was 57 years (SD = 19.30), 45 were male, and 28 were female. A large proportion (n = 37) had no known history of psychiatric or neurological disease, nine had schizophrenia, 10 had bipolar disorder, six had major depression, and 11 had Alzheimer's disease. Samples were derived from frontal (n = 49), temporal (n = 16), and parietal (n = 8) brain tissue.

6.3.2: Relative Allelic Expression

Analysis of cDNA ratios showed good reproducibility, with average coefficients of variation (SD / mean) of 0.06 and 0.10 for assays at rs3751284 and rs348624 respectively. In neither assay was significant correlation observed with sex, age, diagnostic status or brain region assayed.

6.3.3: Full Length mRNA Transcripts

Analysis of full length mRNA transcripts at SNP rs3751284 showed that transcripts tagged by the G allele were on average 11% more abundant than those tagged by the A allele (Figure 6.2), although there was considerable variability with cDNA ratios ranging from 0.74–1.31:1. In total, 15 samples showed an allelic difference of $\geq$20%. The difference between allelic ratios obtained with cDNA and genomic DNA was significant ($P = 2.1 \times 10^{-11}$).

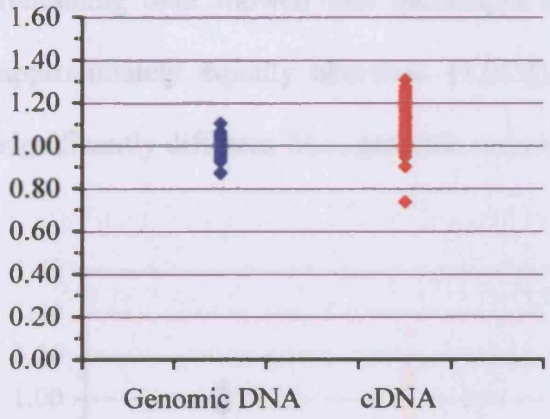

Figure 6.2: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 57 individuals heterozygous for SNP rs3751284. Data are presented as the G allele relative to the A allele (G:A). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

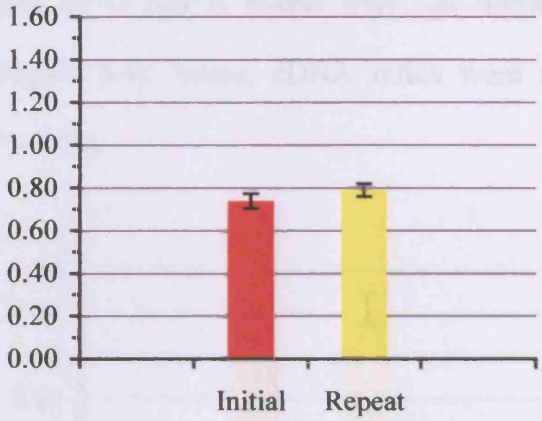

Figure 6.3: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for the individual that initially showed relative under-expression of the G allele. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

There was one clear outlier, a 54-year-old anonymous female with no known history of neurological or psychiatric disorder. For this individual, the average G:A ratio over four replicate cDNA measurements was 0.74:1. When this individual's sample was assayed a further four times, the average G:A ratio over eight replicate cDNA measurements was 0.79:1 (95% C.I. = 0.76–0.82:1; Figure 6.3). Thus, in this individual, expression of the G allele was reduced by 21% (using the A allele as an arbitrary reference point), or expression of the A allele increased by 27% (using the G allele as an arbitrary reference point). Observed cDNA ratios from this individual were significantly different from ratios obtained with genomic DNA ($P = 3.41 \times 10^{-8}$).

6.3.4: Short mRNA Transcripts

In the analysis of short mRNA transcripts at SNP rs348624, allelic cDNA ratios obtained from one individual were extremely unreliable (SD / mean = 0.44), and thus excluded. The

remaining data showed that transcripts tagged by G and A alleles were, on average, approximately equally abundant (1.01:1) (Figure 6.4), hence, cDNA ratios were not significantly different from genomic ratios ($P = 0.25$).

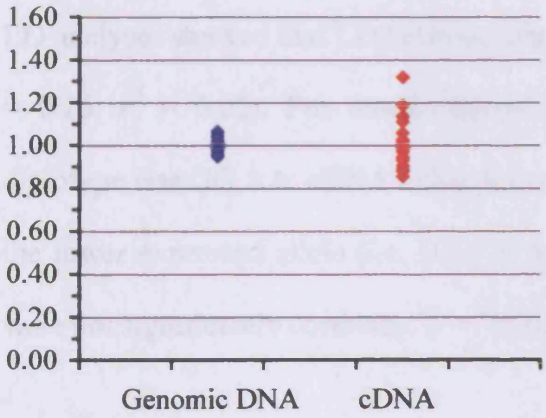

Figure 6.4: Scatter plot showing allelic ratios for genomic DNA and cDNA derived from 27 individuals heterozygous for SNP rs348624. Data are presented as the G allele relative to the A allele (G:A). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

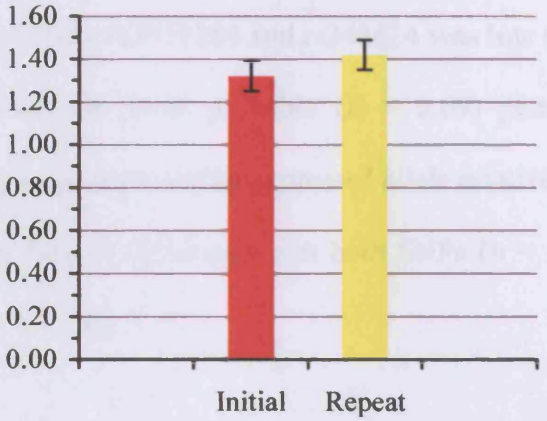

Figure 6.5: Bar chart comparing average allelic ratios obtained in the initial assay (red) with ratios obtained in the repeat assay (yellow) for the individual that initially showed relative over-expression of the G allele. The initial assay average is based on four measurements; the repeat assay average is based on eight measurements.

The individual that displayed a clear allelic difference in expression of the full length transcript was the only one that displayed an allelic expression difference of ≥20% for the short mRNA transcript. For this individual, the average G:A ratio over four replicate cDNA measurements was 1.32:1. When this individual's sample was assayed a further four times, the average G:A ratio over eight replicate cDNA measurements was 1.42:1 (95% C.I. = 1.35–1.49:1; Figure 6.5). Thus, in this individual, expression of the G allele was increased by 42% (using the A allele as an arbitrary reference point), or expression of the A allele reduced by 30% (using the G allele as an arbitrary reference point). Observed

cDNA ratios from this individual were significantly different from ratios obtained with genomic DNA ($P = 6.33 \times 10^{-7}$).

6.3.5: Inter-Transcript Allelic Expression Correlation

LD analyses showed that LD between assayed SNPs rs3751284 and rs348624 was low ($D' = 0.26$, $r^2 = 0.02$). For double heterozygotes, the most probable ($P = 0.69$) phased diplotype was GG,AA. cDNA ratios denoted as the more highly expressed allele relative to the lower expressed allele (i.e. H:L) in brain samples informative at both SNPs ($n = 11$) were not significantly correlated ($r = {}^{+}0.30$; $P = 0.38$).

6.3.6: Effect of SNPs rs1415263, rs4145621 and rs2661818

In the 148 brain samples genotyped in this study, neither of the assayed SNPs was in high LD with any of the three *NOS1AP* SNPs that showed association with schizophrenia in the previous study of Canadian families (Brzustowicz *et al.*, 2004) (Table 6.2). Moreover, cDNA ratios denoted as the more highly expressed allele relative to the lower expressed allele i.e. H:L) did not significantly differ between homozygotes and heterozygotes for any of the three SNPs. This was true for both full length (Figure 6.6; $P \geq 0.50$) and short mRNA transcripts (Figure 6.7; $P \geq 0.73$). These analyses had 80%

	rs1415263	**rs4145621**	**rs2661818**
rs3751284	0.01; 0.00	0.08; 0.01	0.14; 0.01
rs348624	0.10; 0.00	0.04; 0.00	0.35; 0.03

Table 6.2: LD between each assayed SNP (rows) and three putative schizophrenia risk SNPs (columns). D' and r^2 values are shown (i.e. D'; r^2).

power to detect a ratio difference of 0.06 and 0.07 for the full length and short mRNA transcript assays respectively ($P = 0.05$).

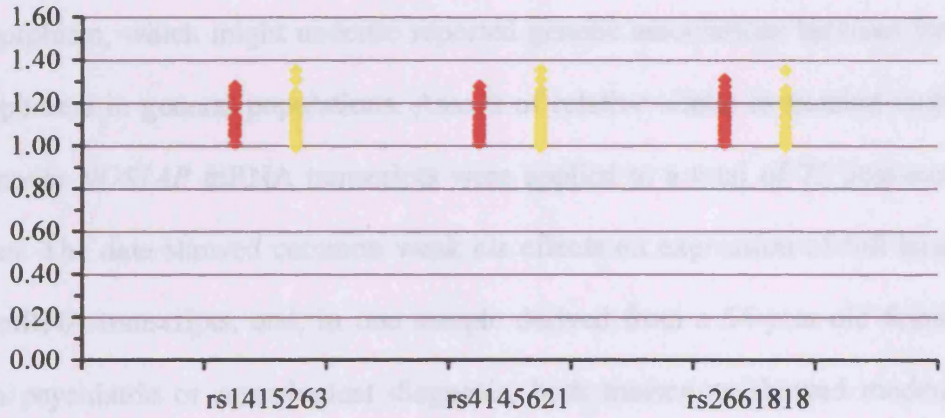

Figure 6.6: Scatter plot showing allelic ratios for cDNA derived from 57 individuals heterozygous for SNP rs3751284 stratified by genotype for three putative schizophrenia risk SNPs. cDNA ratios from individuals that are homozygous for each SNP are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the higher expressed allele relative to the lower expressed allele (H:L). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

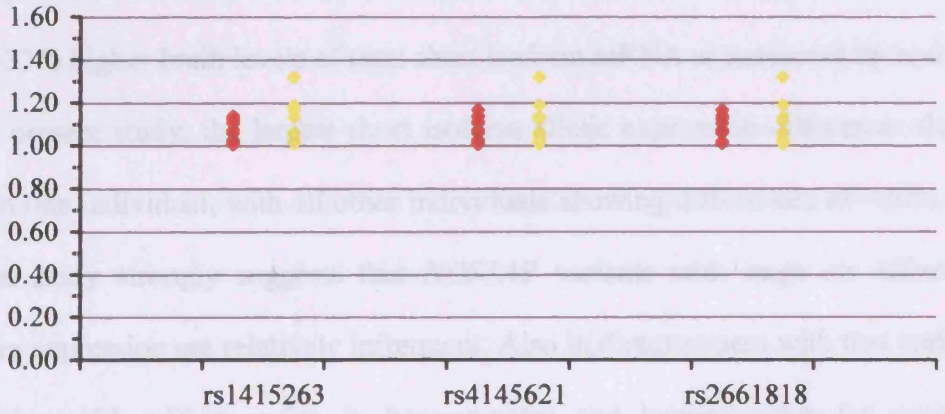

Figure 6.7: Scatter plot showing allelic ratios for cDNA derived from 27 individuals heterozygous for SNP rs348624 stratified by genotype for three putative schizophrenia risk SNPs. cDNA ratios from individuals that are homozygous for each SNP are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the higher expressed allele relative to the lower expressed allele (H:L). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

6.4: Discussion

The present study sought to test the hypothesis that *NOS1AP* contains common regulatory polymorphism, which might underlie reported genetic associations between the gene and schizophrenia in general populations. Assays of relative allelic expression targeted at the two known *NOS1AP* mRNA transcripts were applied to a total of 73 post-mortem brain samples. The data showed common weak *cis* effects on expression of full length but not short mRNA transcripts, and, in one sample derived from a 54-year-old female with no known psychiatric or neurological diagnosis, both transcripts showed moderately large allelic expression differences.

