Genetics in the Pathogenesis and Treatment of Panic and Anxiety

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Abstract

Mental disorders form a broad category of disease that may manifest as emotional or behavioural changes. These disorders are common globally and account for a significant amount of morbidity. The most frequently occurring major category of mental disorder is the anxiety disorders, which manifest as acute (panic attacks) and/or chronic (persistent worry) anxiety.

Mental disorders can be viewed as structural or chemical changes in the brain that result from a combination of genetic and environmental risk factors. Fear and stress pathways are thought to contribute to the development of anxiety disorders, as are specific neurotransmitters and neuromodulators such as serotonin, GABA (γ-amino butyric acid), cholecystokinin and adenosine. Despite this knowledge, there is still much to learn about the pathogenesis of anxiety and pharmacotherapeutic action of the drugs used to treat it.

One means of investigating both of these areas is to study candidate genes. For example, cholecystokinin is a peptide that can induce panic attacks in both normal volunteers and panic disorder patients; the genes for cholecystokinin (*CCK*) and its two receptors (*CCKAR* and *CCKBR*) are, thus, good candidates for anxiety pathogenesis and treatment.

The aims of the present study were to determine whether common variation in any of the cholecystokinin system genes is associated with panic and whether expression levels of any candidate genes were altered in neuroblastoma cell lines following 28-days exposure of antidepressant drugs that have anxiety reducing (anxiolytic) effects.

The three genes from the cholecystokinin system were genotyped in a New Zealand family-based cohort (563 subjects) and analysed for association with panic and related phenotypes. HapMap data was used to select multiple variants within each gene ensuring good coverage of common variation. Significant associations were found with multiple *CCKBR* variants and panic, some of which met the adjusted significance threshold. Further analyses suggested that *CCKAR* variation might be associated with a co-morbid panic and bipolar disorder phenotype, though further research would be needed to confirm this.

Antidepressant drugs are among the most commonly prescribed treatments for the long-term control of anxiety, however, there has been little research into their anxiolytic effects and even less on the anxiety-increasing effects seen in some patients early in treatment. Thus, gene expression changes of a list of 20 candidate genes were investigated in three different neuroblastoma cell lines during a 28-day time course. The two most consistent changes were down-regulations of two-fold or more in the genes *ADORA2A* (encoding an adenosine receptor) and *CCKAR*. Both of these changes began early in the time course, and functional evidence from other studies suggests that the reduced expression may have anxiety-increasing effects. Subsequent protein quantification studies supported a reduction in levels of the adenosine A_{2a} receptor. Thus, changes in one or both of these genes may be involved in the early anxiogenic effects of antidepressants.

A better understanding of both the pathogenesis and treatment of anxiety disorders is important as it may lead to improved diagnosis and treatment of high-risk individuals. This, in turn, should reduce the burden these common diseases cause to both affected individuals and to the broader society.

Acknowledgements

Ko Tarawera te Maunga
Ko Tarawera te Awa
Ko Te Arawa te Iwi
Ko Ngāti Rangitihi te Hāpu
Ko Julia Tōku Ingoa

My name is Julia and I am descended from the North Island iwi Te Arawa. The above is my mihi, a traditional Māori introduction and way of describing where I fit within the world. Māori culture is sometimes described as collectivist, where the achievements of the whānau (family) or iwi (tribe) are more important than the achievements of the individual. But what does this have to do with a scientific thesis, you may ask. New research should build upon the past, not forget or dismiss it, and it should add to a greater whole, more important that the individual study. Knowledge is not created in isolation, by an individual, but built with the support of many.

With this in mind, I would like to thank everyone that has provided support to me during my studies. This includes people who are typically involved in supporting candidates: my friends and family, my supervisors and many of the other staff and students of the Department of Pathology, who have provided emotional or technical support at various times throughout my degree.

For financial support, I have been fortunate to receive a Health Research Council of New Zealand (HRC) Māori Health Career Development Award. This scholarship not only paid for my fees and living expenses for three years, but also included additional funding that allowed me to attend conferences, including the HRC's Hui Whakapiripiri, which I would have otherwise been unable to attend. While the financial assistance from the HRC has been greatly appreciated, the greatest value I place on this scholarship is in the knowledge that the organisation had faith in my ability and I would like to thank the HRC Māori Health Committee for this.

In addition to the HRC, I have received funding from several other sources. The University of Otago Graduate Research Services, the Division of Health Sciences, and the Department of

Preventive and Social Medicine have all provided funding towards attending and presenting at various conferences during my PhD studies. I also received a departmental award from the Department of Pathology to provide for living expenses during my fourth year of study. These additional sources of funding have allowed me to take up opportunities, such as presenting at international conferences, that have enhanced the experience and quality of my PhD.

Most of the physical resources needed to complete my PhD have been provided by the Department of Pathology. This has included laboratory space, a desk and computer and most of the laboratory equipment I used. In addition, Diane Guevremont and the Department of Anatomy kindly allowed me to use their Odyssey imaging system for my protein quantification work. Additional funding for laboratory consumables was provided by: the Dunedin School of Medicine Dean's Bequest Fund, Division of Health Sciences Opportunities for Research Fund and an H.S. and J.C. Anderson Trust Grant.

During my PhD studies I have been a student member of the Brain Health Research Committee (BHRC). Being a member of this group has provided opportunities to network with other staff and students interested in the function of the brain in health and disease. The BHRC kindly runs seminars, conferences and workshops that student members are allowed to attend free of charge. I would like to thank the BHRC for allowing me the opportunity to attend these.

Finally, in addition to those listed above, I have also received support from Māori support groups at the University. This has included: the Māori Centre, who support all Māori students at the University of Otago; MAI ki Otago, the Otago branch of a Māori and indigenous postgraduate support group; and a recently formed group of researchers initiated by Louise Parr-Brownlie to support Māori postgraduates and early career researchers working in neuroscience and related biomedical areas. These groups have provided opportunities to meet other students and to discuss aspects of research in supportive, informal settings.

Contributions of Others

Parts of the research presented here were continuations of previous research in the same laboratory. Consequently, some of the work was conducted by other researchers prior to and during the completion of the current work. These are listed below and a brief description of their use in the current research.

- Recruitment of participants, collection of genetic samples and phenotypic information for the South Island Bipolar Study, followed by pedigree construction and confirmation of uncertain relationships. The resulting genetic samples and phenotypic data were used in the genetic association component of the present study.
- Some *CCK* genotyping (rs1129949, rs10865918 and rs747455) and all *CCK* primer design was conducted by other researchers, as was Sanger sequencing (including design of sequencing primers) for one *CCKBR* variant (rs1042047 - conducted to confirm accuracy of other genotyping attempts). Additionally, I conducted all genotyping and primer design for *CCKAR* as part of an earlier project.
- Some of the primers for gene expression experiments were designed and tested by other researchers and some were designed and tested by me as part of an earlier project. The full list of these primers can be found in Appendix F, with those designed by another researcher shown in grey font colour.

Publications

Sears, C., Wilson, J., Fitches, A. Investigating the role of BDNF and CCK system genes in suicidality in a familial bipolar cohort. *Journal of affective disorders, 151,* 611-617 (2013).

Wilson, J., Markie, D., Fitches, A. Cholecystokinin system genes: Associations with panic and other psychiatric disorders. *Journal of affective disorders, 136,* 902-908 (2012).

Wilson, J., Markie, D., Fitches, A. Analysis of associations for candidate genes with anxiety disorders. *Psychiatry research, 189,* 324-325 (2011).

Table of Contents

Abstract iii
Acknowledgements v
Contributions of Others vii
Publications ix
Table of Contents xi
List of Tables xvii
List of Figures xix
Abbreviations and Nomenclature xxi
1 Introduction 1
1.1 Mental Health 1
1.1.1 Neurobiology 2
1.1.1.1 Cells in the Central Nervous System 5
1.1.1.2 Synapses and Receptors 5
1.1.2 Defining Mental Illness 7
1.1.3 Population Mental Health 8
1.1.3.1 Prevalence 9
1.1.3.2 Impact 11
1.1.3.3 Comorbidity 12
1.1.3.4 Indigenous Mental Health 13
1.1.4 Reducing Mental Illness 15
1.1.4.1 Pharmacotherapy 15
1.1.4.2 Psychotherapy 16
1.1.4.3 Electroconvulsive Therapy 16
1.1.4.4 Prevention 17
1.1.5 Summary 18
1.2 Genetics and Complex Diseases 18
1.2.1 Determining Heritability 18
1.2.2 Identification of Heritable Elements 20
1.2.2.1 Case-Control or Family-Based Analysis 20
1.2.2.2 Candidate Genes 21
1.2.2.3 Linkage and Genome-wide Association Studies 21
1.2.2.4 Haplotypes and the HapMap Project 22
1.2.3 Missing Heritability 24
1.2.3.1 Genetic Factors 25
1.2.3.2 Environmental Factors and Population Variation 27

1.2.4 Summary 28
1.3 Cholecystokinin System *29*
1.3.1 Discovery of CCK 29
1.3.2 Neuronal CCK 29
1.3.2.1 CCK System Expression in the Brain *31*
1.3.3 CCK System Genes 34
1.3.3.1 CCK *34*
1.3.3.2 CCKAR *36*
1.3.3.3 CCKBR *37*
1.3.4 Proteins of the CCK System 39
1.3.4.1 CCK Peptides *39*
1.3.4.2 Receptor Binding *40*
1.3.4.3 Receptor Signalling *41*
1.3.5 Summary 42
1.4 Molecular Biology of Anxiety *43*
1.4.1 Fear, Stress and Anxiety 44
1.4.1.1 Induction of Panic Attacks *48*
1.4.2 Genetics of Anxiety and Panic 50
1.4.3 Neurotransmitters and Neuromodulators 51
1.4.3.1 Serotonin *52*
1.4.3.2 GABA *54*
1.4.3.3 Cholecystokinin *57*
1.4.3.4 Adenosine *58*
1.4.4 Pharmacotherapy for Anxiety 60
1.4.4.1 Anxiolysis and Anxiogenesis with Antidepressants *60*
1.4.4.2 Mechanism of Action of Antidepressants *61*
1.4.4.3 Gabapentin and Pregabalin for Anxiety *64*
1.4.4.4 Mechanism of Action of Gabapentin and Pregabalin *66*
1.4.5 Summary 70
1.5 Aims *70*
2 Methodology 73
2.1 Buffers and Solutions *73*
2.2 Gels *74*
2.2.1 Polyacrylamide Gels 74
2.2.2 Agarose gels 74
2.3 Primer Design *75*
2.4 Association Studies *75*

2.4.1 Subjects 75
2.4.2 Selection of SNPs and Haplotypes with Haploview 76
2.4.3 Genotyping of SNPs 76
2.4.3.1 TaqMan Hydrolysis Probes 77
2.4.3.2 Primers 77
2.4.3.3 Primer Optimisation and Multiplexing 79
2.4.3.4 RFLP Assay 79
2.4.3.5 Sequencing 79
2.4.4 Statistical Analysis 80
2.4.4.1 Power Calculations 80
2.4.4.2 Hardy-Weinberg 81
2.4.4.3 FBAT 81
2.4.4.4 Correction for Multiple Comparisons 82
2.5 Cell Culture 83
2.5.1 Cell lines 83
2.5.2 Culture Conditions 83
2.5.3 Drug Exposure 84
2.6 Gene Expression Analysis 84
2.6.1 RNA Extraction 84
2.6.2 cDNA Production 85
2.6.3 Gene Expression and Normalisation 86
2.6.4 Statistical Analysis 87
2.7 Protein Quantification 88
2.7.1 Protein Extraction 88
2.7.2 Protein Visualisation 88
2.7.2.1 Slot-Blot 88
2.7.2.2 Electrophoresis and Transfer (Western blot) 89
2.7.2.3 Blocking and Washes 90
2.7.2.4 Antibodies 90
2.7.2.5 Imaging 91
2.7.2.6 Quantification 91
3 Genetic Association Studies 93
3.1 Background 93
3.1.1 Cholecystokinin and Panic 93
3.1.2 *ADORA2A* and Panic 97
3.1.3 Aims 98
3.2 Description of Subjects 98

3.3 Technical Results – Genotyping Assays *100*
3.3.1 Selection of SNPs for Analysis 100
3.3.1.1 CCK System Genes *100*
3.3.1.1.1 Order of Genotyping in *CCKBR* 103
3.3.1.2 ADORA2A SNPs *104*
3.3.2 Optimisation of Assays 106
3.3.2.1 Genotyping of SNPs *106*
3.3.2.1.1 TaqMan Hydrolysis Probes 106
3.3.2.1.2 Primer Optimisation 110
3.3.2.1.3 Multiplexing 112
3.3.2.1.4 RFLP Assay 115
3.3.2.2 Confirmation of Genotyping Accuracy *116*
3.3.2.2.1 Haplotypes 117
3.3.2.2.2 Hardy-Weinberg Equilibrium 118
3.3.2.2.3 Mendelian Errors 119
3.4 Associations *120*
3.4.1 Phenotypes of Subjects 120
3.4.2 Associations of CCK System Genes 121
3.4.2.1 Phenotypes Analysed *121*
3.4.2.2 Power of the Sample *122*
3.4.2.3 Variants Analysed *123*
3.4.2.4 Correction for Multiple Comparisons *123*
3.4.2.5 Associations with Any Panic *124*
3.4.2.6 Phenotypes Most Commonly Associated with each Gene. *126*
3.4.3 Association with *ADORA2A* 128
3.5 Discussion *128*
3.5.1 Methodological Strengths and Limitations 129
3.5.1.1 Cohort *129*
3.5.1.2 Candidate Gene vs Genome-wide *130*
3.5.1.3 SNP Selection from HapMap Data *131*
3.5.1.4 Genotyping Methods *132*
3.5.1.5 Analysis with FBAT *132*
3.5.1.6 Power and Correction for Multiple Comparisons *133*
3.5.1.7 Selection of Phenotypes *134*
3.5.2 Genetic Associations with CCK System Genes 135
3.5.2.1 CCK Associations *135*
3.5.2.2 CCKAR Associations *136*
3.5.2.3 CCKBR Associations *137*

3.5.2.4 Differences Between Studies *138*
3.5.3 Genetic Associations with *ADORA2A* 139
3.5.4 Implications of Findings 140
4 Cellular Changes with Antidepressants 143
4.1 Background *143*
4.1.1 Positional Candidates 143
4.1.1.1 ADAM22 ..... *144*
4.1.1.2 CACNA2D1 ..... *144*
4.1.1.3 FZD1 ..... *144*
4.1.1.4 GRM3 ..... *145*
4.1.1.5 LMTK2 ..... *145*
4.1.1.6 SRI *145*
4.1.1.7 TAC1 ..... *146*
4.1.2 Functional Candidates 146
4.1.2.1 ADORA2A *146*
4.1.2.2 BDNF *147*
4.1.2.3 CCK, CCKAR, and CCKBR *148*
4.1.2.4 CDKN1A *148*
4.1.2.5 CRHR2 ..... *149*
4.1.2.6 GABRA1, GABRA2 and GABRA3 ..... *150*
4.1.2.7 NPY and NPY1R *150*
4.1.2.8 TPH2 ..... *151*
4.1.3 Aims 151
4.2 Technical Results - Cell Culture and Drugs *152*
4.2.1 Long Time Course 154
4.2.2 Short Time Course 155
4.3 Technical Results - Gene Expression *155*
4.3.1 Primer Design 155
4.3.2 Quality Control – Technical Replicates 156
4.4 Gene Expression Changes *158*
4.4.1 Short Time Course 159
4.4.2 Long Time Course 165
4.5 Technical Results - A_{2a} Protein Levels *173*
4.5.1 Slot-blot Quantification 174
4.5.2 Western Blot Quantification 178
4.6 A_{2a} Quantification Results *180*
4.7 Discussion *180*

4.7.1 Methodological Strengths and Limitations....181
4.7.1.1 Cell Culture....181
4.7.1.2 Gene Expression Methods....183
4.7.1.3 Protein Level Methods....185
4.7.2 Expression Changes....187
4.7.2.1 Expression Levels Not Analysed....187
4.7.2.2 Small and Inconsistent Changes....189
4.7.2.3 CCKAR....191
4.7.2.4 Adenosine A_{2a}, Encoded by ADORA2A....191
4.7.3 Implications of Findings....192
5 Conclusions....195
5.1 Anxiety Genetics in 2013....195
5.2 The Future of Anxiety Research?....196
References....201
Appendix A – CCK System Custom Blocks....243
Appendix B – Genotyping Details....247
Appendix C – CCK System Association Results....255
Appendix D – *ADORA2A* Association Results....267
Appendix E – RNA and cDNA Measurements....269
Appendix F – qPCR Primers....275

List of Tables

Table 1.1 Twelve-Month Prevalence (%) of Mental Disorders 11
Table 3.1 Previous Association Studies of CCK System Genes and Panic 94
Table 3.2 Parental Ethnicity of Participants. 99
Table 3.3 SNPs that were not in Hardy-Weinberg Equilibrium (Observed - Expected). 119
Table 3.4 Number of Participants with each Phenotype 121
Table 3.5 Estimates of Statistical Power for Any Panic 123
Table 3.6 CCK System Variant Associations with Any Panic. 125
Table 3.7 Strongest Associations For CCK System Variants. 127
Table 3.8 Associations Between *ADORA2A* and Panic. 128
Table 4.1 Consistency of Technical Replicates 157
Table 4.2 Short Time Course Average Expression for Each Drug 159
Table 4.3 Short Time Course Summary for All Cell Lines 160
Table 4.4 Long A Time Course Summary 166
Table 4.5 Long Time Course Average Expression for Each Drug 167
Table 4.6 Long A and B Antidepressant Summary 168
Table 4.7 A_{2a} Relative Levels – Slot-Blot. 177
Table 4.8 Original and Replication Slot-Blot Results. 178
Table 4.9 A_{2a} Relative Levels – Western Blot. 179

List of Figures

All figures are original work and are not copied from another source except figures 1.2, 1.3 and 1.4, which use Allen Human Brain Atlas images of the human brain viewed with Brian Explorer 2 (downloaded on 18/01/12, from http://human.brain-map.org/explorer.html) and images 1.9, 1.10, and 1.11 which used Allen Human Brain Atlas images viewed online (from http://human.brain-map.org/ accessed on 03/06/12). As stated on the Allen Institute for Brain Science website (http://alleninstitute.org/about_us/terms_of_use.html) Allen Human Brain Atlas data is freely available for "research and other noncommercial purposes".

