Gene Expression and Genetic Association in Bipolar Disorder

Catherine S. Sears

A thesis submitted for the degree of Doctor of Philosophy At the University of Otago, Dunedin New Zealand

October 2011
ABSTRACT

Bipolar disorder is a serious psychiatric condition characterised by alternating episodes of major depression with mania or hypomania. The condition is relatively common, affecting 2-3% of the general population, and is generally treated with mood stabilising drugs such as lithium, valproate and carbamazepine. While considerable progress has been made over the last twenty years, both the pathology of bipolar disorder and the mechanisms by which mood-stabilising drugs relieve the symptoms of the disorder are not as yet fully understood. The high heritability of the condition, as evidenced in twin and adoption studies, suggests a genetic aetiology. The time scale of effective clinical treatment of bipolar disorder suggests that mood stabilising drugs may cause long term neural changes mediated by differences in gene regulation. Identifying genes that are differentially regulated by these medications may therefore provide insight into factors contributing to bipolar disorder and the mechanism of action of mood stabilisers.

Transcript quantitation was used to measure the expression of 20 genes across three human neural cell lines, SK-N-SH, SH-SY5Y and SK-N-DZ. Each cell line had been cultured in therapeutic concentrations of two mood stabilising drugs, valproate and carbamazepine. The genes measured were selected from a panel of genes previously established to be significantly up or downregulated after lithium treatment by a previously performed serial analysis of gene expression screen, or alternatively selected from the literature as promising candidates because of significant expression changes after lithium treatment or involvement in a lithium-affected signal transduction pathway. The brain derived neurotrophic factor (BDNF) gene was found to be consistently upregulated across all six cell line/treatment combinations while the other genes showed inconsistent regulation.

Lymphoblast cell lines were used as a model in which to attempt to replicate the changes in expression of four of the 20 initial genes – brain derived neurotrophic factor (BDNF), midkine (MDK), ras-related protein (RAB7A) and 14-3-3γ (YWHAG). Ten lymphoblast cell lines were used, five from
individuals with bipolar disorder and five from unaffected control individuals. These cell lines were treated with a mood stabiliser, either lithium, valproate or carbamazepine, and differences in gene expression between treated cells and controls were measured. No consistent changes in regulation were found in any of the four genes analysed, and no significant differences were found between the control group and the bipolar group.

Single nucleotide polymorphisms from across the genes BDNF, RAB7A and YWHAG were examined for evidence of association with bipolar disorder. The polymorphisms were genotyped in the individuals of the South Island Bipolar Study (SIBS), a resource containing DNA and diagnostic information from individuals with bipolar disorder and their first and second-degree relatives, all sourced from the South Island of New Zealand. The genotypes were analysed for association with bipolar disorder using family based association testing. No polymorphisms of YWHAG showed association with bipolar disorder in the SIBS cohort. Four single nucleotide polymorphisms of BDNF were found to be associated with bipolar disorder after correction for multiple testing. Two of these associations had previously been reported in the literature, while the other two are novel. A previously unreported association with the rs13081864 SNP of RAB7A was also found. In addition, a haplotype of RAB7A consisting of five polymorphisms was found to be associated with bipolar disorder at p=0.00009.

These findings suggest BDNF and RAB7A may be involved in the pathophysiology of bipolar disorder. This study provides the first evidence that the gene RAB7A may be a contributing factor to the disorder.
ACKNOWLEDGEMENTS

I would like to extend my sincere and immense thanks to my supervisor Dr. Alison Fitches for her support and understanding through what has been a long, often interrupted, and (sometimes literally) crazy process. Her encouragement and wise perspective as well as her deft touch in the lab and skill with the editing pen have played a large part in me being able to finish this thesis and the research that went with it.

Many thanks to Dr. David Markie for more active supervision than he probably thought he was signing up for! Discussing theoretical issues with David helped me retain my enthusiasm for science even when things were going pear-shaped and his technical-statistical know-how proved invaluable many, many times.

A mention must be made of Dr. Robin Olds who conceived of this particular piece of research and took a chance on me having a go at it. While his career took him elsewhere while I was working on this project I remain grateful for his sharp wit and his encouragement in the early days.

Much appreciation goes to the members of the Molecular Pathology Group, past and present. Special thanks must go to Dr. Erin Cawston, who taught me a lot, supported me a lot, and provided a lot of friendship and good laughs. I would like to thank everyone at the Pathology Department of the Dunedin School of Medicine. While naming everyone who provided me with assistance over the years would run to many pages, I would especially like to thank Dr. Aaron Jeffs for his expertise with RT-qPCR analysis, Tim Morgan for his assistance with the microsatellite amplifications, Dr. Al Russell and Dr. Hamish Campbell, who added a lot of laughter to my earlier years in the Pathology Department (as well as being helpful in a science-based fashion on the odd occasion), Dr. Tania Slatter for getting me started on the ELISA work, Rhodri Harfoot and Mike Algie for being generally awesome and helpful, and to Lynne Hananeia, who has helped me out over the years in more ways than I can possibly count.
Much appreciation goes to my parents, who forbore with great patience my decision to become an eternal student, and who provided wonderful, recharging summer breaks for us in the last couple of years that make a huge difference to my little family.

Finally I wish to thank my girls – my daughter, Ella, who was born during the course of this PhD and who has made the last couple of years both crazy and wonderful, and my wife, Summer, who didn’t give up on me at any point.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. II

ACKNOWLEDGEMENTS ............................................................................................ IV

TABLE OF CONTENTS .............................................................................................. VI

LIST OF TABLES .......................................................................................................... XII

LIST OF FIGURES ....................................................................................................... XIII

LIST OF ABBREVIATIONS ........................................................................................... XIV

PUBLICATIONS ............................................................................................................ XV

1. INTRODUCTION ...................................................................................................... 1

1.1. BIPOLAR DISORDER .............................................................................................. 1

1.1.1. DIAGNOSTIC FEATURES OF BIPOLAR DISORDER ...................................... 1

1.1.1.1. Bipolar I Disorder ....................................................................................... 2

1.1.1.2. Bipolar II Disorder ..................................................................................... 2

1.1.1.3. Cyclothymic Disorder ............................................................................... 4

1.1.1.4. Bipolar Disorder Not Otherwise Specified .............................................. 4

1.1.2. MODIFIERS OF BIPOLAR DISORDER ........................................................... 5

1.1.2.1. Mixed Episodes ......................................................................................... 5

1.1.2.2. Rapid Cycling ......................................................................................... 5

1.1.2.3. Paediatric and Adolescent bipolar disorder ............................................. 6

1.1.3. PSYCHIATRIC COMORBIDITY ................................................................... 7

1.1.4. MEDICAL COMORBIDITY .......................................................................... 7

1.1.5. THE EPIDEMIOLOGY OF BIPOLAR DISORDER ........................................ 8

1.1.5.1. The epidemiology of bipolar disorder in Aotearoa New Zealand ........... 9

1.2. THE AETIOLOGY OF BIPOLAR DISORDER .................................................... 12

1.2.1. PSYCHOSOCIAL AND ENVIRONMENTAL CONTEXTS ................................. 12

1.2.2. BIOLOGICAL CONTEXTS ............................................................................ 13

1.2.3. EVIDENCE OF A GENETIC BASIS FOR BIPOLAR DISORDER .................. 14

1.2.3.1. Twin Studies ............................................................................................ 14

1.2.3.2. Adoption Studies ..................................................................................... 14

1.2.4. IDENTIFYING GENETIC FACTORS CONTRIBUTING TO BIPOLAR DISORDER 15

1.2.4.1. Linkage Studies ....................................................................................... 15

1.2.4.2. Association Studies ............................................................................... 16
1.2.4.3. Genome Wide Association Studies .......................................................... 17
1.2.4.4. Clues from other psychiatric disorders .................................................. 17
1.2.4.5. Convergent functional genomics .......................................................... 18
1.2.4.6. Heritability ......................................................................................... 19
1.3. THE TREATMENT OF BIPOLAR DISORDER ................................................. 21
1.3.1. MOOD STABILISING DRUGS ................................................................. 21
1.3.1.1. Lithium ............................................................................................ 21
1.3.1.2. Valproate .......................................................................................... 22
1.3.1.3. Carbamazepine ............................................................................... 24
1.3.1.4. Oxcarbazepine ............................................................................... 25
1.3.1.5. Lamotrigine ..................................................................................... 25
1.3.2. OTHER MEDICATIONS .......................................................................... 26
1.3.2.1. Antidepressant drugs ........................................................................ 26
1.3.2.2. Antipsychotic drugs .......................................................................... 27
1.3.3. OTHER TREATMENTS ........................................................................... 28
1.3.3.1. Electroconvulsive Therapy ................................................................. 28
1.3.3.2. Omega-3 Dietary Supplementation .................................................. 29
1.3.3.3. Psychosocial intervention .................................................................. 29
1.4. AETIOLOGICAL CLUES FROM DRUG TREATMENTS ................................... 31
1.4.1. NEUROPROTECTION ............................................................................. 31
1.4.2. MOOD STABILISERS AND SIGNALLING PATHWAYS ................................. 32
1.4.2.1. The Wnt signalling pathway ................................................................ 33
1.4.2.1.1. Glycogen synthase kinase 3 beta .................................................... 34
1.4.2.2. The MAPK/ERK pathways ................................................................ 36
1.4.2.2.1. Brain derived neurotrophic factor ................................................ 36
1.4.2.3. The phosphotidyl inositol pathway ..................................................... 39
1.4.2.3.1. Inositol-1-monophosphatase 1 and inositol-1-monophosphatase 2 .... 40
1.4.2.3.2. Inositol polyphosphate 1-phosphatase ......................................... 42
1.4.2.3.3. Prolyl endopeptidase ..................................................................... 43
1.4.2.3.4. Protein Kinase C A and Protein Kinase C E .................................... 44
1.4.2.4. The Arachidonic Acid cascade .......................................................... 45
1.4.3. SELECTION OF LITHIUM AFFECTED GENES FROM THE LITERATURE .... 46
1.4.3.1. Endoglin .......................................................................................... 46
1.4.3.2. Cyclic phosphodiesterase 4D interacting protein and Ras related protein . 46
1.5. OBJECTIVES OF THIS INVESTIGATION ..................................................... 48

2. MATERIALS AND METHODS ........................................................................ 49
2.1. MATERIALS .............................................................................................. 49
2.1.1. CHEMICALS ........................................................................................................ 49
2.1.2. WATER ............................................................................................................. 49
2.1.3. BUFFERS AND SOLUTIONS ............................................................................. 49
2.1.4. DRUG SOLUTIONS ............................................................................................. 51
2.1.5. CELL LINES ....................................................................................................... 52
2.1.5.1. Neuroblast cell lines....................................................................................... 52
2.1.5.2. Lymphoblast cell lines.................................................................................... 52
2.1.6. DNA ..................................................................................................................... 53
2.1.7. ANTIBODIES ...................................................................................................... 53
2.2. METHODS ............................................................................................................ 54
2.2.1. CELL CULTURE ................................................................................................. 54
2.2.1.1. Neuroblast cell lines....................................................................................... 54
2.2.1.2. Lymphoblast cell lines.................................................................................... 54
2.2.2. POLYMERASE CHAIN REACTION ................................................................... 55
2.2.2.1. Primer Design............................................................................................... 55
2.2.2.2. Standard PCR............................................................................................... 55
2.2.2.3. Allele Specific PCR....................................................................................... 56
2.2.2.4. ARMS Assay ............................................................................................... 57
2.2.2.5. TaqMan........................................................................................................ 58
2.2.3. AGAROSE GEL ELECTROPHORESIS .............................................................. 58
2.2.4. EXTRACTION OF RNA ................................................................................... 59
2.2.5. PREPARATION OF cDNA .............................................................................. 59
2.2.6. RT-QPCR .......................................................................................................... 60
2.2.7. MICROARRAY ANALYSIS ............................................................................... 61
2.2.8. RESTRICITION ENZYME DIGEST .................................................................. 61
2.2.9. SEQUENCING ................................................................................................. 61
2.2.10. PROTEIN EXTRACTION AND QUANTITATION .......................................... 62
2.2.11. POLYACRYLAMIDE GEL ELECTROPHORESIS ............................................. 63
2.2.11.1. Western Blot .............................................................................................. 63
2.2.12. ELISA .............................................................................................................. 64
2.2.13. STATISTICAL ANALYSIS ............................................................................... 65
2.2.13.1. RT-qPCR Analysis ..................................................................................... 65
2.2.13.2. Analysis of protein levels .......................................................................... 66
2.2.13.3. Hardy-Weinberg Analysis ......................................................................... 66
2.2.13.4. Association Analysis .................................................................................. 67

3. GENE EXPRESSION AND PROTEIN LEVELS IN A NEURAL MODEL............... 68
3.1. INTRODUCTION .................................................................................................. 68
3.1.1. EXPERIMENTAL APPROACH ................................................................. 68
3.1.2. SELECTION OF GENES TO SCREEN FOR EXPRESSION CHANGES .............................................. 68
3.1.2.1. Chromosome 7 open reading frame 45 ........................................ 69
3.1.2.2. Fibroblast growth factor receptor 4 .................................................. 69
3.1.2.3. Guanine nucleotide binding protein ................................................. 70
3.1.2.4. Heat shock protein 90kDa beta (Grp94), member 1 .............................. 70
3.1.2.5. Midkine (neurite growth promoting factor 2) ..................................... 71
3.1.2.6. Neural proliferation, differentiation and control 1 ............................... 72
3.1.2.7. Regulator of G-protein signalling 5 ................................................ 72
3.1.2.8. Tumour necrosis factor receptor-associated protein 1 ......................... 74
3.1.2.9. 14-3-3 gamma ................................................................................. 74
3.1.3. SELECTION OF REFERENCE GENES .................................................. 76
3.2. RESULTS ................................................................................................. 78
3.2.1. GENE AND PROTEIN EXPRESSION IN NEURAL CELL LINES ....................................................... 78
3.2.2. PROTEIN STUDIES ............................................................................. 92
3.2.3. CORRELATION BETWEEN BDNF CDNA AND PROTEIN CHANGES ........................................ 93
3.3. DISCUSSION .......................................................................................... 94
3.3.1. EXPRESSION CHANGES IN GENES SELECTED FROM THE SAGE SCREEN ................................ 94
3.3.2. EXPRESSION CHANGES IN GENES SELECTED FROM THE LITERATURE .................................. 95
3.3.3. LACK OF CORRELATION IN DNA AND PROTEIN CHANGES ..................................................... 99
3.3.4. SELECTION OF GENES FOR SUBSEQUENT STUDY ................................................................. 101

4. GENE EXPRESSION IN A LYMPHOCYTE MODEL ............................................ 103
4.1. INTRODUCTION ..................................................................................... 103
4.1.1. LYMPHOBLASTS AS A MODEL .......................................................... 103
4.1.2. SELECTION OF LYMPHOCYTE CELL LINES .......................................... 104
4.1.3. SELECTION OF REFERENCE GENES .................................................. 106
4.1.4. ANALYSIS OF RT-QPCR RESULTS ...................................................... 107
4.2. RESULTS ................................................................................................. 108
4.2.1. GENE EXPRESSION RESULTS ................................................................ 108
4.2.2. GROUP COMPARISONS OF GENE EXPRESSION ...................................... 113
4.2.3. BDNF AND YWHAG PROTEIN LEVELS ................................................ 114
4.2.4. GENE EXPRESSION AND PROTEIN LEVEL CORRELATION .................. 118
4.3. DISCUSSION .......................................................................................... 120
4.3.1. INCONSISTENT RESULTS IN THE NEUROBLAST AND LYMPHOBLAST MODELS .......... 120
4.3.2. LYMPHOBLASTS AS MODEL SYSTEMS .................................................. 121

5. ASSOCIATION STUDIES OF CANDIDATE GENE POLYMORPHISMS IN A COHORT WITH BIPOLAR DISORDER ......................................................... 125
5.1. ASSOCIATION STUDIES ...........................................................................125
  5.1.1. POPULATION-BASED ASSOCIATION TESTING .........................................................125
  5.1.2. FAMILY-BASED ASSOCIATION TESTS .................................................................126
  5.1.3. HAPLOTYPE ASSOCIATION TESTING ..............................................................127
  5.1.4. CORRECTION FOR MULTIPLE TESTING IN ASSOCIATION STUDIES ...............128
5.2. EXPERIMENTAL APPROACH .................................................................130
5.3. DESCRIPTION OF THE SIBS COHORT ..................................................131
5.4. REVISION OF THE SIBS COHORT ........................................................132
5.5. SELECTION OF SNPs FOR ASSOCIATION TESTING .................................134
5.6. GENOTYPING OF CANDIDATE GENE POLYMORPHISMS ............................134
  5.6.1. GENOTYPING OF SNPs USING TARGMan PROBES ...........................................134
  5.6.2. GENOTYPING OF SNPs USING RESTRICTION ENZYME DIGEST .......................135
  5.6.3. GENOTYPING OF RS6265 USING AN ARMS ASSAY ...........................................136
  5.6.4. GENOTYPING OF SNPs USING ALLELE-SPECIFIC PRIMERS ...............................136
  5.6.5. GENOTYPING OF RS1519480 USING INFERENCe FROM HAPLOTYPE ..............137
  5.6.6. CHECKING FOR GENOTYPING ERRORS ..........................................................137
5.7. ASSOCIATION ANALYSIS .........................................................................138
  5.7.1. ASSOCIATION WITH BDNF ............................................................................138
  5.7.2. ASSOCIATION WITH RAB7A ........................................................................140
  5.7.3. ASSOCIATION WITH YWHAG ......................................................................142
5.8. DISCUSSION .........................................................................................144
  5.8.1. IMPROVING THE SIBS COHORT THROUGH PEDIGREE VALIDATION ..................144
  5.8.2. FURTHER STUDY OF RAB7A WARRANTed ........................................................145
  5.8.3. NO EVIDENCE FOR ASSOCIATION BETWEEN YWHAG AND BPD ....................146
  5.8.4. BDNF AS A CANDIDATE FOR INVOLVEMENT IN BPD .....................................147
    5.8.4.1. The ongoing puzzle of BDNF rs6265 and BPD .................................................148
    5.8.4.2. Other SNPs of BDNF ....................................................................................151
    5.8.4.3. BDNF Haplotypes .......................................................................................152
  5.8.5. THE GENETIC DIATHESIS-TRANSACTIONAL STRESS MODEL .....................153
  5.8.6. SEARCHING FOR GENETIC CONTRIBUTION TO COMPLEX DISEASE .............154
  5.8.7. LIMITATIONS .................................................................................................158
5.9. CONCLUSION .......................................................................................159

6. CONCLUSIONS AND FUTURE DIRECTIONS ...........................................160
  6.1. SUMMARY OF FINDINGS ..........................................................................160
    6.1.1. GENE EXPRESSION CHANGES IN NEUROBLAST CELL LINES .........................160
    6.1.2. GENE EXPRESSION CHANGES IN LYMPHOBLAST CELL LINES .......................161
    6.1.3. ASSOCIATION STUDIES .............................................................................161
6.2. FUTURE DIRECTIONS ..................................................................................... 162
6.2.1. REPLICATION OF ASSOCIATION RESULTS ......................................... 162
6.2.2. FURTHER GENE EXPRESSION STUDIES ............................................. 162
6.2.3. FURTHER ASSOCIATION STUDIES WITH THE SIBS COHORT .......... 163
6.3. CONCLUSION ............................................................................................. 164

REFERENCES ..................................................................................................... 165

APPENDIX A ..................................................................................................... 203
APPENDIX B ..................................................................................................... 205
APPENDIX C ..................................................................................................... 207
APPENDIX D ..................................................................................................... 208
APPENDIX E ..................................................................................................... 210
APPENDIX F ..................................................................................................... 216
# LIST OF TABLES

Table 1.1 DSM-IV criteria for major depression and mania .................................................................3
Table 2.1 Cell lines from the Major Affective Disorders Panel ..........................................................53
Table 3.1 Genes chosen for gene expression studies ........................................................................76
Table 3.2 Changes in BDNF expression after 28 days of drug treatment .........................................88
Table 3.3 Changes in MDK expression after 28 days of drug treatment ...........................................89
Table 3.4 Changes in RAB7A expression after 28 days of drug treatment .........................................90
Table 3.5 Changes in YWHAG expression after 28 days of drug treatment .....................................91
Table 3.6 Changes in BDNF protein level after 28 days drug treatment ............................................92
Table 4.1 Lymphoblast source individuals - BPD-I ........................................................................106
Table 4.2 Lymphoblast source individuals - unaffected group ..........................................................106
Table 4.3 Change in BDNF expression levels after 28 days of drug treatment ...............................109
Table 4.4 Change in MDK expression levels after 28 days of drug treatment ...............................110
Table 4.5 Change in RAB7A expression levels after 28 days of drug treatment ............................111
Table 4.6 Change in YWHAG expression levels after 28 days of drug treatment ..........................112
Table 4.7 Gene expression level after 28 days ..............................................................................113
Table 4.8 Change in gene expression after 28 days of treatment ..................................................114
Table 4.9 Change in BDNF protein levels after 28 days of drug treatment ....................................116
Table 4.10 Change in YWHAG protein levels after 28 days of drug treatment ............................117
Table 5.1 BDNF - SNP Association with bipolar disorder ..............................................................139
Table 5.2 BDNF - Full haplotype analysis ......................................................................................139
Table 5.3 BDNF - Haplotype of highest risk ....................................................................................140
Table 5.4 BDNF - Haplotype of highest protection .........................................................................140
Table 5.5 RAB7A - Association with bipolar disorder .................................................................141
Table 5.6 RAB7A - Full haplotype analysis ....................................................................................141
Table 5.7 RAB7A - Haplotype of highest risk ................................................................................142
Table 5.8 RAB7A - Haplotype of highest protection ........................................................................142
Table 5.9 YWHAG - Association with bipolar disorder .................................................................142
Table 5.10 YWHAG - Full haplotype analysis ..............................................................................143
LIST OF FIGURES

Figure 1.1 Twelve-month prevalence rates of bipolar disorder by ethnicity 10
Figure 1.2 The intersection of the Wnt and MAPK signalling pathways 36
Figure 1.3 The phosphoinositide signalling system 40
Figure 2.1 Two-well allele-specific PCR 56
Figure 2.2 Allele specific PCR using a tailed primer 57
Figure 2.3 The ARMS assay for genotype analysis 58
Figure 3.1 Gene expression changes standardised against control after 28 days of drug treatment 79
Figure 3.2 Changes in BDNF expression after 28 days of drug treatment 88
Figure 3.3 Changes in MDK expression after 28 days of drug treatment 89
Figure 3.4 Changes in RAB7A expression after 28 days of drug treatment 90
Figure 3.5 Changes in YWHAG expression after 28 days of drug treatment 91
Figure 3.6 Changes in BDNF protein level after 28 days of drug treatment 92
Figure 3.7 BDNF protein level change vs. BDNF gene expression change after 28-day drug treatment 93
Figure 4.1 Change in BDNF expression levels after 28 days of drug treatment 109
Figure 4.2 Change in MDK expression levels after 28 days of drug treatment 110
Figure 4.3 Change in RAB7A expression levels after 28 days of drug treatment 111
Figure 4.4 Change in YWHAG expression levels after 28 days of drug treatment 112
Figure 4.5 Western blot showing reference (β-actin) and YWHAG bands 115
Figure 4.6 Change in BDNF protein levels after 28 days of drug treatment 116
Figure 4.7 Change in YWHAG protein levels after 28 days of drug treatment 117
Figure 4.8 BDNF protein level change vs. BDNF gene expression change after 28 days drug treatment 118
Figure 4.9 YWHAG protein level change vs. YWHAG gene expression change after 28 days drug treatment 119
Figure 5.1 Pedigree 535 132
Figure 5.2 TaqMan Allelic discrimination plot for BDNF rs1223363 135
Figure 5.3 The BDNF rs6265 SNP ARMS assay 136
Figure 5.4 Schematic diagram of the BDNF gene with significance thresholds 148
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMS</td>
<td>amplification refractory mutation system</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor gene</td>
</tr>
<tr>
<td>BPD-I</td>
<td>bipolar I disorder</td>
</tr>
<tr>
<td>BPD-II</td>
<td>bipolar II disorder</td>
</tr>
<tr>
<td>BPD-NOS</td>
<td>bipolar disorder not otherwise specified</td>
</tr>
<tr>
<td>C7orf45</td>
<td>chromosome 7 open reading frame 45 gene</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ENG</td>
<td>endoglin gene</td>
</tr>
<tr>
<td>FBAT</td>
<td>family-based association test</td>
</tr>
<tr>
<td>FDA</td>
<td>(United States) Food and Drug Administration</td>
</tr>
<tr>
<td>FGFR4</td>
<td>fibroblast growth factor receptor 4 gene</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase gene</td>
</tr>
<tr>
<td>GNG5</td>
<td>guanine nucleotide binding protein gamma 5 gene</td>
</tr>
<tr>
<td>GSK3B</td>
<td>glycogen synthase kinase gene</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>Heat shock protein 90kDa β (Grp94) gene</td>
</tr>
<tr>
<td>IMPA1</td>
<td>inositol-1-monophosphatase 1 gene</td>
</tr>
<tr>
<td>IMPA2</td>
<td>inositol-1-monophosphatase 2 gene</td>
</tr>
<tr>
<td>INPP1</td>
<td>inositol polyphosphate 1-phosphatase gene</td>
</tr>
<tr>
<td>MDK</td>
<td>midkine gene</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribose nucleic acid</td>
</tr>
<tr>
<td>NPDC1</td>
<td>neural proliferation, differentiation and control, 1 gene</td>
</tr>
<tr>
<td>NZMHS</td>
<td>New Zealand Mental Health Survey</td>
</tr>
<tr>
<td>PDE4D</td>
<td>cyclic phosphodiesterase 4d interacting protein gene</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PREP</td>
<td>prolyl oligopeptidase gene</td>
</tr>
<tr>
<td>PRKCA</td>
<td>protein kinase C A gene</td>
</tr>
<tr>
<td>PRKCE</td>
<td>protein kinase C E gene</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>quantitative reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RAB7A</td>
<td>ras-related protein gene</td>
</tr>
<tr>
<td>RG55</td>
<td>regulator of G-protein signalling 5 gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribose nucleic acid</td>
</tr>
<tr>
<td>SAGE</td>
<td>serial analysis of gene expression</td>
</tr>
<tr>
<td>SDHA</td>
<td>succinate dehydrogenase gene</td>
</tr>
<tr>
<td>SIBS</td>
<td>South Island Bipolar Study</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TRAP1</td>
<td>tumour necrosis factor receptor-associated protein 1 gene</td>
</tr>
<tr>
<td>UBC</td>
<td>ubiquitin C gene</td>
</tr>
<tr>
<td>YWHAG</td>
<td>14-3-3γ gene</td>
</tr>
</tbody>
</table>
PUBLICATIONS

Papers


Poster Presentations


Sears, C., Fitches, A. and Olds, R. (2007) "BDNF, INPP1 and RAB7A are upregulated in neural cells by the mood stabilising drugs valproate and carbamazepine" Queenstown Molecular Biology Meeting
1. Introduction

1.1. Bipolar Disorder

Bipolar disorder (BPD) is a common and serious psychiatric illness, characterised by episodes of depression alternating with mania or hypomania. In some individuals symptoms will also include periods of psychosis and disordered thinking (Suppes et al., 2000). Both mania and depression can be highly debilitating and may lead to loss of normal societal functioning, relationship breakdown, loss of employment, hospitalisation and suicide (Jamison, 2000). BPD has been ranked the sixth leading cause of disability worldwide (Murray and Lopez, 1996).

Disorders of the mood have been recognised throughout human history. The ancient Greeks wrote of both ‘melancholia’ and mania, and a connection between them was acknowledged as early as the second century BC by Soranus of Ephesus (Drabkin, 1951). The first clinical description of what is now referred to as BPD was published in 1854 by French psychiatrist Jean-Pierre Falret, who called it ‘folie circulaire’, or ‘circular insanity’. The cyclical and bipolar nature of the disorder has been recognised since the mid 1800s (Pichot, 2004).

The onset of the disorder is usually in the late teens or the twenties. A study performed in the United Kingdom found there were two peaks for age at first episode of mania, one at age 21-25, and another in the late 40s and early 50s (Kennedy et al., 2005).

The rate of suicide due to severe psychiatric distress is elevated in those with BPD. Suicide is the cause of death in around 19% of individuals diagnosed with BPD, compared to 1-2% of the general population (Angst et al., 2002).

1.1.1. Diagnostic features of bipolar disorder

Clinical diagnosis of BPD is usually based on the Diagnostic and Statistical Manual of Mental Disorders (DSM), a diagnostic manual published by the
American Psychiatric Association, currently in its fourth edition. Although there are other well-regarded diagnostic tools available, such as the World Health Organisation’s ‘International Classification of Diseases’ (ICD-10), the DSM is the most widely used diagnostic classification system for psychiatric disorders (Mezzich, 2002).

1.1.1.1. **Bipolar I Disorder**

Bipolar I disorder, also known as classic manic-depressive illness, is the most serious and severe form of the subtypes of BPD. To be diagnosed with BPD-I an individual must have met the criteria for at least one major depressive episode and one manic or mixed episode, with the manic episode not having been caused by another mental disorder, medical condition, or as the result of a medication or a substance of abuse. Psychosis may be present at either pole of the disorder (APAWG, 2000). The diagnostic criteria for both mania and depression are complex and have been summarised in Table 1.1 which is adapted from the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition Text Revision (APAWG, 2000)

BPD-I is considered to be a chronic illness, and the presence of multiple mood episodes is the typical course of the disorder, with most individuals experiencing at least four severe mood episodes over the course of their lives (APAWG, 2000). The median length of these episodes (either manic or depressed) has been reported as 13 weeks, with recovery from mania generally taking a shorter time than recovery from major depression (Solomon et al., 2010).

1.1.1.2. **Bipolar II Disorder**

BPD-II is diagnosed based on the presence or history of one or more major depressive episodes (Table 1.1) and at least one hypomanic episode. The diagnostic criteria for a hypomanic episode are similar to those for a manic episode (see Table 1.1) with differences primarily in duration and severity. The elevated mood state only needs to last four days as opposed to a week, and while there must be definite changes in functioning observable by others who know the person, hypomania must not cause marked impairment or result in hospitalisation. As with mania, the symptoms must not be due to
medication or substance of abuse, and any general medical condition leading to the symptoms must be ruled out.

Table 1.1 DSM-IV criteria for major depression and mania

<table>
<thead>
<tr>
<th>Criteria for a Major Depressive Episode</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> Five (or more) of the following symptoms have been present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.</td>
</tr>
<tr>
<td>• depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful)</td>
</tr>
<tr>
<td>• markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day</td>
</tr>
<tr>
<td>• significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day</td>
</tr>
<tr>
<td>• insomnia or hypersomnia nearly every day</td>
</tr>
<tr>
<td>• psychomotor agitation or retardation nearly every day</td>
</tr>
<tr>
<td>• fatigue or loss of energy nearly every day</td>
</tr>
<tr>
<td>• excessive or inappropriate guilt, feelings of worthlessness</td>
</tr>
<tr>
<td>• diminished ability to think or concentrate, or indecisiveness, nearly every day</td>
</tr>
<tr>
<td>• recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide</td>
</tr>
<tr>
<td><strong>B.</strong> The symptoms do not meet the criteria for a Mixed Episode.</td>
</tr>
<tr>
<td><strong>C.</strong> The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.</td>
</tr>
<tr>
<td><strong>D.</strong> The symptoms are not due to the direct physiological effects of a substance, or a general medical condition.</td>
</tr>
<tr>
<td><strong>E.</strong> The symptoms are not better accounted for by bereavement.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Criteria for a Manic Episode</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least one week (or any duration requiring hospitalisation). During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:</td>
</tr>
<tr>
<td>• inflated self-esteem or grandiosity</td>
</tr>
<tr>
<td>• decreased need for sleep (e.g., feels rested after only three hours of sleep)</td>
</tr>
<tr>
<td>• more talkative than usual or pressure to keep talking</td>
</tr>
<tr>
<td>• flight of ideas or subjective experience that thoughts are racing</td>
</tr>
<tr>
<td>• distractibility (i.e., attention too easily drawn to unimportant or irrelevant external stimuli)</td>
</tr>
<tr>
<td>• increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation</td>
</tr>
<tr>
<td>• excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments).</td>
</tr>
<tr>
<td><strong>B.</strong> The symptoms do not meet the criteria for a Mixed Episode.</td>
</tr>
<tr>
<td><strong>C.</strong> The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalisation to prevent harm to self or others, or there are psychotic features.</td>
</tr>
<tr>
<td><strong>D.</strong> The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication, or other treatments) or a general medical condition.</td>
</tr>
</tbody>
</table>
While the hypomania itself does not cause marked distress or impairment, for a diagnosis of BPD-II the condition as a whole must cause significant problems in one or more important areas of an individual’s life, such as social or occupational (APAWG, 2000).

The diagnosis of BPD-II did not appear in the DSM until the fourth edition in 1994, where it was separated out from BPD-I and ‘BPD not otherwise specified’ (Dunner, 1998).

1.1.1.3. Cyclothymic Disorder

The third major subtype of BPD is cyclothymic disorder, which presents clinically as long-term mood disturbance with hypomanic and depressed episodes that do not meet the criteria for either BPD-I or BPD-II because of reduced severity of symptoms. To be diagnosed with cyclothymic disorder an individual must experience both occurrences of hypomania and episodes of depressed mood. The depression must not be severe enough to meet the full diagnostic criteria for a major depressive episode, and the mood disturbances must continue over the course of two years, with no gaps in symptoms lasting longer than two months. The symptoms must cause clinically significant distress and impairment, and they must not be due to substance abuse or better explained by any of the psychotic disorders. Any fully developed manic, mixed, or major depressive episode within the two year period will rule out cyclothymic disorder and change the diagnosis to one of BPD-I or BPD-II (APAWG, 2000).

1.1.1.4. Bipolar Disorder Not Otherwise Specified

Bipolar Disorder Not Otherwise Specified (BPD-NOS) is the final major subtype recognised by the DSM-IV, and is something of a catchall category for individuals who display bipolar phenotypes without meeting the specific diagnostic criteria of BPD-I, BPD-II, or cyclothymia (APAWG, 2000). An individual might be diagnosed with BPD-NOS if they experience mania or hypomania with only very mild episodes of low mood that do not meet the full criteria for depression. Another manifestation of the disorder to receive a BPD-NOS diagnosis might be the presence of repeated episodes of hypomania and mild low mood over the course of a year, with none long
enough to meet the full criteria for diagnosis of cyclothymia, followed by two years of complete remission. Despite the often less severe presentation of symptoms in BPD-NOS, this subtype of BPD can still cause significant impairment functioning and decrease in quality of life (Judd and Akiskal, 2003).

BPD-NOS is considered likely to be more common than the more specific disorders within the bipolar spectrum. A 2003 study which used the DSM-III criteria to identify individuals with ‘sub-syndromal manic symptoms’ from a cohort of over 18,000 Americans found that these subthreshold cases accounted for over 5% of participants, with 0.8% of participants having experienced a full blown episode of mania, and 0.5% having experienced a hypomanic episode, suggesting symptoms of mania commonly fall below the threshold allowing diagnosis of a specific BPD type (Judd and Akiskal, 2003). A more recent study using DSM-IV criteria found the 12-month prevalence of sub-threshold BPD to be 1.4%, and the lifetime risk to be 2.4%, both higher figures than those found for either BPD-I or BPD-II (Merikangas et al., 2007).

1.1.2. Modifiers of Bipolar Disorder
There are several modifiers of the disorder that can be included in a diagnosis of BPD in order to clarify the course of the disorder in an individual. These are described below.

1.1.2.1. Mixed Episodes
Mixed episodes, sometimes called mixed mania, are where the diagnostic criteria for a major depressive episode and the criteria for mania (other than duration) are satisfied nearly every day for seven days. As with mania, there must be marked impairment and the symptoms must not be caused by a general medical condition or by a medication or a drug of abuse. The presence of a mixed episode leads to the diagnosis of BPD-I and rules out BPD-II (APAWG, 2000).

1.1.2.2. Rapid Cycling
The phenomenon of rapid cycling is a variation in the course of BPD that can be found in some individuals with either BPD-I or BPD-II. Rapid cycling is
diagnosed where an individual experiences at least four episodes within a twelve-month period that meet the criteria for a manic, hypomanic, depressive or mixed episode. These must be discrete episodes separated by either a full or partial remission of at least two months, or a switch to the opposite polarity (APAWG, 2000).

Rapid cycling can be differentiated from cyclothymic disorder by the severity of symptoms, which in rapid cycling must meet the diagnostic criteria of a full-blown episode of mood disturbance. The severity of the rapid cycling course will depend on the severity of the underlying condition modified, either BPD-I or II (Dunner, 1998).

Rapid cycling was added to the DSM-IV on its first publication in 1994 after a multi-site study that pooled and reanalysed data on rapid cycling from four centres in the United States. The outcome of this research was that rapid cycling was a predictive and distinct variation of BPD with a significant variation in demographics, including a higher proportion of females and people from higher socio-economic groups than would be expected by chance. Analysis of the episodic history of over 140 individuals with BPD resulted in the setting of the minimum number of affective episodes over a twelve-month period to four (Bauer et al., 1994).

1.1.2.3. Paediatric and Adolescent bipolar disorder

There has been a growing recognition that BPD can manifest much younger than the standard early-adulthood age of onset. Paediatric BPD has become the subject of much research over the last 15 to 20 years, although the acceptance of a juvenile form of the disorder, and therefore the rates of diagnosis, vary internationally (Soutullo et al., 2005).

The presentation of BPD in children and adolescents often fails to meet the strict conditions of the DSM-IV, and generally follows a broader phenotype that may include severe irritability, labile mood and rapidly changing affective states (Taylor and Miklowitz, 2004). Mick and Faraone (2009) liken the symptoms of BPD in childhood to those of mixed mania in adults (Mick and Faraone, 2009). BPD in children tends not to follow the pattern of sudden,
acute onset characteristic of the disorder in adults, but has a more gradual
onset. Children and younger adolescents with the disorder are more likely to
cycle very rapidly, often several times per day (Geller and Luby, 1997).

Unfortunately, the earlier the age of onset, the less favourable the prognosis is
for the individual. Factors such as frequency and duration of affective
episodes are, on average, more severe the younger the disorder begins to
manifest, and inter-episode recovery is less well delineated. Individuals with
paediatric BPD also tend to have poorer responses to mood-stabilising
treatment than those with adult onset (Taylor and Miklowitz, 2004).