The results of the present study are not consistent with a previous study in which SNPs showing association with schizophrenia were associated with increased *NOS1AP* expression (Xu *et al.*, 2005). In that study, heterozygosity or homozygosity for schizophrenia-associated alleles at SNPs rs1415263, rs4145621 and rs2661818 correlated with ~33% higher brain levels of total short isoform mRNA as measured by real time PCR. In the present study, the largest short isoform allelic expression difference observed was 42% in one individual, with all other individuals showing differences of <20%. Hence the present study strongly suggests that *NOS1AP* variants with large *cis* effects on short isoform expression are relatively infrequent. Also in disagreement with that earlier study is that when H:L cDNA ratios in homozygotes and heterozygotes for each of SNPs rs1415263, rs4145621 and rs2661818 were compared in the present study, no significant differences were found for the short (or full length) mRNA isoforms. Given the statistical power of these analyses (80% to detect a ratio difference of 0.06), this suggests that none of these SNPs have even a relatively modest influence on mRNA abundance, at least not in

the brain tissues analysed. The association of these variants with schizophrenia, if genuine, is therefore unlikely to be mediated by this mechanism.

That common *cis*-regulatory variants affecting expression of the short mRNA isoform were not detected in the present study is also contrary to the findings of a more recent study. In that study, a further common SNP in *NOS1AP*, SNP rs12742393, was found to be associated with schizophrenia in the Canadian families, and was subsequently found to have a functional effect on *NOS1AP* expression in neuronal cell lines, and to be associated with short mRNA isoform abundance in post-mortem brain (Wratten *et al.*, 2009). Although the SNP in question was not genotyped in the present study, given its minor allele frequency of 0.38 in HapMap CEU samples, it is expected that many of the assayed brain samples were heterozygous for it. Therefore, if the SNP indeed has an effect on mRNA abundance, its effect is likely to be very small.

In the assay of full length isoforms, mRNAs tagged by the G allele at SNP rs3751284 were generally slightly (~11% on average) more abundant than mRNAs tagged by the A allele, with 15 samples showing a G allelic increase of $\geq$20%. These findings are consistent with the presence of a *cis*-acting regulatory variant of small effect that is in high LD with the assayed SNP. Although an effect of the putative variant on schizophrenia susceptibility is conceivable, no association was found between SNP rs3751284 and schizophrenia in the Canadian linkage families (Brzustowicz *et al.*, 2004). Furthermore, in a previous report, full length *NOS1AP* mRNA abundance in brain did not significantly differ between individuals with schizophrenia or bipolar disorder and normal controls (Xu *et al.*, 2005), suggesting that full length isoform expression is of little relevance to psychiatric disease. However, the authors noted the possibility that the lack of difference in expression may

have been due to normalisation by antipsychotic drug use. For instance, in the small number of drug-naïve patients, full length mRNA was indeed 24% increased relative to normal controls, although this difference did not quite reach significance ($P = 0.11$).

According to a recent comparative genome hybridisation study, chromosome 1q23 harbours a common ~512 kb deletion polymorphism that encompasses several genes along with the promoter and first exon of *NOS1AP* (de Smith *et al.*, 2007). In the heterozygous state, deletion of the promoter is likely to ablate expression from one gene copy. However, if the deletion is as common in Caucasians as the report suggested (16 observations in 50 individuals), it would be expected to have manifested in the present study as frequent monoallelic expression of the full length isoform. Since this was not found, the present study suggests the reported variant is either far less frequent than reported or does not extend to the *NOS1AP* gene. However, the possibility that the exon 6 SNP assayed in the present study tags an unidentified mRNA transcribed from a novel alternative promoter cannot be excluded.

In brain samples that were informative at both assayed SNPS there was no significant correlation in allelic expression differences between the two transcripts. However, given that allelic expression differences observed in one individual were of comparable size for both transcripts, and, for the full length transcript, in opposite direction to all other individuals, this suggests that the individual may carry a regulatory variant in a sequence element that coregulates both isoforms. Assuming the individual carries the most probable diplotype (GG,AA), the putative variant would be expected to have opposite effects (i.e. up- *and* down-regulation) on expression of the two isoforms. If the individual carries the lesser probable diplotype (GA,AG), the variant would be expected to have the same effect

(i.e. up- *or* down-regulation) on both isoforms. Either way, the variant, if identified, could be a reasonable functional candidate for large case-control association studies of psychiatric disease.

6.4.1: Conclusions

The present study suggests that reports of genetic association between *NOS1AP* and schizophrenia cannot be accounted for by common variants that results in large or even relatively modest changes in short mRNA isoform abundance. The results are, however, consistent with the existence of a common regulatory variant affecting full length isoform expression, and a less common variant affecting both isoforms. If identified, the contribution of these variants to psychiatric disease susceptibility may deserve further investigation.

CHAPTER 7

MICROTUBULE-ASSOCIATED PROTEIN TAU (*MAPT*)

CHAPTER 7: MICROTUBULE-ASSOCIATED PROTEIN TAU (*MAPT*)

7.1: Introduction

7.1.1: The *MAPT* Gene and Tau

The human *MAPT* gene (OMIM: 157140) spans ~134 kb of chromosome 17q21, includes 16 exons, and encodes the microtubule-associated protein tau (Neve *et al.*, 1986; Andreadis, Brown & Kosik *et al.*, 1992). Tau is an abundant protein in the central and peripheral nervous systems (Binder *et al.*, 1985; Georgieff *et al.*, 1991) where it is predominantly expressed in axons of developing and mature neurons (Brion *et al.*, 1988). It was originally recognised for its ability to promote assembly and stabilisation of microtubules (Weingarten *et al.*, 1975; Cleveland *et al.*, 1977a, 1977b; Drubin & Kirschner, 1986), and is now known to play roles in neurite outgrowth and in transduction of signals to the actin cytoskeleton (Shahani & Brandt, 2002).

In human brain, tau exists as six protein isoforms ranging from 352 to 441 amino acids in length generated by alternative splicing of exons 2, 3 and 10 (Himmler *et al.*, 1989). Exons 9–12 each encode an imperfect repeat of a microtubule-binding domain (Lee, Cowan & Kirschner, 1988; Goedert *et al.*, 1989b). When exon 10 is spliced out the resultant protein contains three microtubule-binding domains (3R tau); when spliced in, a protein with an extra such domain is produced (4R tau) (Goedert *et al.*, 1989b). *In vitro* experiments have shown that 4R tau isoforms bind microtubules with higher affinity, and assemble them more efficiently than 3R tau isoforms (Goedert & Jakes, 1990; Butner & Kirschner, 1991). Alternative splicing of exons 2 and 3 generates 3R and 4R isoforms with no (0N), one (1N), or two (2N) additional inserted domains of unknown function (Goedert *et al.*, 1989b). All six tau isoforms can be phosphorylated at several sites (Billingsley & Kincaid,

1997; Buée *et al.*, 2000), and phosphorylation correlates with reduced affinity for microtubules (Biernat *et al.*, 1993; Bramblett *et al.*, 1993) and a slower rate of assembly (Lindwall & Cole, 1984).

The alternative splicing and phosphorylation status of tau are both developmentally regulated. The tau content in human embryonic brain is comprised entirely of the shortest tau isoform (Goedert *et al.*, 1989b) and is highly phosphorylated (Kanemaru *et al.*, 1992; Bramblett *et al.*, 1993; Goedert *et al.*, 1993; Watanabe *et al.*, 1993). In contrast, in adult brain, all six tau isoforms are expressed (Goedert *et al.*, 1989b) with a ratio of 3R and 4R isoforms of ~1:1, and a 0N, 1N and 2N isoform composition of ~37%, ~54% and ~9% respectively (Goedert & Jakes, 1990; Hong *et al.*, 1998). Furthermore, adult brain tau generally shows much lower levels of phosphorylation than embryonic brain tau (Kanemaru *et al.*, 1992; Bramblett *et al.*, 1993; Goedert *et al.*, 1993; Watanabe *et al.*, 1993), and the degree of phosphorylation gradually decreases with age (Mawal-Dewan *et al.*, 1994).

7.1.2: The Role of Tau in the Pathology of Neurodegenerative Disease

The involvement of tau in neurodegenerative disease was first realised when it was found to be the major constituent of the neurofibrillary tangle (Iqbal *et al.*, 1986; Kosik *et al.*, 1986; Goedert *et al.*, 1988), one of the two neuropathologic hallmarks of Alzheimer's disease (AD). Neurofibrillary tangles are intracellular insoluble accumulations of abnormal filaments of hyperphosphorylated tau. In contrast to the distribution of β-amyloid-containing senile plaques, the other hallmark brain lesion in AD, the number of neurofibrillary tangles closely correlates with the degree of neuronal loss and severity of

dementia (Arriagada *et al.*, 1992; Gomez-Isla *et al.*, 1997; Riley, Snowdon & Markesbery, 2002; Giannakopoulos *et al.*, 2003; Guillozet *et al.*, 2003; Ingelsson *et al.*, 2004).