Figure 1.1 Biopsychosocial Model of Mental Health 2
Figure 1.2 Divisions of the Brain and Brainstem. 3
Figure 1.3 Deep Structures of the Brain. 3
Figure 1.4 Superficial Lobes of the Brain. 4
Figure 1.5 Chemical Synapse. 6
Figure 1.6 Activation of Adenylyl Cyclase by G_s. 7
Figure 1.7 Comorbidity Between Common Psychiatric Disorders. 12
Figure 1.8 Factors in Prevalence Differences. 14
Figure 1.9 *CCK* Expression in the Brain. 32
Figure 1.10 *CCKBR* Expression in the Brain. 33
Figure 1.11 *CCKAR* Expression in the Brain. 34
Figure 1.12 *CCK* Splice Variant. 35
Figure 1.13 *CCKAR* Main Transcript and Splice Variant. 36
Figure 1.14 *CCKBR* Variants. 38
Figure 1.15 Activation of CCK Receptors. 42
Figure 1.16 Neurobiology of Fear 45
Figure 1.17 Stress Response. 46
Figure 1.18 Serotonin System. 53
Figure 1.19 $GABA_A$ Receptor. 55
Figure 1.20 Gabapentin Action on Calcium Channels. 69
Figure 2.1 Primer Locations. 78
Figure 2.2 Example of an FBAT Informative Pedigree. 82
Figure 3.1 CCK System Genes. 102
Figure 3.2 *CCKBR* Blocks. 103

Figure 3.3 Genotyping Order.....105
Figure 3.4 Example of an Ideal TaqMan Hydrolysis Probe Result.....107
Figure 3.5 Temperature Gradient for rs2941029.....108
Figure 3.6 Temperature Modification (rs5751876).....109
Figure 3.7 Optimisation Examples.....111
Figure 3.8 Multiplex - Five versus Four SNPs.....113
Figure 3.9 Magnesium Gradient for Multi 3-2.....114
Figure 3.10 Amplification Failure in Multi 3-1.....115
Figure 3.11 Restriction Enzyme Digest.....116
Figure 3.12 Frequency of Haplotypes Defined by rs1112716.....118
Figure 4.1 Small Changes with *FZD1* at 6 hours.....161
Figure 4.2 No Change with *SRI* over 48 hours.....161
Figure 4.3 Variable Expression of *TAC1* over 48 hours.....162
Figure 4.4 Inconsistent Changes with *CDKN1A* over 48 hours.....163
Figure 4.5 *BDNF* Expression in SH-SY5Y Cells over 48 hours.....164
Figure 4.6 Down-regulation of *CCKAR* over 48 hours.....164
Figure 4.7 Down-regulation of *LMTK2* over 32 days.....169
Figure 4.8 Down-regulation of *ADORA2A* over 32 days.....170
Figure 4.9 Down-regulation of *CCKAR* over 29 days.....171
Figure 4.10 Down-regulation of *NPY* over 31 days.....172
Figure 4.11 A_{2a} and γ-actin Band Patterns.....174
Figure 4.12 Relative Protein Concentration Determination.....175
Figure 4.13 Slot-Blot Quantification Pictures.....176
Figure 4.14 Slot-Blot Standard Curve.....177
Figure 4.15 A_{2a} Relative Levels – Slot-Blot.....178
Figure 4.16 Western Blot Quantification Pictures.....179
Figure 4.17 Comparison of A_{2a} Data.....180

Abbreviations and Nomenclature

Abbreviations:

5-HT	5-hydroxytryptamine, the chemical name for serotonin
ADAM22	A disintegrin and metalloprotease 22
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
cDNA	Complementary DNA
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate (adenine, thymine, cytosine and guanine)
DSM	Diagnostic and statistical manual of mental disorders
DSM-IV	Fourth edition of the DSM
EDTA	Ethylenediaminetetraacetic acid
GABA	γ-amino butyric acid
G/C	Guanine/cytosine
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween20
qPCR	quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SIBS	South Island Bipolar Study
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
TBE	Tris-Borate and EDTA buffer
TE	Tris-HCl and EDTA buffer
UTR	Untranslated region

Gene Names: Standardised gene symbols, obtained from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov), were used throughout this thesis. For human genes, the gene abbreviation is written in capital letters and italicised; for rodent genes the first letter is capitalised and the entire abbreviation is italicised. The following list is the protein product corresponding to each gene abbreviation used in this thesis.

ADAM22	A disintegrin and metalloprotease 22
ADORA1	Adenosine A_1 receptor
ADORA2A	Adenosine A_{2a} receptor
CACNA2D1	Calcium channel subunit $\alpha_2\delta$, subtype 1
CACNA2D2	Calcium channel subunit $\alpha_2\delta$, subtype 2
CCK	Cholecystokinin
CCKAR	CCK_A receptor
CCKBR	CCK_B receptor
CDKN1A	Cyclin-dependent kinase inhibitor 1A; also known as p21
CRHR2	Corticotropin releasing hormone receptor 2
FZD1	Frizzled receptor type 1
GABBR1	$GABA_B$ receptor type 1
GABRA1	$GABA_A$ receptor α_1 subunit
GABRA2	$GABA_A$ receptor α_2 subunit
GABRA3	$GABA_A$ receptor α_3 subunit
GABRA5	$GABA_A$ receptor α_5 subunit
GABRA6	$GABA_A$ receptor α_6 subunit
GABRB3	$GABA_A$ receptor β_3 subunit
GAD1	Glutamic acid decarboxylase 1
GAD2	Glutamic acid decarboxylase 2
GRM3	Group II metabotropic glutamate receptor 3
LMTK2	Lemur tyrosine kinase 2
NPY	Neuropeptide tyrosine; also known as neuropeptide Y
NPY1R	Y_1 receptor
SLC6A1	GABA transporter 1 (solute carrier family 6, member 1)
SRI	Sorcin; also known as soluble resistance-related calcium binding protein
TAC1	Tachykinin, precursor 1
TPH2	Tryptophan hydroxylase 2

1 Introduction

Mental illnesses, including anxiety disorders, are poorly understood. These disorders show high rates of comorbidity and are thought to be caused by a mixture of genetic and environmental factors. Panic attacks and panic disorder, collectively referred to here as panic, have a strong physiological basis and respond to both pharmacologic treatment and psychotherapy. However, the understanding of who is most susceptible to panic and the exact mechanism of action of many of the therapies commonly used is still lacking. The present study is focused on improving the understanding of the biological processes associated with panic.

1.1 Mental Health

The World Health Organization defines health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (World Health Organization, 1948). This definition recognises the importance of good mental health in addition to the physical fitness often emphasised in modern healthcare systems. Mental illness is very common with approximately half the population of many countries, including New Zealand, expected to develop a mental disorder within their lifetime (Kessler *et al.*, 2007b). As a consequence, mental illness contributes significantly to disease burden, with neuropsychiatric disorders (particularly depression) estimated to be the leading cause of years of healthy life lost due to disability globally (World Health Organization, 2008).

In modern medicine, mental health is commonly viewed through the biopsychosocial model, in which biological factors, psychological factors and social or societal factors all interact to produce the disease (Figure 1.1) (Engel, 1977). This section will discuss normal brain biology and its contribution to emotion and behaviour, as well as the features of mental illness, and an overview of the strategies to reduce the frequency and impact of these disorders.

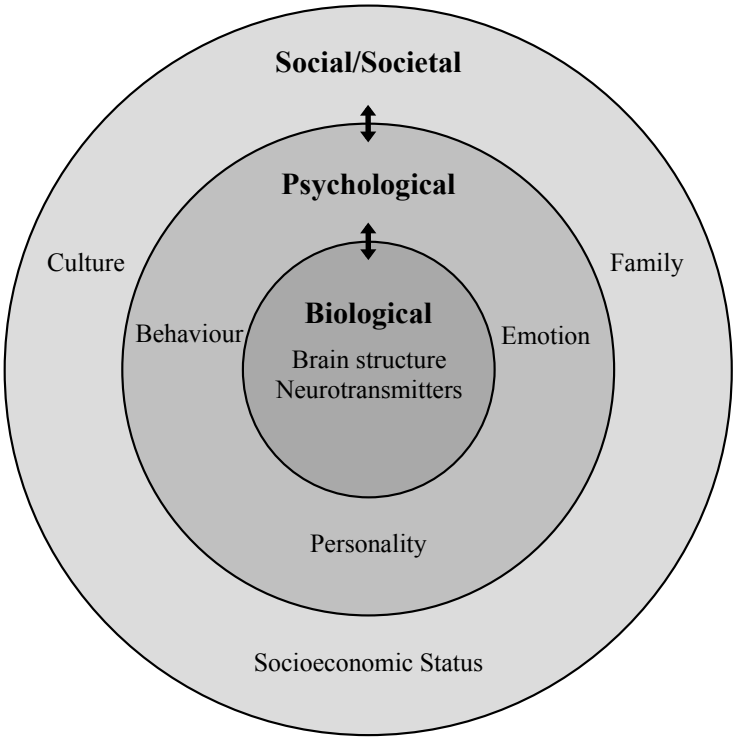


Figure 1.1 Biopsychosocial Model of Mental Health
In the biopsychosocial model of mental health, multiple biological, psychological and societal factors all contribute to normal health or disease.

1.1.1 Neurobiology

Emotions and behaviour predominantly originate from parts of the brain, the three major divisions of which are shown in Figure 1.2 (Clark *et al.*, 2010). The brainstem controls many subconscious bodily functions, such as heart rate (Marieb, 2001), and through these it can produce some of the physical sensations associated with emotion. The cerebellum was once thought to be a relatively simple integration centre (Marieb, 2001), however, recent research has suggested that the cerebellum may also contribute to higher functions including emotion (Schutter & van Honk, 2005). The cerebrum is the main site for the perception and control of emotion and is generally considered the most important brain region in the study of mental health (Carlson & Buskist, 1997).

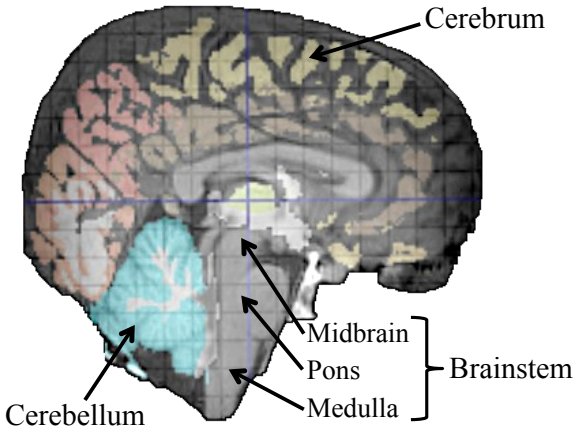


Figure 1.2 Divisions of the Brain and Brainstem.
Sagittal view of a human brain showing the main divisions of the brain (cerebrum, cerebellum and brainstem) and the divisions of the brainstem (midbrain, pons and medulla). Image is of H0351.2001, Allen Human Brain Atlas (Hawrylycz *et al.*, 2012), viewed with Brain Explorer 2, version 2.2.0 (accessed on 18/01/12, from http://human.brain-map.org/explorer.html). Used in accordance with website guidelines (accessed 18/01/12, from alleninstitute.org/about_us/terms_of_use.html).

The cerebrum consists of both superficial and deep structures, the cortex and deep nuclei respectively, which are typically grouped into five main lobes (Figure 1.3 and Figure 1.4). The parietal, occipital and temporal lobes are involved in processing sensory information (Ferrier, 1878a; Salanova *et al.*, 1995). Parts of the frontal lobe are involved in decision making (Ferrier, 1878b) and activity in the prefrontal region is thought to be important for suppression of unwanted or inappropriate emotions and their behavioural responses, such as anger and aggression (Bufkin & Luttrell, 2005). The insular lobe, or insula, processes both sensory and emotional information and associates bodily sensations, such as heart rate, with emotion (Clark *et al.*, 2010; Critchley *et al.*, 2004; Nagai *et al.*, 2007), which may be important in a variety of mental disorders.

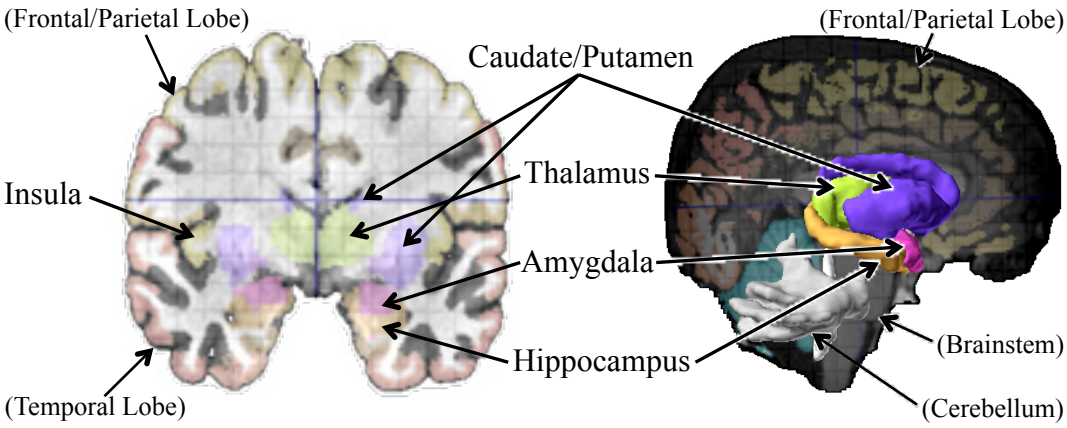


Figure 1.3 Deep Structures of the Brain.
Left: Coronal view of a human brain showing the insular lobe (insula) and the main deep nuclei (thalamus, caudate/putamen, amygdala, and hippocampus). Right: Sagittal view of the human brain with the deep nuclei visible. Images are of H0351.1009 (left) and H0351.2001 (right), Allen Human Brain Atlas (Hawrylycz *et al.*, 2012), viewed with Brain Explorer 2, version 2.2.0 (accessed on 18/01/12, from http://human.brain-map.org/explorer.html). Used in accordance with website guidelines (accessed 18/01/12, from http://alleninstitute.org/about_us/terms_of_use.html).