1.1.3. Psychiatric Comorbidity
The presence of psychiatric comorbidities is very common among individuals
with BPD. A review of 14 studies from across Europe found that around 75%
of individuals diagnosed with a form of BPD also had a comorbid diagnosis
from the DSM-IV (Fajutrao et al., 2009). Primary amongst these are anxiety
disorders and disorders of substance abuse. A National Institute of Mental
Health study in the USA found that individuals with BPD-I had a drug abuse
rate 11 times higher than the general population, and a drug dependence rate
twice as high as those with unipolar depression. The substance abuse
(including alcohol abuse) rate reported in this study for those with BPD-I was
60.7%, compared to 13.2% in the general population (Regier et al., 1990). A
review of studies on comorbid anxiety disorders among individuals with BPD
showed anxiety was associated with longer episodes of abnormal mood, as
well as higher levels of substance abuse. Anxiety occurs as a comorbid factor
more commonly in females than males, and is more widespread among those
with BPD-II rather than BPD-I (McIntyre et al., 2006b). The presence of
comorbidity affects the course of both disease and treatment in affected
individuals and the high overall levels of comorbidity increases the burden of
BPD both socially and economically.

1.1.4. Medical Comorbidity
Individuals with BPD are also at a higher risk of a range of medical conditions
than the general population. Some of these conditions are associated with the
treatment of BPD, such as nephrogenic diabetes insipidus due to the effect of
lithium on the kidneys, or increased levels of polycystic ovarian syndrome in women medicated with sodium valproate. However, the majority are truly comorbid and the reasons for their high levels in people with BPD are not always known (Krishnan, 2005).

A 2006 study by Carney and Jones looked at medical comorbidities in a cohort of 3557 individuals with BPD-I and found they had significantly higher levels of chronic cardiovascular, endocrine, pulmonary and gastrointestinal conditions than the control population. These conditions included congestive heart failure, asthma, migraine, diabetes, stroke and a wide variety of infectious disease including HIV/AIDS (Carney and Jones, 2006).

Several theories have been advanced to explain the high rate of medical comorbidity among those with BPD. McElroy and colleagues believe the elevated rate reflects the higher level of risk factors for poor health generally among those with BPD, factors such as low socio-economic status, lack of access to preventative health care, and poor adherence to medical treatment (McElroy et al., 2002). Both the depressive and the manic phases of BPD generally lead to behaviours considered risky to physical health. During a period of depressed mood, inactivity, smoking, overeating and use of recreational drugs may increase, whereas involvement in risky behaviours such as unprotected sex and activities with high injury potential are more common during a manic episode. These behaviours may partially explain the higher rate of lifestyle-associated conditions among those with BPD (McIntyre et al., 2006a).

1.1.5. The epidemiology of bipolar disorder
Historical figures given for the prevalence of BPD in the population have generally hovered around 1%, but recent research including all subtypes along the spectrum place the figure around the 5% mark (Thomas, 2004). A large study from the United States placed the lifetime risk for BPD-I at 1%, BPD-II at 1.1%, and cyclothymic disorder at 2.4% (Merikangas et al., 2007). Individuals with BPD-I account for the majority of hospitalisations and burden associated with BPD (APAWG, 2000).
Bipolar disorder is equally common in both sexes, however the distribution of subtypes and associated phenotypes does differ by sex. BPD-II is more prevalent among women than men (Judd and Akiskal, 2003), as are the presence of mixed episodes, rapid cycling, and psychiatric comorbidities (Arnold, 2003). Men have an earlier average age of onset for first-episode mania (Kennedy et al., 2005) and a higher rate of comorbid substance abuse (Arnold, 2003).

There are variations in rates of BPD internationally, with the rates reported in one study ranging from a lifetime prevalence of 0.1% in India to 4.4% in the United States. These differing rates across countries, while possibly reflecting actual differences in prevalence caused by genetic or environmental factors, could also be explained by social factors such as the higher false negative diagnostic rate in communities with a greater stigma against psychiatric illness. The large differences in social structure between countries also make it difficult to control for factors such as socio-economic status and education, which may artificially depress rates in some countries (Merikangas et al., 2011).

The prevalence of BPD in children and adolescents, once thought to be less common than among adults, has recently been shown to be quite similar, with a large meta-analysis of epidemiologic studies across seven countries finding a rate of 1.8% (Van Meter et al., 2011). The same study found that there did not appear to be an increase in the prevalence of juvenile and adolescent BPD over the period from 1985 to 2005, despite increasing recognition of the condition by clinicians and researchers.

1.1.5.1. The epidemiology of bipolar disorder in Aotearoa New Zealand

Te Rau Hinengaro, the New Zealand Mental Health Survey (NZMHS), investigated the prevalence and severity of mental health disorders within New Zealand, including affective disorders such as BPD (Wells et al., 2006). The survey found that the level of BPD in New Zealand is similar to that of other developed nations, with a lifetime prevalence of BPD of 3.8% (Oakley Browne et al., 2006b). As with other populations, this figure does not differ significantly by gender. However, lifetime risk of the disorder was found to
be significantly increased in younger age cohorts, ranging from 0.6% in those aged 64 and above, to 5% in 16-24 year olds. The authors of the study theorise that this difference in prevalence can be explained by both differential mortality (those suffering from severe psychiatric disorders are less likely to live to participate in older cohorts) and bias in recall, both factors of which lead to the underestimation of lifetime prevalence.

The NZMHS compared the 12-month prevalence of psychiatric disorders across ethnic groups in New Zealand, finding that rates of BPD are significantly higher in Māori and Pacific Island peoples than in other New Zealanders. This discrepancy remains even after correction for age, sex, household income and education (see Figure 1.1). People with Māori or Pacific Island backgrounds were also significantly less likely to seek treatment for mental health related concerns than people of other ethnicities, adding to the burden of BPD on these communities (Baxter et al., 2006).

![Figure 1.1 Twelve-month prevalence rates of bipolar disorder by ethnicity](image)

A – Adjusted for age and sex  
B - Adjusted for age, sex, educational qualifications and household income  
Figure based on information from Baxter et al., 2006.
The NZMHS did not place a specific figure on the burden of BPD on New Zealand society, but results in a variety of areas show that BPD is of considerable social, occupational and economic impact to the country. The study found that over a twelve-month period, 1.9% of individuals with a diagnosis of BPD attempted suicide, compared to only 0.1% of those without a mental disorder (Beautrais et al., 2006). A separate New Zealand study reported that the lifetime prevalence of suicide attempts among those with BPD was 36% (Joyce et al., 2010). Comorbidity with other psychiatric disorders was found to be common in those with BPD in New Zealand, and the rate of association between BPD and substance abuse disorders was especially marked (Scott et al., 2006b). Higher levels of physical illness (including diagnoses such as cardiovascular disease and respiratory illness) were reported in those with mood disorders, including those with BPD (Scott et al., 2006d). Measures of disability in terms of occupational and social functioning for New Zealanders with mood disorders are on par with those reported for individuals with serious physical ailments such as chronic pain and cancer (Scott et al., 2006c). People with BPD are over-represented in the New Zealand prison population (Brinded et al., 2001) and BPD increases stress on the use of both public and private health services (Oakley Browne et al., 2006a). These findings suggest that BPD is of considerable public health and social significance in New Zealand.
1.2. The Aetiology of Bipolar Disorder

Explanations of the possible causes of BPD have developed with human understanding of the disorder and the cultural context in which the explanation is made. Over the centuries these explanations have ranged from demon possession, through inadequate parenting and childhood deprivation, to the current understanding which encompasses the interaction of genetic, neurochemical and environmental factors (Angst and Sellaro, 2000).

Throughout the first half of the 20th century, the psychosocial explanation for BPD predominated, mostly thanks to the work of Emil Kraepelin (Kraepelin, 1921). Under this model, BPD was thought to be a result of deficiencies in the social and psychological development of an individual, generally due to poor family environment. This changed during the 1950s, when genetic and biological explanations began to predominate as researchers found increasing evidence of BPD clustering in families. Research since over the last 30 years has shown that it is highly unlikely that the disorder is the result of a single gene defect or single major locus (Craddock et al., 1997; Rush, 2003). BPD is now believed to have a multifactorial aetiology, where variation in a number of genes cause a genetic predisposition to the disease and this can also be influenced by external factors (Craddock et al., 1997; Craddock and Jones, 2001).

1.2.1. Psychosocial and Environmental Contexts

Initial modern explanations of the origins of BPD tended to centre strongly on the role of early development, especially on parenting and attachment. Compared to purely biological explanations of BPD, these hypotheses are not widely researched, however the small number of methodologically sound studies tend to show a positive correlation between some styles of parenting and BPD (Alloy et al., 2005a). In particular, bipolar patients tended to report lower levels of maternal affection growing up than controls (Rosenfarb et al., 1994; Geller et al., 2000). Maltreatment in early life is also correlated with development of BPD. Etain et al. (2010) found that individuals with BPD reported higher levels of childhood emotional abuse than unaffected
individuals. Physical, emotional and sexual abuse in early childhood have all been linked to more severe presentations of BPD such as earlier onset, increased psychiatric comorbidity, increased frequency of mood episodes, and higher rates of suicide (Leverich et al., 2002; Garno et al., 2005; Maguire et al., 2008). While a causal relationship between events of early childhood and later development of BPD has not been confirmed, the high rate of correlation between childhood trauma and the disorder pinpoints it as a possible contributing environmental factor. Stress and trauma in adulthood may also have a role to play, as several studies have shown that first and subsequent mood episodes in individuals with BPD are more likely to occur soon after stressful life events (Johnson and Miller, 1997; Alloy et al., 2005a).

An individual’s attributional style is thought to play a role in not only the development but also the ongoing course of BPD. Attributional style describes how an individual explains the causes of a particular event – whether internalising it as a part of their own individual makeup, or attributing to some external factor. Those who have a tendency to internalise the blame for negative events are said to have a pessimistic attributional style (Haugen and Lund, 1998). Studies of unipolar depression have long shown that cognitive styles that include pessimistic explanatory style and concrete thinking increase risk for depressive episodes (Joiner, 2001). Recent research also supports the role of pessimistic attributional style in risk for increased numbers of affective episodes in those with BPD, and an overall difference in cognitive style between those with BPD and controls (Alloy et al., 2005b; Jones et al., 2005; Van der Gucht et al., 2009; Alatiq et al., 2010).

1.2.2. Biological Contexts
Recovery from traumatic head injuries, such as those often caused by motor vehicle accidents, are often complicated by the onset of psychiatric disorders (Kim et al., 2007). Recent head injury is reported to be associated with increased rates of BPD compared to control, however traumatic head injuries only account for a very small percentage of BPD cases (Mortensen et al., 2003).

Obstetric complications have been considered as a possible causative or contributing factor for BPD, and a limited number of studies have reported
that those with BPD are more likely to have had complications occur at around the time of their birth than their unaffected siblings (Kinney et al., 1993; Kinney et al., 1998). However, a systemic review of the literature looking at 22 studies showed that the rates of obstetric complications for those who went on to develop BPD did not differ significantly from those experienced by control individuals (Scott et al., 2006a). While the differing criteria for obstetric complications throughout the studies make an outright conclusion difficult, the current evidence as a whole does not point towards obstetric complications having a large role in the development of BPD, although they may contribute to early life stress.

1.2.3. Evidence of a genetic basis for bipolar disorder
There is strong evidence that there is a genetic component to BPD. Consistent evidence of a genetic link has come from family based research such as twin, adoption and linkage studies (Craddock and Jones, 1999).

1.2.3.1. Twin Studies
Despite a wide variety of methodologies and differences in diagnostic criteria, twin studies have been remarkably consistent in showing that monozygotic twins have a higher concordance of BPD than dizygotic twins (Smoller and Finn, 2003). Because monozygotic twins share both the same genetic makeup and uterine environment compared to dizygotic twins who are genetically as similar as non-twin siblings (i.e. they share 50% of their genetic material), this is good evidence that BPD has a genetic component. A study that pooled available twin study data reported a concordance of 50-60% for monozygotic twins and a 5-10% concordance for dizygotic twins and other first-degree relatives (Craddock and Jones, 1999). A more recent study using a much larger cohort of patients and more explicit diagnostic criteria found the concordance between monozygotic twins to be even higher, at around 85% (McGuffin, 2003).

1.2.3.2. Adoption Studies
Studies of adoptees, their adopted families and their biological families have also shown that genetics is a factor in the development of BPD. An early study showed that the biological parents of bipolar adoptees were
significantly more likely to have BPD (18%) than the adoptive parents (7%) (Mendlewicz and Rainer, 1977). A later study provided a similar result, although due to the size of the study it was not statistically significant (Wender, 1986). Adoption studies involving BPD have historically been small and the diagnostic criteria used are varied, making it difficult to combine data for a more robust statistic.

1.2.4. Identifying genetic factors contributing to bipolar disorder
Many different molecular genetic approaches have been used in order to try to identify genes or variants within genes that contribute to BPD. These approaches have been both positional and functional, and have used a wide range of molecular techniques (Craddock and Forty, 2006). As well as searching for genes that might be contributing to the overall disorder, research has also focussed on possible contributing variants to some of the phenotypes within the disorder, such as rapid cycling, suicidality and age of onset.

Helpful in the search for the genetic causes of BPD have been systems biology techniques that use vast amounts of data from previous studies to identify trends and interesting areas. One such study is that done by Gohlke et al. (2009) who found that candidate genes and variants for neuropsychiatric disorders including BPD tended to cluster on key molecular pathways related to tyrosine metabolism and neuroactive ligand receptor interactions. Studies like this help choose pathways and genes for further research. Investigation into gene expression differences between individuals with BPD and controls can also help pinpoint categories from which to choose candidate genes. For example, postmortem examination has shown that the brains of individuals with BPD have significant upregulation in genes involved in transcription and translation (Iwamoto et al., 2004).

1.2.4.1. Linkage Studies
As both diagnostic and molecular techniques have become more advanced, families with a history of BPD have been analysed for linkage between various chromosomal loci and the disorder, with varying results (Willour, 2003). Linkage studies are designed to pinpoint the location of contributing
genes by studying fragments of the chromosome that are inherited in common with an illness. Most chromosomes have at least one area that has been implicated in BPD, and those with the best evidence include areas on 2p13-16, 4p16, 4q31, 6q, 8q24, 11p13, 12q24, 13q32, 16p13, 16q, 18p11, 18q22, 21q22, 22q12 and Xq22 (Hayden and Nurnberger, 2006). Unfortunately, many of these linkage reports have not been replicated in other studies. A genome-wide meta-analysis performed by Segurado et al. (2003) looked at 18 studies, finding that none of the areas pinpointed by these studies reached genomewide significance. The regions most significantly associated were at 9p22.3, 10q11.21 and 14q24.1, none of which have been replicated in any subsequent studies.

1.2.4.2. Association Studies

Genetic association studies are designed to detect an association between a genetic marker (such as a microsatellite repeat or single nucleotide polymorphism) and a specific trait in a group of individuals. They may be targeted studies of specific genes, or even single polymorphisms. Association studies for a complex disorder such as BPD hope to find polymorphisms that contribute to the phenotype.

One type of association study is the case-control study, which compares the frequency of a specific variant in a group of affected individuals with its frequency in a control group of unaffected individuals. Where these frequencies are significantly different, the variant is said to be associated with the disorder. Also used to find association are family-based studies, where researchers hope to identify a marker or variant that is over-transmitted from parents to affected children, a marker that will then be described as being associated with the condition. While variants found to be associated with a disorder may be causal, contributing in some way to the pathophysiology of the disorder, the more likely scenario is that a highly significantly associated allele is near, or ‘tags’ a nearby mutation that may contribute to the phenotype of the disorder. Well designed association studies of candidate genes can generally find genes of modest effect on the phenotype, which is appropriate given the multifactorial nature of complex diseases such as BPD (Jones and Craddock, 2001). Unfortunately, while there have been
associations found with polymorphisms in many genes, the majority of these have not been replicated in independent cohorts (Kato, 2007).

1.2.4.3. **Genome Wide Association Studies**

In contrast to the more focussed candidate-gene based association studies, genome wide association studies (GWAS) look at the entire genome of a large group of individuals in order to determine if there are any genetic differences that occur significantly more frequently in those with a specific disorder than in control individuals. As costs have decreased and the genotyping process has become quicker and easier, GWAS has become increasingly popular in the search for genetic aetiology in complex disorders where it is useful for finding common genotypic variants of small effect (Manolio, 2010).

Since 2008 there have been over a dozen GWAS trying to identify both variants associated with BPD and variants that may be able to predict specific phenotypes within the disorder, such as treatment response and age of onset. Studies have included populations from Australia (Cichon et al., 2011), Japan (Hattori et al., 2009), Scandinavia (Djurovic et al., 2010; Soronen et al., 2010), North America (Baum et al., 2008; Perlis et al., 2009; Scott et al., 2009; Smith et al., 2009; Oedegaard et al., 2010), Germany (Baum et al., 2008; Belmonte Mahon et al., 2011; Cichon et al., 2011), Bulgaria (Yosifova et al., 2011) and the British Isles (WTCCC, 2007; Ferreira et al., 2008; Sklar et al., 2008). Only a few SNPs have achieved the genome-wide significance threshold of $p \leq 5 \times 10^{-7}$, and few have been replicated between studies. Genes that have been replicated include neurocan (NCAN) (Cichon et al., 2011), cadherin 7 (CDH7) (Sklar et al., 2008; Soronen et al., 2010), diacylglycerol kinase eta (DGKH) (Baum et al., 2008; Yosifova et al., 2011), ankyrin-3 (ANK3) (Ferreira et al., 2008; Smith et al., 2009) and the calcium channel, voltage-dependent, L type, alpha 1C subunit gene (CACNA1C) (Ferreira et al., 2008; Sklar et al., 2008). Research into these genes continues in an attempt to elucidate any contribution to the pathophysiology of BPD.

1.2.4.4. **Clues from other psychiatric disorders**

BPD shares features with other psychiatric disorders such as schizophrenia and major depressive disorder. It is well established that the risk of exhibiting
other psychiatric disorders is higher in people who have a family member with BPD, and that the risk increases the more closely related they are to the proband (Lichtenstein et al., 2009). While there is ongoing scientific debate as to whether or not BPD shares causative factors with other psychiatric disorders, the conditions often appear to share biochemical findings, gene associations and other lines of evidence that suggest they may have overlapping aetiology. A good example of this phenomenon involves the widely studied gene disrupted-in-schizophrenia 1 (DISC1). DISC1 was initially reported as being disrupted by a balanced translocation in a Scottish family who present with a wide range of psychiatric disorders including schizophrenia, BPD, schizoaffective disorder, major depressive disorder and conduct disorder (Millar et al., 2000). Since then DISC1 has been studied in many models in order to determine if it is a contributing factor to any of these disorders in the wider population (Hennah et al., 2006). Several studies have found that polymorphisms of DISC1 are associated with schizophrenia (Hennah et al., 2003; Callicott et al., 2005; Cannon et al., 2005; Thomson et al., 2005; Zhang et al., 2006), while two haplotypes of the gene were reported to be associated with BPD in a Caucasian cohort and lower levels of DISC1 mRNA expression correlate to more severe mania in those with BPD (Maeda et al., 2006).

The potential genetic links between the psychiatric disorders mean that they share possible candidate genes, and that where a genetic link or association is found with one of the other major psychiatric disorders, it is reasonable to then determine whether or not the association also occurs with BPD.

1.2.4.5. Convergent functional genomics

The convergent functional genomics approach is a means of integrating different lines of evidence to assess whether or not a gene is likely to be involved in the aetiology of a complex disease in comparison to other genes.

Le-Niculescu et al. (2009) used a functional genomic approach to identify genes that may be involved in the aetiology of BPD by combining information from GWAS, human blood and postmortem brain gene expression studies, animal model studies, single gene based association studies and linkage
studies. This provided an overview of the evidence for each gene, and a points system was used whereby the data was given a score based on the significance level of the evidence in question. Four genes – brain derived neurotrophic factor (BDNF), aldehyde dehydrogenase 1A1 (ALDH1A1), aryl hydrocarbon receptor nuclear translocater-like (ARNTL) and kruppel-like factor 12 (KLF12) – each received eight points out of a maximum 12 and were considered to have the greatest amount of convergent evidence for having a role in the aetiology of bipolar disorder. A reanalysis of the study to include data from the GWAS performed by Sklar et al. (2008) was reported in 2010, with the genes myelin basic protein (MBP), and ARNTL both receiving 8.5 points (Patel et al., 2010). While the scoring and selection of data available for each gene may be somewhat subjective, the convergent genomics approach may prove to be useful in identifying genes that have a contribution to bipolar disorder but fall short of significance thresholds.

1.2.4.6. Heritability

Heritability is a population measure that expresses the proportion of variation in phenotype between individuals that can be attributed to their genotype. Thus, it can act as a rough guide to how much of the population’s variability in this phenotype is a result of genetic causes, and how much can be attributed to environment. The heritability of BPD is generally stated as being around 80-85% (McGuffin, 2003), which is similar to that of schizophrenia (Cannon et al., 1998) and highly heritable physical disorders such as epilepsy (Kjeldsen et al., 2001) and type-1 diabetes (Hyttinen et al., 2003).

A current concern for those studying the aetiology of BPD and other highly heritable complex disorders is the problem known as the ‘heritability gap’ – the fact that genetic research approaches have so far failed to explain the majority of the heritability of these disorders.

Some of the heritability of BPD may be found in larger polymorphic variants such as inversions and duplications. Other possibilities include rarer alleles of moderate effect, parent of origin effects, and epigenetic inheritance (Eichler et al., 2010).
The complexity of both the disorder and the interaction between possible causative factors ensures that the identification of genetic factors contributing to BPD is an involved process that will require many different strategies and will inform many different areas of genetic research.
1.3. The Treatment of Bipolar Disorder

There is no cure for BPD, but effective management of the disorder is achieved in many individuals through the use of psychiatric drugs, primarily mood stabilisers, antidepressants and antipsychotics (Moller and Nasrallah, 2003).

The administration of a lithium salt (usually lithium carbonate) as a mood stabiliser has been practised for well over half a century and is the best-known and usually frontline treatment for those with BPD (Lenox and Hahn, 2000). More recently, several drugs from the class of anticonvulsants, particularly valproate and carbamazepine, have been shown to have mood stabilising properties and have become common treatments for individuals with BPD (Ketter et al., 2003).

1.3.1. Mood Stabilising Drugs

1.3.1.1. Lithium

The use of lithium salts as a treatment for BPD began in the very early 1950s after Australian doctor John Cade published on the sedative effects of lithium for mania. It was eventually approved by the United States Federal Drug Administration (FDA) for the treatment of mania in 1971 (Serretti, 2002). However, lithium has become more than just a treatment for mania and is now widely used for mania prophylaxis and the long-term treatment of all phases of BPD. Williams et al. (2000) state that ‘lithium is the standard prophylactic agent for bipolar and is taken by 1% of the population’. In 2004 a meta-analysis by Geddes et al. (2004) showed that long-term lithium therapy was significantly more effective than placebo in preventing relapse of mood disturbance. Use of lithium is also associated with a decreased rate of suicidal behaviour in individuals with bipolar disorder (Yerevanian et al., 2003). It is most effective in BPD-I and of limited use in patients with rapid cycling or mixed state bipolar (Freeman et al., 1992).

Despite lithium’s popularity in the treatment of BPD, many individuals have a poor clinical outcome on the drug. Lithium response among individuals
with BPD is about 66% in research situations, but when used in a typical clinical setting positive response to lithium is found only in 33% of patients (Schou, 1997). This efficacy-effectiveness gap may be explained by the side effects of taking lithium. Many patients experience moderate to severe side effects such as increased urination, nausea, tremor and excessive thirst while taking lithium, and this can lead to non-adherence to the drug regimen (Scott and Pope, 2002). One of the most often cited reasons for non-adherence to lithium treatment by people with bipolar disorder are the side effects caused by the drug, or fear of suffering side effects. Non-adherence to a mood stabiliser drug regimen was found to be around 50% among bipolar patients, with compliance reducing the longer the person had been on the prescribed drug (Scott and Pope, 2002). Unfortunately, the therapeutic dose range of lithium (a serum level of 0.8-1.2mM) is very close to the level where lithium becomes toxic (1.5-2.0mM); therefore administration problems can sometimes lead to lithium toxicity (Geddes et al., 2004). Individuals taking lithium carbonate for treatment of BPD are required to have regular blood tests to monitor serum lithium concentration. Despite this, lithium remains the gold standard for both acute and maintenance treatment of BPD (Hirschowitz et al., 2010).

1.3.1.2. Valproate

Valproate, or valproic acid, is a branched chain fatty acid that is structurally similar to gamma-aminobutyric acid, the main inhibitory neurotransmitter found in the human brain (Ketter et al., 2003). Valproate is administered to patients as sodium valproate, the sodium salt of the acid.

First synthesised in 1881, valproate has a long history as an anticonvulsant and is a frontline drug in the treatment of epilepsy (Bowden, 2003). It has been recognised for its mood stabilising effects since as far back as 1966 when it was first trialled on bipolar patients (Lambert et al., 1966).

Two large studies in the early nineties provided the impetus for valproate to receive FDA approval for the treatment of mania. Both studies were double blind and placebo-controlled, and were performed over a three-week period on hospitalised patients. The first, performed by Pope et al. (1991) on lithium
intolerant patients, found that valproate had a 54% improvement over the baseline condition compared to a 5% improvement by those receiving a placebo.

Bowden et al. (1994) showed that valproate was significantly more effective in controlling mania than placebo, with an improvement of 48% over baseline and equivalent to lithium in terms of its mood stabilising effects. Significant improvement with valproate was present from the fifth day of treatment in most patients, and the symptoms that showed the quickest response were those integral to mania – elevated mood, increased activity, and decreased need for sleep. Further studies of valproate monotherapy have confirmed its positive clinical response for the majority of individuals experiencing a manic episode (Vasudev et al., 2000; Macritchie et al., 2003; Ghaemi et al., 2007; McElroy et al., 2010).

Unlike lithium, valproate has been shown to be efficient in treating those patients who have mixed mania, rapid cycling or cyclothymic disorder as well as those with classic mania (Calabrese and Delucchi, 1990; Freeman et al., 1992).

Dosage of valproate in the treatment of mania is individual to the patient, but a minimum serum level of 45-50µg/mL seems to be widely accepted among clinicians (McElroy et al., 1993) and this can be achieved by an initial loading dose of up to 20mg/kg of body weight (Bowden, 1998). Several studies have recommended lower serum levels of valproate for maintenance treatment of BPD, and also for treatment of cyclothymic conditions.

The side effects of valproate are generally better tolerated than those of other mood stabilising drugs, especially those of lithium. Common side effects of valproate are weight gain, tremor and hair loss (Bowden, 2003), and more serious side effects can include reduction in platelets and white blood cells, gastrointestinal problems, and liver failure (Tohen, 1995). Liver function is monitored regularly in patients taking high doses of valproate. Cognitive side effects are very rare but may include mild memory deficit (Goldberg and Burdick, 2001). The benefit of this low side effect profile is that it encourages
patient compliance with the drug regimen, decreasing incidences of relapse. Several studies have shown that discontinuation of mood stabiliser therapy because of drug intolerance is significantly lower among patients being treated with valproate compared to those taking lithium (Bowden et al., 1994; Bowden et al., 2000).

1.3.1.3. Carbamazepine

Carbamazepine is a tricyclic compound that has long been used for seizure control in people with epilepsy and brain injury. Over the last 25 years it has become increasingly popular as a mood stabiliser, prescribed as monotherapy or in combination with lithium or valproate (Keck and McElroy, 2002).

Initial side effects of carbamazepine may include dizziness and sleepiness, however these usually decrease after time at a constant dose. Common side effects of carbamazepine include nausea, vomiting, drowsiness, light sensitivity and mild skin rash (Elphick, 1989).

A few individuals may experience more severe side effects (primarily dermatological conditions caused by increased sensitivity to light, and more rarely, kidney problems) at serum levels considered to be therapeutic for BPD, but this is rare (Delcker et al., 1997).

The initial dosage of the drug is 200-400mg per day, divided into several doses, which can be increased up to 1600mg per day although most patients will find their symptoms controlled at a lower dose than this. Extended release preparations of carbamazepine are available which lower the number of times per day the drug must be taken (Ketter et al., 2004).

Several studies of the efficacy of carbamazepine as compared to lithium in the treatment of BPD found that average patient response to lithium is slightly better, especially in the prophylaxis of mania (Kleindienst and Greil, 2000; Kleindienst and Greil, 2002; Hartong et al., 2003). Thus carbamazepine may be especially useful in patients that do not respond to lithium or are intolerant of its side effects (Hartong et al., 2003). Combination therapy utilizing carbamazepine in conjunction with lithium or valproate has been found to be
highly effective for individuals with rapid cycling bipolar disorder, with Denicoff et al. (1997) reporting that 56% of rapid cycling patients reported no hospitalisation within the course of a year on the combination of carbamazepine and lithium, whereas only 28% (lithium) and 19% (carbamazepine) of patients on monotherapy did the same.

Hartong et al. (2003) also found that patient compliance in those taking carbamazepine was higher than among those taking lithium, and surmise that this is due to the less severe side effects experienced by patients on carbamazepine.

1.3.1.4. Oxcarbazepine
Oxcarbazepine is a tricyclic anticonvulsant drug that was developed by altering the structure of carbamazepine. The resulting molecule has an extra oxygen atom in the centre ring, and is metabolised by the body in a way that results in fewer side effect causing metabolites than is the case with carbamazepine. Oxcarbazepine therefore tends to have a lower side effect profile than carbamazepine, with side effects generally restricted to initial fatigue and, for some individuals, a mild rash (Pratoomsri et al., 2006).

Although oxcarbazepine is widely prescribed for BPD, generally as part of polytherapy in conjunction with lithium, evidence for its efficacy is limited. Only a small number of studies have looked at the drug as part of BPD prophylaxis, and results have been mixed. A small study of oxcarbazepine as adjunct therapy to lithium found oxcarbazepine was superior to placebo at preventing depressive episodes and impulsiveness, but did not reduce time between symptom recurrences significantly (Vieta et al., 2008). Evaluations of oxcarbazepine for maintenance treatment of BPD recommend it only as a secondary treatment to be used in combination with another drug due to lack of evidence of its effectiveness (Mazza et al., 2007).

1.3.1.5. Lamotrigine
Lamotrigine was approved by the United States Food and Drug Administration (FDA) for the treatment of BPD-I in June 2003, and is the only drug other than lithium to have received this approval. Lamotrigine is an
anticonvulsant drug, but bears no structural similarity to either carbamazepine or valproate. Lamotrigine inhibits voltage-sensitive calcium channels, stabilising neuronal membranes and blocking glutamate release (Lees and Leach, 1993).

Lamotrigine was found to be effective against the depressive phase of BPD-I, with a dosage effect where individuals on 200mg/day exhibited a better response than those on 50mg/day (Calabrese et al., 1999). A large study comparing lithium monotherapy, lamotrigine monotherapy and placebo found that lithium and lamotrigine were both significantly better than placebo at preventing or delaying a mood episode, with lithium superior at mania prophylaxis and lamotrigine more effective at prevention of bipolar depression. The side effects of lamotrigine are considered to be less severe than those of lithium (Goodwin et al., 2004). A meta analysis of the data on lamotrigine in the treatment of bipolar depressive episodes, which analysed five studies comprising 1072 individuals, found that lamotrigine was more effective at alleviating depression than placebo, especially among those patients considered to have more severe depression (Geddes et al., 2009).

1.3.2. Other medications

1.3.2.1. Antidepressant drugs
The depressive episodes of BPD may be treated with antidepressant medication, either prophylactically or only during the occurrence of low mood. However, individuals with BPD are at risk of antidepressant-induced mania, which happens in 20-40% of bipolar patients who are treated with antidepressants. This effect has been reported for all classes of antidepressants (Goldberg and Truman, 2003).

It is common for mania induced by antidepressants to be a bipolar individual’s first experience of mania and the first clue that their illness is BPD rather than unipolar depression, although it cannot be diagnosed as such on the basis of a medication-induced manic episode.
Primarily because of the risk of induced mania, the use of antidepressants in BPD is usually as part of polytherapy in combination with a mood stabiliser (Bottlender et al., 2001), however the benefits of this sort of adjunctive treatment are still under debate. A blinded controlled study of 366 bipolar individuals found no difference in mood response between those given paroxetine or bupropion antidepressants in addition to their normal mood stabiliser and those who received a placebo (Sachs et al., 2007). Similarly Nemeroff and colleagues found the addition of paroxetine or imipramine to lithium had no advantage over placebo in 117 bipolar patients experiencing a depressive episode (Nemeroff et al., 2001). More research is required to clarify if antidepressant therapy is truly indicated in BPD.

1.3.2.2. Antipsychotic drugs
Antipsychotic drugs are generally used in BPD where an individual experiences psychosis along with mania (Moller and Nasrallah, 2003). There are two types of antipsychotic drugs, typical (or first-generation) antipsychotics, and second-generation atypical antipsychotics.

The first-generation antipsychotics such as chlorpromazine and haloperidol have long been in use in antimanic treatment, and have been reported to be significantly more effective than placebo in normalising mania and mixed mania (Yildiz et al., 2011). Typical antipsychotics have a range of side effects that many find intolerable, and some individuals develop dangerous conditions such as neuroleptic malignant syndrome, a potentially fatal neurological disorder, and tardive dyskinesia, a condition involving involuntary repetitive movements. Although atypical antipsychotics are not free of side effects they tend to be better tolerated and are now more commonly prescribed than typical antipsychotics. (Liauw and McIntyre, 2010).

Atypical antipsychotics such as olanzapine, risperidone and quetiapine are approved by the FDA for use in acute mania, but an overview of studies on the use of atypical antipsychotics has also shown that they are also superior to placebo in the treatment of the depressive phases of BPD-I (Gao et al., 2005). Atypical antipsychotics are often appropriate for short term stabilisation of
severe episodes of disturbed mood, however recent research has shown that second-generation antipsychotics do not improve long term outcome for BPD when taken prophylactically (Brooks et al., 2011).

1.3.3. Other treatments

1.3.3.1. Electroconvulsive Therapy
Electroconvulsive therapy (ECT) is the induction of seizures by electrical stimulation of the brain in order to treat severe depression, mania, and catatonic schizophrenia. The procedure is performed under anaesthesia, using electrodes placed on the head to deliver measured current to the brain to induce seizure (Fink and Taylor, 2007).

In a recent systematic review, Versiani et al. (2011) reported that while there is a paucity of studies addressing the efficacy of ECT in bipolar disorder, the few appropriately controlled trials show ECT to be better than either placebo or lithium in the treatment of mania, and of similar efficacy to antidepressant medication in the treatment of bipolar depression. The efficacy of ECT in the treatment of unipolar depression is well established (2003), and was found to be equally as effective in bipolar depression in a recent large study (Bailine et al., 2010). Another recent study reported ECT to be effective in individuals with rapid cycling bipolar disorder, significantly decreasing relapse and lessening the duration of time spent in mania or depression 13-fold on average in the course of a year (Minnai et al., 2011).

The major side effect of ECT is memory loss, which can be either transient or permanent. The magnitude of effect on memory varies considerably from individual to individual, with very little discernable loss in some individuals ranging to considerable impairment in others (Crowley et al., 2008).

While ECT is used in New Zealand, it is generally considered to be a treatment of last resort for individuals who have not responded to pharmacotherapy. Reluctance on the part of clinicians to prescribe ECT may be in part to the widespread negative perception of the treatment among the public due to its controversial history in New Zealand (Melding, 2006).
1.3.3.2. Omega-3 Dietary Supplementation

Omega-3 fatty acids, including eicosapentaenoic acid and docosahexanoic acid are often recommended to those with BPD as an addition to their diet. A small study by Stoll et al. (1999) found that individuals with BPD assigned to 6.2g of eicosapentaenoic acid and 3.4g of docosahexanoic acid daily experienced better outcomes on several measures than those assigned to placebo. These outcomes included a significantly longer period of remission, while side effects from the eicosapentaenoic acid and docosahexanoic acid were minimal.

1.3.3.3. Psychosocial intervention

Psychosocial intervention such as counselling, cognitive behavioural therapy, family therapy, group therapy and health education are often used alone or in conjunction with medication to treat individuals with BPD. As the symptoms of BPD can lead to impairment in psychosocial functioning and the breakdown of relationships, psychosocial interventions are designed to protect and enhance interpersonal and environmental functioning, a measure shown to improve long-term outcomes for individuals with BPD (Zaretsky et al., 2007).

Family therapy and psychoeducation have shown high levels of empirical support for their effectiveness (Parikh et al., 1997). Family therapy, which works with the family and caregivers of the affected individual in a group setting, focuses on addressing the interactions between the group members as well as education about the disorder and teaches approaches to lessen the burden of the disorder on the family. A controlled study of the caregivers of individuals with severe BPD found that family-focused cognitive behavioural therapy reduced symptoms of depression in caregivers as well as increasing favourable outcomes for the individual with bipolar disorder (Perlick et al., 2010).

Group therapy in combination with pharmacotherapy was shown to reduce anxiety and depressive symptoms in bipolar patients when compared to pharmacotherapy alone in a recent study by Costa et al. (2011). Similarly, a
randomised controlled study of 84 participants assigned to either group-based psychotherapy alongside their normal drug treatment, or drug treatment alone found that the number and duration of relapses were significantly reduced in the treatment group (Castle et al., 2010).

There are a wide variety of subtypes of psychosocial intervention that may be helpful for those with BPD, however a lack of research on many of these subtypes means empirical evidence for their efficacy is lacking. However as a whole psychosocial intervention has a positive effect on patient outcome in BPD when used in conjunction with pharmacotherapy, and the majority of clinicians recommend a collaborative approach between the two modalities in the treatment of people with bipolar disorder (Berk et al., 2004).
1.4. Aetiological clues from drug treatments

Although clinical use of mood stabilisers is both well established and effective in the management of BPD, the molecular mechanisms by which these drugs normalise mood are only just starting to be understood.

The clinical picture of mood stabiliser treatment shows that patients generally require around four weeks of drug administration before a beneficial effect on mood can be observed. The requirement for chronic exposure of this type has led researchers to theorize that mood-stabilising drugs may induce long-term neuroplastic changes that are mediated by changes in gene regulation (Lenox and Hahn, 2000). Research into the changes effected by lithium on cellular systems, especially in neural cells, have provided important clues as to where the causes of BPD may lie, and provided candidate processes and genes for further investigation.