In addition to AD, abnormal tau filaments are also a prominent pathologic feature of many other neurodegenerative diseases including sporadic forms, such as argyrophilic grain disease (AGD), corticobasal degeneration (CBD), Pick's disease (PiD), and progressive supranuclear palsy (PSP), and familial forms collectively known as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Lee, Goedert & Trojanowski, 2001). These and other diseases that feature tau pathology have collectively become known as 'tauopathies'. Although the phosphorylation status of tau in these tauopathies is similar to that in AD (i.e. hyperphosphorylated), the composition of the filaments are different (Lee, Goedert & Trojanowski, 2001). In AD, neurofibrillary tangles are made up of all six tau isoforms. In contrast, tau filaments in AGD, CBD, PSP and some cases of FTDP-17 are composed primarily of 4R isoforms, whereas in PiD and other cases of FTDP-17, 3R isoforms predominate (Arai *et al.*, 2001; Tolnay *et al.*, 2002; Mott *et al.*, 2005; Yoshida, 2006). Hence, the role of tau in neurodegenerative disease is thought to relate to a dysregulation not just of tau phosphorylation, but also of alternative splicing.

7.1.3: Aberrant Tau Splicing as a Potential Pathogenic Mechanism in Neurodegenerative Disease

The hypothesis that aberrant splicing of exon 10 of *MAPT* can play a primary pathogenic role in neurodegenerative disease is supported by the identification of several rare splice site mutations within or flanking exon 10 that cause FTDP-17 (Goedert, 2005; Goedert & Jakes, 2005). The mutations are believed to destabilise a putative RNA stem-loop structure which is thought to regulate exon 10 splicing (Hutton *et al.*, 1998; Spillantini *et al.*, 1998).

The majority of these mutations increase exon 10 inclusion and raise the 4R:3R mRNA ratio *in vivo* by two- to six-fold (Connel *et al.*, 2005; Hutton *et al.*, 1998; Spillantini *et al.*, 1998). Mutations that markedly reduce or abolish exon 10 inclusion *in vitro* have also been identified in a small number of FTD families (D'Souza *et al.*, 1999; Stanford *et al.*, 2003).

The relative abundance of 4R and 3R transcripts has also been examined in sporadic tauopathies. Consistent with the composition of neurofibrillary tangles, a number of studies have found no differences in abundance of 4R and 3R transcripts between patients with AD and normal individuals (Goedert *et al.* 1989a; Chambers *et al.*, 1999; Boutajangout *et al.*, 2004; Connel *et al.*, 2005; Ingelsson *et al.*, 2006), although others have found a shift towards increased levels of 4R isoforms (Yasojima *et al.*, 1999; Umeda *et al.*, 2004; Ginsberg *et al.*, 2006, Glatz *et al.*, 2006). With regards to the 4R tauopathies, CBD and PSP, the data are consistent, showing increased abundance of 4R mRNAs relative to 3R mRNAs, particularly in brain regions affected by these diseases (Chambers *et al.*, 1999; Takanashi *et al.*, 2002; Umeda *et al.*, 2004). However, in PiD, a 3R tauopathy, the data have been mixed with some cases showing 4R:3R ratios of >1:1 and others with ratios of <1:1 (Umeda *et al.*, 2004; Connell *et al.*, 2005).

That several FTDP-17 mutations that affect splicing do not change the coded amino acid sequence (except through their splicing effect) provides strong evidence that an imbalance of 3R and 4R tau isoforms is sufficient to cause disease. However, despite extensive resequencing, coding or splicing mutations in *MAPT* have not been identified in the overwhelming majority of sporadic tauopathy cases (Bonifati *et al.*, 1999; Roks *et al.*, 1999; Morris *et al.*, 2002; Pastor *et al.*, 2002; Zekanowski *et al.*, 2003; Stanford *et al.*, 2004; Williams *et al.*, 2007). Moreover, the failure of some studies to detect an imbalance

of splice forms in AD, and the inconsistent results regarding PiD, suggests that tau-associated mechanisms not related to alternative splicing may also play an important role in triggering the neurodegenerative process.

7.1.4: *MAPT* as a Risk Factor for Sporadic Neurodegenerative Disease

An association of *MAPT* with sporadic neurodegenerative disease was first reported when one allele (A0) and the homozygous genotype (A0/A0) of a dinucleotide repeat polymorphism in intron 9 were found to be significantly over-represented in patients with PSP when compared with normal controls (Conrad *et al.*, 1997). The association with PSP was then replicated (Bennett *et al.*, 1998; Higgins *et al.*, 1998; Oliva *et al.*, 1998; Hoenicka *et al.*, 1999; Morris *et al.*, 1999), and extended to a haplotype that covered the entire *MAPT* locus (Baker *et al.*, 1999). The haplotype, designated H1, and it allelic counterpart, H2, were found to be definable by eight common SNPs and a 238 bp insertion / deletion polymorphism, all in complete linkage disequilibrium (LD) with each other and the dinucleotide repeat (Baker *et al.*, 1999).

The full extent of LD at the *MAPT* locus has now been defined and found to cover a maximal region of ~1.8 Mb (Pittman *et al.*, 2004). In addition to *MAPT*, this region covers several other genes including *STH* (Saitohin, situated within intron 9 of *MAPT*), *CRHR1* (corticotrophin releasing hormone receptor 1), *NSF* (*N*-ethylmaleimide sensitive factor), *IMP5* (a presenilin homologue), and *LOC284058* (a gene of unknown function). It is believed that the H1 and H2 haplotypes arose approximately 3 million years ago and correspond to a 900 kb inversion on 17q21, which had the effect of suppressing recombination between the two inverted regions (Stefansson *et al.*, 2005). Interestingly, the inverted H2 haplotype is only found in Europeans and populations with a history of

European admixture. In Caucasians, H2 has a frequency of ~20% (Evans *et al.*, 2004; Stefansson *et al.*, 2005), whereas in African, Asian and Native American populations it is either very rare or completely absent (Evans *et al.*, 2004; Stefansson *et al.*, 2005).

The association of H1 with PSP has now been replicated in several independent Caucasian populations with typical allele frequencies of >90% in patients and 70–80% in controls (Pittman, Fung & de Silva *et al.*, 2006). In addition to PSP, the H1 and H2 haplotypes have been analysed in relation to several other neurodegenerative diseases. Similar to PSP, the H1 haplotype is over-represented in patients with CBD (Di Maria *et al.*, 2000; Houlden *et al.*, 2001; Pastor *et al.*, 2004). With regards AD, the data have been largely negative (Roks *et al.*, 1999; Baker *et al.*, 2000; Kwon *et al.*, 2000; Pastor *et al.*, 2000; Russ *et al.*, 2001; Cook *et al.*, 2002; Green *et al.*, 2002; Verpillat *et al.*, 2002b; Clark *et al.*, 2003; Oliveira *et al.*, 2003; Peplonska *et al.*, 2003; Streffer *et al.*, 2003; Forero *et al.*, 2006; Zuo *et al.*, 2006), although two reports describe an increased risk associated with an interaction between H1 and the *APOE* ε4 allele (Lilius *et al.*, 1999; Bullido *et al.*, 2000), and some studies have found an association with H2 (Conrad *et al* 2002, 2004, Combarros *et al.*, 2003; Seripa *et al.*, 2004). Associations with H1 dependent on (Ingelson *et al.*, 2001; Verpillat *et al.*, 2002a) and independent of (Hughes, Mann & Pickering-Brown, 2003) *APOE* genotype have also been reported for sporadic frontotemporal dementia (FTD), although other studies have found no association (Morris *et al.*, 1999; Sobrido *et al.*, 2003; Bernardi *et al.*, 2006). Some support for a risk effect of H1 in AGD has also been found (Ishizawa *et al.*, 2002, Togo *et al.*, 2002, Conrad *et al.*, 2004). Interestingly, there is growing evidence that H1 may also increase risk for Parkinson's disease (PD) (Pastor *et al.*, 2000; Golbe *et al.*, 2001; Maraganore *et al.*, 2001; Martin *et al.*, 2001; Farrer *et al.*, 2002; Healy *et al.*, 2004; Kwok *et al.*, 2004; Levecque *et al.*, 2004; Skipper *et al.*, 2004;

Fidani *et al.*, 2006). Despite reports of conflicting results (Morris *et al.*, 1999; de Silva *et al.*, 2002; Clark *et al.*, 2003; Peplonska *et al.*, 2003; Zappia *et al.*, 2003; Johansson *et al.*, 2005; Rademakers *et al.*, 2006), meta-analysis supports the association, particularly for the H1/H1 genotype (Healy *et al.*, 2004; Zhang *et al.*, 2005a). These findings are surprising as PD is not generally considered a tauopathy, although tau itself is a minor constituent of the Lewy body, the main neuropathologic hallmark of the disease (Arima *et al.*, 1999; Ishizawa *et al.*, 2003).

Although the association of H1 with the mentioned neurodegenerative diseases is not specific to any one gene within the extended region of LD, the prominent tau pathology that characterises the majority of these disorders points directly to *MAPT*. To aid refinement of susceptibility loci, a number of studies have further explored the haplotypic diversity of *MAPT* in representative Caucasian populations. These studies identified a single H2 haplotype and multiple H1 haplotypes, and determined that only one of the common H1 haplotypes, designated H1C (or H1B), is highly significantly associated with PSP (Pittman *et al.*, 2005; Rademakers *et al.*, 2005). In one study, the region of *MAPT* with the strongest association stretches ~54 kb from intron 0 to intron 9 (Pittman *et al.*, 2005). In the other, the strongest association signal came from an overlapping 22 kb region from intron 0 to exon 1 (Rademakers *et al.*, 2005). In both studies, SNP rs242557, a H1-specific variant in intron 0 and part of the H1C haplotype, showed particularly strong evidence for association, with the A allele being over-represented in PSP cases (Pittman *et al.*, 2005; Rademakers *et al.*, 2005).

Recently, the H1C haplotype was suggested also as a risk factor for late-onset AD (Myers *et al.*, 2005). Moreover, efforts to refine the H1 haplotype association in PD in Norwegian

and Greek populations have narrowed the susceptibility locus to a 90 kb region from intron 0 to intron 4 (Skipper *et al.*, 2004; Fidani *et al.*, 2006). However, in French and Belgian populations, early-onset PD was significantly associated with a 3.5 kb region around exon 10, whilst late-onset PD was associated with a 1 kb region upstream of exon 1 (Rademakers *et al.*, 2006). In the Finnish population, an association between PD and the PSP-associated SNP rs242557 was detected (Fung *et al.*, 2006). A significant association between this SNP and CBD has also been reported (Pittman *et al.*, 2005).