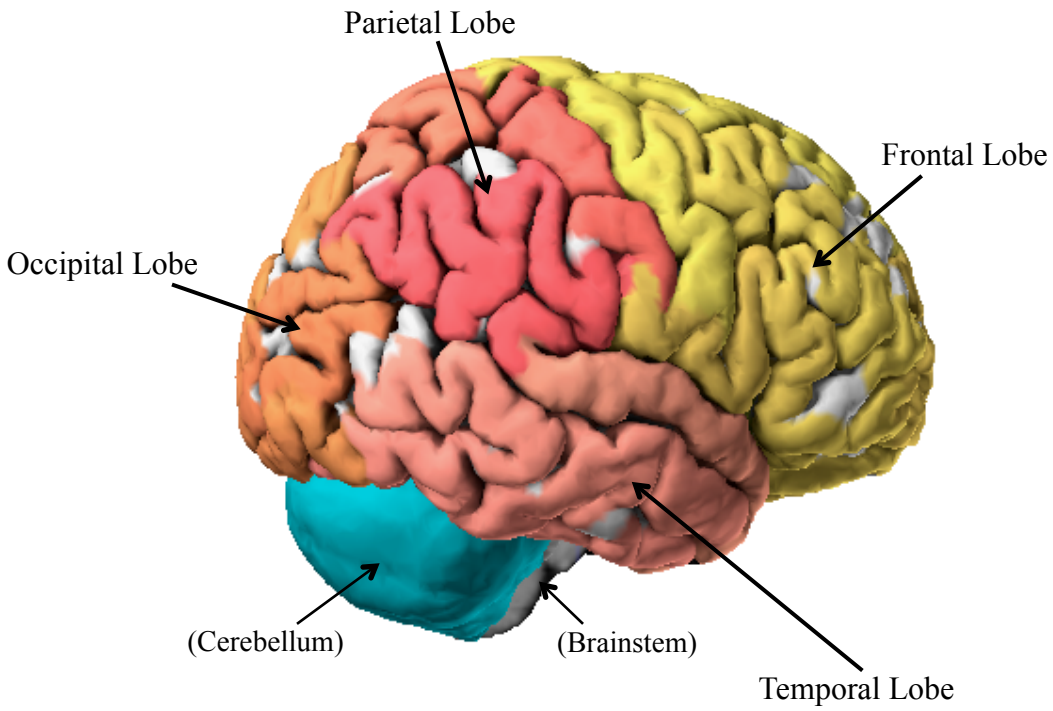


Figure 1.4 Superficial Lobes of the Brain.
Human brain showing the four superficial lobes (frontal, parietal, temporal and occipital). Image is of H0351.1009, Allen Human Brain Atlas (Hawrylycz *et al.*, 2012), viewed with Brain Explorer 2, version 2.2.0 (accessed on 18/01/12, from http://human.brain-map.org/explorer.html). Used in accordance with website guidelines (accessed 18/01/12, from http://alleninstitute.org/about_us/terms_of_use.html).

Among the deep nuclei (Figure 1.3), the amygdala and hippocampus, in the medial region of the temporal lobe are among the most important. The amygdala is involved in the recognition of threatening stimuli and is the key nucleus in the experience of fear (Bufkin & Luttrell, 2005). The hippocampus is important for memory processing and the interaction between the amygdala and hippocampus is a major component of the enhanced memory and recall of emotionally charged events (Banich *et al.*, 2009).

Other important nuclei include the caudate and putamen, which are anatomically divided into two nuclei, but act together as part of the basal ganglia in reward pathways (Chakravarthy *et al.*, 2010), making this region important in addiction. The thalamus acts as a relay site, through which most sensory and other inputs to the cerebral cortex pass, sorting information and providing some basic awareness, such as whether a sensation is pleasant or unpleasant (Marieb, 2001). The hypothalamus controls the secretion of most hormones and regulates several other homeostatic functions, including the autonomic nervous system, through which it can produce many of the bodily sensations associated with emotions (Marieb, 2001) (see also section 1.4.1, page 44).

1.1.1.1 Cells in the Central Nervous System

Although neurons are the main signalling/processing cell within the central nervous system, and are the focus of most research, three types of glial cells are also present and are vital for normal brain function. Oligodendrocytes produce the myelin sheaths that allow rapid action potential conduction; loss or damage of these, as occurs in multiple sclerosis, results in slowed or disordered neuronal signalling (Nestler *et al.*, 2001). Microglia are the resident immune cells in the central nervous system and, as such, they release various signalling molecules in response to infection or damage (Dheen *et al.*, 2007). Microglia also have a role in neurogenesis, with their acute activation generally inhibiting new neuron formation (Cacci *et al.*, 2008), while in other states they can promote neuroblast proliferation and differentiation (Ekdahl *et al.*, 2009).

The third class of glial cells, astrocytes, has several different roles that support or modulate neuronal activity. Although they are not electrically excitable (they do not fire action potentials), astrocytes have a negatively charged resting membrane potential and can alter electrical conductance in response to signalling molecules (Oberheim *et al.*, 2012). Astrocytes may express G-protein coupled receptors for several different neurotransmitters, including glutamate, GABA (γ-amino butyric acid) and noradrenaline, and they can alter the activity of neurons, microglia, blood vessels and other astrocytes through the release of gliotransmitters (Oberheim *et al.*, 2012). Finally, astrocytes are also well known for their role in termination of synaptic signalling by reuptake of some neurotransmitters including glutamate (Ransom & Ransom, 2012). Consequently, astrocytes are not just important for the supply of nutrients to neurons, as previously thought, but also participate in local signalling and regulate neuronal signalling within the central nervous system.

1.1.1.2 Synapses and Receptors

Neurons communicate with each other through direct electrical synapses or via chemical synapses (Nestler *et al.*, 2001). An electrical synapse involves the transfer of ions (current) from one neuron to another. At a chemical synapse the two neurons are separated by a physical gap, the synaptic cleft (Figure 1.5). With a chemical synapse, the cellular response is determined by both the type of neurotransmitter released by the presynaptic cell and the type of receptor on the postsynaptic cell (Nestler *et al.*, 2001). Chemical synapses are the focus of

most research as they are both the most numerous type of synapse in the brain and provide the greatest opportunity for pharmacological intervention.

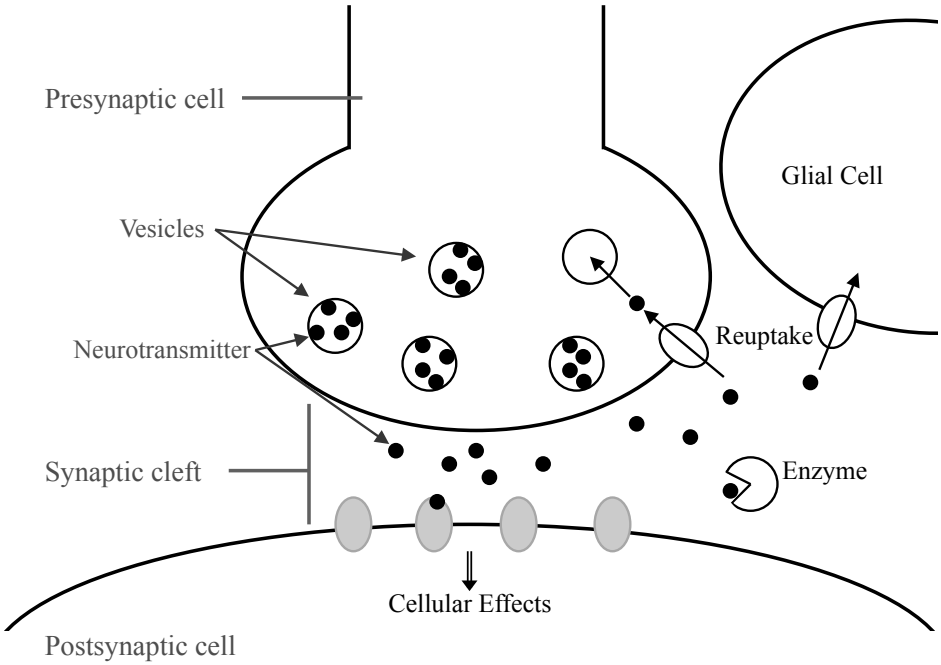


Figure 1.5 Chemical Synapse.
A schematic of a chemical synapse showing the presynaptic neuron, postsynaptic cell and synaptic cleft. Stimulation of the presynaptic neuron results in release of neurotransmitter from vesicles that then stimulate the postsynaptic cell. Signalling is terminated by removing the neurotransmitter from the synaptic cleft, which may be by enzymatic degradation or reuptake.

In addition to synaptic signalling, local and hormonal signalling also occurs in the brain and three main groups of receptors produce the cellular effect(s) of these signalling molecules. The shortest acting receptors are ligand-gated ion channels (ionotropic receptors), which allow ion flux in response to chemical messengers binding (Rang *et al.*, 1999). The longest acting are the cytoplasmic/nuclear receptors, which form part of a transcription factor complex, resulting in gene up- or down-regulation (Rang *et al.*, 1999).

The final group of receptors is the metabotropic receptors; these are predominantly G-protein coupled receptors. These receptors associate with and activate G-proteins, resulting in a cascade of events that produce the changes within the cell (Nestler *et al.*, 2001). Activated G-proteins, in turn, activate or inhibit enzymes and ion channels from inside the cell, an example of which is shown in Figure 1.6. G-proteins are usually described based on the function of their α subunit and are typically grouped into four families: the G_s family stimulate adenylyl cyclase (Figure 1.6); the G_i family inhibits adenylyl cyclase; the G_q family

activates phospholipase C; and the G_{12} family interacts with Rho to produce cytoskeletal changes (Birnbaumer, 2007). Each of these actions can have a variety of cell specific downstream effects, creating a wide range of action for activated G-proteins.

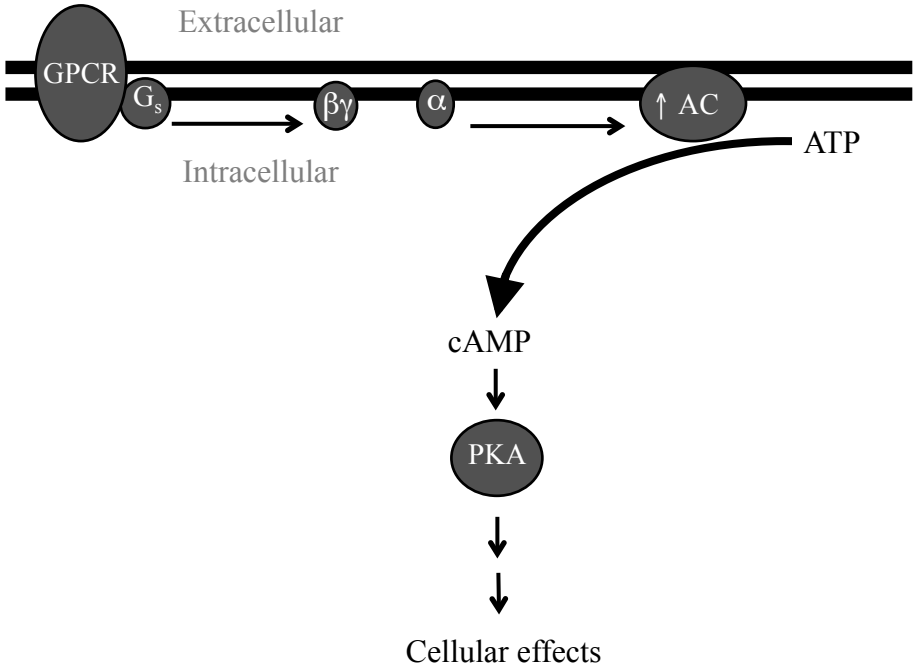


Figure 1.6 Activation of Adenylyl Cyclase by G_s.
The G-protein coupled receptor (GPCR) associates with and activates the G-protein G_s, which causes G_s to dissociate into its α and β/γ subunits. The alpha subunit of G_s activates the enzyme adenylyl cyclase (AC), which catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). A cascade of events is then set off, including the activation of protein kinase A (PKA), that produces specific cellular effects.

Many signalling molecules activate more than one type of receptor. For example, glutamate, the major excitatory neurotransmitter in the brain, activates at least three different types of ionotropic receptors and eight types of metabotropic receptors (Nestler *et al.*, 2001). Hence, the effects of an individual neurotransmitter will vary from cell to cell, depending on the receptors that the target cell expresses. The involvement of specific neurotransmitters in mental disorders will be discussed further in section 1.4.3 (page 51).

1.1.2 Defining Mental Illness

Mental illnesses are disorders of the mind that may manifest as emotional or behavioural abnormalities. There is a wide range of emotional and behavioural variation between people as well as within an individual throughout their lifetime and it would be unreasonable to

consider all of this variation to be "disorder". Typically the distinction between normal emotion and mental disorder is defined by the distress of the individual or by the effect on function – if a change in emotion or behaviour is sufficiently severe to have a negative impact on the individual's daily life it is considered a disorder (American Psychiatric Association, 1994).

A number of different classification systems exist for mental disorders, all of which are artificial constructs based on the current scientific knowledge. Consequently, some patients will not fit ideally into any category, and the systems are regularly re-written as our understanding increases, in an attempt to cluster symptoms in a more meaningful way. One of the most commonly used classification systems is the diagnostic and statistical manual of mental disorders (DSM), which was up to the 4th edition (DSM-IV) when this study was conducted (American Psychiatric Association, 1994). The DSM-IV classifies mental disorders into 17 major diagnostic categories, including:

Substance-Related Disorders – related to use, dependence, abuse, intoxication or withdrawal of drugs, including legally available drugs, such as alcohol or prescription medications, as well as illicit drugs.

Schizophrenia and Other Psychotic Disorders – these are grouped together due to the presence of psychotic features: delusions, hallucinations, disorganised speech, disorganised behaviour or catatonia.

Mood Disorders – grouped into depressive disorders, defined by a reduction in mood, and bipolar disorders, which feature both reduction and elevation (mania or hypomania) in mood.

Anxiety Disorders – a range of disorders that all involve acute and/or chronic anxiety, panic attacks and persistent worry or changes in behaviour intended to reduce apprehension respectively.

1.1.3 Population Mental Health

The World Mental Health Initiative was launched by the World Health Organization to produce statistically robust data on the prevalence of mental disorders following criticisms of the Global Burden of Disease Study (Kessler *et al.*, 2006). The World Mental Health Surveys consisted of multiple large cross-sectional population surveys conducted in over 20 countries throughout the world. Included amongst these is the New Zealand Mental Health Survey,

which was conducted in New Zealand during 2003 and 2004 (Oakley Browne *et al.*, 2006a). These surveys have added a considerable amount of data about mental health globally and will be the main source of information for the discussion below.

1.1.3.1 Prevalence

The New Zealand Mental Health Survey found that mental disorders are very common in New Zealand. The overall twelve-month prevalence was estimated at approximately 20% and New Zealand was among the top four, out of 15 countries surveyed, for twelve-month prevalence of anxiety, mood and substance-related disorders (Wells *et al.*, 2006). Similarly, the projected lifetime prevalence of any mental disorder was estimated to be just below 50% and lifetime prevalence rates in New Zealand of any anxiety, any mood or any substance-related disorders were each in the top four out of 17 countries (Kessler *et al.*, 2007b).

The global and New Zealand twelve-month prevalence estimates are shown in Table 1.1. Lifetime prevalence of any disorder ranged from 12% (Nigeria) to 47.4% (USA), with overlapping ranges of prevalence rates seen among developed and developing nations (Kessler *et al.*, 2007b). There are several possible reasons for the differences in prevalence between countries. As Kessler *et al.* (2007b) described, although the study designs were intended to be comparable, there may have been bias introduced by small differences in the way the questionnaires were conducted in different countries. There may have also been non-response bias. The response rates ranged from 45.9% to 87.7%, with 73.3% response New Zealand (Kessler *et al.*, 2007b). While there was no obvious correlation between response rate and prevalence, if the main reasons for non-response varied by country, this could have added bias. It is also possible that participants felt less comfortable endorsing symptoms of mental illness in the nations with lower rates. However, a study that compared major depressive episode rates between developed and developing nations found no evidence for this (Bromet *et al.*, 2011), so it seems unlikely to have contributed to the overall estimates.