1.4.1. Neuroprotection
BPD causes structural abnormalities in the brain over the long term. Many magnetic resonance imaging studies have confirmed that the brains of individuals with BPD have significantly increased ratio of ventricular space to brain tissue, and this ratio tends to increase over time, especially in untreated patients (Post et al., 2003). Changes in grey matter volume over time, especially in the prefrontal cortex, have been found in both adolescents and adults with BPD (Lisy et al., 2011), and structural changes in the brains of individuals with BPD are primarily found in brain areas thought to be involved in mood regulation and cognition (Frazier et al., 2005). Glial cell density is reduced in the anterior cingulate (Ongur et al., 1998) and the amygdala of those with BPD, however it has been shown that this reduction is ameliorated in individuals treated with lithium or valproate (Bowley et al., 2002). MRI studies show that chronic administration of lithium significantly increases the total amount of grey matter in individuals with BPD compared to untreated individuals, underlining its neuroprotective effect (Manji et al., 2000). Anticonvulsant mood stabilisers have also shown neuroprotective
effects (Li et al., 2002). Valproate, as well as lithium, has been shown to increase levels of the neuroprotective protein bcl-2 in rats (Chen et al., 1999b).

Chronic lithium treatment has been found to be neuroprotective in conditions such as hypoxia, irradiation and chronic stress in both cellular and animal models (Wood et al., 2004). Increase in volume of grey matter has been detected in bipolar patients treated with lithium for four weeks (Moore et al., 2000).

1.4.2. Mood stabilisers and signalling pathways

Signal transduction pathways have been an area of much recent research in the role of mood stabilisers and causes of BPD, primarily because of the direct inhibitory effect lithium has on several of the pathways both extra-cellularly and at the second messenger stage (Ghaemi et al., 1999). Signal transduction pathways are the means whereby signals enter the cell by the initial binding of a ligand to a cell surface or cell membrane integral receptor. The second part of each signal transduction pathway utilises a second messenger to carry signals from the cell-membrane integral receptors to either the cytoplasm or the cell nucleus, depending on site of the pathway target. Most important intracellular processes are controlled by these pathways, and their regulation is a determining factor in how a cell functions and interacts (Fisar and Hroudova, 2010). Genes coding for the proteins and enzymes integral to signalling pathways, especially those directly affected by mood stabilising drugs, are potential candidates for research into the aetiology of BPD. Candidate genes can then be investigated for an association with the disorder. One criticism of the selection of candidate genes for the study of the aetiology of BPD via linkage studies or genome-wide association studies is the lack of plausible functional mechanism of action for their effect. Selecting candidate genes from the pathways affected by lithium has the advantage of already having a functional basis for selection.

The following section provides a brief overview of some of the main signal transduction pathways and how mood stabilisers such as lithium and valproate affect them. It also outlines eight genes from lithium-affected
pathways that are excellent candidates for research into their possible role in the aetiology of BPD or the mechanism of action of mood stabilisers.

1.4.2.1. The Wnt signalling pathway
The Wnt pathway is a highly conserved signal transduction pathway essential to human growth and development, and plays a role in directing important processes such as neuronal migration, embryonic patterning, and cell growth and differentiation (Mikels and Nusse, 2006). As well as being important in embryonic development, it also has critical roles in synaptic plasticity and cell survival in late life, especially in the central nervous system (Gould and Manji, 2002). Because of the importance of the Wnt pathway in neural development, it has long been a candidate for investigation into psychiatric disorders.

The Wnt protein family consists of lipid-modified proteins that are highly conserved across the animal kingdom. Wnt proteins bind to receptors from the Frizzled family on the cell surface, which prevents the proteolysis of β-catenin in the cytosol. β-catenin then enters the nucleus, and forms a complex with transcription factors to activate transcription of Wnt target genes (see Figure 1.2) (Hu et al., 2011). Fine mapping of genes in the Wnt pathway was carried out by Rhoads et al. (1999), and several genes were localised to areas of the genome that had previously been identified through linkage studies as potential susceptibility regions for psychiatric disorders, such as chromosome 22q11.

Mood stabilising drugs have been shown to affect the Wnt signalling pathway, implicating it in the pathophysiology of BPD. Lithium strongly inhibits the central enzyme of the pathway, glycogen synthase kinase 3 (GSK3) (Hedgepeth et al., 1997). This inhibition results in the stabilisation of β-catenin, which then enters the cell nucleus to turn on T-cell factor and lymphoid enhancer element (TCF/LEF) dependent genes. These genes go on to control cell cycle regulation and several other developmental processes (Lenox and Wang, 2003). Valproate has also been shown to indirectly inhibit GSK3 at therapeutically relevant concentrations (Bowden and Singh, 2005;
Kim et al., 2005), but this is not true of carbamazepine (Harwood and Agam, 2003).

Wnt signalling plays an important role in neuroprotection, which may explain the prophylactic effects of mood stabilisers on individuals with BPD. Researchers studying Alzheimer’s disease have found that the activation of the GSK3 protein leads to amyloid-β mediated apoptosis. Where GSK3 is inhibited by lithium, the neurotoxic effects of amyloid-β are reduced (Toledo et al., 2008). The genes involved in Wnt signalling and those that interact with the pathway are strong candidates for research into BPD.

1.4.2.1.1. Glycogen synthase kinase 3 beta

The glycogen synthase kinase 3 (GSK3) protein is a serine/threonine kinase that has two highly homologous isoforms, A and B. These are encoded by two different genes, glycogen synthase kinase 3 alpha and beta. The glycogen synthase kinase 3 beta (GSK3B) gene is located on chromosome three at position q21.1. It is very highly expressed in the human brain and is considered to be very important in the growth and development of the nervous system of most multicellular organisms (Eldar-Finkelman, 2002). GSK3 is part of several signalling pathways including the MAPK and lithium-affected Wnt signalling pathways, and has targets that are involved in cell adhesion, metabolism regulation, transcription, structural functions and cell division (Gould et al., 2004). The GSK3 protein is a negative regulator of the Wnt signalling pathway, phosphorylating β-catenin, which is then degraded by proteosome action. Initiation of Wnt signalling leads to inhibition of GSK3, and therefore the accumulation of β-catenin which then enters the nucleus activating genes targeted by the Wnt pathway (Gurvich and Klein, 2002). As a negative regulator of the signalling pathway, inhibition of GSK3 leads to increased level of Wnt signalling.

The GSK3 enzyme is affected by lithium in both in vitro and in vivo systems. In 1996 it was reported that lithium inhibited the action of GSK3 in Xenopus oocytes (Klein and Melton, 1996) and it has since been found that GSK3 is directly inhibited by lithium in mammals (O’Brien and Klein, 2009).
Valproate was initially reported to directly inhibit expression of the GSK3\(B\) gene at levels clinically relevant for the treatment of BPD (Chen et al., 1999a), however research since has not been able to replicate these results (Harwood and Agam, 2003; Ryves et al., 2005). Valproate also regulates axonal remodelling mediated by GSK3 (Hall et al., 2002).

Blood GSK3 protein levels in individuals during the manic phase have been shown to be significantly higher than those of healthy controls, and antimanic treatment over eight weeks (lithium or valproate either as monotherapy or combined with an antipsychotic such as olanzapine) inhibited GSK3 by serine phosphorylation (Li et al., 2010).

Benedetti et al. (2004) found that a single nucleotide polymorphism of the GSK3\(B\) gene strongly influenced the age of onset of illness in a cohort of 185 individuals with BPD-I. Homozygotes for the common T/T variant of the 50T/C polymorphism were found to have an earlier age of onset than those with the less common T/C genotype. The direct inhibition of the GSK3 protein by lithium, combined with GSK3’s wide role in regulating neuronal cell mechanisms, makes it an excellent candidate for further expression studies.
1.4.2.2. The MAPK/ERK pathways

Mitogen activated protein kinase (MAPK) pathways are complex three-layered signal cascades involved in regulating cell proliferation, controlling cell survival, and regulation of gene expression (Chang and Karin, 2001). Both lithium and valproate have been shown to activate extracellular signal-regulated kinase (ERK), an important component of the MAPK pathway (Yuan et al., 2001; Einat, 2003). Activation of ERK leads to an increase of DNA binding by activator protein 1 (AP-1) transcription factor, regulating gene expression in neural cells (Chen et al., 1997).

1.4.2.2.1. Brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) is a member of the large family of neurotrophic factors (Leibrock et al., 1989), all involved in the development and maintenance of neural cells and the nervous system at large (Maisonpierre et al., 1991). The human BDNF gene is situated on chromosome 11 at 11p14.1. Pruunsild et al. (2007) performed a thorough analysis on the gene and found that it has a complex structure that includes nine functional
promoters and 11 exons, with the 3' exon encoding the majority or all of the protein depending the transcript. Different areas of the brain were found to have various transcript-dependent expression profiles. The hippocampus, for example, had high levels of all transcripts, whereas in the amygdala only transcripts containing exons 1, 4 and 6 occurred at high levels. The combination of multiple tissue-dependent promoter sites and a variety of splice sites indicate BDNF expression is highly regulated at the transcription level and has the potential for very precise regulation throughout development.

The BDNF protein is produced primarily in the brain and spinal cord and plays an important role in the growth, differentiation and maintenance of neural cells (Davies, 1994). BDNF starts off as a larger protein that is processed by pro-protein convertases inside the cell to form the mature secreted BDNF protein, and the sequence of this mature form is conserved across all mammals so far studied (Maisonpierre et al., 1991).

BDNF has long been considered an attractive candidate gene for a role in the aetiology of psychiatric disorders, due to both its recognised importance in the cell survival and regulation of apoptosis in neural cells (Webster et al., 2002), as well as considerable evidence from both animal and human studies. Comparison between levels of BDNF mRNA and BDNF protein have shown marked differences between psychiatric patients and controls, while drug treatment has been shown to affect BDNF levels in several different models. Exposure to stress decreases hippocampal levels of BDNF mRNA in rats (Smith et al., 1995), while long-term administration of antidepressant medications and the induction of an electroconvulsive seizure has the opposite effect (Nibuya et al., 1995), as does the atypical antipsychotic drug quetiapine (Park et al., 2006). It has been shown that long-term administration of lithium to rats leads to a significant increase in the expression of BDNF in both the hippocampus and temporal cortex (Fukumoto et al., 2001). Similarly, Frey et al. (2006b) found that chronic administration of lithium and valproate both caused an increase in BDNF in the hippocampus of a rat model of mania. In humans, postmortem studies of brain tissue have found significant reduction in BDNF mRNA and protein levels in those with BPD in both
hippocampus (Dunham et al., 2009; Thompson Ray et al., 2011) and frontal cortex (Kim et al., 2010) compared to control. Postmortem hippocampal BDNF mRNA and protein levels are lower in those who have committed suicide than in matched non-suicide controls (Dwivedi et al., 2003). Chen et al. (2001) found that BDNF levels were higher in postmortem hippocampal sections of patients with major depressive disorder who were on antidepressant medication at the time of death than those who were unmedicated.

Serum BDNF protein levels are lower in patients who are experiencing a major depressive episode than in control subjects (Karege et al., 2002; Piccinni et al., 2008; Dell’Osso et al., 2010) and treatment with antidepressants is able to increase these levels (Shimizu et al., 2003; Aydemir et al., 2005; Gervasoni et al., 2005; Gonul et al., 2005; Karege et al., 2005; Yoshimura et al., 2007; Huang et al., 2008), as is ECT treatment (Bocchio-Chiavetto et al., 2006). Even depression-related traits such as neuroticism have been found to correlate with lower serum levels of BDNF in healthy individuals (Lang et al., 2004). Many studies have found that plasma and serum BDNF levels are significantly lower in individuals with BPD than in those without mood disorders (Palomino et al., 2006; Machado-Vieira et al., 2007; Monteleone et al., 2008; Fernandes et al., 2009; Tramontina et al., 2009). Studies have also reported that serum BDNF levels tend to be lower in people currently experiencing manic or depressive episode compared to euthymic BPD patients (Cunha et al., 2006; de Oliveira et al., 2009), and these levels decrease over the course of the illness (Kauer-Sant’Anna et al., 2009). People with schizophrenia also have reduced BDNF levels in serum (Toyooka et al., 2002).

In a rat model of depression the introduction of BDNF protein into the hippocampus produced an antidepressant effect of a similar magnitude to antidepressant drugs (Shirayama et al., 2002). In humans, treatment with lithium or other mood stabilisers has been shown to increase plasma BDNF levels in patients undergoing a manic episode (Palomino et al., 2006; de Sousa et al., 2011).

Several BDNF gene targeted knockout mouse models have been created, and these have a wide range of effects on phenotype, from total lethality to
craniofacial abnormalities, depending on the magnitude of the knockout (Bult et al., 2008). Abnormalities of the nervous system and of behaviour are common to all BDNF knockout mice (Davies, 1994; Ernfors et al., 1994; Birling and Price, 1995; Conover et al., 1995; Liebl et al., 1997).

The BDNF val66met polymorphism (rs6265), a non-synonymous single nucleotide polymorphism situated at nucleotide 196, is of special interest because the allele that codes for methionine has been shown to have an effect on the intracellular processing and secretion of the protein, leading to detectable differences in hippocampal function (Egan et al., 2003). The functional nature of this polymorphism makes it a plausible contributing factor for psychiatric disorders such as BPD.

1.4.2.3. The phosphotidyl inositol pathway

The phosphoinositide/protein kinase C signalling pathway is inhibited at several different points by lithium. Lithium inhibits the dephosphorylation of inositol bisphosphates to monophosphates, and similarly the step from inositol monophosphate to inositol. This leads to a reduction in concentration of inositol and an accumulation of diacylglycerol and inositol 1,4,5 triphosphate (InP$_3$). Accumulation of diacylglycerol downregulates the protein kinase C signalling cascade (see Figure 1.3), whereas increased concentrations of InP$_3$ trigger extra Ca$^{2+}$ release from the endoplasmic reticulum (Lenox and Wang, 2003). Chronic valproate administration has the same effect, although without the accumulation of inositol monophosphates (Dixon and Hokin, 1997). It has been suggested that the depletion of inositol causes a series of changes along the protein kinase C pathway that eventually lead to alterations in gene expression producing the clinical benefit given by the drugs (Manji et al., 1999). In addition, Ca$^{2+}$ is an important second messenger that helps regulate enzymatic activity, and changes in cytoplasmic Ca$^{2+}$ concentration may be relevant to BPD pathology. B-lymphocyte cell lines from patients with BPD are known to show abnormalities in Ca$^{2+}$ homeostasis – elevated basal Ca$^{2+}$ is one of the most reproducible markers found among bipolar individuals (Emamghoreishi et al., 1997). Chronic lithium treatment has been shown to attenuate mobilization of intracellular Ca$^{2+}$, further implicating the role of this signalling system in the aetiology of BPD.
Cell lines from bipolar patients have been reported to have significantly lower inositol content than those from controls (Belmaker et al., 2002).

Figure 1.3 The phosphoinositide signalling system

The brain is sensitive to inositol depletion as the blood/brain barrier limits availability of inositol to the brain, making it dependent on inositol recycling and synthesis within the brain (Jope et al., 1996). Three major steps of the recycling of inositol mono- and poly-phosphates back to inositol are directly inhibited by lithium, making the phosphoinositide signalling system an interesting candidate when looking for mechanisms of action of mood stabilising drugs. Six of the genes chosen for screening are part of the phosphoinositide signalling system.

1.4.2.3.1. Inositol-1-monophosphatase 1 and inositol-1-monophosphatase 2
The inositol-1-monophosphatase 1 (IMPA1) and inositol-1-monophosphatase 2 (IMPA2) genes each code for a different version of the IMPase enzyme that plays an integral role in the recycling of inositol within the phosphatidylinositol signalling pathway. IMPase dephosphorylates inositol monophosphates into myo-inositol allowing the downstream activation of protein kinase C (see Figure 1.3). This dephosphorylation is directly inhibited by lithium, which in turn reduces protein kinase C signalling and the release of intracellular secondary messengers (Atack et al., 1995).
This very direct and measurable inhibition by lithium led Berridge et al. (1989) to propose the inositol depletion theory of lithium action, providing another direction of enquiry for researchers interested in the aetiology of BPD and a possible mechanism whereby mood stabilisers were able to reduce the symptoms of the disorder (Atack, 1996).

IMPA1 and IMPA2 are found on different chromosomes (8 and 18 respectively) and do not exhibit a high level of homology (53.5%) (Sjoholt et al., 1997; Sjoholt et al., 2000; Yoshikawa et al., 2000). IMPA1 is expressed at very high levels in the brain and reasonably highly in most other tissues, whereas IMPA2 is mostly expressed in the pancreas, heart and skeletal muscle, and at a much lower level than IMPA1 (Yoshikawa et al., 1997).

Shamir and colleagues (2003) found that Impa1 but not Impa2 was upregulated in mouse brain after the mice were treated with lithium for ten days, and believe that this may be because IMPA1 is expressed at much higher levels than IMPA2 in all brain tissues and may have a greater role in inositol synthesis, requiring it to initiate a higher compensatory response when blocked by lithium. In a similar study, IMPA1 was upregulated 1.22 fold in mouse brain after two weeks of lithium treatment (McQuillin et al., 2007).

Parasarathy et al. (2003) found that IMPase 1 had much higher activity in various rat brain regions (such as the hippocampus, striatum and cerebral cortex) after four weeks of lithium administration at a therapeutic level. Nemanov et al. (1999) looked at the activity of IMPase in the lymphocytes of 36 individuals with BPD who were not on mood stabiliser treatment, and found that it was significantly lower than in control individuals. When the patients were treated with lithium, IMPase activity rose.

Two single nucleotide polymorphisms in the promoter area of IMPA2 have been found to be associated with BPD-I in a cohort of Arab families from Palestine (Sjoholt et al., 2004), and these associations were later replicated in a Japanese cohort (Ohnishi et al., 2007). As yet no associations of BPD with any IMPA1 polymorphisms have been reported.
Given the direct inhibitory effect of lithium on the enzymes coded for by these two genes, both \textit{IMPA1} and \textit{IMPA2} are excellent candidates for analysing changes in gene expression in a model of mood stabiliser administration.

\textbf{1.4.2.3.2. Inositol polyphosphate 1-phosphatase}

The inositol polyphosphate 1-phosphatase gene (\textit{INPP1}) codes for inositol polyphosphate 1-phosphatase (IPPase), which is an integral part of the phospholipase C signalling system and is closely related to IMPase (York \textit{et al.}, 1993).

\textit{INPP1} is found on chromosome 2q32 and consists of six exons spanning 25kb. It is expressed as a 1.9kb mRNA transcript in all body tissues, including the central nervous system (York and Majerus, 1990). Lovlie \textit{et al.} (1999) found that \textit{INPP1} was highly expressed in central nervous system tissue such as the substantia nigra and the cerebral cortex.

At therapeutically relevant concentrations, lithium blocks IPPase, preventing it from dephosphorylating the substrates inositol-1,3,4-trisphosphate and inositol-1,4-bisphosphate leading to a build up of these substances and a downregulation of inositol recycling in the phosphatidylinositol signalling pathway. This direct inhibition has lead researchers to theorise that \textit{INPP1} is a possible target for lithium treatment in bipolar disorder (Steen \textit{et al.}, 1998).

Steen \textit{et al.} (1998) found that none of the four \textit{INPP1} coding region polymorphisms they tested showed association with BPD. However one of the four, the transversion polymorphism C973A, showed a strong association with good lithium response in bipolar patients, with 67% of good responders having the transversion as opposed to only 11% of non-responders. An association has also been found between the rs2064721 SNP of \textit{INPP1} and post-traumatic stress disorder among patients with bipolar disorder (Bremer \textit{et al.}, 2007).

Piccardi \textit{et al.} (2002) found no association between the \textit{INPP1} SNP rs1882891 and bipolar disorder by transmission disequilibrium test of a cohort of 101 bipolar probands and their parents. The C973A polymorphism was similarly found not to be associated with clinical response to lithium prophylaxis in
this cohort (Michelon et al., 2006). Two other known polymorphisms, T51T and V116V, have shown association with autism (Serajee et al., 2003) but have not been looked at for association with BPD.

Bosetti et al. (2002) fed rats therapeutic doses of lithium chloride for 42 days and found by cDNA microarray that INPP1 was downregulated 2.7-fold in brain tissue after seven days and 2.6-fold after 42 days. Thus INPP1 is a very strong candidate for further mood stabiliser related expression studies.

1.4.2.3.3. Prolyl endopeptidase

Prolyl endopeptidase is a serine peptidase coded for by the gene PREP, found on chromosome 6q22 (Goossens et al., 1996). The protein is a monomer composed of 710 amino acids and can be found in both soluble and particulate forms. The enzyme hydrolyses only small peptides (up to 30 amino acids long) due to the action of a seven-bladed β-propeller domain which gates the active site allowing the entry of only small peptides (Polgar, 2002). This particulate form of prolyl endopeptidase is found mainly in the synaptosomal fraction of prepared cellular samples, as opposed to the cytosolic fraction, which indicates that prolyl endopeptidase may be responsible for the degradation of specific proteins in the synaptic cleft (O’Leary et al., 1996).

Irazusta et al. (2002) compared the distribution of prolyl endopeptidase activity in human and rat brains. They found that in humans it has its highest activity in the cerebral cortices, and reduced activity in the cerebellum. In contrast, activity in the rat brain was much lower and relatively homogenous throughout the different areas of the brain. They suggested that prolyl endopeptidase may play a role in higher brain function, such as memory or learning.

Breen et al. (2004) found that prolyl endopeptidase enzyme activity is higher in the blood plasma of individuals with untreated bipolar disorder and schizophrenia than normal controls, with a current manic episode highly correlated with enzyme activity. When treated with lithium, however, bipolar individuals showed a statistically significant drop in enzyme activity that may indicate a change in regulation of this gene by lithium.
Valproate has been found to reduce plasma prolyl endopeptidase enzyme activity, but when prolyl endopeptidase inhibitors are used the effect of these drug is reversed. Depressed subjects also had reduced prolyl endopeptidase activity compared to normal controls, which is a finding that lends weight to the theory that affective disorders may be a spectrum with mania at one end and severe depression at the other (Maes et al., 1995).

Williams et al. (1999) found that loss of the PREP homologue dpoA in the slime mold dictyostelium resulted in the elevation of inositol 1,4,5 trisphosphate (IP$_3$) concentration. Where DpoA activity decreased, inositol pentakisphosphate (IP$_5$) dephosphorylation to IP$_3$ was increased resulting in an IP3 concentration in a dpoA mutant three times higher than in dpoA wildtype cells. Further research has shown that in both dictyostelium and humans, PE acts via multiple inositol polyphosphate phosphatase to mediate the effects of prolyl endopeptidase on lithium sensitivity (King et al., 2010).

A study of nine single nucleotide polymorphisms of the PREP gene found no association with the level of response to lithium as a mood stabilising agent (Mamdani et al., 2007), however the combination of its inhibitory role in the lithium affected phosphatidylinositol signalling pathway and its decreasing plasma activity level under lithium treatment make PREP a good candidate gene for further expression studies.

1.4.2.3.4. Protein Kinase C A and Protein Kinase C E

Protein kinase C (PKC) is an integral part of several signalling pathways. It is activated by diacylglycerol after the hydrolysis of the membrane phospholipid PIP$_2$ (see Figure 1.3). There are 12 structurally similar PKC subtypes, and these are found at varying levels in brain and neural tissue (Hahn and Friedman, 1999). Protein kinase C A (PRKCA) and protein kinase C E (PRKCE) are the genes coding for the A and E subtypes of the protein. PKC signalling is involved in a wide range of cellular activities, including cell proliferation, maintenance of the cell cycle, and apoptosis, and the different gene transcripts are highly expressed throughout the brain (Zarate and Manji, 2009). Because of its integral role in cellular signalling and wide range of functions, PKC has been implicated in a wide variety of human diseases.
PKC levels have been found to be higher in individuals with BPD than controls in several different tissue types, such as whole brain (Wang and Friedman, 1996) and lymphoblasts. Chronic lithium treatment reduces PKC activation (Hahn and Friedman, 1999) and membrane associated PKC levels (Manji et al., 1993) in rat brain. A small study found the level of platelet cytosolic PKC-A protein in a group of lithium treated bipolar individuals was significantly greater than in a control group, suggesting that lithium treatment decreases PKC-A protein levels (Soares et al., 2000). Valproate reduces the level of both PKC-A and PKC-E proteins in cultured rat glioma cells (Chen et al., 1994).

The PRKCA gene has been implicated in association studies with several psychiatric disorders, including schizophrenia and schizoaffective disorder, with a low-frequency four-marker haplotype significantly associated with disorders featuring psychosis in three independent cohorts (Carroll et al., 2010).

1.4.2.4. The Arachidonic Acid cascade

The arachidonic acid cascade mediates secondary messenger pathways in neural tissue. It is cleaved from cell membrane phospholipids after activation of the G-protein initiated cytosolic phospholipase A₂ (cPLA₂) (Axelrod, 1995) and goes on to mediate downstream processes both inside and outside the cell (Quiroz et al., 2010). Mood stabilising drugs have effects on the arachidonic acid cascade; lithium reduces the activity of activator protein-2 (AP2), which then decreases the transcription of cytosolic phospholipase A₂ (cPLA₂), reducing the amount of the enzyme available to cleave arachidonic acid from its source phospholipid. Lithium has been shown to decrease the turnover of arachidonic acid in rat brain phospholipids by 80% (Chang et al., 1996).

Chronic administration of valproate has a similar effect, decreasing arachidonic acid turnover by 33% (Chang et al., 2001), however the
mechanisms by which this occurs appear to be different. Valproate has no effect on cPLA₂, but possibly has its effect on arachidonic acid through coenzymeA and short chain acyl-coenzyme A (Deutsch et al., 2003). Chronic carbamazepine administration has the effect of reducing the turnover of arachidonic acid in a very similar way to lithium, by decreasing cPLA₂ activity through AP2 activity reduction (Bazinet, 2009).

1.4.3. Selection of lithium affected genes from the literature
The literature also shows that lithium affects the expression of a number of genes that are not part of a known signal transduction pathway, and three of these genes are described here.

1.4.3.1. Endoglin
The endoglin (ENG) gene codes for endoglin, a dimeric transmembrane protein that is part of the transforming growth factor β1 receptor complex (Barbara et al., 1999). Mainly found in the vascular endothelium, its structure consists of an extracellular domain of 561 amino acids, a hydrophobic transmembrane section, and a cytoplasmic tail of 47 amino acids (Gougos and Letarte, 1990). Although the endoglin protein is localised primarily to endothelial and stromal cells (St-Jacques et al., 1994), it is present in both neural cells and brain vasculature (Matsubara et al., 2000).

Bosetti et al. (2002) fed rats therapeutic doses of lithium chloride for 42 days and found by cDNA microarray that ENG was downregulated 2.2 fold in brain tissue after seven days and 5.2-fold after 42 days. This makes ENG an interesting candidate gene for gene expression studies to see if this finding is replicated in a human neural cell model.

1.4.3.2. Cyclic phosphodiesterase 4D interacting protein and Ras related protein
Sun et al. (2004) cultured lymphocytes sourced from individuals with lithium responsive BPD and controls in lithium chloride treated media for seven days. A cDNA array was then used to screen for genes with significantly changed expression. Of the seven genes that showed significant changes, five were then verified by northern blotting. The ras-related protein (RAB7A) gene was
found to be downregulated 1.5 times; the cyclic phosphodiesterase 4d interacting protein (PDE4D) gene was downregulated 1.4 times. The fact that none of these genes were regulated by lithium in healthy subjects makes them excellent candidates for further expression studies involving mood disorders.

The RAB7A gene is situated on chromosome three, and consists of six exons and five introns (Suzuki et al., 2003). The gene is highly conserved across mammals, and expressed in a wide variety of tissue types, including neural cells (Vitelli et al., 1996). The RAB7A protein is a small molecular weight GTPase that has been shown to play a role in late endocytosis (Feng et al., 1995) final stage and neural migration (Kawauchi et al., 2010). RAB7A has not so far been reported as playing a part in the mechanism of action of any psychiatric drugs, but has been shown to be associated with more than one neural pathology. RAB7A is significantly upregulated in the cholinergic basal forebrain neurons of individuals with Alzheimer’s disease and mild cognitive impairment compared to controls, and this upregulation is correlated with the degree of cognitive decline (Ginsberg et al., 2011). Four missense mutations of the RAB7A gene are associated with the development of the peripheral neuropathy Charcot-Marie-Tooth syndrome type 2B (Verhoeven et al., 2003). Long-term administration of valproate leads to improvement in neurite formation in both cultured N1E-115 neuroblastoma cells and dorsal root ganglion neurons that carry one of the four mutations. This suggests that valproate may have an indirect regulatory effect on RAB7A, possibly mediated through the actions of c-Jun N-terminal kinase (Ogden et al., 2004; Yamauchi et al., 2010).

The PDE4D protein is a homodimer with high expression in the brain, and is involved with cAMP signalling in the inflammatory response (Verghese et al., 1995). PDE4D is of special interest in psychiatric disorders because it interacts with disrupted in schizophrenia 1 (DISC1). The rs1120303 SNP of PDE4D has been shown to be associated with schizophrenia (Tomppo et al., 2009).
1.5. Objectives of this investigation

The objectives of this investigation are to screen 20 genes that have previously been found to be affected by lithium for expression changes brought on by three mood stabilising drugs in three neural cell lines. Genes that show consistent and replicable changes across the different cell lines and treatments will be further studied in a second model involving lymphocyte cell lines from bipolar individuals and controls. Protein studies will also be undertaken to determine if changes in gene expression are reflected in changes in protein levels within both models.

The candidate genes chosen will also be analysed for association with BPD in the South Island Bipolar Study (SIBS) cohort, a resource consisting of DNA and diagnostic and history information from individuals with BPD and their first and second degree relatives.
2. Materials and Methods

2.1. Materials

2.1.1. Chemicals
Except where otherwise specified, all chemicals used were of molecular biology grade and were sourced from BDH Chemicals NZ Ltd., Palmerston North, New Zealand.

2.1.2. Water
The water used to make the solutions in this chapter was distilled then de-ionised using the Milli-Q Plus system (Millipore).

2.1.3. Buffers and Solutions

PCR Buffer (10x):
- 0.1M Tris-HCl (pH 8.0)
- 0.5M KCl
- 1mM-3.5mM MgCl₂, varied as required

PBS:
- 140mM NaCl
- 3mM KCl
- 8mM Na₂HPO₄
- 1.5mM KH₂PO₄

PBS-T:
- PBS as above
- 0.1% (v/v) Tween-20 (Sigma)

TBE (10x):
- 0.89M Tris-Borate (pH 8.3)
- 20mM EDTA
Acrylamide Gel Separating Buffer:
  1.5M Tris-HCl (pH 8.8)
  0.1% (v/v) SDS

Acrylamide Gel Stacking Buffer:
  0.5M Tris-HCl (pH 6.8)
  0.1% (v/v) SDS

Lysis Buffer:
  10mM Tris-HCl (pH 8.0)
  150mM NaCl
  1mM EDTA
  1% (v/v) Nonidet P40
  0.1% (v/v) SDS
  2x protease inhibitor tabs (Roche) per 100mL

TBS:
  20mM Tris-HCl (pH 7.6)
  150mM NaCl

TBS-T:
  TBS as prepared above
  0.05% (v/v) Tween-20 (Sigma)

TE:
  1M Tris-HCl (pH 8.0)
  0.5M EDTA

Orange G Loading buffer:
  0.005% Orange G (Harleco)
  40% w/v Ficoll 400
  Made up in 1xTBE.
Bromophenol blue Loading buffer:
- 0.005% w/v bromophenol blue
- 40% w/v Ficoll 400
- Made up in 1xTBE

2x Denaturing Loading buffer:
- 0.125M Tris-HCl (pH 6.8)
- 20% v/v glycerol
- 4% v/v 2-mercaptoethanol
- 0.2% w/v bromophenol blue
- 4% w/v SDS

Blocking Solution:
- 5% w/v low fat milk powder diluted in PBS-T

Polyacrylamide Separating gel:
- 8% Acrylamide/Bis Solution 29:1 ratio (Bio-Rad)
- 0.5M Tris-HCl (pH 8.8)
- 0.04% SDS
- 7% sucrose
- 0.2% (w/v) ammonium persulphate (Sigma)
- 0.02% (v/v) TEMED (Invitrogen)

Polyacrylamide Stacking gel:
- 5.5% Acrylamide/Bis Solution 29:1 ratio
- 0.1M Tris-HCl (pH 6.8)
- 0.1% SDS
- 0.16% (w/v) ammonium persulphate
- 0.16% (v/v) TEMED

2.1.4. Drug Solutions
Carbamazepine, lithium chloride and valproic acid were sourced from Sigma-Aldrich, MO, USA. Carbamazepine was prepared as a 0.5M stock solution in 100% dimethyl sulphoxide (DMSO). Lithium was prepared as a 0.1M stock solution of lithium chloride in PBS. Valproate was prepared as a 0.5M stock
solution of valproic acid, also in PBS. All drug solutions were sterilised by filtration.

2.1.5. Cell Lines

2.1.5.1. Neuroblast cell lines
All neuroblast cell lines were sourced from the American Type Culture Collection.

- SK-N-DZ is an adherent neuroblast cell line that was derived from a bone marrow metastasis of a brain tumour in a 2-year-old girl (Sugimoto et al., 1984).
- SK-N-SH is an adherent neuroblast cell line derived from a bone marrow metastasis of a brain tumour in a 4-year-old girl. This line was developed to produce high levels of dopamine β-hydroxylase (Biedler et al., 1973).
- SH-SY5Y is a thrice-cloned neuroblast-derived cell line, produced from the sub-cloning of SH-SY, which originally derived from the SK-N-SH neuroblast cell line. Cultures of SH-SY5Y are comprised of both floating and adherent cells (Biedler et al., 1978).

2.1.5.2. Lymphoblast cell lines
Ten lymphoblast cell lines were sourced from the Coriell Cell Repositories, Camden, NJ, USA. The lines were created by transformation of B lymphocyte cells from peripheral blood by Epstein-Barr virus at the Coriell laboratories. The first five cell lines were selected from the ‘Major Affective Disorders’ panel of the National Institute of General Medical Sciences Human Genetic Cell Repository. The five individuals selected from this panel are male, have a diagnosis of BPD-I, and were stably medicated with lithium carbonate at the time of venesection (see Table 2.1).

The five control cell lines were selected from the ‘Human Variation’ panel of the NIGMS Human Genetic Cell Repository, which consists of cell lines from 200 unrelated, apparently healthy Caucasian individuals. The five individuals selected are male, non-Amish Caucasians and have not been diagnosed with any affective disorder. The age at venesection for these
individuals ranged from 44 to 55. The catalogue identification numbers for the five control cell lines are GM17244, GM17249, GM17212, GM17243 and GM17248.

Table 2.1 Cell lines from the Major Affective Disorders Panel

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>AOO&lt;sup&gt;1&lt;/sup&gt;</th>
<th>#MDD&lt;sup&gt;2&lt;/sup&gt;</th>
<th>#Mania&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Family History&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM05134</td>
<td>15</td>
<td>1</td>
<td>2</td>
<td>Positive</td>
</tr>
<tr>
<td>GM04984</td>
<td>17</td>
<td>2</td>
<td>3</td>
<td>Positive</td>
</tr>
<tr>
<td>GM4986</td>
<td>17</td>
<td>5</td>
<td>2</td>
<td>Brother - BPD-I</td>
</tr>
<tr>
<td>GM07263</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>Father - BPD-I</td>
</tr>
<tr>
<td>GM0490</td>
<td>32</td>
<td>4</td>
<td>Unknown</td>
<td>Positive</td>
</tr>
</tbody>
</table>

<sup>1</sup>Age of onset of BPD-I  
<sup>2</sup>Number of episodes of major depression  
<sup>3</sup>Number of episodes of mania  
<sup>4</sup>Family history of psychiatric disorder

2.1.6. DNA

Human DNA used in the association studies was sourced from the South Island Bipolar Study (SIBS) DNA Database. This database consists of DNA from individuals with BPD and their first-degree relatives, and totals 786 samples. A brief overview of the SIBS can be found in Chapter Five, and full details of the recruitment process and description of the cohort are available in (Joyce et al., 2004).

The DNA had previously been extracted from whole blood by standard phenol-chloroform methods and was stored at -20°C in 100µL aliquots at a dilution of 100ng/µL.

2.1.7. Antibodies

Anti-actin antibody (Sigma): A rabbit polyclonal antibody directed against the 11 residues of the C-terminal, the highly conserved region common to α, β and γ actins in humans. Used for western blot at a 1:5000 dilution.

Anti-14-3-3γ antibody (Santa Cruz): A rabbit polyclonal antibody directed against the C-terminal of the human 14-3-3γ (YWHAG) protein. Used for western blot at a 1:200 dilution.
Anti-rabbit IgG antibody (GE Healthcare): A donkey polyclonal antibody to rabbit IgG, labelled with horseradish peroxidase. Used for western blot at a 1:2500 dilution.

2.2. Methods

2.2.1. Cell Culture

2.2.1.1. Neuroblast cell lines

Neuroblast cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) (Invitrogen) containing 50U/mL penicillin (Invitrogen), 50µg/mL streptomycin sulphate (Invitrogen) and 10% v/v Foetal Bovine Serum (FBS) (Bio International). All neuroblast cell lines were incubated in 10% CO₂ at 37°C.

For the drug studies, carbamazepine stock solution in DMSO (0.5M) was added to a final concentration of 0.05mM. An equal volume of sterile DMSO was added to the carbamazepine control media. Lithium stock solution (0.1M) was added to a final concentration of 1mM. Valproate stock solution (0.5M) was added to a final concentration of 0.5mM. No addition was made to the media for the lithium and valproate control flasks.

Neuroblast cells were harvested with a 0.25% trypsin-EDTA solution (Invitrogen) comprised of 2.5g/l of trypsin, 1.3mM EDTA, and phenol red indicator in Hank’s Balanced Salt Solution. Once the adherent cells had lifted, DMEM with 10% FBS was added to neutralise the trypsin, and the resulting suspension was centrifuged at 400 x g for five minutes. The supernatant was removed, the pellet was then rinsed with PBS and stored at -72°C.