Taken as a whole, the association data suggest that the same variants in *MAPT* may confer risk across diagnoses to several tau-related neurodegenerative diseases. In the absence of coding mutations that alter protein structure, it is assumed that the putative susceptibility variants are regulatory, affecting transcription and / or alternative splicing of *MAPT*.

7.1.5: Genetic Regulation of *MAPT* Expression

Resequencing of the *MAPT* promoter region has identified a series of potentially functional biallelic SNPs and deletion / insertion polymorphisms that segregate with the H1 and H2 haplotypes (Ezquerra *et al.*, 1999; de Silva *et al.*, 2001; Rademakers *et al.*, 2002; Kwok *et al.*, 2004). In luciferase reporter assays, the transcriptional activity of each promoter haplotype was found to be significantly different, with the H1 variant driving ~1.2 fold higher expression than the H2 variant in two human cell lines (Kwok *et al.*, 2004). Given these findings, it is a reasonable hypothesis that variants in H1C, the only H1 haplotype associated with disease, might increase transcriptional activity to an even higher degree.

The H1C-defining SNP rs242557 is located within a 182 bp region that is highly conserved from rats to humans, and is predicted to alter the binding site for two *trans*-regulatory

proteins (Rademakers *et al.*, 2005). In reporter gene assays in which this SNP was cloned upstream of the *MAPT* H1 promoter, it was the non-risk G allele that showed the highest transcriptional activity (Rademakers *et al.*, 2005). However, in a more biologically relevant study in which the SNP was cloned downstream of the H1 promoter, in accordance with expectations, the risk A allele was associated with 2.7 fold higher transcriptional activity than the G allele (Pittman *et al.*, 2006). Moreover, the H1 promoter with the A allele had 4.2 fold higher activity than the H2 promoter with the G allele (Pittman *et al.*, 2006). Together, these results suggest that the pathogenic basis of the H1 and H1C associations with neurodegenerative disease, may, at least in part, be related to an overall increase in *MAPT* expression.

7.1.6: The Present Study

The H1 and H2 alleles of *MAPT* encode identical tau proteins; hence associations between H1, H1C and SNP rs242557 and neurodegenerative diseases may be mediated by changes in *MAPT* expression, including alternative splicing of exon 10. The present study investigated this hypothesis using assays of relative allelic expression. Three assays targeted all *MAPT* mRNAs and a further assay specifically targeted at those containing exon 10 were applied to a total of 39 H1 / H2 heterozygotes. Samples were additionally genotyped for SNP rs242557 and other SNPs that define the H1C haplotype to assess any effect these variants might have. Furthermore, given its importance as a risk factor for many neurodegenerative diseases, an effect of age was specifically checked for as well.

7.2: Materials and Methods

Brain samples and the general methods used for DNA and RNA processing, genotyping, and the relative allelic expression assay are described in more detail in Chapter 2.

7.2.1: Samples

Post-mortem brain tissue derived from frontal, temporal or parietal cortex of 126 unrelated anonymised individuals with no known history of neurodegenerative disease obtained from three sources (the MRC London Brain Bank for Neurodegenerative Diseases, the Stanley Medical Research Institute, and the Karolinska Institute) were used in this study. For each tissue sample, genomic DNA and total RNA were extracted by standard procedures. RNA was then treated with DNAse and reverse transcribed to cDNA.

7.2.2: Genotyping

PCR primers for SNPs used for relative allelic expression analysis (rs1052551, rs1052553, rs17652121 and rs9468), and SNPs rs242557, rs2471738 and rs7521, were designed by use of the Primer3 program (Rozen & Skaletsky, 2000). PCR was carried out in a total reaction volume of 12 μl containing 48 ng of genomic DNA, and consisted of 35 cycles. Genotyping was carried out by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and primers designed by use of the FP Primer program (Ivanov *et al.*, 2004). All primer sequences are shown in Table 7.1, which, where relevant, also shows the annealing temperature used during PCR cycling.

Polymorphism	Primer	Sequence (5'–3')	Ta (°C)
rs1052551*	F:	GGGCTGATGGTAAAACGAAG	60
	R:	CAGAGCTGGGTGGTGTCTTT	
	EXT:	GGTGTCTTTGGAGCGGG	-
rs1052553*	F:	GATCGCAGCGGCTACAG	60
	R:	TTCTTCAGGTCTGGCATGG	
	EXT:	GAGCCCAAGAAGGTGGC	-
rs17652121*	F:	CCATGCCAGACCTGAAGAA	60
	R:	CCCGGGACGTGTTTGATATT	
	EXT:	CCATGCCAGACCTGAAGAA	-
rs9468*	F:	AAGCAGGGTTTGTGATCAGG	58
	R:	AATTAACCGAACTGCGAGGA	
	EXT:	TCTCATTCTCTCCTCTCCAC	-
rs242557	F:	TGATGATGCATGGACCTCTC	58
	R:	CTGTGAGATCATCCCCTGGT	
	EXT:	AAAACCGTGTCCTGGTG	-
rs2471738	F:	GGTTAGAGACCTTGGGGAAA	58
	R:	GAATGAAAGTCACGGTGGTC	
	EXT:	GGAAATAACTCACAGGTGACC	-
rs7521	F:	CGTGTCCCATCTACAGACCT	58
	R:	GCCAGAAATAGTCCTGCTCA	
	EXT:	GTCCCATCTACAGACCTGC	-

Table 7.1: Details of primers used in the study of the *MAPT* gene. Asterisks indicate SNPs used for relative allelic expression analysis. F = forward primer, R = reverse primer, EXT = extension primer, Ta = annealing temperature used during PCR cycling.

7.2.3: Relative Allelic Expression Assay

The SNPs chosen for relative allelic expression analysis were rs1052551, rs1052553 and rs9468. These SNPs are in complete LD with each other, and each defines the H1 and H2 haplotypes perfectly. All three SNPs are located in constitutive exons, and are thus present on all six *MAPT* mRNAs known to be transcribed in human brain (see Figure 7.1).

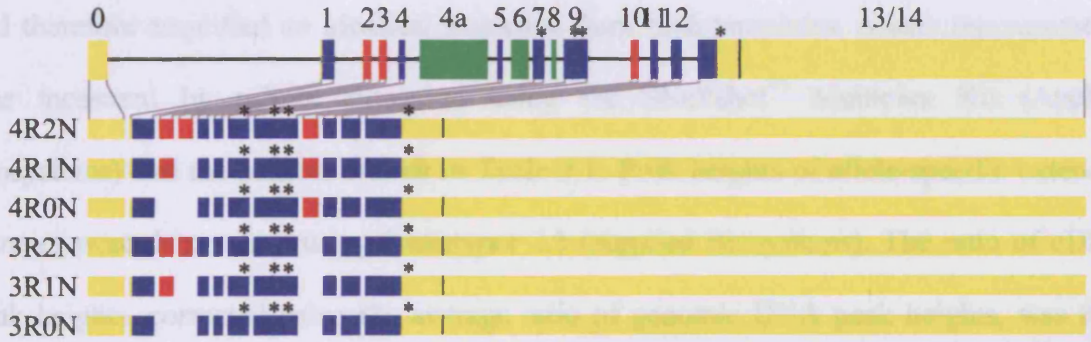

Figure 7.1: The organisation of the *MAPT* gene and its six mRNA transcripts expressed in brain. 4R2N, 4R1N and 4R0N = isoforms containing four repeats and two (NM_005910), one (NM_001123067) or no (NM_016834) N-terminal domains respectively. 3R2N, 3R1N and 3R0N = isoforms containing three repeats and two, one or no (NM_016841) N-terminal domains respectively. Yellow rectangles represent 5' and 3' untranslated regions (UTRs), blue rectangles represent protein-coding exons, red rectangles represent alternatively spliced exons, and green rectangles represent exons not expressed in the brain. The black vertical line indicates a shorter 3' UTR present in some transcripts. Asterisks indicate the positions of assayed SNPs rs1052551 in exon 7, rs1052553 and rs17652121 in exon 9 and rs9468 in exon 13/14.

Genomic DNA from all brain samples was initially genotyped, as described, to identify heterozygotes for the H1 and H2 haplotypes. For each heterozygous sample, cDNA produced from two separate reverse transcription reactions, alongside corresponding genomic DNA, was assayed on two separate occasions (i.e. 2 × {2 cDNA + 1 genomic DNA}) at each of the three SNPS. Genomic DNA and cDNA were assayed under identical conditions. This allowed the average allelic ratio obtained from genomic DNA

(representing a 1:1 ratio) to be used to correct allelic ratios obtained from cDNA for any inequalities in allelic representation specific to the assay.

PCR consisted of 35 cycles and was carried out in a total reaction volume of 12 µl containing 48 ng of genomic DNA or 6 µl of cDNA at the standard cDNA concentration (see Chapter 2, section 2.1.8). Primers, shown in Table 7.1, were based on a single exon and therefore amplified an identical sequence from both templates. Allelic representation was measured by primer extension using the SNaPshot™ Multiplex Kit (Applied Biosystems) and the primers shown in Table 7.1. Peak heights of allele-specific extended primers were determined using Genotyper 2.5 (Applied Biosystems). The ratio of cDNA peak heights, corrected using the average ratio of genomic DNA peak heights, was then used to calculate the relative expression of mRNAs transcribed from each parental chromosome in each individual sample.

An assay of relative allelic expression that was specifically targeted at 4R mRNAs was also carried out. The procedure for this assay was similar to that described above, but instead utilised the exon 9 SNP, rs17652121, which also perfectly defines the H1 and H2 haplotypes. For this assay it was necessary to use a reverse primer that was specific for exon 10. Since the forward and reverse primers targeted different exons, genomic DNA was not assayed, which meant that cDNA ratios could not be corrected. However, this did not prevent comparisons of cDNA ratios being made between brain samples with different genotypes.

To investigate potential influences of the H1C haplotype and the A allele of SNP rs242557 on mRNA abundance, cDNA ratios obtained from samples carrying these variants were

compared with ratios obtained from samples that did not. This comparison was made for ratios obtained in the initial three assays, and the assay that was specific for 4R transcripts.

7.2.4: Transcript Characterisation

To verify that exons 7, 9 and 13/14 (the exons tagged by the three SNPs used for allelic expression analysis) are present on the same mRNA transcripts and not alternatively spliced, RT-PCR and 3' RACE PCR were carried out. High quality human brain RNA (Ambion) was reverse transcribed into cDNA using an oligo$(dT)_{17}$ adapter primer. Using the cDNA as template, *MAPT* transcripts were amplified using the three sets of primers shown in Table 7.2.