It is also possible that the overall rates are similar, but that culture-specific disorders were missed in many nations (Rosenman, 2012). This is supported by the highest lifetime prevalence being found in the USA, the country where the DSM originates, but not by the high rates in Colombia (third highest prevalence and second highest projected lifetime risk for any disorder) (Kessler *et al.*, 2007b). Consequently, it seems likely that prevalence

differences between nations are real, though the magnitude found in the World Mental Health Surveys may be exaggerated. While the cause is unknown, a wide variety of environmental factors that may have contributed. For example, pesticide exposure might increase the risk of depression (Freire & Koifman, 2013), and the association between major depressive episodes with demographic factors, such as age and income, varied between countries (Bromet *et al.*, 2011).

Like most other nations, anxiety disorders were found to be the most common mental disorders in New Zealand, followed by mood disorders (Demyttenaere *et al.*, 2004; Wells *et al.*, 2006). Prevalence rates of psychotic disorders in the general population are difficult to obtain as questionnaires that are not administered by appropriately trained health professionals have been found to overestimate diagnoses (Kendler *et al.*, 1996; Kessler *et al.*, 2005). Consequently, psychotic disorders were not measured in the World Mental Health Surveys. Other studies have found the prevalence of psychotic disorders such as schizophrenia to be low both in New Zealand (Kake *et al.*, 2008) and overseas (Kessler *et al.*, 2005). However they contribute disproportionately to severe mental illness, with high rates of admission, re-admission and long length of stay found at acute psychiatric units in Auckland (Wheeler *et al.*, 2011; Wheeler *et al.*, 2005).

Various groups of people are more likely to experience a mental disorder. In New Zealand, the twelve-month prevalence rates of psychiatric disorders were generally highest in the youngest age group (16-24 years), with the exception of some anxiety disorders and eating disorders (Wells *et al.*, 2006), and an early age of onset for psychiatric disorders is supported by international research (Kessler *et al.*, 2007a). There is also an over-representation of females for most types of anxiety and mood disorders (Table 1.1), while males were significantly over-represented in the substance-related disorders (Seedat *et al.*, 2009; Wells *et al.*, 2006). Lower education levels and lower household income were similarly associated with increased occurrence of mental disorders in the New Zealand Mental Health Survey, as was Māori ethnicity (Oakley Browne *et al.*, 2006a) (see also section 1.1.3.4, page 13).

Table 1.1 Twelve-Month Prevalence (%) of Mental Disorders

Disorder	All NZ (95% CI)	Male : Female	Māori[1] (95% CI)	Global
Any anxiety	14.8 (13.9-15.7)	10.7 : 18.6	19.4 (17.2-21.8)	2.4–18.2
Panic	1.7 (1.4-1.9)	1.3 : 2.0	2.6 (2.0-3.5)	
Agoraphobia[2]	0.6 (0.5-0.8)	0.4 : 0.8	1.0 (0.6-1.5)	
Specific phobia	7.3 (6.8-7.8)	4.3 : 10.1	11.0 (9.6-12.6)	
Social phobia	5.1 (4.6-5.6)	4.5 : 5.6	6.2 (5.1-7.4)	
Generalised anxiety	2.0 (1.7-2.3)	1.4 : 2.6	2.2 (1.6-2.9)	
Post-traumatic stress	3.0 (2.6-3.4)	1.6 : 4.2	4.5 (3.6-5.7)	
Obsessive compulsive	0.6 (0.4-0.9)	0.7 : 0.5	1.0 (0.6-1.6)	
Any mood	8.0 (7.4-8.6)	6.3 : 9.5	11.4 (10.0-13.1)	0.8–9.6
Bipolar	2.2 (1.9-2.5)	2.1 : 2.3	4.6 (3.7-5.7)	
Major depressive	5.7 (5.2-6.2)	4.2 : 7.1	6.9 (5.8-8.1)	
Dysthymia	1.1 (0.9-1.4)	1.0 : 1.3	1.2 (0.8-1.7)	
Any substance-related	3.5 (3.1-4.0)	5.0 : 2.2	8.6 (7.1-10.4)	0.1–6.4
Alcohol abuse	2.6 (2.3-3.0)	3.7 : 1.6	6.7 (5.5-8.1)	
Alcohol dependence	1.3 (1.1-1.5)	1.7 : 0.9	3.9 (3.0-5.0)	
Drug abuse	1.2 (0.9-1.4)	1.6 : 0.8	3.7 (2.8-4.8)	
Drug dependence	0.7 (0.5-0.9)	1.1 : 0.4	1.9 (1.3-2.8)	
Schizophrenia	0.45	0.56 : 0.34	1.06	~0.3
Any disorder	20.7 (19.5-21.9)	17.1 : 24.0	29.5 (26.7-32.5)	4.3–26.4

1. Māori is the indigenous population of New Zealand. For these, and most other surveys, Māori ethnicity was self identified, and does not have strict formal definitions, such as proportion of grandparents that were Māori.
2. Agoraphobia without comorbid panic disorder.
Global range based on Demyttenaere *et al.* (2004), except schizophrenia, based on Kessler *et al.* (2005). New Zealand (NZ) data based on Wells *et al.* (2006) and Māori data based on Baxter *et al.* (2006a); except schizophrenia: calculated based on data in Kake *et al.* (2008).

1.1.3.2 Impact

Mental disorders affect multiple aspects of a person's life. The New Zealand Mental Health survey measured self-reported "interference with life" in the areas of: home, work/study, intimacy, and social life. For the worst month within the past twelve-months, the effects of disorders generally averaged in the moderate range (Wells *et al.*, 2006). There were a few exceptions to this, such as phobic disorders tending to have mild impact and bipolar disorder involving both a depressive and a manic/hypomanic episode during the month having severe impact (Wells *et al.*, 2006). This is supported by global findings that most individual mental disorders resulted in the sufferer being unable to perform their normal function for an average of at least five days per year (Alonso *et al.*, 2011).

Given the high prevalence rates and moderate interference with all aspects of life, it should come as no surprise that mental disorders rated highly on the Global Burden of Disease. Neuropsychiatric disorders were the leading cause of years of healthy life lost to disability,

with depressive disorders the highest amongst the specific disorders (World Health Organization, 2008). Despite the relatively low prevalence, both schizophrenia and bipolar disorder were among the top ten causes of years of healthy life lost to disability globally (World Health Organization, 2008). This highlights the severity and importance of these disorders.

1.1.3.3 Comorbidity

Comorbidity of mental disorders is very common. The New Zealand Mental Health Survey found that approximately 21% of affected New Zealanders experienced two mental disorders and 16% experienced three or more disorders within twelve-months (Scott *et al.*, 2006a). Of the three most common groups of disorder (anxiety, mood and substance-related), mood and anxiety were most likely to co-occur, with approximately 50% of those with a mood disorder also experiencing an anxiety disorder during the twelve months prior to the survey (Figure 1.7) (Scott *et al.*, 2006a). When looking at specific disorders, significant associations were seen for most pairings. The strongest pairings of disorders were seen between the different substance-related disorders and between agoraphobia (without panic) and social phobia (Scott *et al.*, 2006a). Hence, high rates of comorbidity occur both between and within the three most common categories of mental disorder.

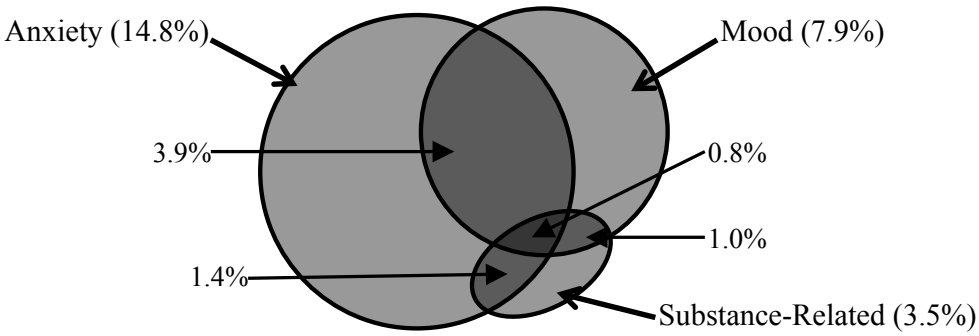


Figure 1.7 Comorbidity Between Common Psychiatric Disorders.
A diagrammatic representation of twelve-month comorbidity in New Zealand of the three most common categories of psychiatric disorder. Based on Scott *et al.* (2006a).

Mental disorders are also commonly comorbid with physical conditions. In the New Zealand Mental Health Survey significant associations were seen for anxiety, mood and substance-related disorders with both chronic pain and respiratory conditions, and for anxiety with high blood pressure and cardiovascular conditions (Scott *et al.*, 2006b). People with anxiety,

mood, or substance-related disorders were more likely to smoke and drink alcohol in a potentially hazardous way, and those with anxiety disorders were also more likely to be obese (Scott *et al.*, 2006b), all of which are risk factors for physical disease. These results were supported by international studies (Buist-Bouwman *et al.*, 2005; Ortega *et al.*, 2006; Scott *et al.*, 2007).

1.1.3.4 Indigenous Mental Health

Minority groups, particularly indigenous populations, are often over-represented among mental disorder statistics (Anderson & Whyte, 2008; Beals *et al.*, 2005; Vos *et al.*, 2007). Within New Zealand, Māori are both the indigenous and the largest minority population and show the expected higher levels of mental disorders seen in many other indigenous populations (Table 1.1, page 11). There are a number of possible reasons for this over-representation, including causes that are difficult to quantify such as disconnect from land and culture, which can reduce ones sense of identity and impair a positive self-image (King *et al.*, 2009). Demographic factors, socioeconomic factors and perceived racism have been studied and will be discussed below. It is also possible that genetic factors may contribute to ethnic differences in prevalence, however, indigenous groups have not been adequately studied in psychiatric genetic research, preventing clear conclusions from being made (Oquendo *et al.*, 2010). Additionally, differing cultural attitudes, beliefs and behaviours between patients and clinicians can result in over- or under-diagnosis of disease (Rosenman, 2012).

Māori generally show similar patterns of mental disorder affliction as the majority of New Zealand, but with higher rates for each disorder (Table 1.1). Part of this increased prevalence is due to demographic differences, such as the different age structure of the Māori population. On average, Māori are younger than the total population of New Zealand (Statistics New Zealand, 2006), and younger age groups are at increased risk of having a mental disorder (Wells *et al.*, 2006). When data were adjusted for age and gender, the proportion of Māori affected with any twelve-month mental disorder declined from 29.5% to 26.4% (Baxter *et al.*, 2006b), compared to 20.7% for New Zealanders overall (Figure 1.8) (Wells *et al.*, 2006).

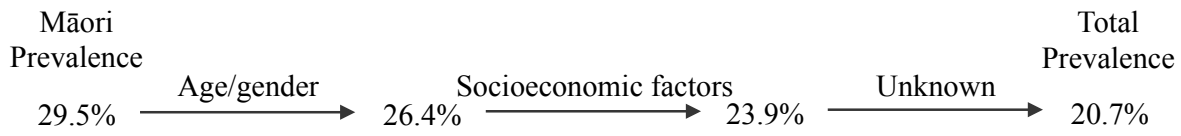


Figure 1.8 Factors in Prevalence Differences.
Twelve-month prevalence of any mental disorder in the New Zealand Mental Health Survey was higher for Māori, compared to the overall (total) New Zealand prevalence. This difference was reduced by adjustment for demographic and socioeconomic factors, but was not eliminated. Unknown signifies factors not quantified in the New Zealand Mental Health Survey, such as experience of racism. Based on data from Wells *et al.* (2006) and Baxter *et al.* (2006b).

Social and economic risk factors for mental illness are also more prevalent among Māori. Māori are less likely to have formal qualifications and more likely to earn below the median income than non-Māori, non-Pacific New Zealanders (Baxter *et al.*, 2006b). These factors are both associated with increased occurrence of mental disorder, though the direction of association is ambiguous. It is likely that effects are bidirectional with the socioeconomic factors increasing the risk of developing a mental disorder and that suffering from a mental disorder increases the risk of adverse socioeconomic outcomes via a reduced ability to work or study full-time (Oakley Browne *et al.*, 2006b). Adjusting for these factors in a cross-sectional survey such as the New Zealand Mental Health survey is, therefore, likely to result in an underestimation of the effects of remaining factors. Despite this, after adjustment for age, sex, educational qualifications and household income, Māori were still more likely to experience any twelve-month disorder (Figure 1.8) and each of the most common groups of lifetime disorders (anxiety, mood and substance disorders) than other New Zealanders (Baxter *et al.*, 2006b; Oakley Browne *et al.*, 2006b). Hence, demographic and socioeconomic factors are contributors to the differences between Māori and non-Māori New Zealanders, but these do not fully explain the variation.

Experience of racism has been found to be significantly associated with poor mental health. In New Zealand, a health survey conducted from 2002 to 2004 found that Māori experienced the highest rates of discrimination at 34% and overall discrimination was associated with worse mental health with an odds ratio of 1.77 (Harris *et al.*, 2006b). Māori experienced between two and thirteen times as much discrimination as European New Zealanders and had significantly worse mental health than Europeans, with an odds ratio of 1.3 after adjustment for age and sex (Harris *et al.*, 2006a). However, further adjustment for deprivation or racial discrimination produced non-significant differences, while adjusting for both eliminated any difference, giving an odds ratio of 1.02 (Harris *et al.*, 2006a). Racism and other forms of

discrimination are likely to contribute to negative mental health outcomes through the stress response and adverse effects on self-esteem (Pascoe & Smart Richman, 2009).

Minority groups, including the Māori population, share a number of risk factors for mental illness. While direct biological factors cannot be ruled out, the studies above indicate that most of this variation can be attributed to psychological and societal level risk factors.

1.1.4 Reducing Mental Illness

Methods to reduce mental illness may include strategies intended to shorten the duration of disease and strategies to prevent the occurrence or re-occurrence of disease. Like the pathogenesis, the strategies to reduce mental illness can be related to a biopsychosocial model, in which pharmacotherapy targets biological factors and psychotherapy targets psychological factors of individuals, while social changes can reduce population risk.

1.1.4.1 Pharmacotherapy

Pharmacotherapy consists of short- or long-term drug treatment that aims to correct underlying biochemical or neurological abnormalities that cause a mental disorder. Four main types of pharmacotherapy exist: antipsychotics, antidepressants, anxiolytics and mood stabilisers (Carson *et al.*, 2000). Although these drug groups are named by the first type of disorder they were used to treat, many are now also used for other disorders. For example, while anxiolytics are the usual treatment for acute anxiety/panic, antidepressants are commonly used to treat chronic anxiety (Zohar & Westenberg, 2000) and low doses of antipsychotics are also increasingly being used for anxiety disorders (Comer *et al.*, 2011; Ravindran & Stein, 2010).

The exact mechanism of action of most of these drugs is not known. The best understood are the quick acting anxiolytics, which are predominantly of the benzodiazepine class of drugs. These agents bind to $GABA_A$ receptors in the brain, enhancing the receptor function upon GABA binding. Since GABA is inhibitory, it is hypothesised that this increased GABA activity reduces signalling in fear pathways (Nestler *et al.*, 2001). Antidepressants and antipsychotics generally target other neurotransmitter systems within the brain, particularly serotonin, dopamine and noradrenaline, altering the signalling pathways (Rang *et al.*, 1999).

However, it is not known whether the effects of these drugs on neurotransmitter systems are necessary or sufficient to produce the therapeutic responses, or if other effects are responsible for the outcome (Petrik *et al.*, 2012). The current understanding of the effects of antidepressants in anxiety is discussed further in section 1.4.4.2 (page 61).

1.1.4.2 Psychotherapy

Psychotherapy includes a variety of treatments that focus on breaking the negative behaviour or thought patterns that often occur in mental disorders. Cognitive-behavioural therapy is one of the most commonly used forms of psychotherapy today and uses a combination of behavioural and cognitive interventions to encourage beneficial thoughts, feelings and behaviours (Carson *et al.*, 2000; Leichsenring *et al.*, 2006). These interventions may include techniques such as systematic desensitisation to anxiogenic (anxiety increasing) stimuli, positive reinforcement, social skills training and stress management (Leichsenring *et al.*, 2006) and have been found to be effective in the treatment of mood, anxiety, psychotic and eating disorders (Butler *et al.*, 2006).

In recent years, modern technology has changed the delivery of cognitive-behavioural therapy with the addition of online computer based programmes that require less clinician involvement. Studies suggest that computer based programmes produce similar therapeutic responses to other treatments (Griffiths *et al.*, 2010) and have high satisfaction rates (Ruwaard *et al.*, 2012). These changes are important as they can reduce the cost and improve accessibility of psychotherapy.