2.2.1.2. Lymphoblast cell lines

Lymphoblast cells were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen) with the addition of 50U/mL penicillin (Invitrogen), 50µg/mL streptomycin sulphate and 10% v/v FBS. All lymphoblast cell lines
were incubated at 37°C and 5% CO₂. Where required mood stabilisers were added to the media as for the neuroblast cell lines.

Lymphoblast cells are non-adherent and were harvested by the centrifugation of the cell suspension at 400 x g for five minutes and removal of the supernatant. The pellet was then rinsed with PBS and stored at -72°C.

2.2.2. Polymerase Chain Reaction

2.2.2.1. Primer Design
Primers for PCR amplification were designed using DNA sequence information from the NCBI GenBank DNA database. Primers were designed to be between 17 and 23 nucleotides in length, and to have a C or G base at the 3’ end where possible. Primers were run through an online DNA Calculator (accessed at http://www.sigma-genosys.com/calc/DNACalc.asp) to check for secondary structures and possible primer dimer. The primers were also run through the UCSC In-Silico PCR online tool (accessed at http://genome.ucsc.edu/cgi-bin/hgPcr) to ensure the correct fragment of DNA was targeted.

Oligonucleotides were synthesised by Invitrogen and supplied desalted and lyophilised. Stock was made from the lyophilised primers to a concentration of 500µM for storage at -20°C. A working solution of 5µM was made, which was also stored at -20°C. All primer sequences for PCR can be found in Appendix E.

2.2.2.2. Standard PCR
A standard PCR reaction was used to amplify fragments of DNA for use in restriction enzyme assays and in sequencing. All PCRs consisted of 1 x PCR buffer, 200µM dNTPs (Roche), 0.2µM of forward and reverse primers, 0.02U/µL Taq polymerase (Invitrogen) and 2.5-5.0ng/µL template DNA. The standard parameters for amplification were as follows: 94°C for three minutes, followed by 30 cycles of 94°C for 15 seconds, a variable annealing temperature (55°C to 65°C, depending on the optimal temperature for the primer set) for 30 seconds, then extension at 72°C for 45 seconds. All PCRs
were performed either on an Eppendorf MasterCycler or a Biometra TProfessional Thermocycler.

PCR conditions for amplification prior to sequencing or restriction digest can be found in Appendix E.

2.2.2.3. Allele Specific PCR
A variation on standard PCR was used to genotype single nucleotide polymorphisms using a three-primer system. Two of the primers were designed to be identical except for the 3' base, which was changed to specifically bind only one of the alleles present in the SNP. The third primer was a common primer. The PCR reaction was performed in two different tubes, one containing the common primer plus the specific primer for allele one, and in the other the common primer and the primer specific for allele 2 (see Figure 2.1). The PCR products were visualised on 1% w/v agarose gel under UV light. The presence of a band in only the first well indicated a homozygous allele one genotype, whereas a band in only the second well indicated a homozygous allele two genotype. Bands in both wells indicated the individual was heterozygous at this site.

Well 1.

![Diagram of Allele 1 Specific Primer Binding](image)

Well 2.

![Diagram of Allele 2 Specific Primer Binding](image)

Figure 2.1 Two-well allele-specific PCR
A second type of allele specific PCR was also used. This assay also involved a common primer and two specific primers varying at the 3’ end, however, the addition of a ‘tail’ to one of the specific primers enabled the reaction to be performed in a single well, with the size of the PCR product being used to differentiate between alleles (see Figure 2.2).

Primers and reaction conditions for allele specific PCR assays can be found in Appendix E.

![Figure 2.2 Allele specific PCR using a tailed primer](image)

### 2.2.2.4. ARMS Assay

The amplification refractory mutation system (ARMS) assay is a four-primer PCR system that uses the production of different size bands to differentiate between alleles (Newton et al., 1989). The two pairs of primers overlap, but the 3’ terminal base of each of the specific primers is complementary to one of the alleles of the SNP. Thus three possible fragments are amplified – a common fragment, using the two common primers, and one fragment for each allele. The two specific fragments are differentiated by their size, and the assay can therefore be performed in a single well (see Figure 2.3).

Primers and reaction conditions for the ARMS assay can be found in Appendix E.
2.2.2.5. *TaqMan*

*TaqMan* SNP Genotyping Assays (Applied Biosystems) were used according to manufacturer’s instructions.

Amplifications were set up in a 96 well plate format, with each well containing a 25µL volume reaction mix consisting of 12.5µL *TaqMan*® Genotyping Master Mix (Applied Biosystems), 20ng of genomic DNA, 1.25µL of SNP Genotyping Assay Mix, and 11.25µL de-ionised water. The reaction was then performed on a thermal cycler with the following parameters: 95°C for 10 minutes, followed by 40 cycles of 92°C for 15 seconds then 60°C for one minute. Information on the *TaqMan* assays and probes can be found in Appendix F.

Plates were read and analysis performed on the Applied Biosystems AB7300 Real Time PCR machine and analysed using the associated AB7300 software.

2.2.3. *Agarose gel electrophoresis*

A 1% w/v agarose gel was prepared by adding agarose (Invitrogen) to 1xTBE and heating in a microwave oven until the agarose was fully dissolved. After the mixture was left to cool to 50°C, 1.3mM ethidium bromide (Invitrogen) was mixed in until thoroughly dispersed.

Gels were set in a casting platform (BioRad) to a thickness of 8mm and dimensions of either 100 x 150mm or 150 x 255mm.
Samples were prepared by the addition of an appropriate amount of either bromophenol blue loading dye or Orange G loading dye to amplified DNA. Around 10µL of sample was loaded per well, alongside a molecular weight marker such as marker V (Invitrogen) or 100bp marker (Invitrogen). Electrophoresis was performed in either a BioRad Wide Mini Sub Cell GT or a Biorad Sub Cell GT in 1xTBE at 10V/cm.

Gels were visualised on a UV transilluminator and images captured on a Panasonic video camera connected to a Macintosh computer running ImageJ software.

2.2.4. Extraction of RNA
RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. The optional on-column DNase digestion step was performed, as the RNA was going to be used to make cDNA for use in sensitive RT-qPCR analysis.

RNAse inhibitor (Qiagen) was added to a sample of extracted RNA from each cell line. The sample was then electrophoresed on a 1% agarose gel stained with ethidium bromide, and then the gel was examined under UV light. This enabled visual assessment of the 18S and 28S ribosomal RNA bands. Where the 28S band was around twice the intensity of the 18S band, RNA integrity was assumed. Concentration of the RNA in ng/µL was measured using the NanoDrop ND-1000 spectrophotometer and NanoDrop software v.3.1.2. For storage, RNAse inhibitor was added to the RNA then it was aliquoted into 100µL volumes and stored at -72°C.

2.2.5. Preparation of cDNA
The SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen) was used to prepare cDNA from RNA. 2µL of RNA was added to 44µL of de-ionised water, 20µL of 2µM dNTPs (Sigma) and 5µL of 50µM random hexamer primer (Invitrogen). This mixture was heated to 65°C for five minutes then cooled to room temperature. 20µL of 5x buffer, 5µL of 0.1M DTT, 2µL of RNase inhibitor, and 2µL of reverse transcriptase enzyme (all Invitrogen) were added to the mixture. This was then heated to 25°C for five
minutes followed by 50°C for one hour, and 70°C for 15 minutes. Once cooled, the 500µL mixture was aliquoted into five 100µL measures and stored at -20°C.

To ensure there was no contamination of the cDNA by genomic DNA, a PCR was run with the primers 5'-GATGCAGCAACCGAGGCTTCC and 5'-CACTCAGCGCAGTCC which amplify a portion of the MDK gene. These primers are designed to amplify across an intron and will therefore amplify a larger fragment (280bp) when amplifying genomic DNA than the 120bp fragment expected from the amplification of cDNA. Amplification was performed in an Eppendorf MasterCycler Gradient using the following cycling parameters: 95°C for 4 minutes, followed by 40 cycles of 95°C for 15 seconds then 60°C for one minute. The PCR products were run on a 1% agarose gel and visualised under UV light and compared against controls of both genomic DNA and cDNA amplified with the same primers and conditions.

2.2.6. RT-qPCR

Quantitative real-time polymerase chain reaction (RT-qPCR) was used to analyse changes in gene expression in both neuroblast and lymphoblast cells after treatment with mood stabilising drugs. Reactions were performed in 20µL volume containing 0.5 x Platinum® SYBR® Green q-PCR Supermix-UDG with ROX (Invitrogen), 0.1mM forward primer, 0.1mM reverse primer, and 5ng/µL template cDNA. Cycling parameters were as follows: two minutes at 50°C and ten minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds then one minute at 60°C. A dissociation cycle was performed at the end of the reaction, consisting of 95°C for 15 seconds, 60°C for one minute, and 95°C for another 15 seconds.

RT-qPCR was performed on the Applied Biosystems AB7300 Real-Time PCR system and analysed using the associated AB7300 software. Primer sequences for RT-qPCR can be found in Appendix A.
2.2.7. Microsatellite analysis
Analysis was performed on four microsatellite markers: D4S2426 (4q33), D7S2423 (7q36), D17S937 (17q25) and D22S1153 (22q13). These markers were amplified by PCR using primer pairs where the forward primer was tagged at the 5’ end with a 6-FAM fluorescent marker. All primers for microsatellite analysis were sourced from Sigma-Aldrich. Primers and conditions for amplification of the microsatellite regions can be found in Appendix E.

Amplified DNA was sent to the Massey Genome Service, Alan Wilson Centre, Palmerston North, New Zealand, for capillary separation. Results were analysed using Applied Biosystems Sequencing Analysis software version 5.2.

2.2.8. Restriction enzyme digest
Restriction enzyme digests were used for the genotyping of some SNPs. Initial amplification of the gene fragment of interest was performed as per section 2.2.2.2, and the amplification product was checked by agarose gel electrophoresis as per section 2.2.3. Once amplification had been confirmed the remaining product was digested with the appropriate enzyme under the appropriate conditions. 15µL of the digestion product was then mixed with 5µL Orange G loading dye and visualised under UV light after electrophoresis at 10V/cm on a 1% agarose gel. Enzymes used and reaction conditions for each SNP can be found in Appendix 2, Table A2.3. All restriction enzymes were sourced from New England BioLabs, MA, USA.

2.2.9. Sequencing
First the fragment to be sequenced was amplified (see section 2.2.2.2) and the product checked by agarose gel electrophoresis as described in section 2.2.3. Once amplification was confirmed, 0.5U of shrimp alkaline phosphatase (Affymetrix) and 10U of exonuclease 1 (NEB) were added to 9µL of the PCR product. This mixture was incubated at 37°C for 15 minutes to digest residual primers and to dephosphorylate residual dNTPs. This was followed by incubation at 80°C for a further 15 minutes to inactivate the enzymes. Sequencing primer to a final concentration of 0.3mM and 1µL of BigDye Terminator v3.1 (Applied Biosystems) was then added to each reaction. After vortexing to ensure a thorough mixing, the reaction mix was heated to 96°C
for one minute. This was followed by 25 cycles of 96°C for ten seconds, 50°C for 15 seconds, and 60°C for four minutes.

After cooling to room temperature, de-ionised water was added to make the mixture up to 20µL. Two µL of sodium acetate (3M), 1µL of EDTA (125mM) and 50µL of 96%v/v ethanol (Scharlau) was added and the mixture was left at room temperature for 15 minutes for the precipitation of the extension products to occur. The mixture was then centrifuged at 9500x g for 30 minutes and the supernatant removed and discarded. The pellet was rinsed with 200µL of 70% ethanol and centrifuged for a further five minutes. The supernatant was again removed, and the pellet allowed to air dry to ensure removal of all the ethanol. 20µL of HiDi formamide (Applied Biosystems) was then added to each well, the mixture was vortexed, spun and then heated to 95°C for two minutes. The product was finally chilled on ice.

Sequencing was performed with an ABI Prism 310 Genetic Analyser and its associated data collection software, version 3.1.0.

Sequence was viewed using 4Peaks sequence viewing software, available at http://www.mekentosj.com/science/4peaks.

2.2.10. Protein Extraction and Quantitation
To extract protein from harvested cells, the pellets were rinsed twice with PBS, centrifuged at 3000g for five minutes, and the supernatant removed. Each pellet was then resuspended in 1mL of lysis buffer and homogenised with a 20G needle and syringe (Terumo). These lysates were shaken overnight at 4°C, after which the homogenisation was repeated.

Total protein concentration was measured for each lysate using the BCA Protein Assay Kit (Pierce) according to manufacturer’s instructions. Absorption was read at 562nm on a BioTek Synergy 2 Plate reader, using BioTek Gen5 software version 1.10.8.

Aliquots of each lysate were refrigerated at 4°C for immediate use. Stocks were frozen at -20°C for longer-term storage.
2.2.11. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins. To prepare the polyacrylamide gel, separating gel was prepared as per section 2.1.2.4 and set in a 1mm cassette (Invitrogen). Stacking gel was prepared as per section 2.1.2.4, and the stacking gel layered on top of the separating gel. A ten-well comb was inserted and the gel left to set.

Once the gel was completely set, the cassette was inserted into the XCell SureLock Mini-Cell (Invitrogen) and the chamber filled with 1xMES SDS running buffer (Invitrogen).

Enough cell lysate to ensure 25µg total protein per well was added to an equal amount of 2xSDS loading buffer, heated to 70°C for ten minutes then cooled to room temperature. The polyacrylamide gel was loaded with one sample per well, plus a control well containing 10µL of 2xSDS loading buffer, one well containing 5µL of western protein standard (Invitrogen), and one containing 10µL of prestained protein marker (Invitrogen). The gel was run at 200V until the proteins of interest would be in the middle section of the gel.

2.2.11.1. Western Blot

Western blotting was used to detect and comparatively quantify specific proteins from cell lysate.

3MM chromatography paper (Whatman) thoroughly wetted with 1x transfer buffer (Invitrogen) was then layered onto the gel and the gel removed from the cassette. A pre-cut piece of polyvinylidene difluoride (PDVF) membrane (GE Healthcare) was prepared by soaking in 100% methanol for 30 seconds followed by soaking in transfer buffer. This membrane was laid on top of the gel and smoothed to remove any air bubbles. A further layer of wetted blotting paper was laid on top of the membrane. The gel/membrane/blotting paper complex was then sandwiched between transfer buffer saturated blotting pads within the XCell II Blot Module (Invitrogen). The module was inserted in the XCell SureLock MiniCell. The space around the blot module
was filled with de-ionised water to absorb any heat created by the transfer process, preventing the blot module and contents from overheating. The transfer was run at 30V for one hour.

Once the transfer was complete the membrane was blocked overnight in blocking solution at 4°C with shaking in order to prevent nonspecific binding of the primary antibody to the PDVF membrane. This was followed by incubation for two hours at room temperature in primary antibody solution composed of blocking solution with the primary antibody added at a 1/200 to 1/5000 dilution. The membrane was then rinsed with PBS-T and washed three times for five minutes in PBS-T with shaking. Once washed the membrane was incubated in blocking solution with a horseradish peroxidase conjugated secondary antibody at a concentration of 1/2500 for approximately two hours with shaking. The PBS-T rinse and washing step was then repeated to remove any unbound secondary antibody.

The ECL Western Blotting Detection System (GE Healthcare) was used to reveal proteins labelled with the HRP-conjugated secondary antibody complex via chemiluminescence. Detection was performed on the ChemiGenius2 (Syngene), using Syngene's GeneSnap software. Image analysis was performed using the GeneTools software, also by Syngene.

2.2.12. ELISA

The BDNF E\textsubscript{max} ImmunoAssay system Kit (Promega) was used according to manufacturer’s instructions to quantitate the amount of BDNF protein in both lymphoblast and neuroblast cell lysates.

Each ELISA was performed in a 96-well plate that was initially coated with anti-BDNF monoclonal antibody in carbonate coating buffer and left overnight for the antibody to bind to the plate. The plate was then rinsed with TBS-T and 200µL 1xBlock and Sample buffer was added to each well and incubated at room temperature for one hour to block the plate in order to prevent non-specific binding. This is followed by a wash in TBS-T.
A standard curve was prepared by performing serial 1:2 dilutions of BDNF protein in 1xBlock and Sample buffer. These were performed in duplicate and provide eight data points for the construction of a standard curve, with concentrations ranging from 0pg/mL to 500pg/mL. Each test sample was prepared in triplicate, with each well containing 5µL protein lysate and 95µL 1xBlock and Sample buffer. The plate was then sealed and incubated at room temperature with shaking for two hours to enable the BDNF protein to bind to the anti-BDNF monoclonal antibody. After the incubation was completed the seal was removed and the plate washed thoroughly five times with TBS-T. Anti-human BDNF monoclonal antibody (raised in chicken) was then added to 1xBlock and Sample buffer and 100µL of the solution was added to each well. The plate was again sealed and incubated for two hours at room temperature with shaking.

After a further rinse step with TBS-T, anti-chicken IgY antibody conjugated to horseradish peroxidase was added to 1xBlock and Sample buffer and 100µL of the solution was added to each well. The plate was sealed and incubated for one hour at room temperature with shaking. After incubation the plate was washed and 100µL of TMB One Solution was added to each well. The plate was then incubated at room temperature with shaking for ten minutes. The reaction was stopped with 100µL 1N HCl and the absorbance of each well was read at 450nm on a BioTek Synergy 2 Plate reader, using BioTek Gen5 software version 1.10.8.

2.2.13. Statistical Analysis

2.2.13.1. RT-qPCR Analysis
Analysis of the RT-qPCR results was performed using QBasePlus software from Biogazelle. Expression changes in each gene for each treatment were normalised against a reference gene and compared to the corresponding control. Statistical analysis of cell line and treatment groupings was also performed on QBasePlus. The reference gene used for the neural cell lines was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is
commonly used as a reference when measuring changes in gene expression in neural cells.

When normalising the gene expression changes in the lymphocytes, three candidate reference genes were used. These were GAPDH, succinate dehydrogenase A (SDHA) and ubiquitin C (UBC). The geNorm algorithm (Vandesompele et al., 2002) was used to determine the most stable of these genes for each RT-qPCR run. The reference genes are ranked according to their expression stability and only the most stable gene or set of genes is used to calculate the gene expression normalisation factor, which is based on the geometric mean of the selected reference genes. Using multiple reference genes in this way reduces the errors that can occur when only one reference gene is used (Thellin et al., 1999). The geNorm applet, which is used within a standard Microsoft Excel platform, can be downloaded at http://medgen.ugent.be/~jvdesomp/genorm/. QBasePlus software from Biogazelle, which has an inbuilt geNorm function, was used to statistically analyse differences in gene expression changes between treatment groups and individuals grouped by affection status.

2.2.13.2. Analysis of protein levels
Measurement of protein levels with and without drug treatment was measured by western blot (YWHAG) or ELISA (BDNF). Once these qualitative measurements had been taken, normalisation was performed in a similar fashion to normalisation of gene expression after RT-qPCR. Densitometric analysis of the chemiluminescent signal from western blot was used to measure levels of YWHAG protein, and these were normalised against β-actin. The level of BDNF protein was calculated by comparing the absorbance of each sample against that of a standard curve, and was normalised against the total protein loaded per well.

2.2.13.3. Hardy-Weinberg Analysis
Each SNP genotyped was assessed for deviation from Hardy-Weinberg equilibrium using a Hardy-Weinberg equilibrium calculator web tool. This tool can be found at http://www.oege.org/software/hwe-mr-calc.shtml (Rodriguez et al., 2009).


2.2.13.4. Association Analysis

All association analyses were performed using the FBAT software program v.2.0.3 (Laird et al., 2000), a family-based association testing program that enables testing of multiple markers individually as well as in haplotypes. FBAT is available from http://www.biostat.harvard.edu/~fbat/fbat.htm. The program uses genotype data from parent-child nuclear families and sibships (or a combination of both) to look for association between a SNP and a given phenotype. Each association test was performed with a dichotomous affection status (each individual classified as either affected or unaffected), and a minimum threshold of ten informative families for a test statistic to be produced. The bi-allelic mode was used for testing of single SNPs, as each of these polymorphisms analysed for association had only two variants. The null hypothesis in all cases was no association in the presence of linkage, and thus the test statistic was calculated using the empirical variance (-e) option.
3. Gene Expression and Protein Levels in a Neural Model

3.1. Introduction
Determining the effect of mood stabilisers on gene expression is a useful tool in selecting genes for further study, not only for the mechanism of action of lithium and other mood stabilising drugs, but also for the genetic contribution to the aetiology of BPD itself. Identification of genes that are differentially regulated by lithium or another mood stabiliser may also highlight cellular process or signal transduction pathways that play an important role in the disorder and may suggest future targets for treatment of the condition.

3.1.1. Experimental Approach
This chapter describes the use of RT-qPCR to determine changes in gene expression in 20 genes in three neural cell lines under treatment from the mood stabilising drugs valproate and carbamazepine. Eleven of the genes were selected because they have been shown to be up or downregulated by lithium in previous studies or are from a lithium-affected signal transduction pathway and these genes were outlined in Chapter 1. The remaining nine genes were chosen from the SAGE screen outlined below. In order to determine if changes in gene expression were mirrored by changes in protein level in these models of mood stabiliser action, the protein corresponding to the gene most consistently regulated by the mood stabilising drugs was also measured and compared to its untreated control.

3.1.2. Selection of genes to screen for expression changes
Nine genes were selected from a serial analysis of gene expression (SAGE) screen previously performed in the Molecular Pathology Group Laboratory, Department of Pathology, Dunedin School of Medicine, University of Otago. SAGE screens determine levels of gene expression using short nucleotide sequence tags that can uniquely identify a transcript, so the level of each transcript can be ascertained by the number of times its tag is present. Full details of the SAGE process can be found in Velescu et al. (1995). The SAGE
screen identified genes that were up or downregulated in SH-SY5Y cells after 28 days of lithium treatment. Analysis of the screen resulted in a list of around 100 genes that had both threefold up or downregulation, combined with significance at p≤0.05 (Fitches and Olds, Unpub). Nine of the genes used in the research described in this chapter were selected from these 100 genes. The following section contains a brief overview of these nine genes.

**3.1.2.1. Chromosome 7 open reading frame 45**
The transcript identified by this tag (C7orf45) is found at chromosome 17p11.12 and is identified by NCBI Unigene as a hypothetical protein. This gene was chosen because it was upregulated nine-fold by lithium in the SAGE screen, the highest level of upregulation found in the screen.

**3.1.2.2. Fibroblast growth factor receptor 4**
Fibroblast growth factor receptor 4 (FGFR4) is a member of a family of receptor tyrosine kinase genes that code for the receptors for the 23 known fibroblast growth factors. The fibroblast growth factors are components of a signal transduction pathway with an important role in several growth related processes such as wound healing, angiogenesis and neurogenesis (Vainikka et al., 1994). FGFR4 has global expression but is most highly expressed in the lungs, kidneys and brain of both humans and mice (Cool et al., 2002). Like the rest of the FGFR family, the FGFR4 protein is a transmembrane protein with three extracellular domains, a transmembrane domain, and a tyrosine kinase cytoplasmic tail (Avraham et al., 1994).

The FGFR4 protein has been found to interact with the inhibitor of nuclear factor kappa B kinase beta subunit (IKKβ), which is an important regulatory protein in the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling pathway (Drafahl et al., 2010). The authors theorise that FGFR4 directly phosphorylates IKKβ, modulating its activity and in this way negatively regulating NFκB signalling.

*FGFR4* was found to be downregulated four-fold by lithium in the SAGE screen at a high level of significance, and this combined with its presence in neural tissue makes it an interesting candidate for drug expression studies.
3.1.2.3. Guanine nucleotide binding protein

Guanine nucleotide binding protein gamma 5 (GNG5) codes for a component of a membrane associated G-protein with a wide tissue distribution that includes the brain. The G-proteins are heterotrimeric, consisting of alpha, beta and gamma subunits. The beta and gamma subunits are also able to form a functional dimer (Liu and Aronson, 1998). The GNG5 gene is composed of four exons and three introns and is six kilobases long. It is one of 11 forms of the gamma subunit and is found on chromosome 1p22 (Ahmad et al., 1995).

G-proteins are important regulators of several signal transduction pathways. The βγ dimer regulates adenylcyclase and phospholipase C, whereas the trimer, including the alpha subunit, induces exchange of bound guanosine triphosphate to guanosine diphosphate, dissociating the G-protein itself. Signal termination occurs at the reformation of the trimer and is reinforced by a regulator of G-protein signalling (RGS) protein.

Willmroth et al. (2002) found that bipolar patients have increased expression of the αs subunit of the G-protein in neutrophils compared to normal controls, independent of treatment by mood stabilisers. This suggests the possibility that there may be differences in the G-protein signalling system that occurring in individuals with BPD.

In the initial SAGE screen, GNG5 was upregulated four-fold in cells treated with lithium compared to untreated cells.

3.1.2.4. Heat shock protein 90kDa beta (Grp94), member 1

Heat shock protein 90kDa beta (Grp94), member 1 (HSP90B1) codes for a glycoprotein that is part of a series of closely related molecules that have a role as molecular chaperones. The HSP90B1 protein is found in the endoplasmic reticulum where it has a role in responding to endoplasmic reticulum stress (the accumulation of unfolded proteins) by refolding and stabilising proteins (Chen et al., 2005).
The gene has 18 exons and a 33bp upstream sequence that is highly conserved. *HSP90B1* was examined for association with bipolar disorder in three independent cohorts – two Japanese and one American - and the single nucleotide polymorphism rs17034977 was found to be associated in both Japanese cohorts (Kakiuchi *et al.*, 2007). In the same study, the authors measured *HSP90B1* mRNA levels in transformed lymphoblasts from 22 individuals with BPD and 19 controls. While there was no significant difference between the two groups, it was observed that those individuals carrying the C allele of rs17034977 had significantly lower expression levels of *HSP90B1* than those individuals homozygous for the A allele. Hayashi *et al.* (2009) reported that addition of substances that cause endoplasmic reticulum stress to lymphoblast cells induces *HSP90B1* expression, and that this response is significantly reduced in individuals with bipolar disorder. This suggests that decreased protective response to stress or damage to the endoplasmic reticulum may contribute to the pathophysiology of BPD. *HSP90B1* was upregulated seven-fold in the SAGE screen following lithium treatment.

### 3.1.2.5. Midkine (neurite growth promoting factor 2)

Midkine (*MDK*) codes for the neurite growth promoting factor 2, a growth factor involved in both cell survival and differentiation of neural cells in embryonic life (Griffith, 1997). *MDK* expression is highest during the mid-gestational period in both mice and humans, but is still expressed in the neural cells of adults (Obama *et al.*, 1995; Kadomatsu and Muramatsu, 2004). Mice deficient in the gene have defects in dopamine function and neurogenesis (Muramatsu, 2010).

*MDK* was downregulated nine-fold after lithium treatment in the SAGE screen, but was found to be significantly over-expressed in rat brain after exposure to imipramine and citalopram (both antidepressant drugs) in a study by Palotas *et al.* (2004). This may indicate a directional effect that would be consistent with a spectrum model of mood disorders, with antimania mood stabilising drugs driving the regulation of *MDK* down, whereas antidepressant drugs push it in the other direction. Confirmation of the SAGE screen with more expression data would help clarify if this may be the case,
and MDK’s expression in neural cells increases its interest as a candidate gene.

3.1.2.6. Neural proliferation, differentiation and control 1

The neural proliferation, differentiation and control 1 gene (NPDC1), which maps to 9q34.3 (Evrard et al., 2004), codes for a transmembrane protein of 325 amino acids, including a signal peptide and transmembrane domain. NPDC1 mRNA is highly expressed in the brain, primarily in the frontal and temporal lobes and the hippocampus, and in both the prostate and mammary glands (Qu et al., 2001). NPDC1 has nine exons and eight introns and maps to chromosome 15 (Galiana et al., 1995).

The NPDC1 protein has been shown to inhibit neural cell proliferation, interacting directly with the transcription factor E2F-1 and the cell cycle protein CDK2 that help regulate the cell cycle and promote apoptosis. NPDC1 suppresses oncogenic transformation in both neural and non-neural cells, giving it an important role in tumour suppression (Dupont et al., 1998).

NPDC1 is not well studied in relation to psychiatric disorders, and other than its presence at high levels in the nervous system there is currently no evidence to link it to psychiatric illness or psychiatric drug response. The results of the SAGE screen, however, show it was downregulated four-fold by lithium with significance of p<0.05, making it a candidate for further studies of regulation by mood stabilising drugs.

3.1.2.7. Regulator of G-protein signalling 5

The regulator of G-protein signalling 5 gene (RGS5) is a member of the large RGS family that are involved in the suppression of G-proteins by promoting deactivation. RGS proteins are responsible for some of the desensitisation that occurs within the G-protein signalling pathway after prolonged stimulation with a drug or biological signalling molecule (Koelle, 1997).

RGS5 was first isolated Seki et al. (1998) from human neuroblastoma cDNA. They were further able to show 90% homology to murine rgs5, and localise the gene to chromosome 1q23. Human tissue studies showed that RGS5
mRNA was highly expressed in heart, lung and skeletal muscle tissue, with lower expression levels in the brain, placenta and liver. Larminie et al. (2004) confirmed the abundance of RGS5 expression in heart muscle, but found that brain tissue had expression levels that were as high as that of the lungs and skeletal muscle.

The RGS5 protein is 181 amino acid residues long, and like all RGS proteins, has a conserved 120-residue domain that binds to the G-protein α-subunit (Seki et al., 1998). This binding accelerates the hydrolysis of the GTP molecule that is also bound to the α-subunit, thus acting as a GTPase. This hydrolysis moves the equilibrium of the system towards the re-association of the α and βγ subunits of the G-protein, thus inactivating signalling (Dohlman and Thorner, 1997). Over-expression of RGS proteins suppresses G-protein mediated cell signalling (Ishii and Kurachi, 2003). Zhou et al. (2001) found that the RGS5 protein binds strongly to five variants of the G-protein α-subunit.

There is some evidence linking RGS5 with psychiatric disorders. A genome wide association study of bipolar disorder in European American and African American cohorts found that there was a considerable difference between the degree of association between BPD and the RGS5 rs4657247 SNP depending on which population was being analysed. This indicates that the association of the SNP with BPD is affected by the genetic background in which it is found (Smith et al., 2009). Another SNP of RGS5, rs10799902, has been shown to be associated with increased severity of schizophrenic symptoms in both an African and a European cohort, as did a haplotype of RGS2, a highly homologous gene from the same family (Campbell et al., 2008). Expression of another RGS family member, RGS4, has been shown to be consistently decreased in the prefrontal cortex of schizophrenic patients (Mirnics et al., 2001). The same study looked at the regulation of ten other members of the RGS family, including RGS5, but failed to find any difference between schizophrenic patients and controls. A study performed by Chowdari et al. (2002) tentatively showed association between several polymorphic variants of RGS4 and schizophrenia. RGS4 was also reported as associated with schizophrenia in a modestly sized family-based study of an Ashkenazi Jewish cohort (Fallin et al., 2005). Within the same homologous family, RGS3 mRNA has been found to be more highly expressed in lithium treated bipolar
individuals than in matched controls (Willmroth et al., 2002). This suggests that lithium, and perhaps other mood stabilising drugs, may have an effect on G-protein regulation by the RGS family.

The results of the SAGE screen, showing that RGS5 is three-fold upregulated by lithium, combined with the fact that RGS5 is involved in an important signal transduction pathway and is expressed in neural tissue make it a promising candidate for research into gene expression changes mediated by mood stabilising drugs.

3.1.2.8. Tumour necrosis factor receptor-associated protein 1
Chen et al. (1996) reported the discovery of the tumour necrosis factor receptor-associated protein 1 (TRAP1), a member of the 90kDa heat shock protein family of molecular chaperones. The TRAP1 protein forms complexes with the retinoblastoma protein during nuclear envelope breakdown and after heat shock, and is partially responsible for the refolding of retinoblastoma protein back to its 3D conformation after denaturation. TRAP1 controls cell cycle activity through the tumour necrosis factor pathways, and probably has a role in cancer proliferation (Liu et al., 2010).

No studies have linked TRAP1 with any neural function or shown any regulation by psychiatric drugs, however the initial SAGE screen showed downregulation of over four-fold after lithium exposure, making it a candidate for further gene expression studies.

3.1.2.9. 14-3-3 gamma
14-3-3 gamma (YWHAG) or the tryptophan 5-monooxygenase activation protein (gamma polypeptide) gene is a member of the large gene family coding for the 14-3-3 proteins, a group of regulatory molecules that have a wide range of roles within eukaryotes. Each 14-3-3 protein is a dimer, with each subunit encoded by one of seven genes (Fu et al., 2000). The family has extremely high sequence conservation and comprises about 1% of the total soluble protein found in the human brain (Aitken et al., 1992). 14-3-3 proteins work as regulators in signal transduction pathways.
One of the seven genes coding for a 14-3-3 subunit, YWHAG was localised to chromosome 7q11.23 by Horie *et al.* (1999). This group also performed expression studies in a variety of tissues, showing YWHAG to be highly expressed in the brain, heart and skeletal muscle, and expressed at a lower level in ovarian and testicular tissue.

Ichimura *et al.* (1987) identified a physiological function of 14-3-3 proteins relevant to the aetiology of psychiatric disorders when they identified the proteins act as activators of tyrosine and tryptophan hydroxylases. This links the family to the neural pathways as tyrosine and tryptophan hydroxylase are the rate limiting enzymes in the synthesis of the neurotransmitters serotonin and catecholamine.

A study of five 14-3-3 isoforms including YWHAG in postmortem brain tissue from individuals with schizophrenia and bipolar disorder as well as controls found no significant differences in expression between the different groups (Wong *et al.*, 2005). However, a 1.2Mb deletion of chromosome 7q11.23 that includes the entire YWHAG gene causes neurological problems in humans, including epilepsy, intellectual disability and behavioural issues such as elevated aggression, hyperactivity and impulsivity, although the phenotype is variable (Ramocki *et al.*, 2010).

YWHAG was upregulated six-fold by lithium in the SAGE screen, which contrasts with its downregulation by the antidepressant drugs imipramine and citalopram in rat cerebral cortex (Palotas *et al.*, 2004). This contrast may indicate that higher levels of YWHAG may be a factor in mania and lower levels in major depression, which would be consistent with the spectrum model that places the two phenotypes at the opposite ends of a continuum with euthymia in the middle.
## Table 3.1 Genes chosen for gene expression studies

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene</th>
<th>Location</th>
<th>Reason chosen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BDNF</strong></td>
<td>brain-derived neurotrophic factor</td>
<td>11p13</td>
<td>Part of MAPK/ERK signaling pathway</td>
</tr>
<tr>
<td><strong>C7orf45</strong></td>
<td>chromosome 7 open reading frame 45</td>
<td>7q32.2</td>
<td>Upregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>ENG</strong></td>
<td>endoglin</td>
<td>9q33</td>
<td>Bosetti <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><strong>FGFR4</strong></td>
<td>fibroblast growth factor receptor 4</td>
<td>5q35.1</td>
<td>Downregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>GNG5</strong></td>
<td>guanine nucleotide binding protein gamma 5</td>
<td>1p22</td>
<td>Upregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>GSK3B</strong></td>
<td>glycogen synthase kinase 3 beta</td>
<td>3q13.3</td>
<td>Part of Wnt signaling pathway</td>
</tr>
<tr>
<td><strong>HSP90B1</strong></td>
<td>heat shock protein 90kDa beta (Grp94), member 1</td>
<td>12q24.2</td>
<td>Upregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>IMPA1</strong></td>
<td>inositol(myo)-1(or 4)-monophosphatase 1</td>
<td>8q21.13</td>
<td>Part of phosphatidylinositol signaling pathway</td>
</tr>
<tr>
<td><strong>IMPA2</strong></td>
<td>inositol(myo)-1(or 4)-monophosphatase 2</td>
<td>18p11.2</td>
<td>Part of phosphatidylinositol signaling pathway</td>
</tr>
<tr>
<td><strong>INPP1</strong></td>
<td>inositol polyphosphate-1-phosphatase</td>
<td>2q32</td>
<td>Bosetti <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><strong>MDK</strong></td>
<td>midkine (neurite growth promoting factor 2)</td>
<td>11p11.2</td>
<td>Downregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>NPDC1</strong></td>
<td>neural proliferation, differentiation and control, 1</td>
<td>9q34.3</td>
<td>Downregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>PDE4D</strong></td>
<td>phosphodiesterase 4D, cAMP-specific</td>
<td>5q12</td>
<td>Sun <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><strong>PREP</strong></td>
<td>prolyl endopeptidase</td>
<td>6q22</td>
<td>Part of phosphatidylinositol signaling pathway</td>
</tr>
<tr>
<td><strong>PRKCA</strong></td>
<td>protein kinase C, alpha</td>
<td>17q22</td>
<td>Part of phosphatidylinositol signaling pathway</td>
</tr>
<tr>
<td><strong>PRKCE</strong></td>
<td>protein kinase C, epsilon</td>
<td>2p21</td>
<td>Part of phosphatidylinositol signaling pathway</td>
</tr>
<tr>
<td><strong>RAB7A</strong></td>
<td>RAB7A, member RAS oncogene family</td>
<td>3q21.3</td>
<td>Sun <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><strong>RGS5</strong></td>
<td>regulator of g-protein signaling 5</td>
<td>1q23.1</td>
<td>Upregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>TRAP1</strong></td>
<td>TNF receptor-associated protein 1</td>
<td>16p13.3</td>
<td>Downregulated in SAGE screen</td>
</tr>
<tr>
<td><strong>YWHAAG</strong></td>
<td>14-3-3γ tyrosine 3-monooxygenase gamma polypeptide</td>
<td>7q11.23</td>
<td>Upregulated in SAGE screen</td>
</tr>
</tbody>
</table>

### 3.1.3. Selection of reference genes

Measurement of changes in gene expression by RT-qPCR requires normalisation against a reference or ‘housekeeping’ gene, a constitutively expressed gene that does not change under the conditions intended for the research. Levels of the reference gene are measured alongside the candidate genes, and the expression levels of the candidate gene are normalised against those of the reference gene. Normalisation of RT-qPCR controls for several
variables, including variations in yield of both RNA and cDNA between samples, and the efficiency of amplification (Bustin et al., 2009).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the reference gene with which to normalise gene expression levels among our candidate genes because it was widely used and had been validated in SH-SY5Y cell lines (Hoerndli et al., 2004), as well as several tissue types (Gorzelniak et al., 2001; Murphy and Polak, 2002; Ullmannova and Haskovec, 2003). While differences were found in GAPDH expression levels between human tissues, levels within a single tissue type had been shown to be reproducible between individuals and within a single individual (Barber et al., 2005). This made GAPDH a reasonable choice for normalisation in this study as all comparisons would be within a single tissue type.