Target	Primer	Sequence (5'–3')	Ta (°C)
Exon 7 – Exon 13/14	F:	GGGCTGATGGTAAAACGAAG	60
	R:	AATTAACCGAACTGCGAGGA	
Exon 7 – Poly(A)	GSP 1:	GGGCTGATGGTAAAACGAAG	60
	GSP 2:	ACCAGGATTCCAGCAAAAAC	
	AP:	GAGTCCAGTCGACACAGAGC	
Exon 9 – Poly(A)	GSP 1:	GATCGCAGCGGCTACAG	60
	GSP 2:	TGGTCCGTACTCCACCCA	
	AP:	GAGTCCAGTCGACACAGAGC	

Table 7.2: Details of primers used to characterise *MAPT* mRNA transcripts. F = forward primer, R = reverse primer, GSP = gene-specific primer, AP = adapter primer, Ta = annealing temperature used during PCR cycling.

To increase specificity, 3'RACE PCR products were diluted 1 in 1,000 and reamplified using a second nested gene-specific primer. Products were then separated and sized by agarose gel electrophoresis, excised from the gel, purified, and sequenced. Sequencing was

performed in the 5' to 3' direction by the fluorescent dideoxy method using the BigDye® Terminator Kit (Applied Biosystems).

7.3: Results

7.3.1: Assayed Samples

A total of 39 genotyped individuals were heterozygous for the expressed H1 / H2-defining SNPs rs1052551, rs1052553, and rs9468 and therefore informative for allelic expression analysis. Of these, 17 were heterozygous for the putative functional variant rs242557, and of these, eight carried the H1C haplotype as further defined by SNPs rs2471738 and rs7521. The mean age at death was 54 years (SD = 20.44, range = 19–102), 28 were male, and 11 were female. None of the individuals had a known history of neurodegenerative disease. Samples were derived from frontal (n = 29), temporal (n = 5), and parietal (n = 5) brain tissue.

7.3.2: Relative Allelic Expression: All *MAPT* mRNA Transcripts

Analysis of cDNA ratios showed good reproducibility, with average coefficients of variation (SD / mean) of 0.05, 0.07, and 0.06 for assays at SNPs rs1052551, rs1052553 and rs9468 respectively. The relative expression of H1 and H2 mRNAs as measured in each SNP assay showed some variation, however, in each the mean ratio was close to 1:1 (Figures 7.2 and 7.3). Individual differences in allelic expression were evident, with some showing an overall higher expression of H1 versus H2 mRNAs, and others showing an opposite allelic pattern. Across the three independent SNP assays, cDNA ratios were significantly positively correlated (Table 7.3).

There was no significant difference in H1:H2 cDNA ratios between individuals carrying the H1C haplotype and individuals carrying other H1 haplotypes in any of the three assays

SNP	rs1052553		rs9468	
Assay	*r*	*P*	*r*	*P*
rs1052551	$^{+}0.61$	5.18×10^{-5}	$^{+}0.62$	3.73×10^{-5}
rs1052553	-	-	$^{+}0.50$	0.002

Table 7.3: Correlation between cDNA ratios as measured at three *MAPT* SNPS.

($P \geq 0.76$; Figure 7.2). Similarly, ratios did not significantly differ according to homozygosity and heterozygosity at SNP rs242557 in any assay ($P \geq 0.20$; Figure 7.3). These analyses had 80% power to detect a ratio difference between H1C non-carriers and carriers of 0.09, 0.20 and 0.10 respectively in each of the SNP rs1052551, rs1052553 and rs9468 assays ($P = 0.05$). Between homozygotes and heterozygotes for SNP rs242557, the corresponding power was 0.08, 0.12 and 0.09 respectively for each assay ($P = 0.05$). Furthermore, cDNA ratios did not significantly differ between the same genotypic

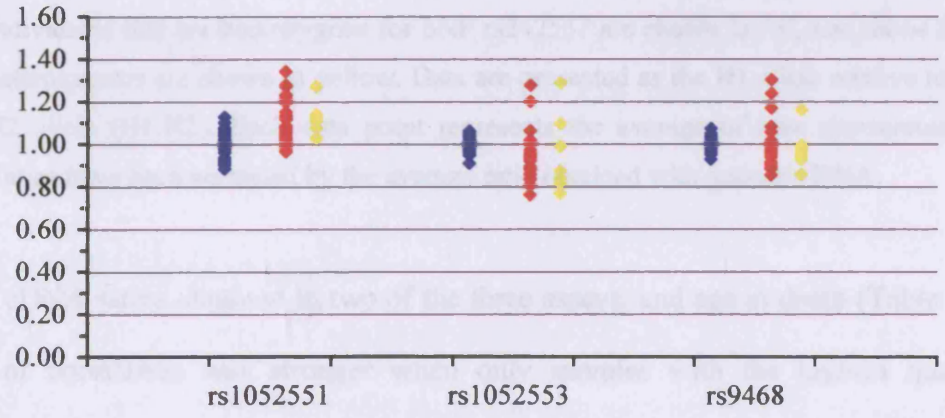

Figure 7.2: Scatter plot showing allelic ratios for genomic DNA (blue) and cDNA derived from 39 individuals heterozygous for the H1/H2 haplotype. cDNA ratios from individuals that do not carry the H1C haplotype are shown in red, and ratios from those that do are shown in yellow. Data are presented as the H1 allele relative to the H2 allele (H1:H2). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

categories when the analysis was confined to older individuals with age at death >50 years ($P \geq 0.68$ for H1C carriers vs. non-carriers; $P \geq 0.14$ for SNP rs242557 homozygotes vs. heterozygotes).

No significant correlation was observed between cDNA ratios and sex or brain region assayed ($P > 0.05$). However, there was a highly significant negative correlation between

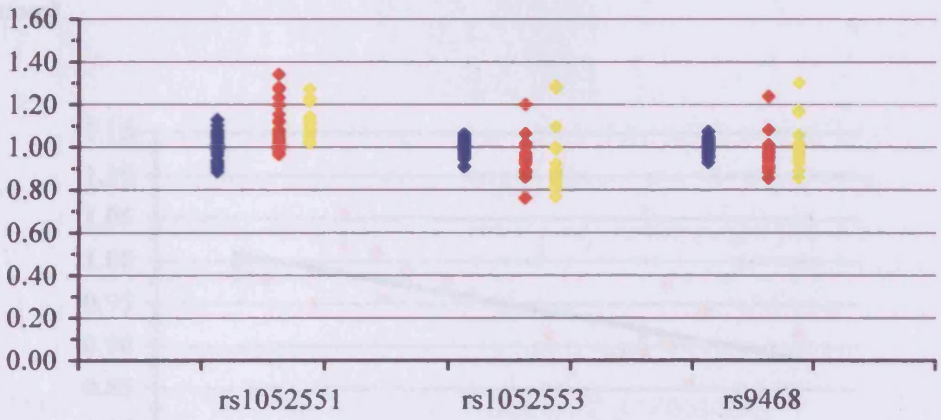

Figure 7.3: Scatter plot showing allelic ratios for genomic DNA (blue) and cDNA derived from 39 individuals heterozygous for the H1/H2 haplotype. cDNA ratios from individuals that are homozygous for SNP rs242557 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the H1 allele relative to the H2 allele (H1:H2). Each data point represents the average of four measurements. Ratios have been corrected by the average ratio obtained with genomic DNA.

H1:H2 cDNA ratios obtained in two of the three assays, and age at death (Table 7.4). The extent of correlation was stronger when only samples with the highest quality data (coefficient of variation of <0.1) were included in the analysis. Furthermore, when only data obtained from samples derived from frontal cortex were included, a significant correlation was observed in all three assays (Table 7.4, Figure 7.4). Significant correlations were not specific to heterozygotes at SNP rs242557.

Samples	*n*	rs1052551		rs1052553		rs9468	
		r	*P*	*r*	*P*	*r*	*P*
All	39	⁻0.26	0.113	⁻0.44	0.006	⁻0.38	0.018
CofV <0.1	35	⁻0.24	0.168	⁻0.53	0.002	⁻0.51	0.003
Frontal CofV <0.1	25	⁻0.45	0.025	⁻0.57	0.005	⁻0.76	3.01×10^{-5}

Table 7.4: Correlation between cDNA ratios as measured at three *MAPT* SNPs with age at death including all samples (top), samples that produced replicate cDNA ratios with coefficient of variation (CofV) <0.1 (middle), and frontal samples with CofV <0.1 (bottom).

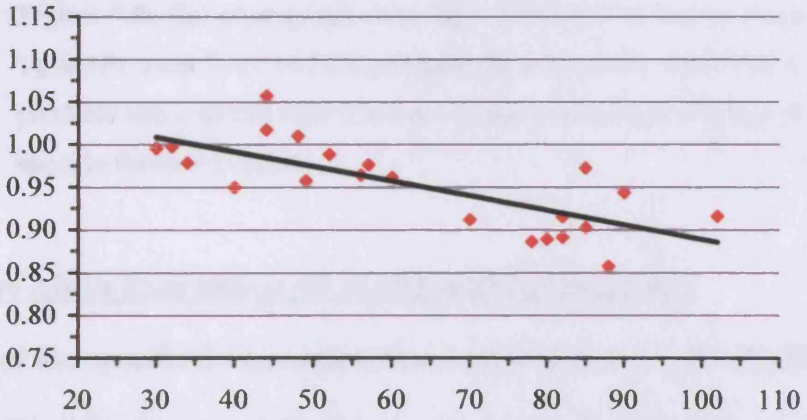

Figure 7.4: Scatter plot showing the correlation of cDNA ratios as measured at rs9468 with age at death (years) in samples derived from frontal cortex that produced replicate ratios with coefficient of variation of <0.1 ($r = ⁻0.76$, $P = 3.01 \times 10^{-5}$).

7.3.3: Transcript Characterisation

PCR using primers to amplify from exon 7 to exon 13/14 of *MAPT* using brain cDNA as template generated two products with sizes that corresponded with the expected sizes for known *MAPT* mRNA isoforms (i.e. 924 and 1,017 bp for 3R and 4R isoforms respectively) (Figure 7.5). Sequencing confirmed their identity and showed that exon 9 was included in both products. 3' RACE PCR using *MAPT*-specific forward primers in exons 7 and 9 also generated two products each that corresponded with known *MAPT* mRNA isoforms (Figure 7.5). Sequencing showed that the two products generated using the exon 7-specific

primer included exons 9 and 13/14, and that the two products generated using the exon 9-specific primer included exon 13/14. All products also included exons 11 and 12.