1.1.4.3 Electroconvulsive Therapy

Perhaps the most extreme form of therapy for mental illness is electroconvulsive therapy. In this treatment, the patient is sedated and an electric current is passed through their head in an attempt to reset the electrical signalling within their brain (Carlson & Buskist, 1997). electroconvulsive therapy is usually reserved for patients with severe, treatment resistant mood or psychotic disorders. Although this is not used for the treatment of anxiety disorders, anxiety symptoms among patients being treated for depression were significantly improved after electroconvulsive therapy (Bayless *et al.*, 2010; Langius-Eklof & Samuelsson, 2009), suggesting a broad spectrum of therapeutic benefit. Like most of the other treatments for

mental illness, the exact mechanism through which electroconvulsive therapy works is unknown.

1.1.4.4 Prevention

Prevention of mental illness may be targeted at individuals or at broader population risk factors, such as socioeconomic status or perceived racism. This section will focus on strategies targeted at individuals as detailed discussion of population risk factors is beyond the scope of this research. Two main groups of people may benefit from preventive strategies: those that have previously had a mental disorder and are at risk of relapse, and those at high risk for developing a new disorder.

Most research has focused on preventing relapse of patients following "recovery" from mental illness, during which many patients continue to experience subclinical disease symptoms. Continuation of existing therapy during this recovery period may be necessary (Glue *et al.*, 2010; Oestergaard & Moldrup, 2011) and patients may benefit from the addition of prevention focused psychotherapy. Well-being therapy and some types of cognitive-behavioural therapy have been found to be effective in reducing residual symptoms and preventing relapse, particularly for anxiety and depressive disorders (Chiesa & Serretti, 2011; Ruini & Fava, 2012)

Increasingly, research has focused on preventing first episodes of disease among high-risk groups. Most of this research is still in the early phases; even in post-traumatic stress disorder, where an obvious risk group exists, only a small number of studies have been conducted, with the strongest support for cognitive-behavioural therapy that begins within weeks of the event (Agorastos *et al.*, 2011). Panic attacks were also found to be preventable with targeted cognitive-behavioural therapy in patients with respiratory problems, with four sessions of cognitive-behavioural therapy significantly reducing the risk of a panic attack in the next 18 months, compared to those that received standard treatment (Livermore *et al.*, 2010). This suggests that identification of risk groups may allow early, targeted intervention to prevent the development of disorder.

1.1.5 Summary

- Emotions and behaviour originate from signalling in multiple parts of the brain. Deep nuclei such as the amygdala are important in the formation of emotions. Other regions, including the insular cortex and hypothalamus, relate bodily sensations such as heart rate to emotions; and the prefrontal cortex can suppress unwanted emotions.
- At a cellular level, the brain activity that causes emotions is produced by signalling between neurons, which involves neurotransmitters, receptors and the normal function of support cells. These are often the targets of pharmacotherapy.
- Mental disorders include a wide variety of conditions that manifest as emotional or behavioural abnormalities. Mental illness has a large impact on global health and affects a high proportion of New Zealanders, with anxiety disorders being the most common form of mental illness.
- The current understanding of the pathogenesis of mental disorders is incomplete, though both environmental and genetic factors have been implicated.
- Although a variety of treatments exist, including psychotherapy and pharmacotherapy, the mechanism of action of most drugs is poorly understood and many patients fail to fully respond to treatment.

1.2 Genetics and Complex Diseases

Mental disorders are complex diseases with a mixture of genetic and environmental predisposing factors. This section discusses the techniques used to determine the genetic contribution to a disorder, methods to identify specific genetic elements and some of the issues with these approaches.

1.2.1 Determining Heritability

To understand who is most at risk of developing a complex disease, the relative contribution of genetic and environmental effects need to be determined. This is more difficult than it sounds as some environmental influences begin very early in life, even *in utero*, while genetic variation can influence all aspects of life, including the environment that the individual resides in. Various methodologies are used to try to determine the genetic contribution of a disease, each with their own advantages and disadvantages.

Before the degree and nature of a genetic contribution to disorder can be investigated, there must first be proof of any genetic involvement. The first studies to provide evidence of this are usually family studies, which ask the question "do biological relatives of an affected individual have an increased risk of developing the disorder" (Carson *et al.*, 2000). If the answer to this question is "no", then it is unlikely that there is any genetic contribution to the disorder. If biological relatives do have an increased risk, this may be due to genetic factors, shared environment, or a combination of both. In which case, further research that can distinguish these should be undertaken.

Adoption or twin studies can identify the relative contribution of genes and shared environment. Theoretically, in adoption studies most risk factors for disease should be similar to those in family studies, but without shared environmental factors. Hence, if biological relatives that have been adopted out of a risk family remain at increased risk, this should be due to genetic factors (Carson *et al.*, 2000). However, there are other possibilities, such as very early (*in utero*) environmental risk factors, and many adoption studies have used retrospective data collection that may have introduced bias (Reiss & Leve, 2007). In twin studies, on the other hand, risk of disorder in monozygotic (identical) and dizygotic (fraternal) twins is compared. In these studies shared environment becomes a constant, while genetic similarity is approximately 50% for dizygotic twins and 100% for monozygotic twins. There are still potential biases, such as the possibility that monozygotic twins are treated more similarly than dizygotic twins; twins that are reared apart would be the ideal study group, but this seldom occurs, so sample sizes would be small (Carson *et al.*, 2000).

While adoption and twin studies can give an indication of the degree of genetic risk, determining the relative contribution of genetic and environmental influences is made more difficult when genetic and environmental factors are not independent. For example, an infant's development is influenced by the parenting style of their primary caregiver (an environmental factor); however, this parenting style is influenced by the child's behaviour, which, in turn, is partly influenced by the child's genes (Boivin *et al.*, 2005). Whether this interaction in the influence of parenting style on a child's outcomes is considered as an environmental or a mixed environmental/genetic factor would depend on the design of the study.

While each type of study has its limitations, when multiple different study types support genetic involvement this is the most likely explanation.

1.2.2 Identification of Heritable Elements

Once a genetic contribution to a disorder has been established further studies are typically conducted to try to identify the specific genetic element(s) involved. Different approaches are used depending on the type of cohort and the region of the genome under investigation. Each of these has advantages and disadvantages, as discussed below.

1.2.2.1 Case-Control or Family-Based Analysis

One of two main approaches is typically used in the recruitment of participants to a genetic association study: case-control, or family-based. In case-control studies, allele frequencies in a cohort of affected individuals (cases) are compared to unaffected individuals from the same population (controls). These studies test for an association between the disease and variant. In family-based studies, affected individuals (probands) and their relatives are recruited and a transmission disequilibrium test is used to determine whether the probands are more likely to have inherited particular alleles than would be expected by chance. Family-based studies test for association in the presence of linkage. Additionally, longitudinal studies that follow individuals over a long period of time, often from birth, may also include a genetic component.

Most genetic association studies have used a case-control design. This design makes it easier to recruit participants, compared to family-based studies, but careful selection of the control cohort is required to avoid false-positive results due to population stratification. In contrast, family-based studies do not require a control cohort, reducing false-positive results (Laird *et al.*, 2000). Another benefit of this is that ethnic minorities do not need to be actively excluded from the analysis, thus increasing the sample size and potential generalisability of the results. However, the need to recruit and genotype multiple members of each family can be a prohibitive factor. Case-control studies became predominant after a study found linkage had less power to detect moderate effect sizes than association studies (Risch & Merikangas, 1996). However, more recent studies have challenged the dominance of case-control studies,

noting that family-based designs have greater ability to detect rare variants, and promoting combination approaches (Ott *et al.*, 2011; Zhu & Xiong, 2012).

1.2.2.2 Candidate Genes

One way of identifying specific heritable elements is to investigate genes that are part of systems or pathways that have been found to be involved in the disorder. These studies may include biochemical changes in patients with the disorder, pathways associated with drug response, or even findings from experimental animals that support the involvement of proteins within a particular pathway. Once a candidate gene has been identified, the frequency of variation within the gene can be compared using case-control, or family-based designs.

Once selected, the candidate gene can be investigated in varying levels of detail. The entire gene, including some surrounding DNA (deoxyribonucleic acid), can be sequenced in all individuals being studied, thus providing detailed information of all variants present in that cohort. While this may be the best method for identifying rare causative variants and new mutations (Myers *et al.*, 2011), it is also relatively expensive, so it is not often done in the first instance. At the other extreme of the scale, some studies have only investigated one variant within the candidate gene, usually a variant with hypothetical support for a functional effect, such as a change in the promoter or protein coding regions of the gene. While this has had some success, this approach has a high risk of missing relevant genetic associations (Lin *et al.*, 2007). Most studies work between these two extremes, investigating several variants within the gene, with each variant included increasing the probability of finding both true and false associations.

1.2.2.3 Linkage and Genome-wide Association Studies

An alternative to the candidate gene approach is to search the entire genome, or large regions of it, to try to narrow down the location of a genetic association. This does not require any previous knowledge about the mechanisms involved in the pathogenesis of the disease and may even result in the discovery of new candidate pathways. Both of the methods described below are based on linkage – that portions of chromosomes are usually inherited together and

the resulting pattern of co-segregating variants allows a small number of variants to be used to represent most common variation in an area.

The early versions of this research were linkage studies. These studies used the transmission within families of a number of sites of genetic variation (loci) to narrow down the location of the causative variant(s). While there has been some success with linkage studies (Gelernter *et al.*, 2004; Sklar, 2002), limits to the number of loci used meant that only a small proportion of common variation was covered. Additionally, the ability to detect risk alleles that only have a small contribution to disease risk is low (Domschke & Deckert, 2010; Risch & Merikangas, 1996); consequently, linkage studies have had limited success in complex disease (Manolio *et al.*, 2009).

Genome-wide association studies have become increasingly common as genotyping cost has decreased and efficiency has improved. These studies include far more variants than linkage studies and aim to cover most common variation (typically variants with a minor allele frequency of 5% or higher). Conversely, this increased number of variants also increases the probability of false positive findings. This can be controlled by adjusting the significance threshold, but requires large numbers of subjects to have sufficient power to find true associations (Liu *et al.*, 2008). The large sample size presents a technical challenge for researchers trying to recruit participants, and may increase the heterogeneity of the resulting cohort (Liu *et al.*, 2008). Thus, the large numbers of variants investigated in genome-wide studies can be beneficial or detrimental, depending on the nature of the genetic risk.

1.2.2.4 Haplotypes and the HapMap Project

Knowledge of the human genome has increased substantially in recent decades. From the sequencing of the first human genome in the early 2000s (International Human Genome Sequencing Consortium, 2004), there are now thousands of sequences available. Two of the largest studies in this area are the International HapMap Project, which focused on phase data for variants with a minor allele frequency greater than 5% (International HapMap Consortium, 2003), and the more recent 1000 Genomes Project, which used high-throughput sequencing with the aim of identify 95% of variants with a minor allele frequency greater than 1% (1000 Genomes Project Consortium, 2010). Since HapMap data was used as part of the present project, this is the focus of the discussion below.

Information gathered as part of the International HapMap Project has aided in the design of both the candidate gene and genome-wide genetic association studies. Haplotypes are sets of alleles that are located within a region of a chromosome; they show strong linkage disequilibrium and are, consequently, inherited together. Knowledge of haplotype structures allows the identification of tag single nucleotide polymorphisms (SNPs) that, through strong correlation (high R^2 value) with other SNPs, allow the maximum genetic information from a region to be determined with minimum genotyping.

The International HapMap Project was instigated to create a haplotype map of the human genome (International HapMap Consortium, 2003). The project intended to characterise 99% of haplotypes with a frequency of at least 5% in four different populations. These initial study populations were:

CEU – 30 parent-child trios from Utah, USA, collected as part of the Centre d'Etude de Polymorphisme Humain, who have Northern and Western European ancestry.

YRI – 30 parent-child trios from the Yoruba people in Ibadan, Nigeria.

JPT – 45 unrelated Japanese individuals from Tokyo.

CHB – 45 unrelated Han Chinese individuals from Beijing.

The CEU population will be the main focus of further discussion as the ancestry of this population is the most similar to that found in the majority of New Zealanders.

The International HapMap Project has been improving haplotype knowledge since its inception. The first phase of the project genotyped just over one million SNPs spaced roughly evenly across the genome (International HapMap Consortium, 2005). The second phase added additional SNPs from regions with low linkage disequilibrium, as well as non-synonymous coding region SNPs. The combined data from these two phases that met quality control standards revealed over three million SNPs that were polymorphic in at least one of the four populations studied (International HapMap Consortium, 2007). Within the CEU population, just over 500 000 SNPs needed to be genotyped to capture all the common variation; in other words, all SNPs with a minor allele frequency of at least 5% would have an R^2 of 0.8 or greater with one of the approximately 500 000 tag SNPs. Since then, the HapMap project has advanced further by including additional populations and different types of genetic polymorphism such as copy number variants (International HapMap 3 Consortium, 2010).

One of the main goals of this detailed characterisation of haplotypes was to reduce the number of variants that would need to be genotyped directly in an association study, without compromising the statistical power to detect an association. A large number of papers have investigated this premise by taking samples genotyped at most SNPs within a region and comparing the coverage of these SNPs with that produced by HapMap derived tag SNPs only. These studies have all shown some reduction in coverage of common variation by using HapMap data to select tag SNPs (Lundmark *et al.*, 2008; Smith *et al.*, 2006; Stathias *et al.*, 2012). However, this was usually in a similar range to that seen if a subset of the study population was used to generate tag SNPs (Mueller *et al.*, 2005; Service *et al.*, 2007; Stankovich *et al.*, 2006), supporting the utility of HapMap data in genetic association studies.

There are some caveats regarding the use of HapMap data to approximate other populations. Although tag SNP coverage is usually high, correlation with other factors, such as block structure (Liu *et al.*, 2004; Pardo *et al.*, 2009) and haplotype frequencies (Sawyer *et al.*, 2005), have been more variable and some regions or populations fail to show expected tag SNP coverage rates (Lundmark *et al.*, 2008; Mueller *et al.*, 2005). The similarity with the HapMap population also needs to be considered; for example, an Estonian sample was well covered by CEU tag SNPs, but not by tag SNPs selected from the combined Asian (JPT and CHB) populations (Montpetit *et al.*, 2006). Hence, the HapMap cohort that is most similar in ancestry to the target cohort should be used. If in doubt, the YRI sample can give good coverage of non-African test samples, but requires high numbers of tag SNPs to be genotyped (Montpetit *et al.*, 2006). Finally, HapMap data is not likely to be useful in the analysis of rare variants, as the correlation between different populations becomes increasingly poor with declining minor allele frequency (Montpetit *et al.*, 2006; Willer *et al.*, 2006; Zeggini *et al.*, 2005). This is one of the major disadvantages of the HapMap data since fitness reducing disease associated variants would be expected to be over-represented among rare variants (Gu *et al.*, 2008).

1.2.3 Missing Heritability

Currently, for most complex diseases, the estimated heritability based on adoption and twin studies is many fold higher than the heritability that has been found through genetic association studies, a phenomenon commonly referred to as “missing heritability” (Manolio *et al.*, 2009). Heritability, for this purpose, is defined as the proportion of total variance in a

phenotype that is the result of genetic factors (Visscher *et al.*, 2008). This ratio is fixed for a particular population at a specific point in time, but may vary both between populations and over time as environmental effects change (Visscher *et al.*, 2008), making it difficult to accurately estimate.

The simplest explanation is that the heritability of the disease has been overestimated. While this is possible, it seems unlikely to be the major problem since heritability estimates from a variety of methods tend to be similar for a particular disease (Manolio *et al.*, 2009). The detection of specific heritable factors, on the other hand, varies depending on the type of genetic risk factor involved, as discussed below. For most complex diseases, there is only a very limited understanding of the mechanisms of its inheritance and, without this knowledge, it is more difficult to identify the risk variants, thus creating a probable gap between estimated and identified heritability.

1.2.3.1 Genetic Factors

In the simplest analysis, two factors need to be considered about each genetic factor contributing to a complex disease: how common is the disease causing variant and how much risk does having this variant confer. This produces four different sets of variants: common variants of large effect, common variants of small effect, rare variants of large effect, and rare variants of small effect. A disease may also be caused by any combination of these four risk variants, further complicating research efforts. Rare variants of small effect would be extremely difficult to detect and, by virtue of being low frequency and low risk, individually would not make a significant impact on population risk. The other three types of variants could be detected by various different studies, as discussed below.