Selection of neural cell lines
Three human neural cell lines, the neuroblast cell lines SK-N-DZ, SK-N-SH and SH-SY5Y, were chosen as a culture model to analyse gene expression changes after treatment with mood stabilising drugs. The cell lines were chosen for their ease of culture for long periods, the ease of drug administration, and their neural source.

There is usually a period of three to four weeks before clinical improvement is seen in patients being treated with mood stabilising drugs. A treatment period of 28 days was used for the neuroblast cell culture model to mirror the time required for clinical efficacy. A shorter, acute treatment may not allow the finding of long-term changes in gene expression. Hence, the cell lines were cultured for a period of 28 days while being provided with media treated with a therapeutically relevant level of mood-stabilising drug. Carbamazepine is generally held at blood levels of around 0.05mM, which is therapeutic in most individuals while minimising side effects (Canterbury, 1986). The therapeutic blood level for sodium valproate in BPD treatment is around 0.5mM (Grimes and Jope, 1999), and lithium blood levels are kept between 0.8 and 1.2mM for most individuals to avoid lithium toxicity.
3.2. Results

3.2.1. Gene and protein expression in neural cell lines
The SK-N-DZ, SK-N-SH and SH-SY5Y cell lines were cultured for 28 days in therapeutically relevant concentrations of carbamazepine and valproate. The cells were then harvested and RNA was extracted from them followed by conversion to cDNA. The expression level of each gene under each treatment was measured by RT-qPCR, and normalised against the reference gene GAPDH. The expression level for each condition was then normalised against its untreated control, generating a result that showed the change in gene expression between treated and untreated control cells. The results are shown below in table and chart form.

Also shown in this section are the results of a similar series of experiments performed by Dr. Alison Fitches that used the same methodology and cell lines, but used lithium as the mood stabilising drug rather than carbamazepine or valproate. These results are presented here because all the data was analysed together and all the results were taken into account when selecting the genes for the subsequent research.

As gene expression levels of treated cells were compared to their untreated controls, the change in expression is shown as a proportion of the control, which is shown in the following figures as one. Thus a change in expression above one is an upregulation, whereas a downregulation will be less than one.
Figure 3.1 Gene expression changes standardised against control after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from a triplet of technical replicates.

**C7orf45**
**ENG**

Gene expression level standardised against untreated control

**FGFR4**

Cell Line

![Diagram showing gene expression level standardised against untreated control for SK-N-DZ, SK-N-SH, and SH-SY5Y cell lines for FGFR4.](image)
Gene expression level standardised against untreated control

**GNG5**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression level standardised</td>
<td><img src="chart1.png" alt="Bar chart for GNG5 showing gene expression levels for SK-N-DZ, SK-N-SH, and SH-SY5Y." /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**GSK3B**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression level standardised</td>
<td><img src="chart2.png" alt="Bar chart for GSK3B showing gene expression levels for SK-N-DZ, SK-N-SH, and SH-SY5Y." /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- Valproate Control
- Carbamaz. Control
- Lithium Control
- Valproate
- Carbamazepine
- Lithium
### HSP90B1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression level standardised against untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### IMPA1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression level standardised against untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- **Valproate Control**
- **Carbamazepine Control**
- **Lithium Control**
- **Valproate**
- **Carbamazepine**
- **Lithium**
**IMPA2**

Gene expression level standardised against untreated control

**INPP1**

Gene expression level standardised against untreated control
**NPDC1**

Gene expression level standardised against untreated control

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1.png" alt="Graph" /></td>
<td><img src="image2.png" alt="Graph" /></td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**PDE4D**

Gene expression level standardised against untreated control

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image4.png" alt="Graph" /></td>
<td><img src="image5.png" alt="Graph" /></td>
<td><img src="image6.png" alt="Graph" /></td>
</tr>
</tbody>
</table>
Gene expression level standardised against untreated control

**PREP**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valproate Control</td>
<td><img src="image1" alt="Valproate Control" /></td>
<td><img src="image2" alt="Valproate Control" /></td>
<td><img src="image3" alt="Valproate Control" /></td>
</tr>
<tr>
<td>Carbamaz. Control</td>
<td><img src="image4" alt="Carbamaz. Control" /></td>
<td><img src="image5" alt="Carbamaz. Control" /></td>
<td><img src="image6" alt="Carbamaz. Control" /></td>
</tr>
<tr>
<td>Lithium Control</td>
<td><img src="image7" alt="Lithium Control" /></td>
<td><img src="image8" alt="Lithium Control" /></td>
<td><img src="image9" alt="Lithium Control" /></td>
</tr>
</tbody>
</table>

**PRKCA**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valproate</td>
<td><img src="image10" alt="Valproate" /></td>
<td><img src="image11" alt="Valproate" /></td>
<td><img src="image12" alt="Valproate" /></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td><img src="image13" alt="Carbamazepine" /></td>
<td><img src="image14" alt="Carbamazepine" /></td>
<td><img src="image15" alt="Carbamazepine" /></td>
</tr>
<tr>
<td>Lithium</td>
<td><img src="image16" alt="Lithium" /></td>
<td><img src="image17" alt="Lithium" /></td>
<td><img src="image18" alt="Lithium" /></td>
</tr>
</tbody>
</table>
**PRKCE**

Gene expression level standardised against untreated control.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-DZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-N-SH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**RGS5**

Gene expression level standardised against untreated control.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-DZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-N-SH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- Valproate Control
- Carbamaz. Control
- Lithium Control
- Valproate
- Carbamazepine
- Lithium
A results table for the previous 16 genes can be found in Appendix B. The remaining four genes (*BDNF, MDK, RAB7A* and *YWHAG*) are presented with tables as they were chosen for subsequent research. The selection of these four genes is discussed in section 3.3.4.
Table 3.2 Changes in \textit{BDNF} expression after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level</td>
<td>95%CI</td>
<td>Level</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.99</td>
<td>1.79 – 2.19</td>
<td>3.16</td>
</tr>
<tr>
<td>Valproate</td>
<td>2.81</td>
<td>2.60 – 3.01</td>
<td>2.45</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.53</td>
<td>1.06 – 2.01</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 3.2 Changes in \textit{BDNF} expression after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95\% confidence interval calculated from of a triplet of technical replicates.
Table 3.3 Changes in *MDK* expression after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level</td>
<td>95% CI</td>
<td>Level</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.81</td>
<td>0.76 – 0.86</td>
<td>0.61</td>
</tr>
<tr>
<td>Valproate</td>
<td>2.11</td>
<td>1.98 – 2.23</td>
<td>2.56</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.80</td>
<td>0.72 – 0.87</td>
<td>2.47</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 3.3 Changes in *MDK* expression after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
### Table 3.4 Changes in RAB7A expression after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level</td>
<td>95% CI</td>
<td>Level</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>2.72</td>
<td>2.42 – 3.01</td>
<td>2.41</td>
</tr>
<tr>
<td>Valproate</td>
<td>1.07</td>
<td>1.04 – 1.10</td>
<td>8.73</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.40</td>
<td>1.32 – 1.48</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

### Figure 3.4 Changes in RAB7A expression after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from a triplet of technical replicates.
Table 3.5 Changes in YWHAG expression after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>95%CI</td>
<td>Level</td>
<td>95%CI</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>2.65</td>
<td>2.32 – 2.98</td>
<td>0.74</td>
</tr>
<tr>
<td>Valproate</td>
<td>0.15</td>
<td>0.13 – 0.17</td>
<td>0.69</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.87</td>
<td>0.77 – 0.96</td>
<td>2.41</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 3.5 Changes in YWHAG expression after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.

QBasePlus was used to analyse between group differences in expression change for each gene. The regulation of each gene was examined for each treatment across the three cell lines, in order to assess the different cell lines as biological replicates. The overall regulation for each cell line was also examined to determine if the three drugs were regulating the gene in common across the cell line. In each case the analysis was a two-sided t-test determining if there was significant difference in the means between the two groups. No significant differences were found between control and treatment, both within cell line and within drug types. The full results of this statistical analysis can be found in Appendix B.
3.2.2. Protein Studies

In order to determine if the changes in gene expression were reflected in protein levels, the protein levels of BDNF were measured for each cell line and treatment type after the 28-day drug treatment. The BDNF protein was chosen because BDNF was the most consistently differentially regulated gene in the gene expression studies. BDNF levels were measured by ELISA, and normalised against total protein to control for differences in supernatant concentration.

Table 3.6 Changes in BDNF protein level after 28 days drug treatment

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>95%CI</td>
<td>Level</td>
<td>95%CI</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.87</td>
<td>1.07-2.67</td>
<td>0.90</td>
</tr>
<tr>
<td>Valproate</td>
<td>0.89</td>
<td>0.69-1.09</td>
<td>0.13</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.62</td>
<td>0.36-0.88</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*Not determined

Protein level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a decrease, and a number above one shows an increase.

Figure 3.6 Changes in BDNF protein level after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from a triplet of technical replicates.
In contrast to the consistent upregulation of the *BDNF* gene, the BDNF protein levels dropped in most cell line/drug pairs. Only one cell line, SK-N-DZ treated with carbamazepine, had a significant rise in BDNF protein level.

### 3.2.3. Correlation between *BDNF* cDNA and protein changes

The standardised changes in gene expression and changes in protein level for BDNF in each cell line/treatment type pair were plotted against each other to determine whether there was any evidence of correlation between the two. A significant positive correlation between the two requires a positive slope and a correlation coefficient of $r^2 \geq 0.95$.

![Figure 3.7 BDNF protein level change vs. *BDNF* gene expression change after 28-day drug treatment](image)

The expression level of *BDNF* after treatment standardised to its control was plotted against the protein level after treatment standardised to its control in order to determine the level of correlation between the two. The slope of the line of best fit indicates a very weak positive correlation with an $r^2$ of 0.07. As there is not enough evidence to reject the null hypothesis of no correlation, it cannot be concluded that there is any relationship between the changes in gene expression and in protein levels for *BDNF*.
3.3. Discussion

3.3.1. Expression changes in genes selected from the SAGE screen

Five of the genes studied in this chapter were upregulated by lithium in the previously performed SAGE screen - \textit{C7orf45}, \textit{GNG5}, \textit{HSP90B1}, \textit{RGS5} and \textit{YWHAG}. Only one of these genes, \textit{RGS5}, was predominantly upregulated in the neural cell screen, with six of nine cell-line/drug combinations showing an increase of expression. This gene also showed upregulation by lithium in all three cell lines, echoing the SAGE screen findings. In contrast, there was no obvious consistent direction of regulation for the other four genes upregulated by lithium in the SAGE screen, with similar numbers of regulation in each direction. \textit{HSP90B1} was significantly upregulated by carbamazepine and significantly downregulated by valproate in all three cell lines, but lithium was inconsistent. \textit{GNG5} showed no regulation pattern at all, with opposite-direction regulation within both cell lines and drug treatments. \textit{C7orf45}, while showing no consistent regulation across all the treatments, was downregulated by all three drugs in the SK-N-SH study. \textit{YWHAG} was significantly downregulated in all three cell lines by valproate, but showed no other consistent pattern. On the whole these five genes were inconsistent in their regulation, and while \textit{HSP90B1} and \textit{RGS5} were more stably upregulated than the others, they still showed downregulation in two or three cell line/drug treatment combinations.

The four genes downregulated by lithium that were chosen from the SAGE screen were \textit{FGFR4}, \textit{MDK}, \textit{NPDC1} and \textit{TRAP1}. All four of these genes showed inconsistent regulation across the nine cell line/drug treatment combinations. \textit{FGFR4} had no consistent regulation across any cell type or drug treatment. \textit{TRAP1} was upregulated in six of nine cell line/drug treatment pairs, including consistent upregulation in SH-SY5Y by all three drugs. Changes in regulation of \textit{NPDC1} showed no particular pattern, with four significantly upregulated and three downregulated cell line/drug treatment pairs, and no common regulation across cell line or drug. \textit{MDK} was the most often downregulated gene, but was a result of only five out of nine
cell line/drug treatment pairs having significantly reduced expression compared to control, so could not be said to be consistent.

3.3.2. **Expression changes in genes selected from the literature**

*ENG* was downregulated by lithium 2.2 fold in rat brain tissue in a study by Bosetti *et al.* (2002), but there is no information on the effect of mood stabilisers on *ENG* in human tissue. There was no consistent direction of regulation for *ENG* in any of the cell line or drug treatments. Overall, upregulation was most common for *ENG* in this research, with six of nine cell line/drug treatment pairs being upregulated at least 1.5-fold. These results suggest that lithium, carbamazepine and valproate do not have common effects on expression of *ENG*.

Also downregulated in the Bosetti *et al.* (2002) study was *INPP1*, which had 2.7-fold reduction in expression in rat brain after seven days of lithium treatment. Again, the opposite was found for *INPP1* in most of the cell line/drug treatment pairs looked at in this research, where seven were significantly upregulated and two were downregulated. The gene was upregulated by both valproate and lithium in all three cell lines.

The findings for both *INPP1* and *ENG* were therefore in direct contrast to those reported by Bosetti *et al.* (2002), which suggests that cultured human neuroblast cell lines and *in vivo* administration to rats are not equivalent models and one or both may not be appropriate for assessing the changes in expression in these genes as experienced by humans medicated with lithium. Another possible explanation for the contrast in results is the differing length of the drug treatment used in the two studies. The neuroblast cell lines were treated for 28 days, a period of time selected to match the typical time required to see clinical improvement in individuals with BPD. In contrast, a seven-day treatment such as that given to the rats in the Bosetti *et al.* (2002) study may be considered acute rather than chronic, and it is possible that there is an initial downregulation of the genes before they are upregulated in the long term. Replication with both models over a varied time course needs to be done to determine if the expression changes seen are stably reproducible.
given the differences between them and the inconsistency across the cell type/drug treatment pairs.

Both \textit{PDE4D} and \textit{RAB7A} were reported to be downregulated by seven days of lithium treatment in cultured lymphoblasts from individuals with BPD (Sun \textit{et al.}, 2004). In contrast, in this research \textit{PDE4D} was upregulated in five out of nine cell type/drug treatment pairs and downregulated in the remaining four. All three drugs increased \textit{PDE4D} expression in SH-SY5Y cells. \textit{RAB7A}, downregulated 1.5-fold by lithium in lymphoblast culture (Sun \textit{et al.}, 2004), was in contrast significantly upregulated between 1.39 and 9.04 times in seven of the nine cell line/drug treatment pairs in this study. One pair was downregulated and the last was unchanged. Again the contrast in findings between the two studies may be a result of the differing lengths of lithium treatment, or the discrepancy may be a result of differences between the neural cell cultures and the lymphoblast culture, as the source cells for each type of culture have very different cellular functions. The ways the different cells are cultured may also have an effect on the outcome, as they require different media and incubation conditions.

The study described in this chapter showed that the gene \textit{PREP} was upregulated in three cell line/drug treatment pairs, and downregulated in four. There was no significant regulation in common in any of the cell lines, nor in any of the drug treatments, and the wide range of changes in expression makes \textit{PREP} the least consistently regulated gene of the 20 genes assayed. While there is no information on the regulation of the gene itself in the literature, the activity of the \textit{PREP} enzyme has been shown to be decreased by lithium (Breen \textit{et al.}, 2004) and valproate (Maes \textit{et al.}, 1995) in the plasma of individuals with BPD. Due to the inconsistent regulation observed, this study is unable to confirm if gene expression in neural cell lines is reduced. The role of prolyl oligopeptidase in the phosphatidylinositol pathway and the increase of the enzyme in the blood of individuals during an episode of mania suggests that \textit{PREP} might be downregulated by lithium, valproate and carbamazepine, but the expression studies here do not support this theory.
The GSK3 protein is directly inhibited by lithium, and this has been shown in several different models (Klein and Melton, 1996; O’Brien and Klein, 2009; Li et al., 2010). The results of this study show downregulation of GSK3B across six of nine cell line/drug treatment combinations, with two significant upregulations. The overall picture is consistent with previous studies on the impact of lithium on GSK3B. This downregulation, in combination with GSK3B’s integral part in the Wnt signalling pathway, confirms GSK3B as a gene worthy of further investigation in the mechanism of action of mood stabilising drugs and the pathophysiology of BPD.

The two IMPase genes, IMPA1 and IMPA2, both displayed varied regulation across all cell line/drug treatment pairs, although IMPA1 was significantly downregulated across all three cell lines by valproate, and upregulated in all three cell lines by lithium, which is consistent with the effects of lithium on IMPA1 in mouse brain tissue as reported by Shamir et al. (2003). The same study did not find upregulation of IMPA2 in mice after lithium treatment, and IMPA2 was inconsistently regulated by lithium across SK-N-DZ, SK-N-SH and SH-SY5Y in this study.

PRKCA was predominantly upregulated across the nine cell type/drug treatment pairs showing significant upregulation from 2.44 to 5.82 fold. This is in contrast to findings reported for the PRKCA protein in a rat glioma cell line, which showed reduction in the PKRCA protein after chronic valproate administration (Chen et al., 1994), and in rat hippocampus, where PRKCA protein levels were reduced after four weeks of lithium administration (Manji et al., 1993). These differences in the direction of change may again be explained by the differences in the models used. Glioma cell lines are derived from glial cells, which provide protection and support to neural cells in the brain, insulating them from each other and supplying their nutrition, giving them a very different role from neural cells. The discrepancy may also be a result of protein levels being unrelated to or affected by more factors than the level of gene expression.

PRKCE was upregulated in seven out of nine cell line/treatment pairs, with the remaining two significantly downregulated. Carbamazepine upregulated
the gene in all three cell lines. There is very little data on the regulation of \textit{PRKCE} by mood stabilising drugs, although the \textit{PRKCE} protein is decreased by valproate in rat glioma cell lines (Chen \textit{et al.}, 1994).

In contrast to the other 19 genes assayed, \textit{BDNF} showed consistent upregulation by all three drugs across all three cell lines, with only one of these cell line/treatment pairs failing to reach significance. This upregulation is in line with previous studies on \textit{BDNF} regulation by mood stabilising drugs, across a variety of models. \textit{BDNF} protein level is increased in the hippocampus of rats by both lithium (Fukumoto \textit{et al.}, 2001; Frey \textit{et al.}, 2006a) and valproate (Frey \textit{et al.}, 2006a).

Group analysis of the expression changes for each gene, both within a particular drug treatment and within a cell line, failed to find any statistically significant difference between the controls and the treated groups. Even for groups such as valproate treated cells, which all show significant, greater than two-fold upregulation of expression of \textit{BDNF} in all three cell lines when compared to their controls, were not significantly different from the untreated cells when grouped. The same was true within a cell line where there was significant upregulation by each drug, such as in the SK-N-SH cell line, where each drug increased expression of BDNF over the controls. When grouped, these also failed to reach statistical significance.

There are several different possible explanations for this phenomenon. Firstly, failure to reach significance despite the three members of the group having individual significance is likely to be a result of the compounding of the standard errors over the normalisation of the result against a reference gene, followed by the normalisation against the control, then the grouping. This leads to wide confidence intervals requiring greater numbers of samples to find significance.

In most of the genes analysed, however, there is no significant difference between the treated and untreated cells because the individual regulation within the group is itself not significant, and often in the opposite direction despite identical drug treatment. The fact that there are no significant
regulation changes across the cell lines even by single drugs, suggests that the cell lines SK-N-DZ, SK-N-SH and SH-SY5Y are not good biological replicates despite being the same neuroblastoma cell type. As all three of these cell lines have been under culture for 30-40 years, it is possible that they have evolved differences that change how they interact with the three treatments used. The separate origins of SK-N-DZ from the other two cell lines (see section 2.1.5.1) may also explain the lack of consistency of their response to the drugs.

The lack of any significant difference between regulation in control and treated cells within the one cell line could be explained by the three drug treatments having different mechanisms of action that regulate the genes in different ways. The most likely explanation, however, is that most of the genes studied do not play a part in the mechanism of action of any of these three drugs, resulting in mostly random expression changes that are not relevant to how valproate, lithium and carbamazepine work in the treatment of BPD.

3.3.3. Lack of correlation in DNA and protein changes
There is a complete lack of correlation between the changes in expression of BDNF and changes in BDNF protein levels, as shown in Figure 3.7.

The generally accepted wisdom at the time of the design of these experiments was that protein levels should correlate in some measure to gene expression, and thus gene expression changes should lead to equivalent changes in protein levels, an admittedly intuitive assumption. However, studies in the intervening time have shown quite convincingly that there are wide ranges of gene expression and protein correlation depending on a variety of factors such as organism, cell type, sample timing, and gene/protein being assessed. In a review article on the subject, Maier et al. (2009) describe mRNA-protein level correlation as being ‘notoriously poor’.

The first evidence of this phenomenon was found in 1999, when a study compared protein abundance and mRNA levels in yeast and found that mRNA only predicted protein levels in the most common of the proteins (Gygi et al., 1999). Studies of more complex tissue such as human cancers have
shown no correlation in a wide range of genes (Chen et al., 2002; Lichtinghagen et al., 2002; Pascal et al., 2008; de Sousa Abreu et al., 2009), and even within the same prostate cancer cell line rates of reported correlation have ranged from 32% (Waghray et al., 2001) to 84% (Lin et al., 2005).

It is not yet firmly established as to whether or not the lack of correlation between gene expression and protein level reflects the underlying biological reality. Both biological and methodological reasons have been offered to explain the phenomenon. Schwanhausser et al. (2011) found that the level of protein in cells is controlled mostly by the level of translation rather than transcription, and that even where a specific mRNA and its corresponding protein correlate, their half-lives tend to be unrelated and the stability of an mRNA has no bearing on the stability of the protein. Where the gene expression is affected by an external factor, such as the administration of a drug, the protein level may or may not be similarly affected, as shown by Yang et al. (2001), who reported that administration of a dioxin to rats decreased carboxylesterase mRNA levels in a dose dependent fashion, but the protein level was increased in groups of rats who received a sub-lethal dose, and decreased in those who received a lethal dose. Because of the differing stability and production rates of mRNA and protein, it is unlikely that there will be any consistent differences between the two, either in magnitude or direction of change at any given point after the introduction of an external factor.

The lack of correlation between changes in gene expression and changes in protein level could also be explained by one or more experimental factors. While the RT-qPCR for BDNF achieved high levels of amplification, any less than optimally efficient amplification of the gene would have contributed to the lack of correlation between changes in gene expression and protein levels. This would also be the case where the antibodies used in the ELISA measuring BDNF protein levels were not maximally efficient. While the primers used for amplification of BDNF were designed to amplify the section of the gene common to the majority of variants, there was also the possibility of non-amplification of variants that would however be translated to protein
and measured by the ELISA. These variants would then also contribute to the discrepancy in the changes between gene expression and protein level.

It is possible that the lack of correlation between the changes of expression of in the BDNF gene and the levels of the BDNF protein found in this study are a result of both experimental and biological factors, and may not reflect what is actually happening in the cultured cells when mood stabilising drugs are administered.

3.3.4. Selection of genes for subsequent study

BDNF, MDK, RAB7A and YWHAG were chosen for further expression analysis in a human lymphoblast model. The initial rationale for the selection of genes for further study was to choose the two most consistently upregulated genes and the two most consistently downregulated genes to attempt to replicate the changes in regulation in a second model. While selecting the two most consistently upregulated genes was straightforward, selection of the remaining two genes was more complicated, primarily because most genes lacked any real consistency in their changes in regulation across the nine experimental conditions.

BDNF, being upregulated between 1.53 and 3.16 fold by all three drugs across all three cell lines, had the most consistent changes in regulation that were in line with the literature. RAB7A showed significantly increased expression across seven cell line/drug treatment combinations, and only one significant downregulation.

MDK was the most consistent of the genes that were predominantly downregulated across the nine treatments, with five cell line/drug pairs significantly downregulated between 1.28 and 2.00 times. While GSK3B had seven significant downregulations, the low expression level of the mRNA, even without drug treatment, resulted in technical difficulties carrying out the RT-qPCR analysis hence it required many more replications to achieve an analysable result. For these reasons GSK3B was not included in the four genes for further study.
As there was no obvious choice for the fourth gene, YWHAG was chosen for further study because of its interesting background in the literature, including being part of the 14-3-3 family which has been implicated in psychiatric disease, and its role as an activator of tyrosine and tryptophan hydroxylase the rate limiting enzymes in the synthesis of the neurotransmitter serotonin (Ichimura et al., 1987).
4. Gene Expression in a Lymphocyte Model

4.1. Introduction
In the previous chapter, 20 genes were analysed for changes in gene expression in neural cell lines brought about by the mood stabilising drugs carbamazepine, valproate and lithium. Four of these genes, BDNF, MDK, RAB7A and YWHAG, were chosen to see if the gene expression changes caused by these drugs could be replicated in another model.

4.1.1. Lymphoblasts as a model
Immortalised lymphocytes were chosen as a model in which to attempt to replicate the gene expression changes found in previous chapter. While lymphoblasts are not neural in origin, they have been used in many studies looking at gene expression as it relates to psychiatric disorders (Karege et al., 2004a; Karege et al., 2004b; Sun et al., 2004; Philibert et al., 2007; Tseng et al., 2008; Hu et al., 2009). The advantages of using lymphoblasts as a model include ease of collection, transformation, and the minimally invasive nature of the cell collection (peripheral venesection). Large cohorts of people can have lymphocytes collected in a short time, making it easier to sample groups such as families with a positive pedigree history of a psychiatric disorder, or individuals from a specific demographic group. This compares favourably to the previous model used, neuroblastoma cells, which are not generally available from individuals with selected disorders. Lymphoblast cultures are also used for pharmacogenetic studies because of the ease of drug administration to the culture and ease of culture maintenance, among other factors (Shukla and Dolan, 2005).

Liew et al. (2006) found that 81.9% of genes expressed in brain tissue were also expressed in peripheral blood, the source of lymphocytes, and suggested that this co-expression combined with the ease of obtaining blood made peripheral blood cells a good surrogate for other tissues, including central nervous system tissue. Tsuang et al. (2005) found that gene expression profiles from peripheral blood could be used to differentiate between individuals
with schizophrenia, individuals with BPD and normal controls. Gene expression profiles in peripheral blood are altered in other complex diseases, such as hypertension (Bull et al., 2004), juvenile-onset arthritis (Barnes et al., 2004), and systemic lupus erythematosus (Rus et al., 2002). Importantly, B-lymphoblasts have been shown to maintain the transcriptional profile of the B-cells from which they have originated, showing that expression studies are not likely to be affected by the immortalisation process (Morello et al., 2004). Previous research using lymphoblast cells have found changes in gene expression and protein levels after treatment with mood stabilising drugs, and also differences in expression between individuals with bipolar disorder and control individuals (Nemanov et al., 1999; Belmaker et al., 2002; Sun et al., 2004). In a small study utilising B-lymphoblasts from individuals with lithium responsive BPD-I and an unaffected control group, the lymphoblasts from the bipolar individuals were found to have a 36% lower level of BDNF protein (Tseng et al., 2008).

4.1.2. Selection of lymphocyte cell lines
Ten cultures of immortalised B-lymphocytes were sourced from the Human Genetic Cell Repository at the Coriell Institute. Five of these cell lines were from individuals who have been diagnosed with BPD-I (the BPD-I group), and five were control individuals who have no history of psychiatric problems (the unaffected group).

Several factors were taken into account when selecting the ten cell lines. The Coriell Institute provides a limited amount of information on the individuals from whom the cell lines originated, allowing a certain amount of matching between the BPD-I and the control individuals along with the ability to control for a narrow range of other variables. Racial background, gender and drug status were taken into account, and five Caucasian males, ranging in age from 24 to 38 years and all stably medicated on lithium at the time of venesection were chosen. Males were chosen in order to control for the potential effects of the cycling hormone levels experienced by females as a part of the menstrual cycle, which have been shown to have an effect on both symptoms and drug response (Hendrick et al., 1996). All five individuals chosen have some form of family history of psychiatric disorders. No
individuals from Old Order Amish families were selected. While the Coriell Institute carries many cell lines sourced from individuals from these families, several of which have a BPD-I diagnosis, the extremely high prevalence of both BPD and other psychiatric conditions among the Old Order Amish may mean that the cause of BPD in these families is not the same as in bipolar individuals from the general non-Amish population.
### Table 4.1 Lymphoblast source individuals - BPD-I

<table>
<thead>
<tr>
<th>ID</th>
<th>Coriell ID</th>
<th>Sex</th>
<th>AAV(^1)</th>
<th>AAO(^2)</th>
<th>Ethnicity</th>
<th>Medication</th>
<th>Family History</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GM05134</td>
<td>M</td>
<td>24</td>
<td>15</td>
<td>Caucasian</td>
<td>Lithium</td>
<td>Positive</td>
</tr>
<tr>
<td>B</td>
<td>GM04984</td>
<td>M</td>
<td>20</td>
<td>17</td>
<td>Caucasian</td>
<td>Lithium</td>
<td>Positive</td>
</tr>
<tr>
<td>C</td>
<td>GM04986</td>
<td>M</td>
<td>28</td>
<td>17</td>
<td>Caucasian</td>
<td>Lithium</td>
<td>BPD-I</td>
</tr>
<tr>
<td>D</td>
<td>GM07263</td>
<td>M</td>
<td>22</td>
<td>20</td>
<td>Caucasian</td>
<td>Lithium</td>
<td>BPD-I</td>
</tr>
<tr>
<td>E</td>
<td>GM04950</td>
<td>M</td>
<td>38</td>
<td>32</td>
<td>Caucasian</td>
<td>Lithium</td>
<td>Positive</td>
</tr>
</tbody>
</table>

\(^1\) Age at venesection.  
\(^2\) Age of onset of BPD-I.

Five individuals with no history of psychiatric disorders were chosen for the unaffected control group. As with the BPD-I group these individuals were all Caucasian males, however their ages were different, ranging from 44 to 55 years. Older individuals were chosen to reduce the risk that any of the control group would go on to develop bipolar disorder, which is statistically more likely to appear before 40 years of age (Merikangas et al., 2011).

### Table 4.2 Lymphoblast source individuals - unaffected group

<table>
<thead>
<tr>
<th>ID</th>
<th>Coriell ID</th>
<th>Sex</th>
<th>AAV(^1)</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>GM17244</td>
<td>M</td>
<td>44</td>
<td>Caucasian</td>
</tr>
<tr>
<td>G</td>
<td>GM17249</td>
<td>M</td>
<td>44</td>
<td>Caucasian</td>
</tr>
<tr>
<td>H</td>
<td>GM17212</td>
<td>M</td>
<td>51</td>
<td>Caucasian</td>
</tr>
<tr>
<td>I</td>
<td>GM17243</td>
<td>M</td>
<td>52</td>
<td>Caucasian</td>
</tr>
<tr>
<td>J</td>
<td>GM17248</td>
<td>M</td>
<td>55</td>
<td>Caucasian</td>
</tr>
</tbody>
</table>

\(^1\) Age at venesection.

### 4.1.3. Selection of reference genes

While a single gene, *GAPDH*, was used as the reference in the last chapter, it was decided to increase the number of references genes to three for the analysis of gene expression in the lymphoblast cell lines. Several studies validating reference genes found that *GAPDH* had unacceptably high variability for their particular model (Hamalainen et al., 2001; Bustin, 2002; Glare et al., 2002; Tricarico et al., 2002; Dheda et al., 2004), and while *GAPDH* has been validated in neuroblastoma cells (Hoerndli et al., 2004), it has not been validated in lymphocyte or lymphoblast cell lines.
In an attempt to improve normalization of RT-qPCR, Vandesompele et al. (2002) presented a strategy that uses the geometric average of multiple internal control genes. The associated software, geNorm, performs the geometric averaging allowing the user to select the most appropriate combination of reference genes for each normalisation. Along with GAPDH, succinate dehydrogenase (SDHA) and ubiquitin C (UBC) were selected to use in conjunction with geNorm to normalise gene expression change in the lymphoblast cell line analysis.

4.1.4. Analysis of RT-qPCR results
Analysis of the RT-qPCR results was performed using the geNorm applet within Microsoft Excel. Biogazelle QBasePlus software was used to perform Mann-Whitney U tests in order to determine if there was significant difference between expression changes in grouped data, for example, whether carbamazepine had a greater effect on gene expression in the group of individuals with BPD-I than in the group of controls.
4.2. Results

4.2.1. Gene expression results
The ten lymphoblast cell lines were initially grown in drug-free media for a period of 28 days, after which the expression of the genes BDNF, MDK, RAB7 and YWHAG were measured for all ten cell lines by RT-qPCR, normalised against a set of reference genes. These measurements provided a baseline to compare gene levels in the unaffected and BPD-I groups before drug treatment. The lymphoblasts were then cultured for a further 28 days in media containing a mood stabilising agent (lithium, carbamazepine or valproate). As in the previous chapter, the concentration of the drug in the culture media is the same as the therapeutically relevant concentration in blood serum, and as before the 28-day period mimics the chronic exposure (3-4 weeks) usually required for mood stabilising drugs to show clinical effect. Each culture treated with a mood stabiliser had a corresponding untreated control. Gene expression was again measured by RT-qPCR, and normalised against a set of reference genes, GAPDH, SDHA and UBC. Unfortunately, the cell line GM04984, designated individual B, was of poor quality and did not grow well in either the treated or untreated media. Eventually this cell line failed in culture and was not able to be recovered, and this led to several results missing for this cell line.
Table 4.3 Change in *BDNF* expression levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level¹</td>
<td>95% CI</td>
<td>Level¹</td>
</tr>
<tr>
<td>A</td>
<td>0.96</td>
<td>0.68 – 1.24</td>
<td>0.22</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>1.08</td>
</tr>
<tr>
<td>C</td>
<td>9.00</td>
<td>8.28 – 9.72</td>
<td>1.52</td>
</tr>
<tr>
<td>D</td>
<td>0.19</td>
<td>0.18 – 0.20</td>
<td>2.66</td>
</tr>
<tr>
<td>E</td>
<td>2.01</td>
<td>1.41 – 2.61</td>
<td>3.74</td>
</tr>
<tr>
<td>F</td>
<td>0.23</td>
<td>0.17 – 0.29</td>
<td>1.27</td>
</tr>
<tr>
<td>G</td>
<td>1.29</td>
<td>1.13 – 1.45</td>
<td>0.51</td>
</tr>
<tr>
<td>H</td>
<td>0.55</td>
<td>0.53 – 0.57</td>
<td>1.09</td>
</tr>
<tr>
<td>I</td>
<td>0.62</td>
<td>0.54 – 0.70</td>
<td>0.88</td>
</tr>
<tr>
<td>J</td>
<td>1.00</td>
<td>0.98 – 1.02</td>
<td>10.69</td>
</tr>
</tbody>
</table>

¹Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 4.1 Change in *BDNF* expression levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
### Table 4.4 Change in MDK expression levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level¹</td>
<td>95% CI</td>
<td>Level¹</td>
</tr>
<tr>
<td>A</td>
<td>0.48</td>
<td>0.47 – 0.49</td>
<td>0.77</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>1.46</td>
</tr>
<tr>
<td>C</td>
<td>0.02</td>
<td>0.02 – 0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>D</td>
<td>0.45</td>
<td>0.44 – 0.46</td>
<td>9.40</td>
</tr>
<tr>
<td>E</td>
<td>5.72</td>
<td>5.71 – 5.73</td>
<td>0.27</td>
</tr>
<tr>
<td>F</td>
<td>2.21</td>
<td>2.15 – 2.27</td>
<td>0.03</td>
</tr>
<tr>
<td>G</td>
<td>0.03</td>
<td>0.02 – 0.04</td>
<td>2.51</td>
</tr>
<tr>
<td>H</td>
<td>8.66</td>
<td>8.62 – 8.70</td>
<td>5.26</td>
</tr>
<tr>
<td>I</td>
<td>0.53</td>
<td>0.39 – 0.67</td>
<td>1.25</td>
</tr>
<tr>
<td>J</td>
<td>0.37</td>
<td>0.36 – 0.38</td>
<td>7.04</td>
</tr>
</tbody>
</table>

¹Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

### Figure 4.2 Change in MDK expression levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
Table 4.5 Change in RAB7A expression levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level(^1)</td>
<td>95% CI</td>
<td>Level(^1)</td>
</tr>
<tr>
<td>A</td>
<td>0.65</td>
<td>0.51 – 0.79</td>
<td>3.02</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>0.89</td>
</tr>
<tr>
<td>C</td>
<td>0.64</td>
<td>0.62 – 0.66</td>
<td>0.38</td>
</tr>
<tr>
<td>D</td>
<td>0.46</td>
<td>0.44 – 0.48</td>
<td>3.92</td>
</tr>
<tr>
<td>E</td>
<td>0.71</td>
<td>0.65 – 0.77</td>
<td>0.47</td>
</tr>
<tr>
<td>F</td>
<td>0.38</td>
<td>0.36 – 0.40</td>
<td>0.83</td>
</tr>
<tr>
<td>G</td>
<td>0.62</td>
<td>0.56 – 0.68</td>
<td>1.42</td>
</tr>
<tr>
<td>H</td>
<td>0.18</td>
<td>0.17 – 0.19</td>
<td>4.27</td>
</tr>
<tr>
<td>I</td>
<td>0.08</td>
<td>0.07 – 0.09</td>
<td>0.54</td>
</tr>
<tr>
<td>J</td>
<td>0.14</td>
<td>0.13 – 0.15</td>
<td>5.81</td>
</tr>
</tbody>
</table>

\(^1\)Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 4.3 Change in RAB7A expression levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
Table 4.6 Change in YWHAG expression levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level</td>
<td>95% CI</td>
<td>Level</td>
</tr>
<tr>
<td>A</td>
<td>5.9</td>
<td>5.84 – 5.96</td>
<td>2.61</td>
</tr>
<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>0.19</td>
</tr>
<tr>
<td>C</td>
<td>0.83</td>
<td>0.82 – 0.84</td>
<td>0.10</td>
</tr>
<tr>
<td>D</td>
<td>0.07</td>
<td>0.06 – 0.08</td>
<td>0.91</td>
</tr>
<tr>
<td>E</td>
<td>0.58</td>
<td>0.52 – 0.64</td>
<td>0.26</td>
</tr>
<tr>
<td>F</td>
<td>0.72</td>
<td>0.71 – 0.73</td>
<td>1.73</td>
</tr>
<tr>
<td>G</td>
<td>1.09</td>
<td>1.08 – 1.10</td>
<td>0.70</td>
</tr>
<tr>
<td>H</td>
<td>9.41</td>
<td>9.33 – 9.49</td>
<td>2.16</td>
</tr>
<tr>
<td>I</td>
<td>2.38</td>
<td>2.36 – 2.40</td>
<td>1.29</td>
</tr>
<tr>
<td>J</td>
<td>5.75</td>
<td>5.73 – 5.77</td>
<td>3.39</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.