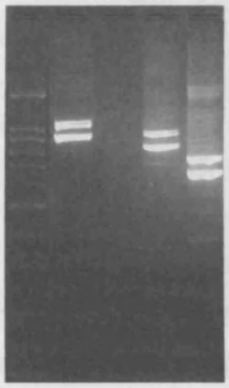

Figure 7.5: Gel photograph showing a 100 bp DNA ladder (lane 1), *MAPT* exon 7–13/14 PCR products (lane 2), and 3' RACE PCR products using nested *MAPT* exon 7- (lane 4) and exon 9- (lane 5) specific forward primers.

7.3.4: Relative Allelic Expression: 4R *MAPT* mRNA Transcripts

In an assay that was specific for transcripts that contained exon 10, H1:H2 cDNA ratios did not significantly differ between individuals carrying the H1C haplotype and individuals carrying other H1 haplotypes ($P = 0.58$; Figure 7.6). Similarly, ratios did not significantly differ according to homozygosity and heterozygosity at SNP rs242557 ($P = 0.11$; Figure 7.7). These analyses had 80% power to detect a ratio difference of 0.10 between H1C non-carriers and carriers, and 0.08 between rs242557 homozygotes and heterozygotes ($P = 0.05$).

No significant correlation was observed between cDNA ratios and sex or brain region assayed ($P > 0.05$). However, in samples with the highest quality data (coefficient of variation of <0.1), there was a significant positive correlation between H1:H2 ratios and age at death ($n = 17$, $r = {}^{+}0.52$, $P = 0.031$; Figure 7.8).

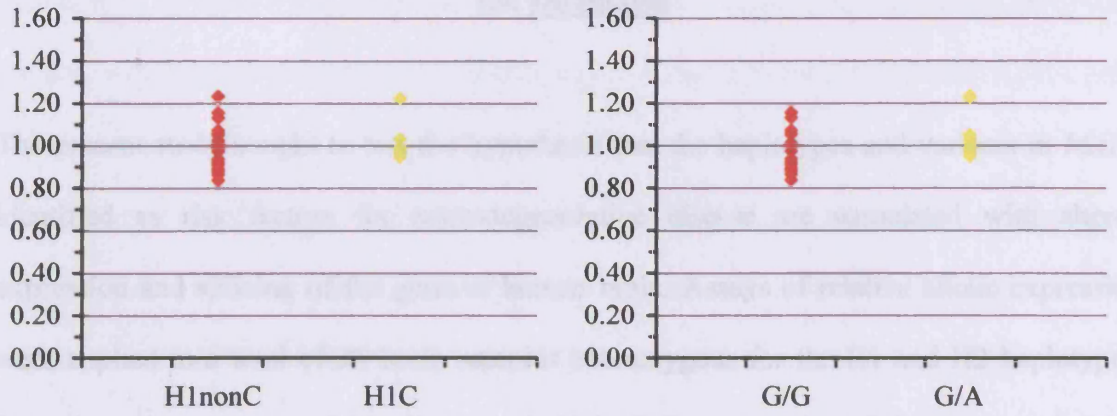

Figure 7.6 (left) & 7.7 (right): Scatter plots showing 4R-specific allelic ratios for cDNA derived from 33 individuals heterozygous for the H1/H2 haplotype. In Figure 7.6, ratios from individuals that do not carry the H1C haplotype are shown in red, and ratios from those that do are shown in yellow. In Figure 7.7, ratios from individuals that are homozygous for SNP rs242557 are shown in red, and ratios from heterozygotes are shown in yellow. Data are presented as the H1 allele relative to the H2 allele (H1:H2). Each data point represents the average of four measurements. Ratios have not been corrected for uneven allelic representation and should thus be considered arbitrary.

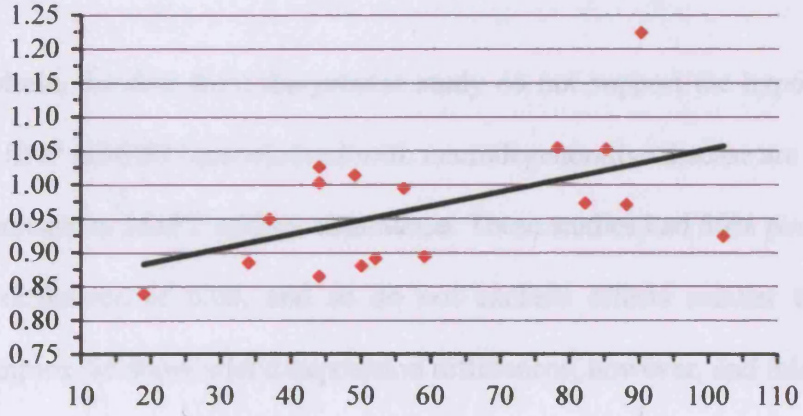

Figure 7.8: Scatter plot showing the correlation of 4R-specific cDNA ratios as measured at rs17652121 with age at death (years) in samples that produced replicate ratios with coefficient of variation of <0.1 (n = 17, r = $^{+}$0.52, P = 0.031). Ratios have not been corrected for uneven allelic representation and should thus be considered arbitrary.

7.4: Discussion

The present study sought to test the hypothesis that the haplotypes and variants in *MAPT* identified as risk factors for neurodegenerative disease are associated with altered expression and splicing of the gene in human brain. Assays of relative allelic expression were applied to a total of 39 brain samples heterozygous for the H1 and H2 haplotypes. Data from three assays did not show marked allelic expression differences, and mean H1:H2 ratios for each assay were close to 1:1. There was no significant difference in ratios obtained from samples carrying the risk H1C haplotype and samples carrying other H1 haplotypes, and no effect of the putatively functional SNP rs242557 was detected. This was apparent not just in assays targeted at all brain-expressed *MAPT* mRNAs, but also in an assay specifically targeted at transcripts containing exon 10 (4R mRNA isoforms).

Taken as a whole, the data from the present study do not support the hypothesis that the H1, H1C and SNP rs242557 associations with neurodegenerative disease are mediated by a pathogenic increase in *MAPT* mRNA abundance. These studies had 80% power to detect a cDNA ratio difference of 0.08, and so do not exclude effects smaller than that size. Individual samples did show allelic expression differences, however, and this may indicate either the presence of *cis*-acting loci that are not well captured by the H1 / H2 division or may reflect inter-individual variance in measurement. The observation that cDNA ratios were significantly positively correlated across the three independent SNP assays suggests the observed differences between individuals are unlikely to be entirely attributable to measurement error.

The assay targeted at 4R mRNAs was carried out to investigate possible genetic influences on alternative splicing of exon 10. The assay did not include genomic DNA, meaning cDNA ratios could not be not corrected, which thus precluded any conclusion about which of the H1 and H2 alleles produced more abundant 4R mRNAs. However, the data from this assay was amenable to between-samples comparisons to assess possible effects of the H1C haplotype and SNP rs242557. That cDNA ratios did not significantly differ between carriers and non-carriers of the H1C haplotype, or between heterozygotes and homozygotes for SNP rs242557, suggests that the putative pathogenic effect of these variants is not related to alternative splicing of exon 10.

The results of the present study are consistent with some of the findings of a recent study that also used measures of relative allelic expression to assess *cis*-acting effects on *MAPT* expression in human brain (Caffrey *et al.*, 2006). In that study, which used primer extension and matrix-assisted laser desorption / ionisation time of flight mass spectrometry, H1 and H2 haplotypes showed no difference in total *MAPT* expression in healthy post-mortem brain. However, the authors additionally found that the H1 haplotype expressed, on average, 29% and 43% more 4R mRNA than H2 in frontal cortex and globus pallidus respectively, although the assay did not fully control for possible allelic biases in PCR efficiency.

A limitation of the methodologies used in that earlier study and in the present work is that neither provides a direct measure the 4R:3R ratio of mRNAs transcribed from each haplotype, which may also be an important factor. Nonetheless, if the reported finding of a 4R-specific effect is genuine, it would suggest that the pathogenic basis of the H1 association with neurodegenerative disease might be, as postulated, related to alternative

splicing of exon 10. More recently, the same research group also reported that H1 expresses ~50% less 2N mRNA (containing exons 2 and 3) than H2 (Caffrey *et al.*, 2008), which may indicate a further effect on splicing of exon 3. Consistent with the present findings, and challenging the pathological relevance of the expression changes, however, in neither of their studies did they detect any significant effect on *MAPT* expression of the H1C haplotype or SNP rs242557.

An effect of the H1C haplotype on brain *MAPT* expression has, however, been detected in one other study, which employed a relative allelic expression assay based on real-time PCR (Myers *et al.*, 2007b). In that study, H1 expression of total *MAPT* mRNA and specifically of 4R mRNA isoforms was found to be, respectively, 11–13% and 25% higher in H1C / H2 heterozygotes than in heterozygotes carrying other H1 haplotypes, suggesting effects on both transcription and alternative splicing. The different results between the studies may possibly be explained by differences in the specific brain regions studied.

Although, taken together, the data generated in the initial three SNP assays of the present study clearly suggest that H1 and H2 produce approximately equal levels of mRNA, the cDNA ratios were spread about a slightly different mean value for each one. A plausible explanation for this was that one or more of the SNPs did not tag all the same transcripts as the others. However, investigation of this possibility by RT-PCR and 3' RACE did not support this hypothesis, but was instead consistent with early studies that showed exons 7, 9 and 13/14 to be constitutively expressed in all brain-expressed *MAPT* mRNAs (Goedert *et al.*, 1989a, 1989b; Andreadis, Brown & Kosik, 1992). An alternative hypothesis is that there are minor differences in RT efficiency that are dependent on the base composition at each SNP site, but this is speculative. Nevertheless, a similar phenomenon was also

observed in the study described above (Caffrey *et al.*, 2006). For instance, whereas the average H1:H2 cDNA ratios for 4R mRNAs was 1.43:1 as measured at SNP rs1052553, the average at SNP rs17652121 in the same tissue was ~1.30:1 (Figure 4 in Caffrey *et al.*, 2006).