The common disease, common variant hypothesis postulates that complex disease that is common in a population is likely to be due to multiple contributing variants, each of which is also common in the population (minor allele frequency of greater than 1%) (Chakravarti, 1999). One of these common variants would not be sufficient to cause disease, requiring additional common variants and/or environmental risk factors to also be present, which is consistent with the lack of Mendelian inheritance of the disorder. This scenario could consist of a small number of contributing variants, each with a large to moderate effect on risk, or it could include a large number of variants, each contributing a small increased risk of disease.

If the variants have a large effect, they should segregate strongly with the disease, making them easy to detect in association studies. Thus, common variants of large effect are unlikely to contribute to "missing heritability". Common variants of small effect, however, would be much more difficult to detect as they will be common in both affected and unaffected individuals. Increasing the cohort size of association studies increases the ability to detect common variants of small effect (Moonesinghe *et al.*, 2008), with meta-analyses of genome-wide studies potentially including more than ten thousand participants (for examples, see Anderson *et al.*, 2011; Arking *et al.*, 2011; de Moor *et al.*, 2012).

Rare variants with moderate to large effect may also contribute significantly to disease risk. One of the main arguments in favour of rare variants is the evolutionary argument that any allele that causes a reduction in fitness should be selected against; this is consistent with findings that SNPs occur at a lower frequency in coding regions than non-coding (Cargill *et al.*, 1999). Statistical modelling has also shown that rare variants can be inherited with one form of a common variant, producing a "synthetic association" that may appear to be an association with the more common variant (Dickson *et al.*, 2010). When such synthetic associations exist, the estimated contribution to disease risk is likely to be lower than the actual risk, accounting for some of the missing heritability. Direct detection of rare variants requires DNA sequencing, which is usually not feasible for the number of individuals that would need to be studied, thus making rare variants difficult to detect.

In addition to the above considerations for individual risk variants, there is also the possibility of interactions with other genes. Most genetic studies in complex disease assume an additive model of genetic interaction, such that each risk variant confers a specific increase in disease susceptibility, regardless of other genetic or environmental risk factors (Manolio *et al.*, 2009). However, from the initiation of transcription, genes and their products usually interact with multiple other molecules in complex molecular pathways – one of the major arguments in favour of gene-gene interactions (epistasis) being common (Moore, 2003). There is theoretical support for epistatic interactions having a large impact on the apparent contribution of a risk allele, particularly for minor alleles (Song *et al.*, 2010). In practise, epistatic interactions are difficult to detect, but new statistical methods are improving this, with one analysis of genome-wide data that found associations with numerous small networks of interacting SNPs, which were not predicted by significant effects of individual SNPs (Hu *et*

al., 2011). Thus, epistatic interactions may potentially explain a significant amount of the missing heritability.

1.2.3.2 Environmental Factors and Population Variation

As well as gene-gene interactions affecting the expression of risk variants, interactions with environmental variables are likely to be important for many complex diseases. The concept that genotype A may perform better than genotype B in one environment, but worse than genotype B in another environment has been around for decades (Haldane, 1946). Gene-environment interactions have been particularly popular in psychiatric genetics as it fits with the diathesis-stress model, in which the affected individual has a pre-existing risk (in this case, genetic) that only results in disease following exposure to environmental stressors (Rende & Plomin, 1992). There are some studies showing support for the importance of gene-environment interactions (Binder *et al.*, 2008; Xie *et al.*, 2012), though, like gene-gene interactions, this is an area where statistical approaches are still developing (Nugent *et al.*, 2011).

As well as interactions between genetic and environmental risk factors, genetic factors can directly influence environmental variables. These are often referred to as gene-environment correlations as, instead of two independent variables interacting to produce the risk, the genetic risk factor directly influences the environmental risk factor (Rende & Plomin, 1992). An example for this relationship is genetic effects on personality, which then influences environment, such as a happy child receiving positive responses from adults or an extroverted child forming strong social networks (Carson *et al.*, 2000). While the existence of these correlations is generally accepted, the contribution they make to phenotypic risk is difficult to quantify and it is difficult to separate these effects from gene-environment interactions (Reiss & Leve, 2007).

Finally, genetic risk variants may differ between populations. This may be associated with specific gene-environment relationships, or it may simply be due to genetic drift or new mutations producing population specific risk alleles. This has been described as “clan genomics”, in which it is argued that most of the risk for common diseases results from variants that are rare in the global population, but may be found at higher rates in specific pedigrees (Lupski *et al.*, 2011). The rare variants proposed by clan genomics are consistent

with negative selection effects and research into associated alleles that have been identified by genome-wide association studies found that these tended to be more recently derived, rarer variants (Lachance, 2010).

For population specific variants, including variants that only act in specific environments, the associations are most likely to be detected in small homogenous cohorts. As cohort sizes increase, so will the genetic and environmental heterogeneity of the participants, which will then dilute population specific effects, making them less likely to be detected. This variation between populations would also explain why variants that were significantly associated in one study often fail to replicate in other studies (Moonesinghe *et al.*, 2008) or show associations in the opposite direction (Lin *et al.*, 2007). Since most association testing is indirect, variable linkage disequilibrium with the causative variant might also present as population specific associations.

1.2.4 Summary

That genetic factors are involved in complex disease has been well established, however, an understanding of the mechanisms of the genetic risk is lacking for most diseases. Studies to identify specific heritable elements have resulted in the conundrum of "missing heritability". Potential major contributors to this and strategies to improve the chance of detecting them are:

- Common variants of small effect – detected by large genome-wide association studies.
- Rare variants of large effect – detected by direct sequencing.
- Epistatic/gene-gene interactions – detected by specialised statistical analysis of association data.
- Gene-environment interactions/correlations – detected by longitudinal studies that monitor environmental variables or association studies with more homogenous populations.
- Population specific variants – detected by association studies that focus on homogenous populations.

These different possibilities are not mutually exclusive; hence, a variety of different techniques are indicated for the future study of complex disease.

1.3 Cholecystokinin System

The cholecystokinin (CCK) system is one potential candidate system for genetic association with anxiety and panic attacks. CCK is a peptide signalling molecule found in various regions of the body. It has two known receptors: the CCK_A receptor, also known as CCK_1 receptor, is predominantly found in the gastrointestinal system; and the CCK_B receptor, also known as CCK_2 or gastrin receptor, is the main receptor in the brain. High homology is seen for these three proteins, with most mammals having an identical carboxy-terminal octapeptides of CCK (de Weerth *et al.*, 1993; Lee *et al.*, 1993; Rehfeld, 2006). These three components comprise the CCK system and are described further below.

1.3.1 Discovery of CCK

In 1928, Ivy and Oldberg described a series of experiments using an intestinal extract that caused contraction of the gall bladder, they proposed naming the substance cholecystokinin: "that which excites or moves the gall bladder". By crossing the arteries of two dogs so that blood passed from one animal to the other, this study also demonstrated that CCK travels in the blood and is, hence, a hormone (Ivy & Oldberg, 1928). Initially, the effects of CCK were thought to be fairly specific for gall bladder contraction, however, later studies also established a role in pancreatic enzyme secretion (Harper & Raper, 1943; Mutt & Jorpes, 1971; Mutt & Jorpes, 1968).

1.3.2 Neuronal CCK

In addition to the hormonal signalling within the gastrointestinal system, CCK is also found in the brain. The first study that detected CCK in the brain tested various human tissues from autopsy with an antibody raised against gastrin (Vanderhaeghen *et al.*, 1975). The highest levels of immunoreactivity were found in the gastric antrum, followed by cerebral lobes and blood. Although anti-gastrin antibodies were used, the brain peptide detected was suspected to be CCK as it was not the size expected for gastrin and, since CCK and gastrin share significant homology, antibodies raised against gastrin often cross-react with CCK. This was later confirmed as CCK (Dockray, 1976), followed by a rapid growth of research into CCK functions in the brain and the realisation that it is one of the most abundant neuropeptides in mammals (Crawley, 1985).

CCK is also found in peripheral nerves, particularly those associated with the gastrointestinal tract. The myenteric plexus, a network of nerves that controls the muscle layers of the gastrointestinal tract, is one region where CCK neurotransmission has been found, acting via CCK_A receptor (Corsi *et al.*, 1994; Schutte *et al.*, 1997). The vagus nerve is another major peripheral nerve and some of the gastrointestinal functions originally attributed to hormonal CCK have been found to be partially mediated by vagal CCK release (Chang *et al.*, 2000). Both types of CCK receptors were also found on vagal neurons in rats and humans (Moriarty *et al.*, 1997). Thus, it has been hypothesised that CCK released from enteroendocrine cells, acting in a paracrine fashion, can activate the myenteric plexus, which then activates vagal afferent neurons. This is supported by the finding that intraperitoneal administration of CCK increases neuronal activation in both the myenteric plexus around the duodenum and in brainstem nuclei associated with the vagus nerve (Cooper *et al.*, 2008). Hence, the vagus nerve may provide a pathway for CCK signalling in both directions between the central and peripheral nervous systems.

Many experiments on CCK's functions in the brain use peripherally administered CCK. This raises the question of whether this administration directly affects the brain or if it acts via peripheral nerves, like vagus. More specifically, does the peripherally administered CCK also cross the blood-brain barrier to directly affect central nerves? Evidence against this includes a study that administered intravenous and intrathecal injections of CCK to dogs and then measured cerebrospinal fluid and plasma levels. Significant changes in the peptide were only detected in the compartment that it was administered to, suggesting that it did not cross the blood-brain barrier (Zhu *et al.*, 1986). It should, however, be noted that the study used relatively crude detection methods and did not test the smaller CCK peptides. The smallest CCK peptide commonly administered, CCK-4, has been tested for uptake into monkey brain. Labelled CCK-4 injected intravenously was detected in various regions of the brain, suggesting that this peptide is small enough to cross the blood-brain barrier (Sandell *et al.*, 1997). Additionally, unidirectional transport of CCK-8 from the cerebrospinal fluid to plasma has been found in rabbits (Passaro *et al.*, 1982), suggesting that there may be some individual or species differences in blood-brain barrier transport of CCK. Hence, it seems that the smallest CCK peptides can cross the blood-brain barrier, slightly larger peptides may be transported out of the brain in some species, and the largest peptides are unlikely to cross at all.

Moreover, CCK does not need to cross the blood-brain barrier to directly affect some central functions. In some regions of the brain the blood-brain barrier is incomplete, allowing large signalling molecules to pass between central and peripheral regions (Ganong, 2000). At least one of these regions, the area postrema (located by the fourth ventricle, within the medulla/lower brainstem), contains CCK nerve fibres (Fodor *et al.*, 1994) and shows changes in activity in response to peripherally administered CCK (Blevins *et al.*, 2009; Cooper *et al.*, 2008). Consequently, this site and others like it may be involved in the initiation of central effects from peripherally administered CCK.

1.3.2.1 CCK System Expression in the Brain

CCK is widely distributed within the central nervous system and both CCK_A and CCK_B receptors are also found in the brain. In the human central nervous system, levels of *CCK* messenger ribonucleic acid (mRNA) are highest in some spinal and brainstem nuclei, the amygdala, hippocampus, insula, and frontal lobe (based on donors H0351.2001 and H0351.2002, http://human.brain-map.org/) (Hawrylycz *et al.*, 2012) (Figure 1.9). Immunohistochemical studies to detect CCK protein also generally support this distribution in humans (Emson *et al.*, 1980; Emson *et al.*, 1982; Studler *et al.*, 1982).

CCK_B is the most widespread CCK receptor in the brain, with human *CCKBR* expression highest in some spinal and brainstem nuclei, the amygdala, hippocampus, and temporal lobe, as well as moderate levels in some cerebellar nuclei and in the frontal lobe (based on donors H0351.2001 and H0351.2002, http://human.brain-map.org/) (Hawrylycz *et al.*, 2012) (Figure 1.10). As the amygdala and hippocampus are associated with fear and memory respectively, CCK_B receptor knockout mice would be expected to show behavioural abnormalities. Although they appear physically normal (Nagata *et al.*, 1996), several studies have found reduced anxiety-like behaviour from CCK_B receptor knockout mice, though this is gender and context specific (Abramov *et al.*, 2004; Areda *et al.*, 2006). Further studies into these mice indicate that some of the behavioural changes may be due to altered levels or responses of other neurotransmitters, particularly dopamine and opioid systems (Koks *et al.*, 2001; Pommier *et al.*, 2002; Runkorg *et al.*, 2003). These changes may be abnormalities of brain development due to the lack of normal CCK signalling or they may be compensatory changes, to minimise the adverse effects of CCK_B receptor loss.

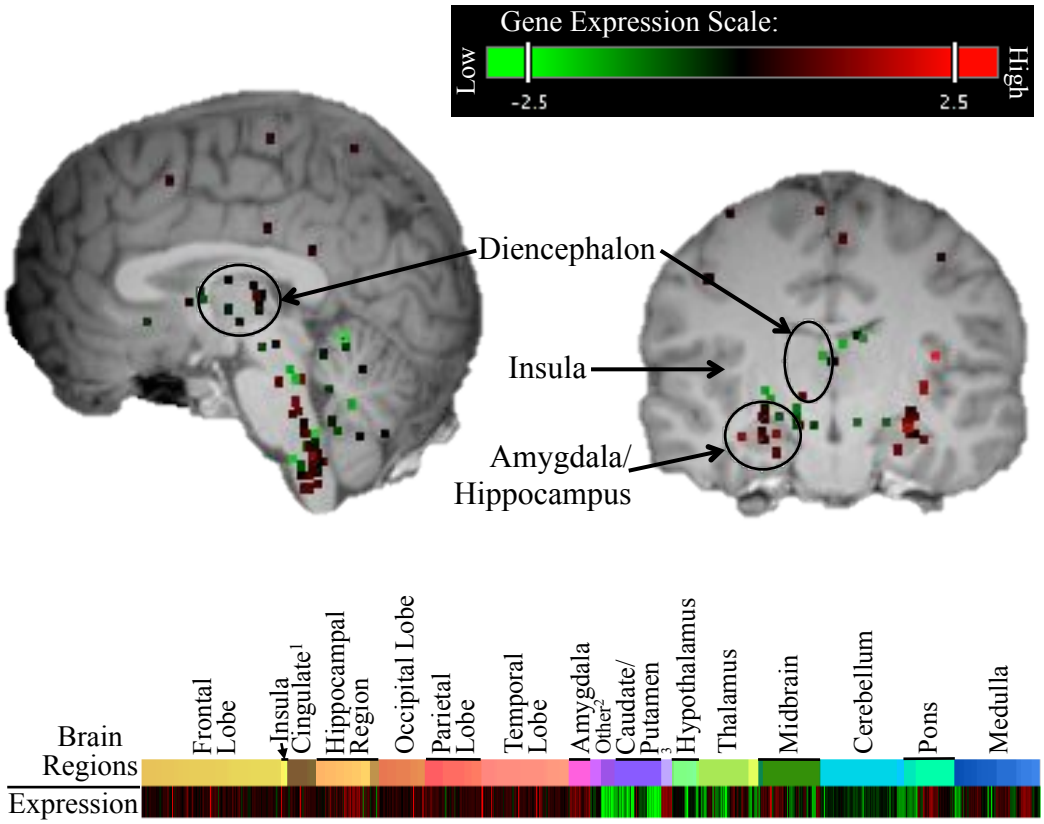


Figure 1.9 *CCK* Expression in the Brain.
Relative levels of *CCK* mRNA are shown for the various brain regions tested. As indicated by the scale, bright red represents high relative expression and bright green low. Data displayed are from donor H0351.2002, probe A_23_P425681 (from http://human.brain-map.org/ accessed on 03/06/12). 1. Cingulate refers to a region of cortex on the medial surface that includes parts of the frontal and parietal lobes. 2. Other includes deep nuclei (parts of the basal forebrain and the globus pallidus). 3. The claustrum – a thin strip of gray matter between the putamen and the insular cortex. Used in accordance with Allen Institute for Brain Science website guidelines (from http://alleninstitute.org/about_us/terms_of_use.html accessed 18/01/12).