Figure 4.4 Change in YWHAG expression levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
4.2.2. **Group comparisons of gene expression**

Group differences were analysed using the Mann-Whitney U test, which compares the means of two groups and uses their distribution to determine whether the means are significantly different. The resulting p-value describes the likelihood that the means are different, a p-value less than 0.05 provides 95% confidence that the two means are different. P-values greater than one indicate that there is no evidence to discard the null hypothesis that the means of the two groups are not significantly different.

The first analysis compared the gene expression levels of the unaffected group to the BPD-I group. This analysis did not first standardise each cell type/treatment against its own untreated control, so the results of the analysis specifies the difference in raw gene expression level between the two groups. This analysis also was performed on the untreated cells of both groups after the initial 28-day drug-free incubation to provide a baseline difference in gene expression between the unaffected group and the BPD-I group.

<table>
<thead>
<tr>
<th>Table 4.7 Gene expression level after 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Valproate</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Lithium</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

While no significant difference was found between the unaffected control group and the bipolar group for the expression levels of any of the genes, the difference is much closer to significant in the untreated cells than after any of
the drug treatments. This pattern is most noticeable in YWHAG and does not seem to occur in MDK.

The second analysis compared the gene expression levels of the unaffected control group to the BPD-I group after standardising each cell type/treatment against its own untreated control. The results of this analysis therefore specify any difference in change of gene expression levels between the two groups after drug administration.

Table 4.8 Change in gene expression after 28 days of treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Unaffected Group</th>
<th>BPD-I Group</th>
<th>p-value Mann-Whitney</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>Carbamazepine</td>
<td>1.00</td>
<td>0.077</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>1.00</td>
<td>0.582</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>1.00</td>
<td>0.686</td>
<td>0.730</td>
</tr>
<tr>
<td>MDK</td>
<td>Carbamazepine</td>
<td>1.00</td>
<td>0.080</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>1.00</td>
<td>0.686</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>1.00</td>
<td>0.111</td>
<td>0.086</td>
</tr>
<tr>
<td>RAB7A</td>
<td>Carbamazepine</td>
<td>1.00</td>
<td>0.279</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>1.00</td>
<td>0.339</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>1.00</td>
<td>0.370</td>
<td>0.429</td>
</tr>
<tr>
<td>YWHAG</td>
<td>Carbamazepine</td>
<td>1.00</td>
<td>0.800</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>1.00</td>
<td>0.966</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>1.00</td>
<td>0.333</td>
<td>0.730</td>
</tr>
</tbody>
</table>

None of these comparisons found any significant difference between the changes in gene expression level for the unaffected group and the BPD-I group. The difference in regulation of MDK between the unaffected group and the BPD-I group was almost significant, with the bipolar group being downregulated almost ten fold compared to the control group and generating a p-value of 0.086.

Results for gene expression changes for untreated vs. all treatments combined can be found in Appendix C.

4.2.3. BDNF and YWHAG protein levels

Measurements of changes in BDNF and YWHAG protein levels between the untreated and treated lymphoblast cultures were carried out in order to determine if the lack of correlation between changes in gene expression and changes in protein level found in the neuroblast cell lines in the previous
chapter were cell type or protein specific. The original plan was to measure the levels of all four proteins before and after treatment in order to have a wider range of proteins to compare, however repeated failure of antibodies to the MDK and RAB7A proteins meant that their levels could not be consistently analysed.

BDNF protein levels were measured by ELISA and normalised against total protein, as in the previous chapter. YWHAG levels were quantitated by chemiluminescent measurement after western blot, and the changes in protein level between control and drugged cell lysate were normalised against actin protein levels (see Figure 4.5). Unfortunately, the slow growth of the E cell line (BPD-I) meant that the cell harvest was low and as a result of this YWHAG protein levels could not be measured for this line.

![Figure 4.5 Western blot showing reference (β-actin) and YWHAG bands](image)

The lane labelled ‘M’ is the ‘Magic Mark’ protein marker from Invitrogen. Band sizes are labelled in kDa.
Table 4.9 Change in BDNF protein levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level¹</td>
<td>95% CI</td>
<td>Level¹</td>
</tr>
<tr>
<td>A</td>
<td>0.69</td>
<td>0.49 – 0.89</td>
<td>0.66</td>
</tr>
<tr>
<td>B</td>
<td>ND²</td>
<td>ND</td>
<td>0.63</td>
</tr>
<tr>
<td>C</td>
<td>1.24</td>
<td>0.86 – 1.62</td>
<td>1.72</td>
</tr>
<tr>
<td>D</td>
<td>0.96</td>
<td>0.66 – 1.26</td>
<td>3.9</td>
</tr>
<tr>
<td>E</td>
<td>ND</td>
<td>ND</td>
<td>1.00</td>
</tr>
<tr>
<td>F</td>
<td>1.54</td>
<td>1.40 – 1.68</td>
<td>1.33</td>
</tr>
<tr>
<td>G</td>
<td>1.45</td>
<td>1.19 – 1.71</td>
<td>0.95</td>
</tr>
<tr>
<td>H</td>
<td>1.18</td>
<td>1.14 – 1.22</td>
<td>0.96</td>
</tr>
<tr>
<td>I</td>
<td>0.88</td>
<td>0.68 – 1.08</td>
<td>0.72</td>
</tr>
<tr>
<td>J</td>
<td>0.5</td>
<td>0.42 – 0.58</td>
<td>0.55</td>
</tr>
</tbody>
</table>

¹Protein level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a decrease, and a number above one shows an increase

²Not determined

Figure 4.6 Change in BDNF protein levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from a triplet of technical replicates.
Table 4.10 Change in YWHAG protein levels after 28 days of drug treatment

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Carbamazepine</th>
<th>Valproate</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level &lt;sup&gt;1&lt;/sup&gt;</td>
<td>95% CI</td>
<td>Level &lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>1.45</td>
<td>1.43 – 1.47</td>
<td>1.3</td>
</tr>
<tr>
<td>B</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td>ND</td>
<td>0.95</td>
</tr>
<tr>
<td>D</td>
<td>1.25</td>
<td>1.11 – 1.39</td>
<td>0.92</td>
</tr>
<tr>
<td>E</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F</td>
<td>0.6</td>
<td>0.50 – 0.70</td>
<td>0.62</td>
</tr>
<tr>
<td>G</td>
<td>0.78</td>
<td>0.74 – 0.82</td>
<td>0.59</td>
</tr>
<tr>
<td>H</td>
<td>0.8</td>
<td>0.74 – 0.86</td>
<td>1.01</td>
</tr>
<tr>
<td>I</td>
<td>0.96</td>
<td>0.94 – 0.98</td>
<td>ND</td>
</tr>
<tr>
<td>J</td>
<td>0.68</td>
<td>0.67 – 0.69</td>
<td>0.58</td>
</tr>
</tbody>
</table>

<sup>1</sup>Protein level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a decrease, and a number above one shows an increase.  
<sup>2</sup>Not determined

Figure 4.7 Change in YWHAG protein levels after 28 days of drug treatment

All cell line/treatment pairs are standardised to their own untreated control, which has been set at one. Error bars represent the 95% confidence interval calculated from of a triplet of technical replicates.
4.2.4. Gene expression and protein level correlation

The standardised changes in gene expression and changes in protein level for each BDNF and YWHAG cell line/treatment type pair were plotted against each other to determine whether there was any evidence of correlation between the two. A significant positive correlation between the two requires a positive slope and a correlation coefficient of $r^2 \geq 0.95$.

No evidence of correlation was found between changes in $BDNF$ gene expression level and changes in BDNF protein after 28 days of drug treatment. The correlation coefficient is $r^2 = 0.03$.

![Figure 4.8 BDNF protein level change vs. BDNF gene expression change after 28 days drug treatment](image)
Figure 4.9 YWHAG protein level change vs. YWHAG gene expression change after 28 days drug treatment

No evidence of correlation was found between changes in YWHAG gene expression level and changes in YWHAG protein after 28 days of drug treatment. The correlation coefficient is $r^2=0.02$. 
4.3. Discussion

The replication of results is an important part of research, and therefore a second model system was used to attempt to replicate the changes in gene expression found in the treated neuroblast cell lines for four genes – BDNF, MDK, RAB7A and YWHAG. Lymphocytes were chosen for their ease of culture and the fact that lymphoblast cell lines from both individuals with bipolar disorder and control individuals were readily available.

The gene expression results from the neuroblast cell lines could not be replicated in the ten lymphoblast cell lines. There was no consistent up- or downregulation of any of the mRNA expression by any of the three drug treatments. Changes in gene expression levels did not match those that occurred in the neuroblast cell lines in any consistent way. Results comparing the unaffected control group and the BPD-I group were likewise negative. No significant differences were found between the unaffected group and the BPD-I group both when changes in gene expression and raw gene expression before and after treatment were analysed. However, it is interesting to note that for the genes BDNF, RAB7A and YWHAG, the difference in gene level between the two groups is much closer to being statistically significant in the undrugged, treatment naïve condition than after treatment. In the case of BDNF and RAB7A, the difference is consistent with data from the previous research, with the genes being close to significantly lower in the bipolar cell lines than the control cell lines, and then no difference found between them after treatment. This suggests that the drug treatments may normalise the levels of gene expression of BDNF and RAB7A in bipolar individuals, however this would have to be repeated in a much larger group to see if significance could be found.

4.3.1. Inconsistent results in the neuroblast and lymphoblast models

There are several factors that may explain the inconsistencies in the results between the neuroblast and lymphoblast models. Firstly there is the possibility that the discrepancy is a result of the regulation being random in one or both of the models, indicating that there is no real effect on regulation
of any of these genes by the three mood stabilising drugs. While this may be true for the genes MDK, RAB7A and YWHAG, the gene expression changes in BDNF in the neuroblast cell lines are consistent with the literature and the consistent upregulation in this gene across SK-N-DZ, SK-N-SH and SH-SY5Y is unlikely to be a spurious finding. A second explanation for the inconsistencies between the models might be the limited sample sizes, which may simply be too small to allow a consistent pattern to emerge. Thirdly, lymphoblasts may not be an appropriate model for assessing changes in expression caused by psychiatric drugs. While lymphoblasts may be a good model for brain tissue for some purposes (see section 4.1.1), their different function may lead to emphasis on different signalling pathways and possibly different gene regulation profiles than those of neural cells, and it is changes in neural cells that are thought to be behind the bipolar phenotype.

4.3.2. Lymphoblasts as model systems
DeLuca et al. (2008) examined the ratio of BDNF Val/Met-specific mRNA from heterozygous individuals in both postmortem brain tissue and B-lymphoblasts. They found no difference in the Val/Met transcript ratio between individuals with BPD and controls in both models. However, the ratio was significantly lower in the brain tissue than in the lymphoblasts. This suggests that while transcription level of a gene overall may be equivalent between central nervous system tissue and lymphoblasts, there may be differences in the proportions of different alleles of a gene between the two cell types which may not show up in an expression assay such as described in this chapter. Lymphocytes, then, may not be an appropriate model where functional polymorphisms have a possible influence on phenotype.

The use of lymphocytes to look at gene expression has been performed more frequently in research on schizophrenia, but the results may be relevant to studies on bipolar disorder as well. A study by Matigian et al. (2008) compared lymphocyte gene expression profiles from eight individuals with schizophrenia to a matched group of seven unaffected controls. They were unable to find more genes differentially expressed between the two groups than would be expected by chance, and concluded that lymphocytes had not yet been shown to be appropriate for gene expression studies in
schizophrenia, although this finding may have been affected by the small size of the study. Gene expression profiles from brain tissue, on the other hand, can differentiate between different forms of psychiatric illness, as well as between individuals with no psychiatric conditions and those with bipolar disorder (Iwamoto et al., 2004).

Lymphoblasts, while a useful model in terms of accessibility and the ability to easily recruit a cohort of individuals with a given disorder, may be inappropriate as an alternative to neural cells in studies of psychiatric disorders, so other models may be preferred.

Accessing human brain and neural tissue is difficult and generally involves either a brain or spinal biopsy, or more commonly for research purposes, postmortem removal of neural tissue. Very uncommonly, central nervous system tissue may be obtained from metastases of malignancies that begin in neural tissue, such as the metastasised neuroblastoma source of the SK-N-DZ and SK-N-SH cell lines used in the research presented in Chapter Three. None of these options allow for the large numbers of individuals often required for significant findings, and can make matching of features such as diagnosis, age, race and gender difficult. Issues such as cause of death and postmortem treatment of the body, both of which may have an effect on gene expression and protein levels, further complicate the use of postmortem tissue. These factors have led to considerable interest in trying to find a more easily available tissue surrogate for central nervous system tissue. An appropriate surrogate for use in gene and protein studies hoping to elucidate the genetic and molecular contributors to psychiatric disorders would enable larger and better controlled studies that would hopefully produce more robust results.

Other models that have been investigated include whole blood, cultured fibroblast cells, and olfactory epithelium. Both cultured fibroblast cell lines and whole blood have limits as models for analysis of the pathophysiology of psychiatric disorders. In common with other models not derived from neural cultures, there is not a complete correlation between gene expression in these models and in the central nervous system, and differences are not yet fully explored. These differences may be altered between individuals, a factor that
would have to be taken into account. Fibroblasts are relatively easy to culture, however the media type and handling of the cells have been shown to effect enzymatic activity, which must then be controlled for in the experimental design. Fibroblasts from older individuals do not live as long in culture, and may not be appropriate for drug studies requiring extended culture times (Mahadik and Mukherjee, 1996). Fibroblasts from individuals with schizophrenia have been shown to have decreased adhesion efficiency in the short term when compared to fibroblasts from controls when cultured in vitro (Miyamae et al., 1998). Sullivan et al. (2006) used microarray data to compare gene expression in brain tissue with 60 other body tissues in an attempt to determine if more easily available tissue could provide a surrogate for brain tissue for the study of psychiatric disorders. They reported that whole blood showed significant similarities in gene expression to brain tissue, especially the prefrontal cortex, the amygdala, and whole brain. Whole blood achieved a gene expression correlation with brain tissue of 0.5 out of a maximum of 0.85, about the same as muscle tissue. Using a 50th percentile threshold, 47% of all genes analysed were expressed in both whole blood and prefrontal cortex tissue. The authors, while expressing caution and a need for further study, conclude that whole blood may be a useful surrogate for central nervous system tissue in gene expression studies if it has been established that the gene or genes concerned are expressed in both tissues.

Olfactory epithelium, the epithelial layer of the upper nasal passages, is a source of neurons that can be accessed in a less invasive manner than other neural tissues. Olfactory neurons, one of the four cell types found in the olfactory epithelia, have historically been collected under general anaesthesia, with the procedure being less invasive than brain or spinal biopsy but still considerably more involved and painful than peripheral venesection. However, a comparatively non-invasive procedure for collecting olfactory neurons has been developed very recently by Benitez-King et al. (2011). Collecting the cells involves exfoliation of the back of the medial lateral concha, a short distance up the nasal passage. Neural cells from these epithelial scrapings have been successfully grown, maintained and differentiated in culture. Thus cultured olfactory neurons as a model look to
benefit from an ease of collection similar to that of lymphocytes, while still being fully neural in origin.

McCurdy *et al.* (2006) used microarray to investigate gene expression in olfactory neurons sourced from individuals with schizophrenia and bipolar disorder and found that expression was significantly different from controls in both cell cycle markers and genes involved in the phosphatidyl signal transduction pathway. Research with olfactory neurons has shown that calcium signalling in individuals with bipolar disorder is significantly different from normal individuals, with drug treatment normalising the intracellular calcium levels (Hahn *et al*., 2005).

Thus while using cultured olfactory neurons to investigate psychiatric conditions such as BPD is still in its infancy, it appears quite promising and with the recent improvements in collection methods, may prove to be a convenient and effective model.
5. Association Studies of Candidate Gene Polymorphisms in a Cohort with Bipolar Disorder.

5.1. Association Studies

Genetic association studies are designed to detect an association between a genetic marker (such as a microsatellite repeat or single nucleotide polymorphism) and a specific trait in a group of individuals. Association studies for a complex disorder such as BPD hope to find polymorphisms that contribute to the phenotype. They may be targeted studies of single genes or even single polymorphisms, or genome wide association studies designed to find common genotypic variants of small effect (Manolio, 2010).

The two main categories of association studies are those performed on groups of unrelated individuals and controls, and those that use family groups. Each of these approaches has its own merits and drawbacks, but when tested against well-established genetic associations both have been found to give similar results (Ackerman et al., 2005; Evangelou et al., 2006).

5.1.1. Population-based association testing

Population-based, or ‘case-control’ association studies involve genotyping a cohort of unrelated affected individuals for a particular polymorphism and comparing the allele frequencies of the cohort with a matched group of unaffected individuals. Statistical analysis of any difference in frequencies between cases and controls can then be performed using chi-squared or log-linear tests. Where allele frequencies are significantly different between the two groups, the polymorphism is said to be associated with the trait in question, either by directly contributing to or protecting from it, or being in linkage disequilibrium with a polymorphism that does.

An advantage of population-based studies is the ability to pool DNA for both case and control groups to determine overall allele frequency, a much quicker and less technically involved process than genotyping large numbers of individuals (Cardon and Bell, 2001).
The primary limitation associated with case control studies is that of population stratification. Stratification is a result of the presence of multiple sub-groups within the study population. Differences in ethnicity, for example, may mean that there are sub-groups of individuals in a study cohort that differ from the overall study population in both marker allele frequency and the frequency of the trait being assessed (Gorroochurn et al., 2004). Where the cases and controls draw unequally from these sub-groups, false-positive associations may be found (Heiman et al., 2004; Rodriguez-Murillo and Greenberg, 2008). There are several approaches for controlling for population stratification. Genomic control involves using an inflation factor to correct statistics at each marker, however differences in ancestral population allele frequency variation can mean that some markers will be over-corrected while some will be insufficiently corrected (Price et al., 2010). Principle components analysis infers population structures, and like genomic control, adds an inflation figure to marker analysis, however, the inflation figure changes depending on what is known about the differences in allele frequency between populations (Price et al., 2006). Structured association uses clustering software to form multiple random subgroups of samples, and association analysis is performed within these clusters to determine the level of variation in association between sub-groupings within the overall cohort. Once this variation is established it can be incorporated into the analysis of the final full-cohort marker-phenotype association (Sillanpaa, 2011). The use of these techniques reduces the number of false-positive associations associated with stratification.

5.1.2. Family-based association tests

Family-based association studies utilise genotype and phenotype data from nuclear families to look for association without the need for external controls. While there are other ways to analyse family-based genotype data, such as the haplotype relative risk method (Knapp et al., 1993), most statistical tests are based around the transmission disequilibrium test (TDT). This test compares the observed number of transmissions of an allele from parent to affected offspring with the number of transmissions expected under the laws of Mendelian inheritance (Spielman and Ewens, 1996). The TDT as originally designed required genotype and phenotype data from probands and both
their parents, but there have been many additions made to the basic analysis to allow for multiple siblings, missing parental data, multiple markers and quantitative rather than binary traits (Cardon and Bell, 2001).

The advantages of family-based association tests are that they do not require the selection of a set of controls, and that they are not affected by population stratification and admixture, ruling out two of the known causes of type I (false-positive) errors (Lange et al., 2008).

While family-based association testing is not subject to issues of population stratification, it does have its own set of limitations. Because the TDT analyses the transmission of alleles from parent to child, parents that are homozygous at the polymorphism under study are uninformative for the purposes of determining association. This can result in the number of informative families for any given analysis being much smaller than the actual cohort, and cohorts need to be large to provide enough power to detect association (Morton and Collins, 1998). Due to the necessity of collecting both genotype and phenotype data from the affected individual and their parents for a classic TDT, or the affected individual and any available parents and siblings for the more extended analyses, family-based studies can be time consuming and expensive to organise (Laird and Lange, 2006). Some adult-onset disorders may not be suitable for family-based testing due to the unavailability of genotypes from deceased parents. The cohort size in family-based studies also tends to be smaller than that of case-control studies; where it may be possible to recruit 1000 individuals with a given condition, it is much more difficult to do so when their family members must also be recruited. Thus the family-based association test has more purely logistical limitations than association studies performed using a case control design (Cardon and Bell, 2001).

5.1.3. Haplotype Association testing
As well as analysing single polymorphisms for association with a trait or disorder, it is possible to combine markers together to form a single block, known as a haplotype block, and to analyse association between the block and the trait. A haplotype block is “a combination of alleles at different markers along the same chromosome that are inherited as a unit.” (Crawford
Analysis of a haplotype is possible because of the phenomenon of linkage disequilibrium, the non-random association of alleles at two or more loci. Where loci are either so close together that recombination between them is a rare event, or there is of selection pressure selecting for the combination, the alleles at these loci can effectively be treated as one unit (Reich et al., 2001; Cardon and Abecasis, 2003).

Haplotype blocks allow individual SNPs to act as proxies for other SNPs within the block, a process known as haplotype tagging. Using haplotype tagging, several SNPs can be chosen from a haplotype block that will capture most of the genetic variation right across the block. This cuts down on the effort and expense required to genotype groups of individuals for multiple markers. Several programs have been designed that use algorithms to determine the ‘tag’ SNPs, or the SNPs that need to be genotyped to determine the variation across the haplotype block.

While using haplotype blocks in association studies can make the genotyping process easier as well as less expensive, it has drawbacks that need to be taken into account. Tag SNPs tend to be population specific, depending on the ethnicity of the group being assessed; therefore it is important to choose appropriate tags for the study population. More limitations are that some areas of the genome may have very high numbers of alleles with low allele frequency for one haplotype block (more than 20 with no frequency greater than 0.05 in one example) (Crawford et al., 2004), and that the algorithms used to define haplotypes may impose artificially simple block structure in areas of more complex LD (Carlson et al., 2004).

Despite these limitations, haplotype blocks provide a useful tool for association studies, especially in terms of choosing which polymorphisms to genotype to extract maximum information with the limited resources available for any one study.

5.1.4. Correction for multiple testing in association studies
Where an association study looks for association between a phenotype and more than one SNP or haplotype, it is necessary to correct for multiple testing.
The more tests that are performed, the more likely it becomes that one will find a significant association with the phenotype by chance, and the primary means of dealing with this error is by recalculating the threshold of significance for each polymorphism to take into account that multiple tests are being performed. The threshold for genome-wide significance has been calculated at \( p \leq 5 \times 10^{-7} \) (Iles, 2008), and any SNP reaching this threshold can be considered to be associated with the phenotype with a good degree of certainty that the association is not by chance.

Correction for multiple testing is a simple process when the polymorphisms in question are independent of each other. Adjusting the threshold of significance can be done by performing a Bonferroni correction, which involves taking the standard threshold for significance (\( p \leq 0.05 \)) and dividing it by the number of polymorphisms being tested for association. Thus a study involving four SNPs that are not in LD with each other would generate a significance threshold of \( p \leq 0.0125 \) (i.e. \( 0.05/4 = 0.0125 \)). To be found to be significantly associated a SNP from this example would have to generate a p-value less than or equal to 0.0125.

When the polymorphisms being assessed are in linkage disequilibrium, however, the Bonferroni correction is considered overly conservative (Gao et al., 2008) and may therefore result in polymorphisms that actually are associated not meeting the corrected significance threshold. In these cases, randomised, or Monte Carlo, permutations of the data to calculate the true significance threshold are preferred. Such permutation testing, however, is computationally intensive and currently available software is not able to deal with multi-generation extended pedigrees.
5.2. Experimental Approach

The initial screen of gene response to mood stabilising drugs as outlined in chapter three identified a number of genes that are potentially involved in the mechanism of action of mood stabilising drugs and may therefore have a role in the aetiology of BPD. One means to further investigate these genes for an association with BPD is by analysing polymorphisms in the DNA sequence of each gene in a cohort with BPD. This chapter describes the use of the South Island Bipolar Study, a cohort of bipolar individuals and their first- and second-degree relatives, to investigate *BDNF*, *RAB7A* and *YWHAG* for association with the bipolar phenotype using family-based association testing.
5.3. Description of the SIBS cohort

Bipolar probands and their first- and second-degree relatives were recruited from the South Island of New Zealand as part of the South Island Bipolar Study (SIBS). Ethics approval for the study was given by the Multi-Region Ethics Committee of the Ministry of Health, New Zealand.

Subjects were initially recruited from the Otago Bipolar Register, a database of individuals with BPD in the Healthcare Otago catchment area. Further subjects were then recruited from all over the South Island of New Zealand by approaching clinicians to identify potential participants who were then invited to take part in the study by letter. Once written informed consent had been received from an individual who was willing to participate, other family members were approached by letter.

The South Island Bipolar Study database is comprised of DNA and phenotypic information from 786 individuals from the South Island of New Zealand. The cohort is made up of 153 family groups containing a proband with a diagnosis of BPD-I or BPD-II, plus 57 individuals similarly diagnosed but without family members. It consists primarily of Caucasians with ancestry from Western Europe but also includes individuals of Polynesian descent (2.5%), Chinese descent (0.1%) and African descent (0.1%). Full details of the cohort can be found in Joyce et al. (2004). Probands were interviewed using the Diagnostic Interview for Genetic Studies, and family members of the probands were assessed using the Family Interview for Genetic Studies (Nurnberger et al., 1994). The diagnoses of probands were made using standard DSM-IV criteria. A research psychiatrist assessed the diagnostic interview questionnaires and the clinical notes for each individual to confirm the diagnosis. Where the diagnosis was unclear, two psychiatrists re-evaluated the information separately then came to a consensus. The psychiatrists compared diagnoses to ensure inter-rater reliability (Edmonds et al., 1998). The author of this thesis did not take part in the setting up of the SIBS, and had no involvement in subject recruitment, interviewing, blood collection or DNA extraction.
5.4. Revision of the SIBS cohort

Examination of the results of genotyping performed previously on the SIBS cohort showed that Mendelian errors were appearing repeatedly in the same pedigrees. This caused concern that the family structures were not exactly as documented in the original pedigree files. There were also several individuals in the cohort who were designated a ‘niece’ or ‘nephew’ of the proband without specifying who of the proband’s siblings were their parent (see Figure 5.1). Both pedigree errors and the omission of possibly informative individuals can lower the power of the test to detect true associations, so it was important to increase the accuracy of the pedigrees before performing association analysis.

**Pedigree 535**

![Pedigree 535](image)

**Figure 5.1. Pedigree 535**

Shows four possibly informative individuals (two nieces and two nephews of the Proband) whose parents were not clearly identified in the SIBS data.

The FBAT programme (Horvath et al., 2001) was used to identify Mendelian errors in the genotyping across 50 previously typed SNPs. An upper allowable limit of three Mendelian errors per pedigree was set, as it was important not to rule out pedigrees where Mendelian errors were a result of genotyping error rather than problems with the family structure as
documented. Hence the 17 pedigrees that had four or more Mendelian errors were selected for further investigation into their accuracy. Where possible, genotyping was repeated to ensure that the Mendelian error was not a result of a genotyping mistake. After all possible genotype clarification had been performed and the number of Mendelian errors reassessed, each individual from the 15 remaining inconsistent pedigrees, plus individuals from pedigrees with unclear parentage (105 individuals in total) was typed for two polymorphic microsatellite markers (D4S2426 and D22S1153). The high number of possible alleles in these polymorphic markers (11 for D4S2426 and eight for D22S1153) made them ideally suited to assess pedigree accuracy. The results were used to attempt to rule out the family relationships documented in the study notes. The initial microsatellite results were inconclusive for ten individuals, and these people were further typed for a second set of two microsatellite markers - D7S2423 (nine alleles) and D17S937 (ten alleles).

On completion of the analysis six full families were removed from the cohort due to multiple Mendelian errors involving more than two people within the family. Two genetically incompatible fathers were removed from congruent mother and sib-ship groups, and six individuals were removed from the cohort because their SNP genotypes and microsatellite markers were not consistent with those of the rest of the family. Not enough information was available to identify the parent of any of the individuals designated ‘niece’ or ‘nephew’ of proband, and taking into account the possibility of parents of these individuals not being part of the SIBS study, it was decided to leave them out of the association analysis altogether.

The amended cohort contained 139 pedigrees that break down into 200 individual nuclear families. After the addition of place-holding individuals, which are used to help define sib-ships and missing parents in FBAT, the cohort ready for association analysis was comprised of 795 individuals, 565 for whom direct genotype information was available.
5.5. Selection of SNPs for association testing

Raw genotype data for BDNF, MDK, RAB7A and YWHAG was sourced from the public HapMap database (http://www.hapmap.org, HapMap release #22). Haploview 4.0 (Barrett et al., 2005) was used to examine the linkage disequilibrium between the SNPs across the gene and thus the haplotype block structures, where the blocks were defined according to criteria proposed by Gabriel et al. (2002). A single haplotype block was chosen for BDNF that spanned the whole gene as well as incorporating 0.75kb upstream and 1.25kb downstream. The blocks selected for RAB7A and YWHAG spanned 97.25% and 93% of each gene respectively. The small size of MDK and the paucity of SNPs entered into HapMap for that gene at the time of block selection meant that MDK was not included in studies of association.

The ‘Tagger’ tool (de Bakker et al., 2005) within Haploview was used to select tag SNPs to represent each block, ensuring that the SNPs captured at least 80% of the variation within the block. Detailed haplotype block information can be found in Appendix D.

5.6. Genotyping of candidate gene polymorphisms

Several strategies were used to genotype the SIBS cohort for the 21 selected SNPs, as no one approach was suitable for every SNP.

5.6.1. Genotyping of SNPs using TaqMan probes

The majority of the SNPs investigated for association with BPD in this study were genotyped using commercial TaqMan SNP genotyping assays (Applied Biosystems). These provided a simple and efficient way to genotype SNPs for a cohort of this size using a minimum amount of DNA for each individual. TaqMan uses a two-probe approach, where each probe is specific to one of the alleles of the SNP and is tagged with a specific fluorophore. After qPCR amplification, software is then used to plot the intensity of signal from each specific fluorophore (McGuigan and Ralston, 2002). As depicted in Figure 5.2, the signals will cluster in four groups. One cluster shows samples where the signal from both fluorophores is at a background level and the second cluster
indicates where both are elevated over a specific threshold, identifying heterozygotes. The third and fourth clusters show where only one of the two signals is elevated, identifying individuals with a homozygous common or homozygous rare genotype. The SNPs genotyped using the TaqMan approach were rs10835210, rs11030107, rs12273363, rs11030119 and rs7103873 from BDNF; rs9820753, rs2712402 and rs13081864 from RAB7A; and rs2961034, rs13247572 and rs12531257 from YWHAG. Information on the TaqMan assays and probes can be found in Appendix F.

![Figure 5.2 TaqMan Allelic discrimination plot for BDNF rs1223363](image)

Blue diamonds represent an individual homozygous for the common allele, green triangles represent heterozygous individuals, and red circles represent those who carry two rare alleles at this location. Black crosses show individuals that cannot be typed - in this case two blank controls to the left, and one ambiguous individual to the right.

5.6.2. Genotyping of SNPs using restriction enzyme digest

Restriction enzyme digests, while not well suited to genotyping large numbers of individuals, were used where commercial TaqMan assays were not available and where allele specific primers had failed to provide a consistently readable genotype. This occurred for five of the 21 SNPs genotyped - BDNF SNP rs11030104, RAB7A SNPs rs4927892, rs4333102 and rs7372263, and YWHAG SNP rs11763069.
Amplification and restriction enzyme digest conditions and details for genotyping these five SNPs can be found in Appendix E.

5.6.3. Genotyping of rs6265 using an ARMS assay
The ARMS assay involves the use of four primers to produce different-sized bands to differentiate between each allele. This allows the genotyping of an individual in a single reaction. Figure 5.3 shows the ARMS assay used to type rs6265, which amplifies a control band of 308bp, a band of 128bp for the G allele (Val) and a 239bp band for the A (Met) allele. This assay was designed by Tony Harley of the Gene Structure and Function Laboratory, Christchurch School of Medicine, University of Otago Christchurch.

![BDNF Exon 1](image)

**Figure 5.3 The BDNF rs6265 SNP ARMS assay**
A. Design of the rs6265 ARMS assay. B. Agarose gel showing results for three individuals (23, 26 and 28). Each individual has the large control band (308bp) and the 128bp band indicating the G allele. Individuals 23 and 26 also have the 239bp band for the A allele, indicating that they are heterozygotes. Individual 28 is homozygous for the G allele.

5.6.4. Genotyping of SNPs using allele-specific primers
SNPs RAB7A rs4384970 and YWHAG rs6465098 were typed using sets of three primers - one common primer and two allele specific primers. Each individual was typed in two separate reactions, with well one containing the common primer and the primer specific to the high frequency allele, and the other well containing the common primer and the low-frequency allele specific primer.

Genotyping of YWHAG rs1046304 was also performed with a three primer set, but included the addition of a 15bp GC-rich tail to the 5’ end of the primer for the low frequency T allele. Amplification of this allele led to the presence of a 170bp band, clearly distinguishable from the smaller 155bp band that indicated the higher frequency G allele. Addition of this primer tail to
differentiate the two amplification products meant each individual could be genotyped with a single reaction.

5.6.5. **Genotyping of rs1519480 using inference from haplotype**
The *BDNF* SNP rs1519480 was partially genotyped by inference from the genotypes present at the seven other SNPs for this gene. Where this was not possible, rs1519480 was genotyped by DNA sequencing. Primer sequence and amplification conditions for sequencing this SNP can be found in Appendix E.

5.6.6. **Checking for genotyping errors**
In order to screen for errors in genotyping, each SNP was assessed for deviation from Hardy-Weinberg equilibrium using a Hardy-Weinberg equilibrium calculator web tool (Rodriguez *et al.*, 2009). All SNPs except *BDNF* rs6265 were found to be in Hardy-Weinberg equilibrium. To ensure the accuracy of genotyping of rs6265, genotypes were compared with genotyping of rs6265 performed on this cohort by another research group. The genotypes were 99.3% concordant. The non-concordant genotypes were sequenced and confirmed.
5.7. Association analysis

Individuals were included in the statistical analysis where they were part of a family that had a proband and at least one parent, or a proband and at least one sibling. Individuals with a diagnosis of BPD-I, BPD-II, or cyclothymic disorder were classed as affected, all other individuals were classed as unaffected. Families with apparent Mendelian errors after genotyping were excluded from analysis for each SNP.

Family-based association testing was performed for each SNP using FBAT (Horvath et al., 2001), with a null hypothesis of no association in the presence of linkage. Each pedigree is broken down into all possible nuclear families and each trio is analysed separately. The FBAT looks at the transmission of alleles to affected individuals to determine if any are significantly over- or under-transmitted compared to chance.

5.7.1. Association with BDNF

Analysis of BDNF in the HapMap CEU population (residents of Utah, USA, with ancestry from northern and western Europe) with Tagger (de Bakker et al., 2005) showed that the eight SNPs chosen captured 88% of the possible information genotyped in the HapMap CEU population across this haplotype block (where the minor allele frequency was ≥0.05 and the $r^2=0.89$), and therefore provided good coverage of the variation in this block.

A suggestion of association with BPD was found (individual p<0.05) for six of the eight SNPs tested (see Table 5.1). To provide further clarification, an individual threshold of p<0.00625 was chosen to provide a family-wise p-value of <0.05, correcting for multiple testing (Bonferroni). This statistic is appropriate for independent markers, and therefore very conservative for the eight SNPs investigated because they are in high LD. Four of the SNPs met this threshold for significance - rs1519480, rs11030104, rs11030107 and rs11030119.
Table 5.1 BDNF - SNP Association with bipolar disorder

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position 1</th>
<th>Location</th>
<th># IF2</th>
<th>MAF3</th>
<th>p-value</th>
<th>Allele4</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1519480</td>
<td>27675712</td>
<td>Downstream</td>
<td>49</td>
<td>0.287</td>
<td>0.004</td>
<td>C*</td>
</tr>
<tr>
<td>rs6265</td>
<td>27679916</td>
<td>Exon 2</td>
<td>36</td>
<td>0.173</td>
<td>0.04</td>
<td>A</td>
</tr>
<tr>
<td>rs11030104</td>
<td>27684517</td>
<td>Intron 1</td>
<td>40</td>
<td>0.206</td>
<td>0.004</td>
<td>C*</td>
</tr>
<tr>
<td>rs11030107</td>
<td>27694835</td>
<td>Intron 1</td>
<td>46</td>
<td>0.249</td>
<td>0.006</td>
<td>A*</td>
</tr>
<tr>
<td>rs10835210</td>
<td>27695910</td>
<td>Intron 1</td>
<td>54</td>
<td>0.433</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>rs7103873</td>
<td>27700317</td>
<td>Intron 1</td>
<td>53</td>
<td>0.468</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>rs11030119</td>
<td>27728102</td>
<td>Intron 1</td>
<td>49</td>
<td>0.28</td>
<td>0.003</td>
<td>G*</td>
</tr>
<tr>
<td>rs12273363</td>
<td>27744859</td>
<td>Upstream</td>
<td>36</td>
<td>0.184</td>
<td>0.008</td>
<td>T</td>
</tr>
</tbody>
</table>

1Positions on chromosome 11 are based on dbSNP build 131/human genome build 37.1. GRCh37 NM_170731.4, isoform c.
2Number of informative families for this SNP in the SIBS cohort.
3Minor allele frequency in SIBS cohort.
4Allele associated with BPD at $p \leq 0.05$.
5Allele associated with BPD at corrected threshold of $p \leq 0.00625$.

The haplotypes of the BDNF block as represented by the tag SNPs were also examined for association with BPD. When full haplotype analysis was performed one significant protective association was identified at $p \leq 0.05$ (Table 5.2).