A notable difference between the present study and those described above is that the present study included brain samples from both younger and older individuals (range = 19–102 years). By definition, age is a risk factor for neurodegenerative diseases, and it is conceivable that part of its contribution to susceptibility is related to temporal changes in gene expression rather than simply the lifetime accumulation of abnormal processes. For this reason, the present finding that age is correlated with allelic expression of *MAPT* is intriguing. It is well-documented that alternative splicing of exon 10 is modified from foetal stages of life to adulthood (Goedert *et al.*, 1989a, 1989b; Kosik *et al.*, 1989; Goedert & Jakes, 1990). In the present study, the three assays targeted at all *MAPT* mRNA isoforms (i.e. both 3R and 4R isoforms) showed a negative correlation between H1:H2 cDNA ratios and age at death. Thus, with increasing age, H1 produced relatively less mRNA encoding total tau as compared with H2. Conversely, in the assay that specifically targeted 4R mRNAs, H1:H2 cDNA ratios showed a positive correlation with age. Thus, with increasing age, H1 produced relatively more mRNA specifically encoding 4R tau isoforms as compared with H2. Although no equivalent assay targeted specifically at 3R mRNA isoforms was performed, the present data suggest that the H1 haplotype produces a relative increase in 4R tau compared with all other tau isoforms with age. A progressive increase in the 4R:3R ratio of mRNAs generated from H1 may be of relevance to the risk effect of this haplotype on susceptibility to tauopathies such as CBD and PSP, which are associated with neurofibrillary tangles comprised mainly of 4R tau isoforms. Importantly, carriers of H1C

and heterozygotes for SNP rs242557 had similar age ranges as non-carriers and homozygotes, suggesting that the age correlation did not affect the failure to detect effects of these variants.

It is important to emphasise that the results of the present study do not rule out alteration of *MAPT* mRNA abundance as the pathogenic basis of the reported associations. For example, the present study does not exclude effects that are specific to brain regions not tested, particular cell types, or particular mRNA isoforms. However, it is possible that the associated variants may actually influence the regulation of other genes within the vicinity of *MAPT*. Of note is the *STH* gene of unknown function nested within intron 9 of the *MAPT* gene (Conrad *et al.*, 2002). The expression of this gene has been reported to be increased in post-mortem brain from individuals with PSP (Ezquerra *et al.*, 2007) and PD (Tobin *et al.*, 2008) and positively correlated with the ratio of 4R:3R *MAPT* mRNA (Ezquerra *et al.*, 2007). Hence, it is not difficult to imagine that the identified risk variants could have a *cis* effect on *STH* expression, which in turn may act in *trans* to alter splicing of *MAPT* exon 10. A similar *cis* × *trans* effect on *MAPT* transcription is also plausible given the recent identification of natural antisense transcripts that map to the promoter region of the gene (D'Souza *et al.*, 2006; Guo *et al.*, 2006).

An alternative hypothesis is that the identified risk variants might only increase susceptibility in the context of additional disease-specific *trans*-acting factors that interact with *MAPT*. For example, risk variants for AD on the H1 background were recently found to be associated with increased *MAPT* expression, but only in individuals with evidence of β-amyloid deposition (Kauwe *et al.*, 2008). A further hypothesis, with particular reference to the rare tauopathies, CBD and PSP, is that these disorders are caused by pathogenic

regulatory mutations that may have arisen in the context of the identified risk variants but are too rare to have been detected in the present study. Consistent with this, resequencing of *MAPT* has identified intronic mutations on the H1 background in sporadic CBD and PSP cases that were not found in healthy controls (Cruts *et al.*, 2005; Rademakers *et al.*, 2005). Relative allelic expression assays of *MAPT* in brain samples from relevant patient groups could be useful in testing these possibilities.

7.4.1: Conclusions

The present study suggests that haplotypes H1 and H1C and SNP rs242557 do not have a major *cis* effect on total *MAPT* mRNA abundance in normal human brain. The association between these variants and AD, CBD, PSP, PD and other tau-related neurodegenerative diseases may relate to altered regulation of nearby genes, disease-specific *trans* factors, rare mutations, or possibly an effect on specific *MAPT* mRNA isoforms. An effect of age on the relative amount of *MAPT* mRNA produced by H1 compared with H2 was observed, and warrants further investigation.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8: GENERAL DISCUSSION

8.1: Introduction

The series of studies described in this thesis investigated the possibility that genes implicated in psychiatric and neurodegenerative diseases contain regulatory variants that could contribute to disease pathogenesis. In total, 10 genes were investigated. These genes had previously been found to be genetically associated with one or more psychiatric or neurodegenerative diseases, and, in several instances, to show altered expression in post-mortem analyses of brain tissue from individuals affected by such disorders. None of the genes were known to contain protein-coding variants that could fully account for reported disease associations.

Each gene was investigated by one or more assays of relative allelic expression, which are well-suited for the detection of *cis*-regulatory variants with effects on mRNA abundance and for testing individual variants or haplotypes for *cis*-regulatory effects. These assays were applied to a large number of post-mortem brain samples. Although *cis*-regulatory variation was detected in most of the genes assayed, only in two genes (i.e. *RELN* and *MAPT*) was there evidence that this is related to variants associated with disease. In the following sections, the principal findings of each study will be briefly reiterated followed by a more general discussion of the methods and avenues for future work.

8.2: Principal Findings

8.2.1: Disrupted in Schizophrenia 1 (*DISC1*)

The *DISC1* gene was selected for study based on several findings of association with schizophrenia, schizoaffective disorder, and bipolar disorder (Hennah *et al.*, 2006; Chubb *et al.*, 2008). Although *DISC1* is known to contain variants that alter the amino acid sequence of the encoded protein, the majority of reported associations are not fully explicable in terms of these changes. For example, where *DISC1* protein-coding changes have been associated with a psychiatric phenotype, it has usually been as part of a haplotype including non-coding SNPs (as described in Chapter 3, section 3.1.2), suggesting that effects on gene expression or splicing may be involved. A pathogenic role for altered *DISC1* expression was suggested by the finding that *DISC1* expression in lymphoblastoid cells of *DISC1* t(1;11) translocation carriers is approximately halved, which is consistent with haploinsufficiency (Millar *et al.*, 2005).

Out of 65 brain samples assayed in the study described in Chapter 3, only one showed a robust allelic expression difference. In that sample, one gene copy was expressed ~50% higher than the other. Sequencing of the *DISC1* promoter in that sample's genomic DNA revealed the heterozygous presence of an insertion / duplication variant and a TG repeat polymorphism. However, both variants were detected in samples that did not show allelic expression differences, indicating that these variants were not in themselves sufficient to alter expression. That this study did not detect common variants that markedly alter expression of *DISC1* suggests that common *DISC1* haplotypes associated with schizophrenia and bipolar disorder, the HEP3 haplotype in particular (Hennah *et al.*, 2003, 2005; Palo *et al.*, 2007), do not exert their pathogenic effect by altering mRNA abundance.

8.2.2: Reelin (*RELN*)

The *RELN* gene was principally selected for study on the basis of the fairly robust findings of a ~50% reduction in expression of this gene's mRNA and protein in brains of schizophrenic and bipolar patients (Torrey *et al.*, 2005). A similar sized reduction had also been observed in the brains of autistic individuals (Fatemi *et al.*, 2005a). Moreover, *RELN* is located within a consistent linkage region (7q22) for autism (Badner & Gershon, 2002; Trikalinos *et al.*, 2006), and non-coding polymorphisms in the gene had been associated with autism as well as schizophrenia (as described in Chapter 4, section 4.1.4). Together, these findings highlighted the possibility that genetic effects on *RELN* expression may influence susceptibility to these disorders.

Out of 66 brain samples assayed in the study described in Chapter 4, three showed allelic expression differences of ≥20%, of which two were clear outliers. Interestingly, the repeat number of a 5' UTR CGG polymorphism that had previously been associated with autism in some studies (Persico *et al.*, 2001; Zhang *et al.*, 2002; Skaar *et al.*, 2005) was found to be correlated with *RELN* allelic expression differences, suggesting a pathogenic mechanism in the disorder. However, variants in linkage disequilibrium (LD) with this polymorphism were not found to be associated with schizophrenia or bipolar disorder using imputed genotype data, suggesting that any alterations in *RELN* expression that result from the CGG repeat are not relevant to susceptibility to those disorders (with caveats, noted in Chapter 4, about power to detect association). In addition, a SNP reported to be associated with schizophrenia in females (Shifman *et al.*, 2008) was not found to have any influence on allelic expression.

8.2.3: $GABA_A$ Receptor Subunit Genes

A selection of six $GABA_A$ receptor subunit genes (*GABRB1*, *GABRA4*, *GABRA5*, *GABRB3*, *GABRG2* and *GABRG3*) were selected for study based on findings of association with bipolar disorder, and more specifically, RDC-defined schizoaffective disorder, bipolar type (Craddock *et al.*, 2008). This family of receptors plays a major role, directly or indirectly, in probably all brain functions, and alterations in their expression had previously been implicated in schizophrenia and bipolar disorder (as described in Chapter 5, section 5.1.4).

Although, in the study described in Chapter 5, *cis*-regulatory variation in most of these genes was detected, no evidence that the putative susceptibility variants had any influence on mRNA abundance was obtained. The *cis*-regulatory variants that were detected may, however, be relevant to several other disorders in which this family of genes is thought to be important. For example, if identified, the variants in some of the genes may represent good candidates for case-control association studies for autism and epilepsy.

8.2.4: Nitric Oxide Synthase 1 Adapter Protein (*NOS1AP*)

The *NOS1AP* gene was selected for study based on linkage and association findings that implicated it in schizophrenia in a Canadian family sample (Brzustowicz *et al.*, 2000, 2002, 2004). Resequencing had failed to identify any protein-coding variants in the gene (Brzustowicz *et al.*, 2004), and post-mortem analyses had found the gene's short mRNA isoform to show increased expression in schizophrenic and bipolar disorder brain (Xu *et al.*, 2005). Moreover, increased expression of the short, but not the full length, mRNA isoform in brain had been found to be associated with risk alleles of three putative susceptibility SNPs identified in the Canadian families (Xu *et al.*, 2005).

In the study described in Chapter 6, analysis of this gene in 57 brain samples for the full length mRNA isoform and 28 for the short mRNA isoform identified one that showed unequal allelic expression of both isoforms. Evidence was also obtained for a common variant in high LD with the assayed SNP that has a small effect on expression of the full length isoform, but no evidence for common *cis*-regulatory variation that influences expression of the short isoform was found. Genotyping of the three SNPs that showed association with schizophrenia in the Canadian families did not support the hypothesis that those variants influenced expression of either isoform. Based on these findings, it was concluded that the association of those SNPs in *NOS1AP* is probably not mediated by direct effects on mRNA abundance.