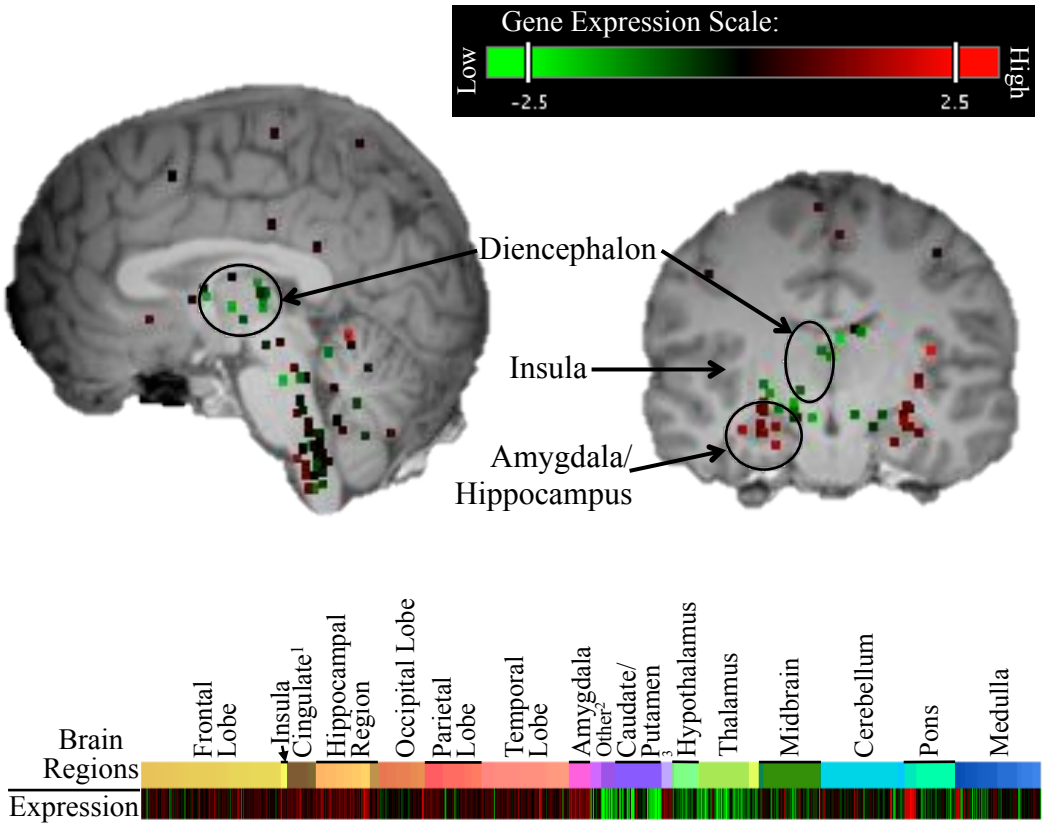


Figure 1.10 *CCKBR* Expression in the Brain.
Relative levels of *CCKBR* mRNA are shown for the various brain regions tested. As indicated by the scale, bright red represents high relative expression and bright green low. Data displayed are from donor H0351.2002, probe A_23_P162010 (from http://human.brain-map.org/ accessed on 03/06/12). 1. Cingulate refers to a region of cortex on the medial surface that includes parts of the frontal and parietal lobes. 2. Other includes deep nuclei (parts of the basal forebrain and the globus pallidus). 3. The claustrum – a thin strip of gray matter between the putamen and the insular cortex. Used in accordance with Allen Institute for Brain Science website guidelines (from http://alleninstitute.org/about_us/terms_of_use.html accessed 18/01/12).

Although overall expression is lower than that of CCK_B, CCK_A receptor mRNA levels are high in the corpus callosum, some spinal and brainstem nuclei, the hypothalamus, and the basal ganglia of the human brain (based on donors H0351.2001 and H0351.2002, http://human.brain-map.org/) (Hawrylycz *et al.*, 2012) (Figure 1.11). The basal ganglia are involved in movement, so it should not be surprising that rats with a naturally occurring mutation in CCK_A receptor, demonstrated lower locomotor activity in the open-field test than the wild-type strain they developed from (Kobayashi *et al.*, 1996). This change was further supported as being due to CCK_A receptor loss as blockade of CCK_A receptor in the wild-type rats produced similar reductions in activity.

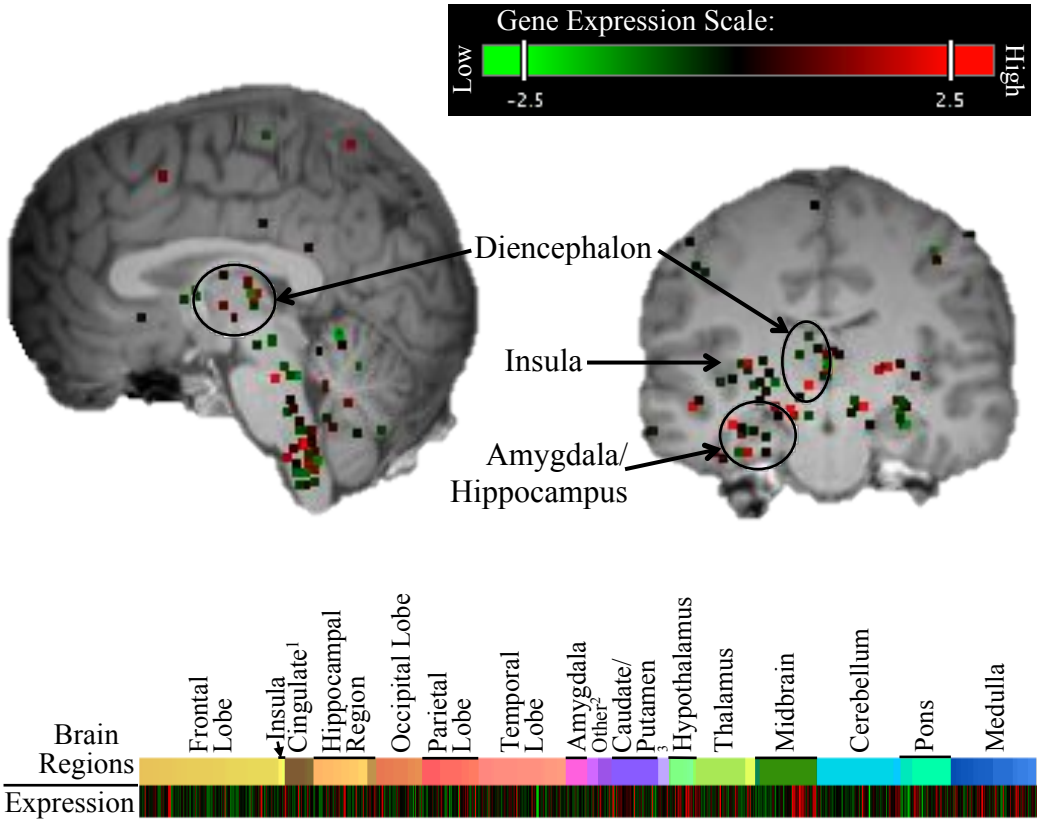


Figure 1.11 *CCKAR* Expression in the Brain.
Relative levels of *CCKAR* mRNA are shown for the various brain regions tested. As indicated by the scale, bright red represents high relative expression and bright green low. Data displayed are from donor H0351.2002, probe A_23_P41395 (from http://human.brain-map.org/ accessed on 03/06/12). 1. Cingulate refers to a region of cortex on the medial surface that includes parts of the frontal and parietal lobes. 2. Other includes deep nuclei (parts of the basal forebrain and the globus pallidus). 3. The claustrum – a thin strip of gray matter between the putamen and the insular cortex. Used in accordance with Allen Institute for Brain Science website guidelines (from http://alleninstitute.org/about_us/terms_of_use.html accessed 18/01/12).

1.3.3 CCK System Genes

The three proteins of the CCK system are each encoded by a single gene and the same initial protein product is produced in both neuronal and gastrointestinal tissue. Control of transcription and translation of these genes may directly affect the amount of functional protein produced and may be important in the pathogenesis of some disorders.

1.3.3.1 *CCK*

The first described human *CCK* sequence spanned 7 kb and had three exons: the first was non-coding, the second contained the translation start site, and the third exon contained the

sequence for functional peptide (Takahashi *et al.*, 1986; Takahashi *et al.*, 1985) (Figure 1.12). Hybidisation with genomic DNA indicated that only one copy of *CCK* exists in the genome. Parts of the promoter region of this *CCK* transcript have been studied, providing some understanding of the control of gene transcription. Multiple sites in the *CCK* promoter region are thought to be important in control of transcription and some of these sites have been confirmed to bind transcription factors. For example: an enhancer box element has been found that binds upstream stimulatory factor; a combined CRE/TRE site that binds cAMP response element-binding protein and activator protein 1; and a guanine/cytosine (G/C) rich region that binds Sp1 (Nielsen *et al.*, 1996; Pedersen *et al.*, 1994). The enhancer box and CRE/TRE elements are further supported as active sites since mutation of either of these results in lower activity of promoter-reporter gene constructs (Nielsen *et al.*, 1996). Hence, multiple different transcription factors are important for controlling *CCK* gene transcription.

In addition to the transcript described above, *CCK* has a common splice variant. In rats, alternative transcription start sites have been described that are upstream of the major start site and appear to be utilised in the brain, but not intestine (Katsel & Greenstein, 1997; Kuwano *et al.*, 1992). A human splice variant was found in the Mammalian Gene Collection Program (Strausberg *et al.*, 2002), which, similar to the rat variant, was found in brain samples and starts before the previously described transcription start site. When this variant is transcribed, three non-coding exons make up the 5' untranslated region (5'-UTR) and the normal exon 1 is spliced out, while the coding exons (2 and 3 in main transcript) remain unchanged (Figure 1.12). Little is currently known about this alternative transcript or its regulation.

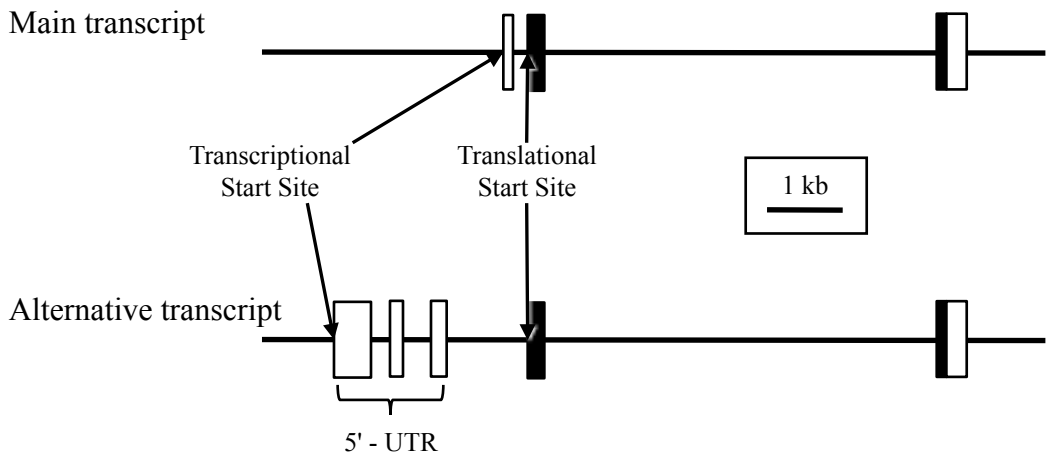


Figure 1.12 *CCK* Splice Variant.
The two variants of human *CCK* transcripts, located at 3p22.1, are shown (based on data from http://www.ncbi.nlm.nih.gov/gene/885 accessed on 03/06/12). They differ only in their 5'-UTR, with the main transcript having one exon for this region, and the alternative transcript having three exons.

1.3.3.2 *CCKAR*

The human complementary DNA (cDNA) sequence for *CCKAR* was first described in 1993 by two independent research groups. The sequence included 428 amino acids and was consistent with a G-protein coupled receptor, (de Weerth *et al.*, 1993; Ulrich *et al.*, 1993). The *CCKAR* gene contains five exons and spans approximately 9 kb (Miller *et al.*, 1995) (Figure 1.13), it exists as a single copy and was localised to chromosome 4 (de Weerth *et al.*, 1993; Huppi *et al.*, 1995).

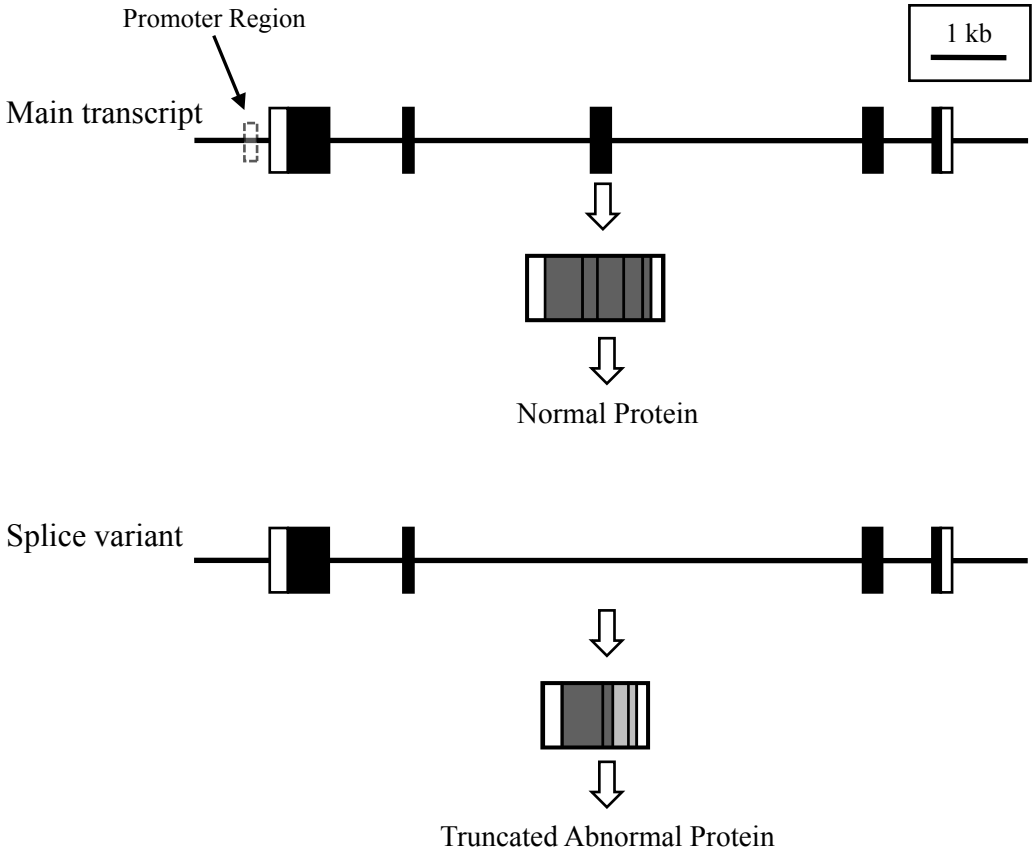


Figure 1.13 *CCKAR* Main Transcript and Splice Variant.
The main transcript of *CCKAR,* located at 4p15.2 (based on http://www.ncbi.nlm.nih.gov/gene/886 data, accessed on 03/06/12), and an abnormal splice variant that predisposes people to gallstones (Miller *et al.*, 1995) are shown.

The control of *CCKAR* transcription has not been as thoroughly researched as that for *CCK*. The transcription initiation site was found to be 205 base pairs (bp) upstream of the translation start site (Funakoshi *et al.*, 2000) and reporter constructs indicated that 197 to 31 bp upstream of the transcription start site contained most of the cis-elements important for transcription (Takata *et al.*, 2002). Variations within this region have been identified and

postulated to alter transcription factor binding sites (Tachikawa *et al.*, 2001), though these sites have not been directly investigated.

Splice variants of *CCKAR* seem to be uncommon, but may be important in human gastrointestinal disease. An obese patient with gallstones was found to have a splice variant of *CCKAR* that removes exon 3, causing a frameshift mutation, an early stop signal and a non-functional protein (Figure 1.13) (Miller *et al.*, 1995). Miyasaka *et al.* (2002) also found a truncated *CCKAR* transcript (assumed to be a splice variant of exon 5) in a patient with gallstones, as well as increased gallstone formation in CCK_A receptor knockout mice.