Table 5.2 BDNF - Full haplotype analysis

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs1519480</th>
<th>rs6265</th>
<th>rs11030104</th>
<th>rs11030107</th>
<th>rs10835210</th>
<th>rs7103873</th>
<th>rs11030119</th>
<th>rs12273363</th>
<th># Inf. Fam.1</th>
<th>Direction2</th>
<th>HapFreq3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>42</td>
<td>-</td>
<td>P</td>
<td>0.475</td>
<td>0.5</td>
</tr>
<tr>
<td>A2</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>23</td>
<td>P</td>
<td>0.15</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>24</td>
<td>-</td>
<td>0.143</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>11</td>
<td>-</td>
<td>0.05</td>
<td>0.7</td>
</tr>
<tr>
<td>A5</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>12</td>
<td>-</td>
<td>0.042</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1Number of informative families for this haplotype in the SIBS cohort.
2Direction of effect of the haplotype. P=protective, R=risk.
3Frequency of the haplotype in the SIBS cohort. As there is a long tail of haplotypes with very low frequencies, none of which were associated, for clarity only haplotypes with a frequency of over 4% are shown.
BDNF was also examined to find the haplotypes with the most statistically significant effect. All the haplotypes of between two and seven SNPs were systematically investigated for their association with BPD and the most significant risk haplotype and the most significant protective (i.e. under-transmitted to affected individuals) were found. In the case of BDNF, both the most significant risk haplotype and the most significant protective haplotype were represented by only two tag SNPs. It is unlikely that either of these associations would remain significant after correction for multiple testing.

Table 5.3 *BDNF* - Haplotype of highest risk

<table>
<thead>
<tr>
<th>rs1519480</th>
<th>rs11030119</th>
<th>#IF</th>
<th>HapFreq</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>G</td>
<td>50</td>
<td>0.721</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

1Number of informative families for this haplotype in the SIBS cohort.
2Frequency of the haplotype in the SIBs cohort.

Table 5.4 *BDNF* - Haplotype of highest protection

<table>
<thead>
<tr>
<th>rs1519480</th>
<th>rs11030104</th>
<th>#IF</th>
<th>HapFreq</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>A</td>
<td>47</td>
<td>0.267</td>
<td>0.009</td>
</tr>
</tbody>
</table>

1Number of informative families for this haplotype in the SIBS cohort.
2Frequency of the haplotype in the SIBs cohort.

5.7.2. Association with *RAB7A*

The tag SNPs chosen captured 80% of the possible information genotyped in the HapMap CEU population across this haplotype block (where the minor allele frequency was ≥0.05 and the $r^2$=0.8), providing reasonable coverage of the 31 SNP block.

One SNP of *RAB7A*, rs13081864, was found to be significantly associated with BPD at $p≤0.05$, and this significance remained after Bonferroni correction of the significance threshold to $p≤0.007$. 
Table 5.5 RAB7A - Association with bipolar disorder

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position¹</th>
<th>Location</th>
<th>#IF²</th>
<th>MAF³</th>
<th>p-value</th>
<th>Allele⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9820753</td>
<td>128446192</td>
<td>Intron 1</td>
<td>24</td>
<td>0.114</td>
<td>0.92</td>
<td>-</td>
</tr>
<tr>
<td>rs4384970</td>
<td>128446412</td>
<td>Intron 1</td>
<td>18</td>
<td>0.082</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>rs2712402</td>
<td>128455073</td>
<td>Intron 1</td>
<td>27</td>
<td>0.099</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>rs4927892</td>
<td>128473225</td>
<td>Intron 1</td>
<td>52</td>
<td>0.432</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>rs13081864</td>
<td>128490273</td>
<td>Intron 1</td>
<td>37</td>
<td>0.139</td>
<td>0.004</td>
<td>C*</td>
</tr>
<tr>
<td>rs4333102</td>
<td>128491001</td>
<td>Intron 1</td>
<td>22</td>
<td>0.057</td>
<td>0.64</td>
<td>-</td>
</tr>
<tr>
<td>rs7372263</td>
<td>128499195</td>
<td>Intron 1</td>
<td>47</td>
<td>0.334</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Positions on chromosome 11 are based on dbSNP build 131/human genome build 37.1, GRCh37 NM_170731.4, isoform c.
²Number of informative families for this SNP in the SIBS cohort.
³Minor allele frequency in SIBS cohort.
⁴Allele associated with BPD at p≤0.05.
*CAllele associated with BPD at corrected threshold of p≤0.007.

One of the full haplotypes from the RAB7A block, as tagged by all seven SNPs, was significant at p≤0.05. This haplotype was found in just over 7% of the individuals genotyped (see Table 5.6).

Table 5.6 RAB7A - Full haplotype analysis

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs9820753</th>
<th>rs4384970</th>
<th>rs2712402</th>
<th>rs4927892</th>
<th>rs13081864</th>
<th>rs4333102</th>
<th>rs7372263</th>
<th>#Inf. Fam.¹</th>
<th>Direction²</th>
<th>HapFreq³</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>47</td>
<td>-</td>
<td>0.512</td>
<td>0.5</td>
</tr>
<tr>
<td>A2</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>22</td>
<td>-</td>
<td>0.100</td>
<td>0.09</td>
</tr>
<tr>
<td>A3</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>23</td>
<td>-</td>
<td>0.088</td>
<td>0.08</td>
</tr>
<tr>
<td>A4</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>22</td>
<td>R</td>
<td>0.073</td>
<td>0.008</td>
</tr>
<tr>
<td>A5</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>10</td>
<td>-</td>
<td>0.056</td>
<td>0.4</td>
</tr>
<tr>
<td>A6</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>18</td>
<td>-</td>
<td>0.056</td>
<td>0.4</td>
</tr>
</tbody>
</table>

¹Number of informative families for this haplotype in the SIBS cohort.
²Direction of effect of the haplotype. P=protective, R=risk.
³Frequency of the haplotype in the SIBS cohort. As there is a long tail of haplotypes with very low frequencies, none of which were associated, for clarity only haplotypes with a frequency of over 4% are shown.

RAB7A was examined for the haplotypes of most significant effect. The haplotype of highest risk incorporated the variation represented by five of the tag SNPs (see Table 5.7), whereas the haplotype of highest protection was comprised of only two SNPs (see Table 5.8).
5.7.3. Association with YWHAG

SNPs chosen captured 92% of the possible information genotyped in the HapMap CEU population across this haplotype block (where the minor allele frequency was ≥0.05 and the r²=0.8).

No association was found between any of the tag SNPs of YWHAG and BPD.

Similarly, no risk or protective haplotypes of YWHAG were found to be associated with BPD.
Table 5.10  YWHAG - Full haplotype analysis

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs1046304</th>
<th>rs2961034</th>
<th>rs645098</th>
<th>rs11763069</th>
<th>rs13247572</th>
<th>rs12531257</th>
<th># Inf. Fam.</th>
<th>Direction</th>
<th>HapFreq</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>49</td>
<td>-</td>
<td>0.347</td>
<td>0.4</td>
</tr>
<tr>
<td>A2</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>39</td>
<td>-</td>
<td>0.222</td>
<td>0.4</td>
</tr>
<tr>
<td>A3</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>18</td>
<td>-</td>
<td>0.116</td>
<td>0.1</td>
</tr>
<tr>
<td>A4</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>24</td>
<td>-</td>
<td>0.108</td>
<td>0.5</td>
</tr>
<tr>
<td>A5</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>22</td>
<td>-</td>
<td>0.101</td>
<td>0.6</td>
</tr>
<tr>
<td>A6</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>14</td>
<td>-</td>
<td>0.051</td>
<td>0.2</td>
</tr>
</tbody>
</table>

1Number of informative families for this haplotype in the SIBS cohort.
2Direction of effect of the haplotype. P=protective, R=risk.
3Frequency of the haplotype in the SIBs cohort. As there is a long tail of haplotypes with very low frequencies, none of which were associated, for clarity only haplotypes with a frequency of over 4% are shown.
5.8. Discussion

5.8.1. Improving the SIBS cohort through pedigree validation

One of the primary strengths of this study is the SIBS cohort, consisting of 139 multiplex families that break down into 200 trios. While this is a fairly small sample, it is well characterised and has been through the validation process to exclude samples that consistently showed Mendelian errors by using microsatellite analysis.

Consistent confirmed Mendelian errors within a putative nuclear family can have three possible causes. Firstly, and probably the most common, is the mislabelling or mishandling of individual samples either before or after the DNA extraction from whole blood. Secondly, pedigree information can be recorded incorrectly by those collecting the data, by accident or through a misunderstanding of the information provided. Thirdly, the information provided by the family or individual may be incorrect, whether the informant is aware of this or not. Any of these occurrences can lead to genotypes not being consistent with the pedigree information as recorded. This was the case with several of the pedigrees in the SIBS, and while the cause of mismatch between the DNA and the pedigree as revealed by the presence of multiple Mendelian errors could not be determined, it was certainly possible to improve the quality of the pedigrees by excluding individuals found to be inconsistent with their families.

Microsatellite analysis of two families in the SIBS cohort showed that the recorded father of a sibship could not in fact be the biological father of one (or more) of the children. This finding, which works out to a mistaken paternity rate of 1.4%, is in line with the results of studies that look at paternity in high paternity confidence situations (Bellis et al., 2005; Anderson, 2006).

Improving the accuracy of the pedigrees in the cohort leads to more accurate results in transmission disequilibrium based association studies such as the FBAT, as it removes instances of the analysis including random transmission
of alleles between unrelated people. This decreases noise in the analysis and improves its ability to find true associations.

5.8.2. Further study of RAB7A warranted

RAB7A has not been studied for any possible association with a psychiatric disorder to date. While four mutations of RAB7A are known to be causal variants in the periphery neuropathy Charcot-Marie-Tooth type 2B (Spinosa et al., 2008), the association of RAB7A rs13081864 with a psychiatric disorder is a novel one and will need to be replicated in an independent cohort.

While risk and protective haplotypes of low p-value were found for RAB7A, inability to correct for multiple testing means that it is not known if these associations are significant. The standard way to correct for multiple testing in haplotypes is to run a Monte Carlo simulation, repeating the analysis multiple times with randomised data. Within the context of a case-control study this can be accomplished by randomising the affection status of each individual for each run, however randomisation within a family-based analysis is more complicated. There is software available to run such Monte Carlo analyses within family-based studies performed as a standard TDT using parent-child trios, such as PBAT (Lange et al., 2004) and FAMHAP (Becker and Knapp, 2004; Becker et al., 2005), unfortunately these programs cannot be used with the more extensive family-based data that is available through the SIBS. Excluding all individuals from the SIBS cohort that are not part of a classic parent-child trio results in the disappearance of all associations previously found and it is evident that the power of the cohort to detect associations lies in the multigenerational pedigrees that FBAT breaks down into all possible trios when analysing association.

One simple way to correct for multiple testing in the haplotype analysis is to use the figure for genome-wide significance used in GWAS. To be significant at a genome-wide scale the p-value must be $p \leq 5 \times 10^{-7}$ (Iles, 2008). None of the haplotypes of RAB7A found to be associated meet this figure, with the haplotype of highest risk at $p = 9 \times 10^{-5}$ and the haplotype of highest protection at $p = 5 \times 10^{-3}$. The genome-wide significance threshold as a correction figure for multiple testing is likely to be wildly conservative for a
small candidate gene study such as described here, so the haplotype of highest risk, associated at a p-value of $9 \times 10^{-5}$, is an interesting finding that also needs to be replicated in an independent cohort.

5.8.3. **No evidence for association between YWHAG and BPD**

Several of the genes that code for proteins in the 14-3-3 protein family that includes YWHAG have been investigated for association with other psychiatric disorders, primarily schizophrenia. YWHAE is an interesting candidate gene for schizophrenia, not only because of the interaction of the YWHAE protein with the Disrupted-in-Schizophrenia1 protein but also because it maps to a part of chromosome 22q that has been highlighted in genome wide linkage studies (Schwab et al., 1995; Vallada et al., 1995). No association, however, was found between polymorphisms of YWHAE and schizophrenia in a case-control association study performed by Bell et al. (2000). A larger study found a significant association between YWHAE SNP rs28365859 and schizophrenia (Ikeda et al., 2008), leaving the question of YWHAE association undetermined.

YWHAH was found to be associated with schizophrenia in an Ashkenazi Jewish population at a p value lower than 0.01 (Fallin et al., 2005), and a recent large family based study showed that three SNPs of the gene were significantly associated with BPD with psychotic features (Grover et al., 2009). The research presented here found no association between any of the SNPs of YWHAG studied and BPD, nor was their any association with haplotypes tagged by the YWHAG SNPs studied. This lack of association echoes a study where three polymorphisms of YWHAG – rs2961037, rs11765693, and rs17149177 – were tested for association with schizophrenia in a case-control study of an Irish cohort, but no association was found (Sun et al., 2011). While none of these three polymorphisms were repeated in this research it adds to the evidence suggesting that there is no association between YWHAG and BPD.

Despite the failure to implicate YWHAG in the aetiology of BPD in the SIBS cohort, it remains a possible candidate given its similarity to the genes coding for to other members of the 14-3-3 protein family. While the overall picture
for the 14-3-3 family is patchy, the combination of the positive associations found so far and the integral part they play in tryptophan metabolism (Ichimura et al., 1987) mean that further research into their role in the aetiology of psychiatric disorders such as BPD is warranted.

5.8.4. **BDNF as a candidate for involvement in BPD**

Polymorphisms in the *BDNF* gene have been shown to affect hippocampal volume (Szeszko et al., 2005) and activity (Hariri et al., 2003), lithium response (Rybakowski et al., 2005), cognition (Tsai et al., 2004) and lethality in suicide attempts (Schenkel et al., 2010), and to be associated with other psychiatric disorders such as schizophrenia (Neves-Pereira et al., 2005), unipolar depression (Schumacher et al., 2005; Licinio et al., 2009), obsessive-compulsive disorder (Hall et al., 2003), bulimia nervosa (Koizumi et al., 2004), and psychosis in unipolar depression (Iga et al., 2007). In genome wide association studies, rs6265 has been shown to be associated with the initiation of tobacco smoking (TAGC, 2010), however no SNPs of *BDNF* have reached significance in GWAS with bipolar disorder.

We examined eight single nucleotide polymorphisms of *BDNF* and found six with evidence of association with bipolar disorder at a significance level of *p* ≤ 0.05. Like several previous studies, we were able to show association between the *BDNF* Val66Met polymorphism (rs6265) and BPD (Neves-Pereira et al., 2002; Sklar et al., 2002; Lohoff et al., 2005; Kremeyer et al., 2006). We were also able to confirm a previously described association between BPD and rs1519480 (Liu et al., 2008).

Four of the SNPs remained significant when assessed against a lower *p*-value threshold (*p* ≤ 0.00625) that was generated by performing a Bonferroni correction (Figure 5.4). This correction is appropriate to multiple testing with independent markers and therefore very conservative where the markers are in high LD. Using such a conservative correction reduces the chance of false positive associations and increases confidence that those associations found are real. It also however increases the likelihood of false negatives and thus association cannot be totally ruled out in the two *BDNF* SNPs that meet the *p* ≤ 0.05 threshold while not meeting the corrected threshold. The first of these,
rs12273363, has a p-value of 0.008 and is therefore very close to significance at the more conservative threshold. This SNP was found to be significantly associated with bipolar disorder by Liu et al. (2008) and remains a strong candidate.

The Val66Met polymorphism was also shown to be associated at p≤0.05 but not p≤0.00625.

Figure 5.4 Schematic diagram of the BDNF gene with significance thresholds

Shown are the positions of the BDNF SNPs of interest and \(-\log_{10}(P)\) value for each SNP. Threshold lines show a p-value of 0.05 and the conservative p-value of 0.00625 calculated using a Bonferroni correction. Positions on chromosome 11 are based on dbSNP build 131/human genome build 34.1. NM_170731.4, isoform c

5.8.4.1. The ongoing puzzle of BDNF rs6265 and BPD

The BDNF rs6265 SNP (also known as BDNF Val66Met) is non-synonymous, with the presence of the rarer A allele rather than the common G allele changing the amino acid coded for from valine to methionine. The polymorphism also is functional, affecting the trafficking of the protein in neuronal cells (Chen et al., 2004). For these reasons this SNP is the most widely studied of the polymorphisms of BDNF. Results of previous association studies between BPD and rs6265 have been mixed. The first research to show a positive association between rs6265 and BPD was that of Neves-Pereira et al. (Neves-Pereira et al.). This has been replicated in other studies using family-based association testing, finding association with either
BPD itself (Sklar et al., 2002; Kremeyer et al., 2006) or a sub-type of the disorder such as juvenile onset (Geller et al., 2004), rapid cycling (Muller et al., 2006), or lithium response (Rybakowski et al., 2011). Other family-based studies, however, have been unable to replicate these associations (Liu et al., 2008; Mick et al., 2009).

The situation with case-control studies is similar. Several studies reported no association between rs6265 and BPD (Hong et al., 2003; Nakata et al., 2003; Kunugi et al., 2004; Oswald et al., 2004; Skibinska et al., 2004; Neves-Pereira et al., 2005; Green et al., 2006; Liu et al., 2007; Kim et al., 2008; Ye et al., 2009; Dmitrzak-Weglarz et al., 2010; Neves et al., 2010). Zai et al. (2007) found that rs6265 was not associated with antidepressant induced mania in a cohort of bipolar individuals. Positive findings in case control studies have mostly involved subtypes or phenotypic groupings within the disorder. These have included age of onset (Tang et al., 2008), suicidality (Kim et al., 2008; Vincze et al., 2008) and rapid cycling (Green et al., 2006).

A 2007 meta-analysis of 11 case-control studies found that the pooled data showed no evidence of association (Kanazawa et al., 2007), however their study was somewhat skewed by the addition of a research paper that used a cohort of individuals who presented with a child-onset mood disorder, rather than a definitive diagnosis of bipolar disorder (Strauss et al., 2004). A second meta-analysis, which analysed nine case control and five family-based studies all published prior to May 2007, found a statistically significant over-representation of the G allele (Fan and Sklar, 2008). The presence of an association between bipolar disorder and rs6265 has therefore neither been confirmed nor ruled out despite considerable research addressing the question.

The direction of effect is another ambiguity that has not been resolved. The majority of studies that have found significant association between BPD and rs6265 have found the common allele, G, to be the associated allele (Neves-Pereira et al., 2002; Sklar et al., 2002; Geller et al., 2004; Lohoff et al., 2005; Green et al., 2006). However, a recent large case-control study found a positive association with the rarer A allele, with the authors of the study
hypothesizing that this discrepancy may be a result of the ethnic background of the populations involved (Xu et al., 2010). The cohort used in the Xu et al. (2002) study was exclusively of Han Chinese ethnicity whereas the majority of studies that have found association with the G allele have had predominantly Caucasian cohorts. There have been positive findings for schizophrenia at this locus in Caucasians (Neves-Pereira et al., 2005), however findings in Asian populations have been predominantly negative (Chen et al., 2006; Naoe et al., 2007; Kawashima et al., 2009), which adds to the possibility that ethnic background may be an important confounding factor. The frequency of the A allele varies widely by population (Petryshen et al., 2010) and is significantly higher in populations of Asian descent than in Caucasian populations (for example, 0.616 in Han Chinese and 0.372 in Japanese in the International HAPMAP Study, compared to 0.195 in the Caucasian CEPH population and 0.173 in the SIBS), which makes comparisons between the studies problematic. However, while the SIBS cohort used in this research is primarily composed of Caucasians of western European descent, it also showed association with the A allele, suggesting that there may be factors other than ethnicity at play. The differences in direction, whether or not directly related to ethnicity, suggest that BDNF rs6265 is very unlikely to be a causal variation, but may be linked to a polymorphism that contributes to BPD.

The marked lack of consistency between these studies means that association between BPD and Val66Met remains ambiguous. The differences in results could be caused by any of a variety of factors – differences in ethnicity, differences in power due to cohort size, and genetic loading due to strong family histories of BPD in some of the families used in the family-based studies (Lohoff et al., 2005).

Association analysis using the SIBS cohort has unfortunately been unable to provide any further clarification as to whether or not rs6265 is associated with BPD. We found association at \( p \leq 0.05 \), suggesting the possibility of a role for the SNP, but this significance disappears when the stricter corrected threshold for significance is applied. However, given the functional nature of this polymorphism and the wide range of studies associating it with various
psychiatric disorders (Rybakowski et al., 2003; Neves-Pereira et al., 2005; Rosa et al., 2006), we still believe this SNP is a strong candidate for a role in BPD and justifies further investigation.

5.8.4.2. Other SNPs of BDNF

The seven other SNPs investigated for association in this study are not as well represented in the literature as Val66Met. A 2008 study by Liu et al. (2008) investigated association with BPD in ten SNPs across BDNF included the SNPs rs1519480, rs11030104 and rs12273363 as well as rs6265. The study cohort of 250 families of European American origin was used to examine association with BDNF SNPs using three stacked affection statuses.

This study replicates the association found by Liu et al. between the C allele of rs1519480 and BPD, with this association remaining significant after correction for multiple testing in both studies (Foroud, 2011). Also found to be significantly associated in both studies was the SNP rs12273363, however the direction of effect was not concordant, with Liu et al. (2008) finding over-transmission of the C allele (Foroud, 2011), whereas analysis of the SIBS cohort showed over-transmission of T. The SNP rs11030104 was found to be associated in the SIBS cohort even after Bonferroni correction, but Liu et al. found negative results for this SNP across all three of their models.

The discrepancies in the findings between the two studies could be explained by slight differences in ethnic makeup, cohort size or criteria for positive affection status. Liu et al. (2008) used three hierarchical definitions of affected when determining affection status, with the first being that only those diagnosed with BPD-I were classed as affected, the second also including those with BPD-II, and the third encompassing BPD-I and II with the addition of those individuals with recurrent unipolar depression. How affection status was defined appeared to affect the degree of association found across all ten of their SNPs, with the most conservative definition of affected (BPD-I only) having the lowest p-values in SNPs shown to be associated. In contrast, this study used only a single positive affected status that combined all individuals diagnosed with BPD-I, BPD-II or cyclothymia into the affected category. This allowed us to avoid further correction for multiple testing while ensuring the
positive affected status category was robust, well described, and included all those individuals with a bipolar phenotype.

The four other BDNF SNPs investigated - rs11030107, rs10835210, rs7103873 and rs11030119 - have not appeared in any previous association studies with BPD, although rs10835210 has been investigated in several studies of other mood disorders. A study of over 500 Chinese undergraduates found that rs1083510 was significantly associated with the development of depression and anxiety (Meng et al., 2011), and a similar result was found in an earlier study (Meng et al., 2009). In contrast, the SNP was reported to not be significantly associated with depression only in a family-based Chinese study (Liu et al., 2009). The SNP rs10835210 has also been associated with phobic disorders (Xie et al., 2011), but was not found to be associated with BPD in the SIBS cohort.

The associations found involving BDNF rs11030107 and rs11030119 are novel and will need to be replicated hence further investigation is warranted.

5.8.4.3. BDNF Haplotypes

While both risk and protective haplotypes of BDNF were found at the p≤0.05 level in the SIBS cohort, the conundrum of correcting for multiple testing remains. The haplotype of highest risk was associated at p=9 x 10^{-4}, that of highest protection at p=9 x 10^{-3}. Again, neither of these SNPs are significant at the genome-wide level of p=5 x 10^{-7}, which is conservative for this study.

As in the study by Liu et al. (2008), we found an unusual pattern of association across the BDNF haplotype block. The SNPs rs10835210 and rs7103873, both of which showed no evidence of association, were situated between SNPs that had p-values of less than 0.01. The explanation for this pattern of association became apparent in both cases when the r^2 statistic for LD was examined instead of the D'. The two SNPs that show no association are in high LD with each other (r^2=0.86), but in much lower LD with the other six SNPs (r^2<0.33) that do show association. Thus in both studies the SNPs with no association form their own block and are not in high LD with the SNPs surrounding them. Those with evidence of association were shown to be in high LD despite being separated by the SNPs with no association. This
particular set of SNPs illustrates an instance where the $r^2$ measure of LD is more appropriate than the $D'$ measure. Each measure has a different interpretation – $D'$ can be used to examine the probability of historical recombination in a population, whereas $r^2$ acts as an overview of the history of both recombination and mutation. Estimates of $D'$ depend strongly on sample size, and are inflated in small samples, especially in SNPs with rare alleles. The $r^2$ measurement, while dependent on the difference of minor allele frequencies of the two loci being compared, is not as susceptible to distortion by small numbers and is the preferred measurement when comparing LD in the context of both fine mapping and association studies (VanLiere and Rosenberg, 2008).

5.8.5. The Genetic Diathesis-Transactional Stress Model
One possible explanation for the discrepancies in the results of association studies between BDNF and BPD was supplied by Hosang et al. (2010), who investigated the role of stressful life events in individuals with BPD. They found that individuals who carry at least one Met allele have a significantly higher correlation between the experience of stressful life events and the development of bipolar disorder, suggesting that there is important gene-environment interaction at work in the development of BPD (Hosang et al., 2010). This finding echoes similar results for unipolar depression (Bukh et al., 2009). The failure of association studies to control for environmental interaction is a likely factor in the inconsistent results so far, both in terms of whether there is an association, and if so, with which allele. A 2007 study reported that bipolar patients with a history of traumatic life events had higher levels of comorbid anxiety and alcohol abuse than those who had experienced fewer traumas. The authors also found that those with the increased trauma history had lower serum BDNF levels than the rest of the study group (Kauer-Sant'Anna et al., 2007). A recent study on rats was able to show that early life stress in the form of maternal deprivation decreases BDNF levels in the hippocampus in adulthood (de Lima et al., 2011).

In light of the conflicting results of association studies between BDNF and BPD, and the growing awareness of the importance of environment in terms of the experience of acute or chronic stress, Richard Liu has put forward a
possible model for the development of BPD, the genetic diathesis-
transactional stress model (Liu, 2010). The model outlines how a
neurobiological predisposition in the form of a specific allele, such as the G
variant of rs6265, may exacerbate the results of abuse or stress in early life.
This can lead to significant decreases in the expression of neurotrophic factors
like BDNF, which can cause neural cell death in particularly sensitive brain
areas such as the hippocampus, further increasing the risk for BPD. While this
model only deals with the interaction between two possible contributing
aetiological factors, it is reasonable that the theory can be extended to other
possible predisposing genes that have shown association with bipolar
disorder. It is also a possible explanation for the inconsistent results that have
plagued association studies of BDNF rs6265 with BPD.

5.8.6. Searching for genetic contribution to complex disease
The search to elucidate genetic contributions to complex disease has thus far
had two major tools: linkage analysis and the association study.

Previous to the widespread use of association studies, linkage analysis was
successfully used to locate causative genes for Mendelian conditions such as
Huntington’s disease, but this approach fell out of favour for diseases
considered to be complex because of the large effect any given variant would
require to be detectable (Risch and Merikangas, 1996).

The common-disease common-variant model has predominated over the last
twenty or so years, leading to large-scale and eventually genome-wide
association studies that have the ability to detect genes of small and additive
effect, but limited ability to detect rare variations of larger effect (McClellan
and King, 2010).

The common-disease common-variant model posits that complex diseases are
a result of the additive effect of common variants of small effect. These
deleterious variants are thought to be able to stay in the population due to
balancing selection, where they are advantageous or protective in other
conditions. Changing environmental conditions may also have meant
previously advantageous common alleles have become liabilities in modern environmental conditions.

While GWAS has been incredibly useful in finding genetic variation contributing to many somatic disorders such as Crohn’s disease, it has been less successful at elucidating the genetic contributions to psychiatric and neurological disorders such as schizophrenia and BPD (Manolio, 2010). While there have been some limited replication of GWAS findings in bipolar disorder (Soronen et al., 2010), the first five GWAS of schizophrenia did not find any genetic variants that were significant at a genome-wide level (Manolio, 2010).

The use of GWAS to look for causes of complex disease under the common-disease common-variant model has recently been criticised on several different grounds. McClellan and King (2010) point out that of the associations found so far, the majority have no known functional or biological relevance.

Dickson et al. (2010) argued that a large portion of positive GWAS findings were due to ‘synthetic associations’, where common variants found to be associated with disease were actually in association with rare genetic variants, and that multiple rare variants across large blocks of the genome may lead indirectly to spurious positive findings. Their study was able to demonstrate the tendency of rare variants to create multiple independent synthetic associations.

Researchers asserting that GWAS studies support the common-disease common-variant theory argue the predominance of positive results in non-coding areas are not false associations but are in LD with functional variants, and cite the large number of positive associations found in promoter regions (Klein et al., 2010). They also put forward the point that the existence of positive associations that do not lie within a currently described coding area may be more of a reflection of our lack of knowledge rather than a false positive association. Similarly, Wang et al. (2010) argue that GWAS were not
designed to find the exact functional variant but to pinpoint areas where they might be found.

An alternate hypothesis to the common-disease common-variant model is one that posits that multiple rare variants of larger effect may be causative in common complex disease. GWAS approaches are not optimal for detecting SNPs with a minor allele frequency of less than approximately 1%, with only around 12% of the tag SNPs in the most commonly used GWAS system able to detect variants occurring at the 1% level (Gershon et al., 2011). Detection of associated rare variants by GWAS would require markers at similar frequencies as well as incredibly large study cohorts, which would make the research both very expensive and logistically difficult.

The primary historical argument against the multiple rare variant model, especially in common complex disorders, is the perceived lack of fitness of such mutations, leading to their inability to persist in the population. New mutations would therefore be needed to replenish these alleles of larger effect. An important assumption of this argument is that the level of de novo mutation in humans is too low to account for the required level of new mutation needed for common disease (Mitchell and Porteous, 2011). Studies over the last 12-15 years, however, have shown that rates of mutation are higher than once suspected (Crow, 2000), which may overcome the problem of lack of fitness for highly penetrant alleles of large effect.

Sequencing of individuals with neurological disorders such as schizophrenia and autistic spectrum disorder has uncovered the role that de novo mutation makes in complex common disease. Selecting individuals on the basis of these disorders significantly enriches for de novo exonic mutation, as in the recent study by Awadalla et al. (2010). Another recent study sequenced the exomes of a group of individuals with schizophrenia along with their parents, and found 15 de novo mutations in eight probands (Girard et al., 2011). This figure represents a de novo mutation rate of $2.59 \times 10^{-8}$ base substitutions per site per generation compared to the rate of $\sim 1.1 \times 10^{-8}$ established by the 1000 Genomes Project (2010). The authors point out that their analysis was specific to exonic coding areas only, and that genome–wide sequencing of the same
individuals is likely to show a de novo mutation rate within the normal range. However, the new mutations in the schizophrenic individuals have, by chance, occurred preferentially in coding regions, which may be part of their underlying pathology.

Sequencing large numbers of individuals can discover rare alleles of major effect. This approach is becoming more common as the costs for gene, exome, and whole genome sequencing decrease.

Myers et al. (2011) used a ‘deep re-sequencing’ approach to identify genes involved in neurological disorders. The group sequenced 408 brain-expressed genes in 143 individuals with schizophrenia and 142 individuals with autistic spectrum disorders. 285 control individuals were then sequenced for the 38 genes that had turned up de novo mutations or variations with the potential to disrupt proteins in the affected individuals. This large-scale sequencing approach found a much greater number of rare variants in the affected individuals than the controls. This evidence supporting the idea that rare variants of larger effect are an important component of schizophrenia and autistic spectrum disorders is likely to also apply to other complex neurological conditions such as BPD.

Structural variations, in particular copy number variants, have been studied less frequently than single nucleotide polymorphisms as they have been more difficult to detect and describe accurately. Sequencing, however, can pick up these variants far more easily and copy number variants may supply some of the missing heritability (Cichon et al., 2009).

The decreasing cost of sequencing is allowing for detection of increasing numbers of SNPs and copy number variants (Gershon et al., 2011) which can be assessed for association with psychiatric disorders. While the statistical demands of such large quantities of data will be huge, this approach should go some way towards finding the associations that are part of the heritability gap for disorders such as BPD.
5.8.7. Limitations
One possible limitation of this study is related to the limits of the diagnostic criteria. Diagnoses were based on the current standard DSM criteria. There is growing evidence, however, that BPD exists as a wide spectrum, the lowest extremes of which are not diagnosable by the current criteria (Angst et al., 2003). This means that while the diagnoses of BPD-I and II are robust and replicable, there may be people in the SIBS cohort with very mild bipolar forms that remain undiagnosed, or are misdiagnosed as unipolar depression. Coding these individuals as unaffected for the purposes of analysing genetic association will necessarily skew results unless the genetic variants responsible for the softer end of the bipolar spectrum are vastly different from the genetic variation that causes the more classical bipolar phenotype.

The size of the SIBS cohort is a limitation on the study. A larger sample may have allowed us the power to detect smaller effects and may have provided more information on the borderline associated BDNF SNPs rs6265 and rs12273363.

The presence of comorbidity in the sample is a further possible limitation. The level of comorbidity, especially with other psychiatric disorders, is high in individuals with BPD, and the vast range of possible comorbidities made it difficult to control for in any meaningful way. Unfortunately, comorbidity has been shown to be a possible cause of artificial correlation between a genetic marker and a particular trait within transmission disequilibrium tests (Robins et al., 2001). Replicating the associations found in this study in a population based study, which is not subject to this particular limitation, would provide some measure of assurance that the associations found in the SIBS cohort are true associations despite the presence of comorbidity in the sample.
5.9. Conclusion

Four SNPs from *BDNF* and one SNP from *RAB7A* are associated at a 95% significance level after correction for multiple testing. No SNPs from *YWHAG* were found to be associated, and further studies on this gene in the SIBS cohort are not recommended. While a low p-value was found for the haplotype of highest risk in the *RAB7A* haplotype block, the inability to correct for multiple haplotype testing in the SIBS cohort means it is not currently possible to determine if this association is significant.

In light of the results outlined in this chapter, the next step is to attempt to replicate the associations found in an independent sample, preferably using a case-control based study that would control for the possibility of false association due to comorbidity. Assuming the *RAB7A* associations could be replicated, it would be prudent to extend the *RAB7A* SNP genotyping in the SIBS outside the boundaries of the block genotyped in this study to determine if association could be found with upstream or downstream variants.
6. Conclusions and Future Directions

6.1. Summary of findings

Bipolar disorder is a complex condition that is likely to be a result of the interaction of a number of genes and the effect of the environment on their regulation. Bipolar disorder has heritability of around 80%, which indicates a strong genetic component. While there has been significant progress made in identifying possible genetic contributions to the disorder over the last twenty years, the exact pathogenesis remains unclear. Mood stabilising drugs require a three to four week time period to achieve clinical effectiveness, and this time lag suggests that their effect on phenotype may be a result of long-term biochemical alteration in neural cells mediated by changes in gene expression. For this reason, examining changes in regulation of genes caused by the action of mood stabilising drugs is a promising means of identifying genes that may be contributing factors in the pathophysiology of BPD.

6.1.1. Gene expression changes in neuroblast cell lines

Twenty genes were selected from either lithium-affected signal transduction pathways, a previously performed serial analysis of gene expression screen, or from genes reported to be differentially regulated by lithium, and had their expression in three neuroblast cell lines measured after 28 days exposure to valproate or carbamazepine at therapeutically relevant concentrations. While the majority of the genes were inconsistent in their differential regulation, four genes – BDNF, RAB7A, PRKCE and INPP1 – were significantly upregulated in at least seven of the nine cell line/treatment type pairs. BDNF was the most consistent gene of the twenty, being upregulated under all nine conditions, with only one condition not reaching statistical significance. The two most frequently downregulated genes were GSK3B and MDK, with GSK3B being significantly downregulated in seven of the nine cell line/treatment pairs, and MDK in five.
Changes in BDNF protein level after treatment with valproate and carbamazepine were also assessed but were inconsistent and did not replicate the direction and magnitude of gene expression changes.

6.1.2. Gene expression changes in lymphoblast cell lines
Lymphoblast cell lines were used in an attempt to replicate the changes of expression in the four genes BDNF, MDK, RAB7A and YWHAG. Five of the cell lines were sourced from individuals with BPD-I, and five came from control individuals with no psychiatric disorder. Again, gene expression was measured after the cell lines were cultured for 28 days in the presence of lithium, valproate or carbamazepine. There were no consistent changes of regulation in any of the lymphocyte cell lines or under any of the drug treatments, and the results did not replicate the findings in the neuroblast cell lines.

There were no statistically significant differences between the changes in expression of any of the genes in the group of control individuals compared to the cell lines from the bipolar individuals. However, the difference in gene expression between the untreated control cells and the untreated cells from individuals with bipolar disorder was much closer to significance than the difference after treatment with any of the drugs, except in the gene MDK. This suggests that the drug treatments may normalise the expression levels of BDNF, RAB7A and YWHAG in bipolar patients, but this would likely require a larger sample to reach significance.

Lymphocytes have the distinct advantage of being easy to access, allowing for larger samples, larger demographic choices and better matching of controls. However, the lack of replication in lymphoblasts of the results found in the neuroblast cell lines suggest that lymphoblasts may be an inappropriate choice for studies of the effects of mood stabilising drugs on gene expression.

6.1.3. Association studies
The South Island Bipolar Study database of DNA from individuals with bipolar disorder and their relatives was used to look for association between BPD and the three genes BDNF, RAB7A and YWHAG. Microsatellite analysis
was first used to clarify family relationships where Mendelian errors from previous genotyping cast doubt upon the accuracy of some of the pedigrees in the SIBS, then the cohort was genotyped for 21 single nucleotide polymorphisms that were chosen to represent variation across the haplotype blocks spanning the three genes. No significant association was found with any SNPs or haplotypes of YWHAG. Six SNPs of BDNF were found to be associated with BPD at p≤0.05, and four of these remained significant after correction for multiple testing. One SNP of RAB7A was associated with BPD after correction, and one haplotype of RAB7A was associated with BPD in the SIBS cohort at a p-value of 0.00009.

6.2. Future Directions

6.2.1. Replication of association results
Two of the significant associations found between SNPs of BDNF and BPD in this study have not previously been reported, and need to be replicated in an independent cohort. This is also applies to the association found between RAB7A rs13081864 and BPD. The novel RAB7A risk haplotype finding, while not considered to be significant on a genome wide basis, is significant enough to warrant further investigation, especially considering the small size of the study performed here.

YWHAG, while remaining a possible candidate for involvement in the aetiology of BPD, does not justify further association studies at this stage as none of the SNPs or haplotypes of the gene examined were found to be associated with the disorder.

6.2.2. Further gene expression studies
RAB7A’s upregulation in neural cell lines after treatment with mood stabilising drugs runs counter to a previously study that reported downregulation of RAB7A in lymphocytes after seven days of lithium treatment. The reason for this discrepancy may be made clear by performing a time course analysis of RAB7A in the three neural cell lines in order to determine if RAB7A expression is downregulated in the short term by acute
mood stabiliser exposure and then upregulated after longer exposure to medication as the research presented here suggests.