8.2.5: Microtubule-Associated Protein Tau (*MAPT*)

The *MAPT* gene was selected for study on the basis of reported association of an extended haplotype (denoted H1) and sub-haplotype (denoted H1C) in this gene with progressive supranuclear palsy (PSP) and other neurodegenerative diseases in which aggregation of the tau protein is a characteristic neuropathologic feature (described in Chapter 7, section 7.1.4). The association data could not be explained by protein-coding changes, as both H1 and its allelic counterpart, H2, encode identical tau proteins and do not include such polymorphisms. Furthermore, resequencing of the gene failed to identify protein coding mutations in the vast majority of cases (as described in section 7.1.3).

In the study described in Chapter 7, a direct relationship between disease-associated haplotypes and variants and *MAPT* expression was not found. However, total *MAPT* expression from the H1 haplotype was found to reduce with age relative to that from H2, while the proportion of transcripts containing exon 10 (encoding 4R tau, the isoform that is

prone to aggregation in PSP and other tauopathies) was found to increase with age from H1 relative to H2. This finding may be of relevance to the aetiology of 4R tauopathies, but requires further investigation.

8.3: Potential Confounds and Limitations

Assays of relative allelic expression were the principle method used in the studies reported in this thesis. Although they are a particularly powerful means of studying *cis*-regulatory phenomena, a number of potential confounds and limitations should be noted.

8.3.1: Relative Allelic Expression Assays Only Measure Effects on mRNA Abundance

It is important to emphasise that the studies presented in this thesis only examined effects on mRNA abundance. It is possible that putative disease susceptibility variants in these genes operate via other effects on gene expression, such as RNA splicing, mRNA transport or translational efficiency, which were not examined in detail in the present series of studies.

Another limitation of relative allelic expression assays is that they measure only the *relative* expression of each gene copy in heterozygotes. Findings of differential allelic expression may not necessarily indicate changes in total expression levels. For instance, it is quite possible that if transcription is more or less efficient from one allele of a gene, *trans*-acting homeostatic mechanisms might restore total expression levels to those required for proper cell function. However, where *cis*-regulatory variants are associated with a disorder, this implies that levels are not adequately restored, at least not in some cells. Indeed, in a polygenic disorder, it is possible that the *trans*-regulators are themselves affected by susceptibility variation, which will impact on their capacity to restore optimal expression.

8.3.2: Genetic or Epigenetic?

Where allelic expression differences are seen, it is not immediately obvious whether this is the result of genetic sequence variation or allele-specific epigenetic modification. However, where differences in allelic expression are found to associate with a specific genetic polymorphism, these changes can be reasonably attributed to a primary genetic change, although the effects may additionally be mediated by epigenetic modification. Additional methods such as bisulphite sequencing may indicate epigenetic mechanisms.

8.3.3: Potential Allelic Bias in Reverse Transcription

In the investigations reported in this thesis, potential allelic biases in PCR or primer extension efficiency when assaying cDNA were controlled for by assaying genomic DNA under identical conditions. However, it was not possible to apply an equivalent measure to control for potential allelic biases in RT efficiency. Since this would manifest as a general shift in allelic expression in all samples heterozygous for the assayed SNP, it would be difficult to distinguish from regulatory effects arising from the SNP itself or from *cis*-regulatory variation in high LD with it. Gene assays showing this pattern of allelic expression should therefore be interpreted with this caveat.

8.3.4: The Choice of SNP

The specific mRNA isoforms targeted in relative allelic expression assays is entirely dependent on the choice of SNP used to distinguish between mRNAs transcribed from each gene copy and the primers that are used to amplify it in PCR. Therefore if *cis*-regulatory variants affect an mRNA isoform that does not contain the SNP, then the effects of those variants will not be detected. In contrast, if *cis*-regulatory variants have an effect on specific mRNA isoforms, then the simultaneous assay of additional mRNA isoforms

that are not influenced by those variants may cause the size of their effect to be under-estimated or indeed to go undetected.

A further limitation with the use of SNPs in exons is that if an allelic expression difference is detected, it is usually unclear whether the difference is a result of an effect on transcription, mRNA stability or alternative splicing. In theory, this limitation can be partly addressed by using a SNP within an intron. Since this will assay only unspliced RNA, any differences in relative allelic expression will most likely arise from effects on transcription. However, assays that target pre-mRNA are reportedly more variable and more likely to fail probably due to the fact that pre-mRNA represents only a small fraction of total RNA preparations (Pastinen & Hudson, 2004). This approach may also be more susceptible to bias towards a 1:1 ratio arising from any residual genomic DNA contamination if present.

8.3.5: Context Specificity of Allelic Expression Differences

The detection of a *cis*-regulatory variant that affects expression of a gene in a tissue or cell is dependent not only on expression of that gene, but also its regulation by *trans* factors that act at the variant regulatory sequence. Since regulation of a gene can be tissue- or cell type-specific and may be highly dynamic, it is expected that many *cis*-regulatory polymorphisms will only give rise to an allelic expression difference in certain tissues or cell types or under certain conditions. This is illustrated in a study of the *BMP5* gene, in which relative allelic expression was analysed across a variety of mesenchymal synovial joint tissues within a number of individuals (Wilkins *et al.*, 2007). Out of 16 individuals assayed, 11 showed relative allelic expression differences that were seen in some but not all of the tissues analysed. Where relative allelic expression was seen in more than one tissue, the allelic ratios were often significantly different between them, and in one

individual, the allele that was most abundant in ligament and synovial tissue was the least abundant in cartilage tissue.

In light of these findings, since, in the studies reported in this thesis, tissue from only one brain region was assayed in each individual and that tissue from only three different brain regions was assayed in total, it is quite possible that there were *cis*-regulatory variants in the samples assayed that were not detected. Furthermore, brain tissue is made up of several cell types, including glia as well as neurons, and it is not known from which of these types allelic expression differences arose. It is also possible that allelic expression differences in one type of cell might be 'diluted out' by the simultaneous assay of mRNAs from the other cell types. Moreover, brain samples from different individuals, as well as differing in brain region, may also differ in the cellular makeup of the tissue. Whilst this will not give rise to false positive results, it may mean that true effects of haplotypes on expression when compared between groups of individuals with and without that haplotype may go undetected due to confounding.

As well as being tissue- or cell type-specific, *cis*-regulatory effects can also be specific to certain developmental time points (e.g. Parker-Katiraee *et al.*, 2008). This may be particularly relevant to neurodevelopmental disorders such as autism and schizophrenia. Similarly, *cis*-regulatory variants might only have an effect in the presence of certain *trans* factors, which may be elevated in disease in the form of additional susceptibility genes or environmental risk factors. The use of control individuals, which constitute the majority of subjects in the current studies, may have reduced power to detect presence of regulatory variation in such circumstances.

8.4: Further Work and Future Prospects

Given the context-specific nature of relative allelic expression, for some genes, it may be necessary to perform relative allelic expression analyses across multiple discrete regions of the human brain, perhaps also at different developmental stages, in order to capture the effects of *cis*-regulatory variation. That allelic expression can differ between brain regions has recently been demonstrated in an assay of the *RGS4* gene (N. Bray, personal communication, 2009). Assay of this gene across 10 discrete brain regions within 12 individuals showed clear regional differences, with the most pronounced effects seen in sub-cortical regions of the brain. It may be possible to localise the effects of *cis*-regulatory variation even further using techniques for isolating particular cell populations, such as laser capture microdissection.

It may also be instructive to compare relative allelic expression between normal individuals and those affected with disease. According to the common disease common variant hypothesis, it is unlikely that affected individuals will carry *cis*-regulatory variants that are absent in reasonable sized samples of unaffected individuals, but examining cases may bring to light *cis*-regulatory variants that are only active in the disease state, perhaps as a result of gene × environment or gene × gene interactions.

An interesting observation from the work presented in this thesis is that nearly all the genes tested had at least one brain sample that showed allelic expression differences, although in several genes (e.g. *DISC1*, *NOS1AP*, *RELN*, *GABRA4*) such variants were uncommon. Where just one observation of allelic expression difference is seen, it is difficult to discern its relevance to disease susceptibility, since it is unlikely to strongly correlate with the

variants showing disease association in current studies, which typically have minor allele frequencies of >0.05. In such circumstances, extensive sequencing may reveal the causative regulatory variants, although, unless they have a fairly profound effect on sequence (e.g. a copy number variant), they may be difficult to recognise as such. If identified, such variants could be tested for disease association in large samples of cases and controls to test their pathogenic role. Alternatively, under a multiple rare variant hypothesis, the relative allelic expression method could be used to screen candidate genes for *cis*-regulatory variants of strong effect in large case-control samples, with the frequency of these variants compared between groups.

In addition to testing association between specific haplotypes and gene expression, relative allelic expression assays are of demonstrated utility for *defining* common regulatory haplotypes (Pastinen *et al.*, 2005). Thus, allelic expression can be assessed in a series of samples and this then compared with genotype at SNPs across the gene. The identification of functional regulatory variation will enable direct tests of association with disease and related phenotypes (Hudson, 2003; Bray, 2008).

An important step for relative allelic expression assays would be to scale-up the process for simultaneous assay of multiple genes. Although microarrays have been used to assay large numbers of genes in parallel (e.g. Lo *et al.*, 2003), these generally have a large level of noise, and can therefore only detect larger allelic expression differences. With recent developments in next generation sequencing technologies, large scale allelic expression analyses are now possible. The so called RNA-Seq approach (reviewed in Wang, Gerstein & Snyder, 2009) enables transcriptome-wide analysis of gene expression. Each RNA molecule is digitally quantified, meaning highly accurate absolute quantification of

individual RNA isoforms is possible with little noise. Furthermore, because it is based on sequencing, mRNAs from each chromosome can be distinguished in SNP heterozygotes, allowing for highly accurate measures of relative allelic expression.

8.5: Concluding Remarks

The series of studies presented in this thesis explored *cis*-regulatory variation in 10 candidate susceptibility genes for psychiatric and neurodegenerative disease in human brain tissue. Differences in allelic expression were observed in nearly all of these genes, consistent with previous observations that *cis*-regulatory variation affects expression of a large proportion of human genes. However, in only a small proportion of the genes analysed was allelic expression found to be related to variants or haplotypes associated with disease, making the pathological significance of most of the detected regulatory variation unknown. Future studies, using higher throughput technologies, may allow the allelic expression method to be applied on a genome-wide scale in multiple tissues. The data that this approach would produce could then be integrated with genome-wide association data based upon some of the principles applied in this thesis.

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