The results of the gene expression studies in the neuroblast cell lines suggest that further investigation into \textit{INPP1} and \textit{PRKCE} may be warranted, as they were both predominantly upregulated across the nine cell line/drug treatment pairs. Both genes are involved in the lithium-affected phosphatidyl inositol signal transduction pathway and remain interesting candidates, as does \textit{GSK3B}, the gene that codes for the central enzyme of the Wnt signal transduction pathway. \textit{GSK3B} was significantly downregulated in seven out of nine cell line/drug treatment pairs, but is expressed in a very low level in the neuroblast cell lines studied, leading to technical difficulties in measuring expression changes after drug treatment. For this reason \textit{GSK3B} was not chosen for the lymphoblast gene expression studies, but it certainly warrants further investigation. Replicating these gene expression results in another model, such as olfactory epithelial cells, would add to the possibility of their involvement in the pathophysiology of BPD.

\textbf{6.2.3. Further association studies with the SIBS cohort}

The SIBS cohort, while of modest size, is well characterised and a useful resource for preliminary association studies of candidate genes.

An association study between BPD and the \textit{BDNF}-linked complex polymorphic region as described by Okada \textit{et al.} (2006) and considered to be potentially functional, would be a rational next step in investigating \textit{BDNF} in this cohort.

The \textit{MDK} gene is now well enough characterised to perform SNP and haplotype association studies with the SIBS, and the first step would be to type \textit{MDK} SNPs representative of a haplotype block encompassing the gene. Other interesting association studies might involve SNPs that have emerged with highly significant associations out of GWAS and functional convergence studies, such as \textit{CACNA1C, DGKH, MBP} and \textit{ARNTL}, as well as attempting to replicate associations found for BPD in other studies such as with the 50T/C
polymorphism of GSK3B (Benedetti et al., 2004) or with the two SNPs of the promoter region of IMPA2 (Sjoholt et al., 2004; Ohnishi et al., 2007).

Another interesting further direction that would make good use of the SIBS cohort would be to perform exome sequencing on ten to twenty of the individuals with BPD-I and their unaffected parents to identify de novo mutations or rare transmitted variants that may be associated with bipolar disorder and provide new directions for research.

6.3. Conclusion

Recent evidence has made it clear that research into the genetic contribution into bipolar disorder must extend to looking at the interaction of genetic and environmental factors. The genetic diathesis-transactional stress model proposed by Liu (2010) provides an interesting framework around which to base gene-environment interaction analysis, and has already been validated by studies like that performed by Hosang et al. (2010). The causes of bipolar disorder are likely to involve numerous genes of varying levels of effect that interact with each other and the environment, a process that is going to be very difficult to discover and characterise. The future of the search for the causes of BPD may be in the direction of exome or whole genome sequencing to find rarer polymorphic variants of moderate risk and then to examine them in the context of environmental background over long periods of time. Large, well-designed multi-disciplinary prospective studies may be a way to achieve this, but such studies will require enormous resources and are unlikely to provide any answers within the short term.

Better understanding of the pathophysiology of BPD and how different factors contribute to the phenotype will enable improvements in treatment and the longitudinal course of the disorder for individuals with BPD, and may one day be able to determine individual risk factors leading to early diagnosis, individualised treatment and a better lifetime outcome for people with BPD.
REFERENCES


Ichimura, T., Isobe, T., Okuyama, T., Yamauuchi, T. Fujisawa, H. (1987). Brain 14-3-3 Protein Is an Activator Protein That Activates Tryptophan 5-Monoxygenase and Tyrosine 3-Monoxygenase in the Presence of
Ca2+, Calmodulin-Dependent Protein Kinase II. *FEBS letters* **219**(1): 79-82.


Analysis of the Rat Cortex Following Treatment with Imipramine and Citalopram. *Int J Neuropsychopharmacol* 7(4): 401-413.


## APPENDIX A

Table 1.A Primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>5'-AGCGGTGTGTGACAGTATTAGTG-3'</td>
<td>5'-AGAAGTATTGCTTCAGTTGGCC-3'</td>
</tr>
<tr>
<td>C7orf45</td>
<td>5'-TTGCGTACCCACTGCAGTAGG-3'</td>
<td>5'-GGCTCCTGGCTCCAATACTC-3'</td>
</tr>
<tr>
<td>ENG</td>
<td>5'-TCAGCAAGTATGATCAGCAAT-3'</td>
<td>5'-GAAAAGAGAGGTGTCCATGGTT-3'</td>
</tr>
<tr>
<td>FGFR4</td>
<td>5'-AGTGTAGAAGCTGATCGG-3'</td>
<td>5'-ACTCCACGATCACGTACAG-3'</td>
</tr>
<tr>
<td>GNG5</td>
<td>5'-CTGGAGGGCGGACTCAACC-3'</td>
<td>5'-GTAATTCAAGATACTCCAGTC-3'</td>
</tr>
<tr>
<td>GSK3B</td>
<td>5'-TGCTCGGATTCAAGCAGCTGC-3'</td>
<td>5'-ATGCAAGAAGCAGATTATTGTT-3'</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>5'-GGAGGTGTGAGGATCCGAAC-3'</td>
<td>5'-AACTTCATCGTACGCTGTAC-3'</td>
</tr>
<tr>
<td>IMPA1</td>
<td>5'-AAGAGACAGGTGCGGAACA-3'</td>
<td>5'-CAAGGATCAGCCATCTCTT-3'</td>
</tr>
<tr>
<td>IMPA2</td>
<td>5'-AGCTCGACATCTTGTGACAG-3'</td>
<td>5'-CGGCGCTTCTTGCAATGAAC-3'</td>
</tr>
<tr>
<td>INPP1</td>
<td>5'-GATGTACTGGTACAGGTT-3'</td>
<td>5'-GAACACACACCTCAAGGTAAT-3'</td>
</tr>
<tr>
<td>MDK</td>
<td>5'-GATGCAGCACCAGGCTTC-3'</td>
<td>5'-CACTCACGCAGCTCGTCC-3'</td>
</tr>
<tr>
<td>NPDC1</td>
<td>5'-TCCAGGAGGACAGCAAGG-3'</td>
<td>5'-AAGTCAATCTCATCTCCTCAGTC-3'</td>
</tr>
<tr>
<td>PDE4D</td>
<td>5'-TGCTGGACCAGCTAGACAGGAC-3'</td>
<td>5'-CCAGACCGACTCATTTTCAGAG-3'</td>
</tr>
<tr>
<td>PREP</td>
<td>5'-TTATATGGCTATGGCGGCTTC-3'</td>
<td>5'-CCTCTGTATGTTGGCCACTGCC-3'</td>
</tr>
<tr>
<td>PRKCA</td>
<td>5'-GTGGTCCACAAGAGGTGCC-3'</td>
<td>5'-CACAGTGTACGCAAGGTTG-3'</td>
</tr>
<tr>
<td>PRKCE</td>
<td>5'-TGTATGTGATCAGTCTCAGTC-3'</td>
<td>5'-ATGAACTTGCGGTGACGACC-3'</td>
</tr>
<tr>
<td>RAB7A</td>
<td>5'-CACAGGCGCTGTGCTACAGC-3'</td>
<td>5'-TCTTTGTAAGCTCCACCTCGC-3'</td>
</tr>
<tr>
<td>RGS5</td>
<td>5'-TACCTAAAGCGGAGGAGCTAAGG-3'</td>
<td>5'-CCTTACTTAGCTTAATCTCC-3'</td>
</tr>
<tr>
<td>TRAP1</td>
<td>5'-GAGATTCCACTTGCAAGAATG-3'</td>
<td>5'-CAGGTTGGACACAGCTCTTC-3'</td>
</tr>
<tr>
<td>YWHAG</td>
<td>5'-GAGCGCTACGAGCACAGGC-3'</td>
<td>5'-GTCTTGTAGGCCACAGACAG-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>5’-CCATTTGATGACAAGCTTCACG -3’</td>
<td>5’-GTGAAGGTCGGAGTCAACCG-3’</td>
</tr>
<tr>
<td><strong>SDHA</strong></td>
<td>5’-TGGGAACAAGGGGCATCTG-3’</td>
<td>5’-CCACCACTGCATAAATTCATG-3’</td>
</tr>
<tr>
<td><strong>UBC</strong></td>
<td>5’-ATTTGGTGCCTCCTGTT-3’</td>
<td>5’-TGCTTGACATTCTGATGGT-3’</td>
</tr>
</tbody>
</table>
**APPENDIX B**

Table B.1 Gene expression changes after 28 days drug treatment

<table>
<thead>
<tr>
<th>Drug</th>
<th>SK-N-DZ</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change</td>
<td>95% CI</td>
<td>Change</td>
</tr>
<tr>
<td>C7orf45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>1.87</td>
<td>1.80 – 1.94</td>
<td>0.30</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.01</td>
<td>0.94 – 1.08</td>
<td>0.15</td>
</tr>
<tr>
<td>ENG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.39</td>
<td>0.37 – 0.41</td>
<td>3.66</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.27</td>
<td>2.09 – 2.46</td>
<td>8.36</td>
</tr>
<tr>
<td>FGFR4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.77</td>
<td>0.73 – 0.82</td>
<td>1.00</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.33</td>
<td>1.22 – 1.43</td>
<td>5.65</td>
</tr>
<tr>
<td>GNG5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.56</td>
<td>0.54 – 0.59</td>
<td>5.25</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.26</td>
<td>2.05 – 2.46</td>
<td>0.84</td>
</tr>
<tr>
<td>GSK3B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.89</td>
<td>0.82 – 0.95</td>
<td>2.41</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.11</td>
<td>1.90 – 2.32</td>
<td>0.55</td>
</tr>
<tr>
<td>HSP90B1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>2.12</td>
<td>2.06 – 2.18</td>
<td>2.25</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.20</td>
<td>0.17 – 0.23</td>
<td>0.56</td>
</tr>
<tr>
<td>IMPA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.86</td>
<td>0.81 – 0.90</td>
<td>0.12</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.35</td>
<td>2.15 – 2.55</td>
<td>1.42</td>
</tr>
<tr>
<td>IMPA2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.40</td>
<td>0.39 – 0.42</td>
<td>1.67</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.70</td>
<td>0.65 – 0.76</td>
<td>0.66</td>
</tr>
<tr>
<td>INPP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>8.61</td>
<td>8.11 – 9.12</td>
<td>2.66</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.27</td>
<td>2.21 – 2.34</td>
<td>0.30</td>
</tr>
<tr>
<td>NPDC1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>3.93</td>
<td>3.85 – 4.01</td>
<td>0.48</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.04</td>
<td>2.01 – 0.27</td>
<td>0.17</td>
</tr>
<tr>
<td>PDE4D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.16</td>
<td>0.15 – 0.17</td>
<td>0.52</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.90</td>
<td>0.83 – 0.97</td>
<td>1.65</td>
</tr>
<tr>
<td>PREP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.30</td>
<td>0.27 – 0.33</td>
<td>2.31</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.99</td>
<td>1.92 – 2.07</td>
<td>4.96</td>
</tr>
<tr>
<td>PRKCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.66</td>
<td>0.61 – 0.70</td>
<td>4.08</td>
</tr>
<tr>
<td>Lithium</td>
<td>5.83</td>
<td>5.64 – 6.02</td>
<td>0.74</td>
</tr>
<tr>
<td>PRKCE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>1.69</td>
<td>1.49 – 1.89</td>
<td>3.66</td>
</tr>
<tr>
<td>Lithium</td>
<td>2.32</td>
<td>2.06 – 2.58</td>
<td>4.37</td>
</tr>
<tr>
<td>RGS5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>0.49</td>
<td>0.44 – 0.55</td>
<td>8.65</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.67</td>
<td>1.64 – 1.70</td>
<td>1.38</td>
</tr>
<tr>
<td>TRAP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbemaz.</td>
<td>3.70</td>
<td>3.21 – 4.19</td>
<td>2.34</td>
</tr>
<tr>
<td>Lithium</td>
<td>1.15</td>
<td>1.06 – 1.24</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Expression level is expressed as a proportion of its untreated control, where the control equals 1. Thus a level of 1 implies no change; a number below 1 shows a downregulation, and a number above one shows an upregulation.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Ratio&lt;sup&gt;1&lt;/sup&gt;</th>
<th>p-val&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Cell Line</th>
<th>Ratio</th>
<th>p-val&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>Carbamazepine</td>
<td>0.4880</td>
<td>0.4840</td>
<td>SK-N-DZ</td>
<td>0.4890</td>
<td>0.5870</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.4030</td>
<td>0.6460</td>
<td>SK-N-SH</td>
<td>0.4230</td>
<td>0.5900</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.0290</td>
<td>0.0860</td>
<td>SH-SYSY</td>
<td>0.3570</td>
<td>0.4940</td>
</tr>
<tr>
<td>C7orf45</td>
<td>Carbamazepine</td>
<td>1.4980</td>
<td>0.8130</td>
<td>SK-N-DZ</td>
<td>0.6010</td>
<td>0.8130</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.5970</td>
<td>0.8260</td>
<td>SK-N-SH</td>
<td>3.3970</td>
<td>0.5230</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.1630</td>
<td>0.7810</td>
<td>SH-SYSY</td>
<td>0.7180</td>
<td>0.8310</td>
</tr>
<tr>
<td>ENG</td>
<td>Carbamazepine</td>
<td>0.5460</td>
<td>0.8760</td>
<td>SK-N-DZ</td>
<td>0.8340</td>
<td>0.8980</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.6510</td>
<td>0.7920</td>
<td>SK-N-SH</td>
<td>0.3220</td>
<td>0.2850</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6290</td>
<td>0.7720</td>
<td>SH-SYSY</td>
<td>0.8300</td>
<td>0.9490</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Carbamazepine</td>
<td>1.1660</td>
<td>0.9620</td>
<td>SK-N-DZ</td>
<td>0.8080</td>
<td>0.8510</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.2160</td>
<td>0.2280</td>
<td>SK-N-SH</td>
<td>0.2160</td>
<td>0.2280</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.8820</td>
<td>0.5730</td>
<td>SH-SYSY</td>
<td>0.4510</td>
<td>0.5850</td>
</tr>
<tr>
<td>GNG5</td>
<td>Carbamazepine</td>
<td>0.9000</td>
<td>0.8850</td>
<td>SK-N-DZ</td>
<td>0.4940</td>
<td>0.7820</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.4480</td>
<td>0.6550</td>
<td>SK-N-SH</td>
<td>0.6610</td>
<td>0.6760</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6350</td>
<td>0.7660</td>
<td>SH-SYSY</td>
<td>0.7850</td>
<td>0.8510</td>
</tr>
<tr>
<td>GSK3B</td>
<td>Carbamazepine</td>
<td>0.8310</td>
<td>0.7990</td>
<td>SK-N-DZ</td>
<td>1.7730</td>
<td>0.8980</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>2.5690</td>
<td>0.3700</td>
<td>SK-N-SH</td>
<td>1.0280</td>
<td>0.9840</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>5.1690</td>
<td>0.7570</td>
<td>SH-SYSY</td>
<td>1.8060</td>
<td>0.8320</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>Carbamazepine</td>
<td>0.3740</td>
<td>0.3360</td>
<td>SK-N-DZ</td>
<td>1.7300</td>
<td>0.6310</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>3.2600</td>
<td>0.1800</td>
<td>SK-N-SH</td>
<td>0.5440</td>
<td>0.7140</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6990</td>
<td>0.7420</td>
<td>SH-SYSY</td>
<td>0.8050</td>
<td>0.7920</td>
</tr>
<tr>
<td>IMPA1</td>
<td>Carbamazepine</td>
<td>2.7020</td>
<td>0.2240</td>
<td>SK-N-DZ</td>
<td>1.2080</td>
<td>0.9640</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>1.2140</td>
<td>0.9510</td>
<td>SK-N-SH</td>
<td>1.4100</td>
<td>0.8390</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>1.7380</td>
<td>0.6670</td>
<td>SK-N-DZ</td>
<td>1.2240</td>
<td>0.9100</td>
</tr>
<tr>
<td>IMPA2</td>
<td>Lithium</td>
<td>0.7440</td>
<td>0.8450</td>
<td>SH-SYSY</td>
<td>0.9220</td>
<td>0.9280</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.7350</td>
<td>0.9030</td>
<td>SK-N-SH</td>
<td>0.8430</td>
<td>0.8880</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.7440</td>
<td>0.8450</td>
<td>SH-SYSY</td>
<td>0.9220</td>
<td>0.9280</td>
</tr>
<tr>
<td>INPP1</td>
<td>Carbamazepine</td>
<td>0.2930</td>
<td>0.6500</td>
<td>SK-N-DZ</td>
<td>0.2780</td>
<td>0.6090</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>2.3580</td>
<td>0.0700</td>
<td>SK-N-SH</td>
<td>0.9790</td>
<td>0.9840</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.3500</td>
<td>0.8020</td>
<td>SH-SYSY</td>
<td>1.3950</td>
<td>0.8800</td>
</tr>
<tr>
<td>M6K</td>
<td>Carbamazepine</td>
<td>0.8340</td>
<td>0.8020</td>
<td>SK-N-DZ</td>
<td>0.9020</td>
<td>0.9900</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.7180</td>
<td>0.7610</td>
<td>SK-N-SH</td>
<td>0.6380</td>
<td>0.7170</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.9550</td>
<td>0.7970</td>
<td>SH-SYSY</td>
<td>0.9940</td>
<td>0.9900</td>
</tr>
<tr>
<td>NPDC1</td>
<td>Carbamazepine</td>
<td>0.4790</td>
<td>0.7660</td>
<td>SK-N-DZ</td>
<td>0.9420</td>
<td>0.9610</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>2.4600</td>
<td>0.7080</td>
<td>SK-N-SH</td>
<td>2.1500</td>
<td>0.8270</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.8450</td>
<td>0.8880</td>
<td>SH-SYSY</td>
<td>0.4920</td>
<td>0.2500</td>
</tr>
<tr>
<td>PDE4D</td>
<td>Carbamazepine</td>
<td>1.8390</td>
<td>0.6380</td>
<td>SK-N-DZ</td>
<td>2.0790</td>
<td>0.5750</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.5030</td>
<td>0.3750</td>
<td>SK-N-SH</td>
<td>0.8900</td>
<td>0.9330</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6760</td>
<td>0.8110</td>
<td>SH-SYSY</td>
<td>0.3380</td>
<td>0.3870</td>
</tr>
<tr>
<td>PREP</td>
<td>Carbamazepine</td>
<td>1.1540</td>
<td>0.8210</td>
<td>SK-N-DZ</td>
<td>1.2850</td>
<td>0.8400</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.4950</td>
<td>0.3930</td>
<td>SK-N-SH</td>
<td>0.4950</td>
<td>0.5370</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>1.3670</td>
<td>0.6170</td>
<td>SH-SYSY</td>
<td>1.2220</td>
<td>0.7910</td>
</tr>
<tr>
<td>PRKCA</td>
<td>Carbamazepine</td>
<td>0.5350</td>
<td>0.8130</td>
<td>SK-N-DZ</td>
<td>0.6330</td>
<td>0.6230</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.3600</td>
<td>0.3120</td>
<td>SK-N-SH</td>
<td>0.6550</td>
<td>0.8700</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.9250</td>
<td>0.9220</td>
<td>SH-SYSY</td>
<td>0.4300</td>
<td>0.3840</td>
</tr>
<tr>
<td>PRKCE</td>
<td>Carbamazepine</td>
<td>0.5000</td>
<td>0.6110</td>
<td>SK-N-DZ</td>
<td>0.7540</td>
<td>0.9460</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.9490</td>
<td>0.9720</td>
<td>SK-N-SH</td>
<td>1.6990</td>
<td>0.6440</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.8950</td>
<td>0.9620</td>
<td>SH-SYSY</td>
<td>0.3310</td>
<td>0.3840</td>
</tr>
<tr>
<td>RAB7A</td>
<td>Carbamazepine</td>
<td>0.4320</td>
<td>0.5460</td>
<td>SK-N-DZ</td>
<td>0.6270</td>
<td>0.7510</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.2280</td>
<td>0.2150</td>
<td>SK-N-SH</td>
<td>0.4200</td>
<td>0.4310</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.7850</td>
<td>0.8790</td>
<td>SH-SYSY</td>
<td>0.2900</td>
<td>0.4130</td>
</tr>
<tr>
<td>RGS5</td>
<td>Carbamazepine</td>
<td>0.7010</td>
<td>0.6280</td>
<td>SK-N-DZ</td>
<td>1.2780</td>
<td>0.8750</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>0.8430</td>
<td>0.9000</td>
<td>SK-N-SH</td>
<td>0.3180</td>
<td>0.1550</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6210</td>
<td>0.8640</td>
<td>SH-SYSY</td>
<td>0.9020</td>
<td>0.9410</td>
</tr>
<tr>
<td>TRAP1</td>
<td>Carbamazepine</td>
<td>0.1120</td>
<td>0.5930</td>
<td>SK-N-DZ</td>
<td>5.8270</td>
<td>0.4840</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>12.7530</td>
<td>0.2510</td>
<td>SK-N-SH</td>
<td>0.3040</td>
<td>0.6450</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6850</td>
<td>0.4000</td>
<td>SH-SYSY</td>
<td>0.3550</td>
<td>0.7520</td>
</tr>
<tr>
<td>YWHAG</td>
<td>Carbamazepine</td>
<td>0.6880</td>
<td>0.8190</td>
<td>SK-N-DZ</td>
<td>1.4230</td>
<td>0.8830</td>
</tr>
<tr>
<td></td>
<td>Valproate</td>
<td>2.3670</td>
<td>0.2660</td>
<td>SK-N-SH</td>
<td>0.9330</td>
<td>0.9480</td>
</tr>
<tr>
<td></td>
<td>Lithium</td>
<td>0.6660</td>
<td>0.7920</td>
<td>SH-SYSY</td>
<td>0.8160</td>
<td>0.7290</td>
</tr>
</tbody>
</table>

<sup>1</sup>Ratio of treated group to untreated control group

<sup>2</sup>Result of t-test comparing the mean of the untreated control group to the treated group. P-values ≤0.05 indicate the means of the two groups are significantly different.

Table B.2 Grouped gene expression changes after 28 days drug treatment
## APPENDIX C

### Table C.1 Gene expression results - untreated vs. combined treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Untreated</th>
<th>Combined Treatments&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mann-Whitney p-value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.3950</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>0.8170</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>0.3950</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>0.3070</td>
</tr>
<tr>
<td>C</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.4220</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>27.2950</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>1.2360</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>5.7200</td>
</tr>
<tr>
<td>D</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.6200</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>1.1830</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>1.4910</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>1.1480</td>
</tr>
<tr>
<td>E</td>
<td>BDNF</td>
<td>1.00</td>
<td>1.2190</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>0.8750</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>2.0220</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>3.1390</td>
</tr>
<tr>
<td>F</td>
<td>BDNF</td>
<td>1.00</td>
<td>1.9300</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>0.1320</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>1.4880</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>0.4700</td>
</tr>
<tr>
<td>G</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.7950</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>2.9510</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>1.1670</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>2.0030</td>
</tr>
<tr>
<td>H</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.2070</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>0.2450</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>4.1540</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>0.5920</td>
</tr>
<tr>
<td>I</td>
<td>BDNF</td>
<td>1.00</td>
<td>1.4610</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>0.3380</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>6.0220</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>1.7150</td>
</tr>
<tr>
<td>J</td>
<td>BDNF</td>
<td>1.00</td>
<td>0.3450</td>
</tr>
<tr>
<td></td>
<td>MDK</td>
<td>1.00</td>
<td>1.2330</td>
</tr>
<tr>
<td></td>
<td>RAB7A</td>
<td>1.00</td>
<td>1.4280</td>
</tr>
<tr>
<td></td>
<td>YWHAG</td>
<td>1.00</td>
<td>1.1600</td>
</tr>
</tbody>
</table>

<sup>1</sup>This value is the combination of all drug treatments standardised against all untreated controls (which are expressed as one), for each individual cell line.

<sup>2</sup>The Mann-Whitney U test calculates if the means of two groups are significantly different. A p-value of ≤0.05 indicates the means of the two groups are significantly different.
APPENDIX D

Figure D.1 Haplotype block linkage disequilibrium plot for BDNF
Tag SNPs are circled

Figure D.2 Haplotype block linkage disequilibrium plot for MDK
Tag SNPs are circled
Figure D.3 Haplotype block linkage disequilibrium plot for YWHAG
Tag SNPs are circled
### Table E.1  Microsatellite amplification conditions and primer sequence

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Reagents</th>
<th>Cycling Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4S2426F</td>
<td>5'-[6FAM]CCTGAGATGCCGTAAAGCTA-3'</td>
<td>1 x PCR buffer, 1.5mM MgCl₂, 100μM dNTPs, 0.2pmol/μL forward primer, 0.2pmol/μL reverse primer, 1U Taq</td>
<td>94°C 2min (94°C 30s, 55°C 30s, 72°C 45s) x 30, 72°C 5min</td>
</tr>
<tr>
<td>D4S2426R</td>
<td>5’-CCTATTTAAAAATACCTCAATATGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S2423F</td>
<td>5'-[6FAM]CTTCAGACCTTCAGTTGATGAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7S2423R</td>
<td>5’-GCTTCTCAGACACATTTTCCA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17S937F</td>
<td>5’-6FAM]CATGGAGGGACTTGCG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D17S937R</td>
<td>5’-TTCCAGAACACCCTTTT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D22S1153F</td>
<td>5’-[6FAM]TGAGGCAATGCAGCACCAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D22S1152R</td>
<td>5’-CCAAGTTTTGATTCCAGAAACACC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table E.2 SNP Genotyping - Restriction Digest Conditions

<table>
<thead>
<tr>
<th>SNP</th>
<th>Restriction Enzyme</th>
<th>Incubation Temperature</th>
<th>NEB Buffer¹</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11030104</td>
<td>Rsal</td>
<td>37°C</td>
<td>Buffer 4</td>
<td>2 hours</td>
</tr>
<tr>
<td>rs4927892</td>
<td>MstI</td>
<td>37°C</td>
<td>Buffer 4</td>
<td>4 hours</td>
</tr>
<tr>
<td>rs4333102</td>
<td>BsrI</td>
<td>65°C</td>
<td>Buffer 3</td>
<td>3 hours</td>
</tr>
<tr>
<td>rs7372263</td>
<td>DraI</td>
<td>37°C</td>
<td>Buffer 4</td>
<td>Overnight</td>
</tr>
<tr>
<td>rs11763069</td>
<td>Avall</td>
<td>37°C</td>
<td>Buffer 4</td>
<td>2 hours</td>
</tr>
</tbody>
</table>

¹New England Biosystems standard buffers
### Table E.3 ARMS Assay Primers and reaction conditions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reagents</th>
<th>Cycling Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Forward</td>
<td>5'-AGGTGAGAAGAGTGATGCACCATCCTTTT-3'</td>
<td>1 x PCR buffer</td>
<td>94°C 3min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5mM MgCl₂</td>
<td>(94°C 30s, 65°C 20s) x 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100µM dNTPs</td>
<td>68°C 7min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL common forward primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL common reverse primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4pmol/µL specific forward primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1pmol/µL specific reverse primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1U Taq</td>
<td></td>
</tr>
<tr>
<td>Common Reverse</td>
<td>5'-ACTACTGAGCATTCAACCCTGGACGTGTAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific Forward</td>
<td>5'-TGACATCATGGGCTGACACTTTTGAAGCAAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific Reverse</td>
<td>5'-CTGTCCTCATTCAAGCTCTTCTATCCT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table E.4 Primer sequences and conditions for PCR amplification

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primers</th>
<th>Reaction Mix</th>
<th>Cycling Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 x PCR buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100µM dNTPs</td>
<td></td>
</tr>
<tr>
<td>rs1519480</td>
<td>5'-GAGGAAACCATGTTGATG-3'  R 5'-GTACCCAATCTAAGATTG-3'</td>
<td>0.2pmol/µL forward primer</td>
<td>94°C 3min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL reverse primer</td>
<td>(94°C 15s, 58°C 30s, 72°C 45s) x 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1U Taq</td>
<td>72°C 5min</td>
</tr>
<tr>
<td>rs6265</td>
<td>5'-GTTATTTCATACCTTGGTGGC-3'  5'-CATGTGTCAGCATCTAG-3'</td>
<td>1 x PCR buffer</td>
<td>94°C 3min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5mM MgCl₂</td>
<td>(94°C 15s, 55°C 30s, 72°C 45s) x 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100µM dNTPs</td>
<td>72°C 5min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL forward primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL reverse primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1U Taq</td>
<td></td>
</tr>
<tr>
<td>rs11030104</td>
<td>5'-CCTAAAGCAGAATTGACAGG-3'  5'-CAAGGACAGCTGCTTCTG-3'</td>
<td>1 x PCR buffer</td>
<td>94°C 3min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5mM MgCl₂</td>
<td>(94°C 15s, 58°C 30s, 72°C 45s) x 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100µM dNTPs</td>
<td>72°C 5min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL forward primer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2pmol/µL reverse primer</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>5'-Sequence 1</td>
<td>5'-Sequence 2</td>
<td>1 x PCR buffer</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>rs11030107</td>
<td>5'-GTGTGATTTGGTCTGGAAGATC-3'</td>
<td>5'-CCAAGATAAGCATGCTGTG-3'</td>
<td>1 x PCR buffer</td>
</tr>
<tr>
<td>rs10835210</td>
<td>5'-GTAGAAACTCAGCATTCTGAG-3'</td>
<td>5'-CACATGGCTCATTATGC-3'</td>
<td>1 x PCR buffer</td>
</tr>
<tr>
<td>rs7103873</td>
<td>5'-GTCGTGCTACACAGATAGGC-3'</td>
<td>5'-CAGATGACTACATGTGGAAAG-3'</td>
<td>1 x PCR buffer</td>
</tr>
<tr>
<td>rs11030119</td>
<td>5'-CCACTACTTGTCTCAAGATCC-3'</td>
<td>5'-GATGGAGAACAGCTGCTTAC-3'</td>
<td>1 x PCR buffer</td>
</tr>
<tr>
<td>rs12273363</td>
<td>5'-CAGTTGTTATGGAGATGTCC-3'</td>
<td>5'-CTTATGTCTGTGCTTTAATAC-3'</td>
<td>1 x PCR buffer</td>
</tr>
</tbody>
</table>
| rs9820753 | 5’-CCTCTGAAGTACCACCTGTG-3’  
|           | 5’-TCGGTGACACTACCAAC-3’  | 1 x PCR buffer  
|           | 1.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 55°C 30s, 72°C 45s) x 30  
|           | 72°C 5 min  |
| rs4384970 | 5’-CCTCTGAAGTACCACCTGTG-3’  
|           | 5’-GCTGGACAGCCATCAAC-3’  | 1 x PCR buffer  
|           | 1.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 60°C 30s, 72°C 45s) x 30  
|           | 72°C 5 min  |
| rs4927892 | 5’-GAAGGAAGTCATGAAGGTTG-3’  
|           | 5’-CTGCCAATCTGTCTTGG-3’  | 1 x PCR buffer  
|           | 1.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 58°C 30s, 72°C 45s) x 30  
|           | 72°C 5 min  |
| rs13081864| 5’-GTCATCTTCAAGAGTGAGG-3’  
|           | 5’-GATGGTAAGATGCAGAC-3’  | 1 x PCR buffer  
|           | 1.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 58°C 30s, 72°C 45s) x 30  
|           | 72°C 5 min  |
| rs4333102 | 5’-GACTGTGAGGACTCTTGTA-3’  
|           | 5’-CATGTAACCTATTAGGACATGTAGG-3’  | 1 x PCR buffer  
|           | 1.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 58°C 30s, 72°C 45s) x 30  
|           | 72°C 5 min  |
| rs7372263 | 5’-GATAGGAGAGAACTGCACATC-3’  
|           | 5’-CACAAACTCCAGTCAAAC-3’  | 1 x PCR buffer  
|           | 2.5mM MgCl₂  
|           | 100 µM dNTPs  
|           | 0.2pmol/µL forward primer  
|           | 0.2pmol/µL reverse primer  
|           | 1U Taq  | 94°C 3 min  
|           | (94°C 15s, 57°C 30s, 72°C 45s) x 30  
<p>|           | 72°C 5 min  |</p>
<table>
<thead>
<tr>
<th>rs1046304</th>
<th>5'-GGAAGAGCTATATCCTTAACC-3' 5'-GTGAGACAAGCCAATCTC-3'</th>
<th>1 x PCR buffer 2.5mM MgCl&lt;sub&gt;2&lt;/sub&gt; 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</th>
<th>94°C 3min (94°C 15s, 58°C 30s, 72°C 45s) x 30 72°C 5min</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2961034</td>
<td>5'-CAATCTCCGTATTTGTG-3' 5'-CTGGACAACGTTACTCCTAC-3'</td>
<td>1 x PCR buffer 1.5mM MgCl&lt;sub&gt;2&lt;/sub&gt; 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</td>
<td>94°C 3min (94°C 15s, 54°C 30s, 72°C 45s) x 30 72°C 5min</td>
</tr>
<tr>
<td>rs6465098</td>
<td>5'-CTTCTGAAAGTGCTGAATG-3' 5'-CATACTTGCTTAGGCTCAATTAG-3'</td>
<td>1 x PCR buffer 1.5mM MgCl&lt;sub&gt;2&lt;/sub&gt; 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</td>
<td>94°C 3min (94°C 15s, 60°C 30s, 72°C 45s) x 30 72°C 5min</td>
</tr>
<tr>
<td>rs11763069</td>
<td>5'-CTTGCCCTCAATAAGCTGG-3' 5'-AGCAGTAGGCTCCATTAG-3'</td>
<td>1 x PCR buffer 1.5mM MgCl&lt;sub&gt;2&lt;/sub&gt; 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</td>
<td>94°C 3min (94°C 15s, 55°C 30s, 72°C 45s) x 30 72°C 5min</td>
</tr>
<tr>
<td>rs12531257</td>
<td>5'-CTGATAATAAAGCCAGCC-3' 5'-GAATGTGTCGAATGAG-3'</td>
<td>1 x PCR buffer 2.5mM MgCl&lt;sub&gt;2&lt;/sub&gt; 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</td>
<td>94°C 3min (94°C 15s, 54°C 30s, 72°C 45s) x 30 72°C 5min</td>
</tr>
<tr>
<td>SNP</td>
<td>Primer</td>
<td>Primer Sequence</td>
<td>Reagents</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Common Primer</td>
<td>5’-CCTCTGAAGTACACTGGTG-3’</td>
<td>1 x PCR buffer 1.5mM MgCl₂ 100µM dNTPs 0.2pmol/µL forward primer 0.2pmol/µL reverse primer 1U Taq</td>
</tr>
<tr>
<td></td>
<td>HFA Primer</td>
<td>5’-CTGCCGGTATTCAGAGCT-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFA Primer</td>
<td>5’-CTGCCGGTATTCAGAGCG-3’</td>
<td></td>
</tr>
<tr>
<td>rs4384970</td>
<td>Common Primer</td>
<td>5’-GGAAGAGCTATATCCTTAACC-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HFA Primer</td>
<td>5’-GTTCCTGCTCATAAGGAGTTCC-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFA Primer</td>
<td>5’-GGCCGAGGCGGCGGCGCTCAGCTAAGAGGAAT-3’</td>
<td></td>
</tr>
<tr>
<td>rs1046304</td>
<td>Common Primer</td>
<td>5’-CTTCTGAAAGTGCTGAAGTG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HFA Primer</td>
<td>5’-GAGAGTGCTGGATCATAGA-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFA Primer</td>
<td>5’-GAGAGTGCTGGATCATAGA-3’</td>
<td></td>
</tr>
<tr>
<td>rs6465098</td>
<td>Common Primer</td>
<td>5’-CCTCTGAAGTACACTGGTG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HFA Primer</td>
<td>5’-CTGCCGGTATTCAGAGCT-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFA Primer</td>
<td>5’-CTGCCGGTATTCAGAGCG-3’</td>
<td></td>
</tr>
</tbody>
</table>

1High frequency allele primer
2Low frequency allele primer
# APPENDIX F

## Table F.1. TaqMan Probe Information

<table>
<thead>
<tr>
<th>SNP</th>
<th>Assay ID</th>
<th>Context Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12273363</td>
<td>C__31701018_10</td>
<td>GATGCTGCAGAAGA[C/T]GGTGGATAGCTCTTAAG</td>
</tr>
<tr>
<td>rs10835210</td>
<td>C__1751795_10</td>
<td>AAAGCACAGGGAAAGTAG[A/C]TCATTACTTGTAGCT</td>
</tr>
<tr>
<td>rs13247572</td>
<td>C__394723_10</td>
<td>TCCCTTTGACTACC[A/G]AGCATAGAAAGTGGTCC</td>
</tr>
<tr>
<td>rs12531257</td>
<td>C__32056407_10</td>
<td>TATACAGTGGT[C/G]AAGTCCATAACTTTTTAAGT</td>
</tr>
<tr>
<td>rs2961034</td>
<td>C__15957053_10</td>
<td>TGTGCCACACACTC[C/T]GTCTGGGACCTTCTG</td>
</tr>
<tr>
<td>rs11030119</td>
<td>C__31701027_10</td>
<td>CACTCAGACTTTTCTC[A/G]TAGCAAAAAGATCAGA</td>
</tr>
<tr>
<td>rs7103873</td>
<td>C__1751797_10</td>
<td>CCCCCAAATGTGA[A/G]ACTAAATTGAAAAACCA</td>
</tr>
<tr>
<td>rs2712402</td>
<td>C__15834025_10</td>
<td>AGCCACTGTTGTAGTAG[A/G]GACTGGGACTAG</td>
</tr>
<tr>
<td>rs11030107</td>
<td>C__31701054_10</td>
<td>GTGATGCAACTGCAATTC[A/G]TTGGCCCTAAAG</td>
</tr>
<tr>
<td>rs9820753</td>
<td>C__115672_10</td>
<td>TCAGACTCCAGACACACT[A/G]TATTAATTTTCTA</td>
</tr>
<tr>
<td>rs13081864</td>
<td>C__461959_10</td>
<td>TAGCAGGTAAATAAGGGG[C/T]TTACTCCATTTT</td>
</tr>
<tr>
<td>rs12273363</td>
<td>C__31701018_10</td>
<td>AGCGATGCTGCAGAAGA[C/T]GTTGGGATAGCTC</td>
</tr>
</tbody>
</table>