1.3.3.3 *CCKBR*

Human *CCKBR* is located on chromosome 11, has five exons and shows high homology with the CCK_B receptors of other species (Huppi *et al.*, 1995; Lee *et al.*, 1993; Pisegna *et al.*, 1992; Song *et al.*, 1993) (Figure 1.14). Like *CCK*, multiple transcription factor binding sites have been identified upstream of the *CCKBR* 5'-UTR, and deletion of this region or mutation of any of the binding sites reduced basal promoter activity (Ashurst *et al.*, 2008). Different pathways, including protein kinase C and MEK (Ashurst *et al.*, 2008), as well as microRNAs (Chen *et al.*, 2010b; Song *et al.*, 2012) have been found to be involved in the regulation of *CCKBR* expression, and some of these regulatory factors may be important in the development of gastrointestinal cancers (Hori *et al.*, 2003; Song *et al.*, 2011).

A number of splice variants of *CCKBR* have been described (Figure 1.14), some of which alter receptor levels or function. One of these variants is produced by alternative splice sites at the end of exon 4 and increases the number of amino acids in the third intracellular loop by five (Song *et al.*, 1993). This produced no change in agonist binding or function of the receptor (Ito *et al.*, 1994), but the long variant produced lower levels of functional membrane bound protein when transfected into a mouse cell line (Morton *et al.*, 2003). Another splice variant is produced by use of an alternative exon 1, named exon 1b. This variant has the normal carboxy-terminal amino acids, but is truncated at the amino-terminus extracellular domain and has been reported to have a stronger binding affinity for gastrin than the originally described transcript (Miyake, 1995). A related transcript has also been found that has both the normal exon 1 and the exon 1b; this splice variant results in a short, non-

functioning peptide (Monstein *et al.*, 1998). Both of these variants were found to be expressed in human brain, stomach and pancreas.

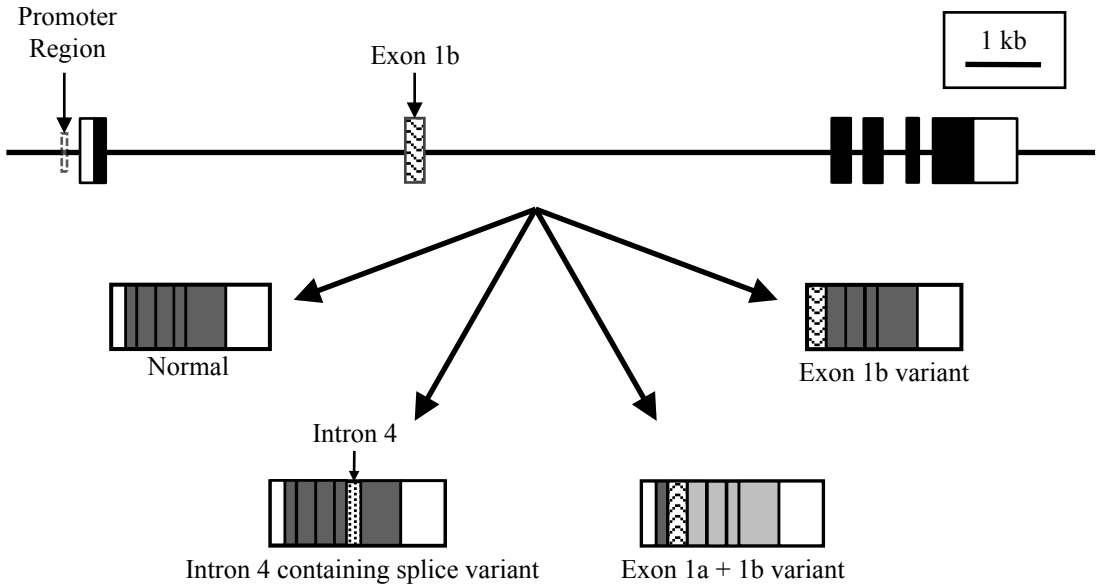


Figure 1.14 *CCKBR* Variants.
The gene for *CCKBR*, located at 11p15.4 (based on data from http://www.ncbi.nlm.nih.gov/gene/887 access on 03/06/12), is shown with three of the splice variants described in the text above. The inclusion of both the normal exon 1 and exon 1b results in an early stop signal.

The most commonly studied splice variant of the CCK_B receptor is produced when intron 4 is retained, resulting in additional amino acids in the third intracellular loop (Figure 1.14). This variant has been found in a number of gastrointestinal cancer samples, including pancreatic cancer (Smith *et al.*, 2004; Smith *et al.*, 2002) and colon cancer (Biagini *et al.*, 1997), as well as non-cancerous colonic polyps (Hellmich *et al.*, 2000) and normal blood mononuclear cells (Schmitz *et al.*, 2001b). Its high occurrence in cancer has resulted in the name CCK_C receptor, though it is also known as CCK-BRi4sv (CCK_B receptor intron 4 containing splice variant). There is some evidence that the retention of intron 4 produces increased tumour growth (Chao *et al.*, 2007; Hellmich *et al.*, 2000; Smith *et al.*, 2002), possibly related to gastrin activation of the receptor (Smith *et al.*, 2004). The splicing failure appears to result from a suboptimal 3′ splice site combined with low levels of the protein U2AF35, which forms part of the 3′ spliceosome complex (Ding *et al.*, 2002).

A number of other mutants and splice variants have also been described. This includes variants that have been found among colon cancer and pancreatic cancer samples that may increase the growth of the tumour (Ryberg *et al.*, 2011; Schmitz *et al.*, 2001a). The early

studies of *CCKBR* cDNA also typically found two different sized fragments with different tissue distributions (de Weerth *et al.*, 1993; Lee *et al.*, 1993; Pisegna *et al.*, 1992), suggesting that the splicing of *CCKBR* is highly variable.

1.3.4 Proteins of the CCK System

CCK is a member of the gastrin-cholecystokinin family of peptides and shares significant carboxy-terminal homology with gastrin, caerulein, phyllocaerulein and cionin, with identical pentapeptides and sulphated tyrosine residues located six or seven amino acids from the amidated carboxy-terminus (Rehfeld, 2006).

1.3.4.1 CCK Peptides

CCK is produced as a preproprotein that undergoes several processing steps within the cell prior to secretion (Beinfeld, 2003). As part of this, various sized peptides may be produced through tissue specific processing (Rehfeld *et al.*, 2003). These peptides are named based on the number of amino acids they have, with amino acids cleaved or retained from the amino-terminus. Some of the important CCK peptides are:

- CCK-83 is the largest biologically active, amidated form of CCK (Eberlein *et al.*, 1992).
- CCK-58 is found in tissues of a number of different species (Eysselein *et al.*, 1990; Eysselein *et al.*, 1982; Tatemoto *et al.*, 1984), is capable of both hormonal and neuronal receptor activation (Cooper *et al.*, 2008; Eysselein *et al.*, 1983), and is one of the major forms of CCK secreted into human blood (Eberlein *et al.*, 1987).
- CCK-33 was the first form sequenced (Mutt & Jorpes, 1971) and comprised the exogenous CCK used in most of the early studies.
- CCK-8 is the major form found in the brain (Dockray *et al.*, 1978; Rehfeld, 1978). Following confirmation of its biological activity (Rubin *et al.*, 1969), this peptide rapidly became the most commonly used for experimental investigation of CCK's effects.
- CCK-5, also known as pentagastrin, is sometimes used experimentally as a selective CCK_B receptor agonist. It has been detected in brain homogenates at approximately ten fold lower levels than CCK-8 (Harro *et al.*, 1997; Lofberg *et al.*, 1996) and may be a breakdown product of CCK-8 (Durieux *et al.*, 1985).
- CCK-4 is the smallest active peptide and is found in the brain of many mammals (Larsson & Rehfeld, 1979; Rehfeld, 1978) at levels approximately ten fold lower than

CCK-8 (Harro *et al.*, 1997; Lofberg *et al.*, 1996). Like CCK-5, CCK-4 is sometimes used as a selective CCK_B receptor agonist.

Other peptides, such as CCK-22, CCK-12 and CCK-7, have also been found (Rehfeld, 1987; Rehfeld & Hansen, 1986; Ryder *et al.*, 1980).

Peptides larger than seven amino acids have similar effects but differ in half-life and receptor affinity. Shorter peptides like CCK-8 act faster, while the longer peptides like CCK-33 have a longer duration of action and are degraded more slowly (Deschodt-Lanckman *et al.*, 1981; Rubin *et al.*, 1969). Additionally, while CCK-8 and CCK-58 were shown to have similar effects at the CCK_A receptor, including changes in cytosolic calcium levels and rate of receptor internalisation, the affinity of CCK-8 was found to be approximately five times higher (Wu *et al.*, 2008). These differences support their function as the larger, longer lasting peptides are the main forms released as hormones, while the shorter, faster acting peptides are released in neurotransmission.

The two shortest naturally occurring peptides, CCK-4 and CCK-5, differ from the larger peptides as they have little to no activity at the CCK_A receptor (Rubin *et al.*, 1969). As mentioned above, these peptides are used experimentally as selective agonists for the CCK_B receptor. The pentapeptide sequence of CCK-5 is also shared by related compounds such as gastrin, allowing them to activate the CCK_B receptor with similar potencies to CCK (Rehfeld, 2006).

1.3.4.2 Receptor Binding

The two types of cholecystokinin receptors are both G-protein coupled receptors from the rhodopsin family (also known as class A). Phylogenetic analysis groups these two receptors together, with *NPY5R* the only other receptor on that branch (Fredriksson *et al.*, 2003). The high homology between these two receptors explains many of their similarities.

Consistent with other work, fluorophore binding research suggests that only the carboxy-terminal region of CCK binds to CCK_B receptor, while most of CCK-8 interacts directly with CCK_A receptor (Harikumar *et al.*, 2005), allowing smaller and related peptides to selectively activate the CCK_B receptor. Ligand binding to G-protein coupled receptors typically stimulates changes to reduce the activity of the receptor. For CCK_A receptor, ligand binding

has been shown to induce receptor internalisation, with some of these internalised receptors later recycled back to the plasma membrane (Rao *et al.*, 1997; Roettger *et al.*, 1995). Ligand binding also induces phosphorylation of the CCK_A receptor, with multiple different kinases, including protein kinase C and β-ARK, likely to be involved (Gates *et al.*, 1993; Rao *et al.*, 1997). CCK_B receptor seems to require phosphorylation of residues in the carboxy-terminus for ligand-induced internalisation to occur (Pohl *et al.*, 1997).

1.3.4.3 Receptor Signalling

Both of the CCK receptors couple to the G-protein G_q, activating the inositol phosphate system (Archer-Lahlou *et al.*, 2005; Ferrand *et al.*, 2005; Wu & Wang, 1996). Activation of G_q triggers a cascade of events beginning with the activation of phospholipase C and results in the activation of protein kinase C and other calcium dependent proteins within the cytoplasm (Nestler *et al.*, 2001) (Figure 1.15).

In addition to the activation of G_q, CCK_A receptor can also activate G_s, increasing the formation of cAMP (Yule *et al.*, 1993). This is a property that is not shared by CCK_B receptor and amino acids in the first intracellular loop have been shown to be the main source of this activity (Wu *et al.*, 1997). In fact, changing a single amino acid in CCK_B receptor, the serine at position 82 for asparagine - the equivalent amino acid in CCK_A receptor - produced a significant increase in cAMP production when stimulated by CCK-8 or gastrin (Wu *et al.*, 1999).

The ultimate cellular effect of activating phospholipase C or adenylyl cyclase depends on the cell involved. For example, the activation of CCK_B receptor in isolated rat medium spiny neostriatal neurons produced a reversible dose-dependent depolarisation that was likely to be due to the opening of non-selective cation channels (Wu & Wang, 1996). In other neuronal cell lines, CCK_B receptor activation increased phosphorylation of ERK1/2 and increased binding of activator protein 1 to DNA (Langmesser *et al.*, 2007). Hence, both short-term (ion flux) and long-term (protein production) changes can be induced by activation of the same receptor in different cells.

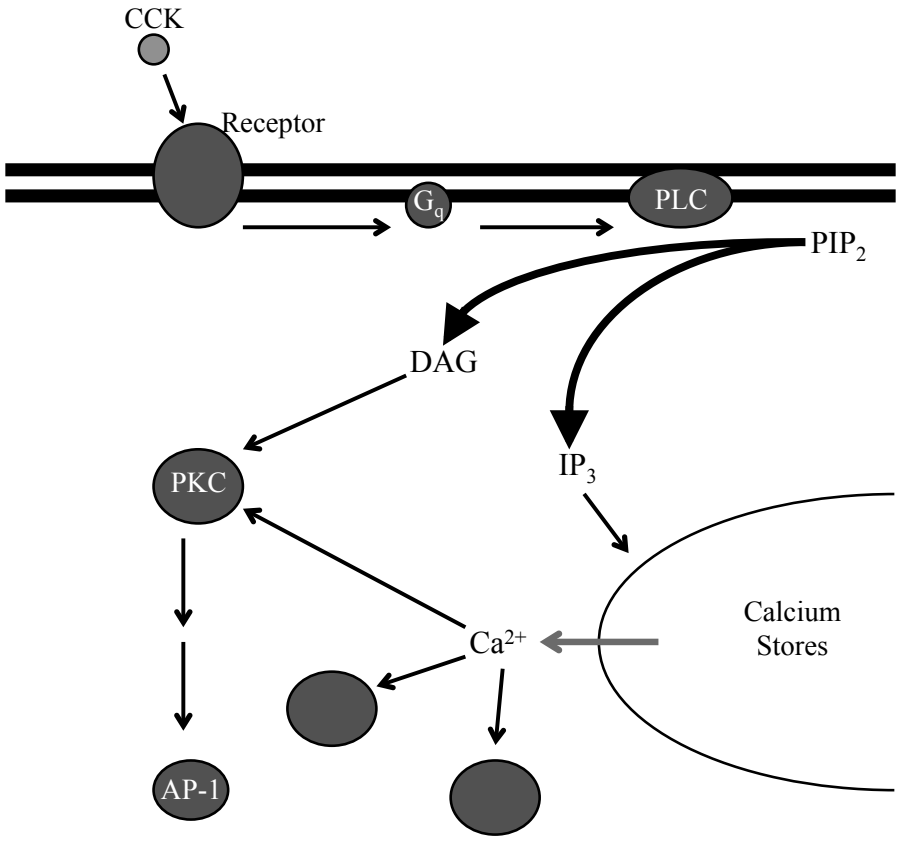


Figure 1.15 Activation of CCK Receptors.
CCK binding to CCK_A or CCK_B receptors activates the G-protein G_q, this then activates the enzyme phospholipase C (PLC), which catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) directly. IP_3 stimulates calcium release from intracellular stores, which increases the activity of calcium dependent proteins including PKC. Various cellular effects can be produced, such as activation of activator protein 1 (AP-1), which alters gene transcription.

1.3.5 Summary

Since its discovery in 1928 as a hormone that produces gallbladder contraction, the known functions of cholecystokinin have increased dramatically. The gastrointestinal functions of CCK were the first to be studied and CCK is still most commonly described as a gastrointestinal hormone; however, an increasing number of roles for CCK in the brain have been discovered and are proving to be highly important in human health and disease.

- CCK signalling can transmit information between the central and peripheral tissues, for example, via the vagus nerve.
- Both CCK_A and CCK_B receptors are found in gastrointestinal tissues, peripheral nerves and central nerves. This can make it difficult to determine which pool(s) of receptors are mediating effects of CCK.

- The two CCK receptors are closely related and both receptors produce their effects by G_q activation of phospholipase C. CCK_A receptor also activates G_s under some circumstances.

1.4 Molecular Biology of Anxiety

Anxiety disorders are the most common of all the DSM-IV categories of mental illness, having the highest twelve-month prevalence (Demyttenaere *et al.*, 2004) and lifetime prevalence (Kessler *et al.*, 2007b) in most countries that have been studied. In the DSM-IV, the major types of anxiety disorders recognised are panic disorder with/without agoraphobia, agoraphobia without history of panic disorder, specific phobia, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder, acute stress disorder and generalised anxiety disorder (American Psychiatric Association, 1994). For the purposes of this thesis most of these categories will be followed, except that panic disorder and agoraphobia (fear of situations from which escape may be difficult) will be considered two separate disorders. The different anxiety disorders include a wide range of symptoms, from excessive fear of a specific object or situation (specific phobia) to compulsively performing specific acts (obsessive-compulsive disorder) or re-experiencing a traumatic event through dreams and flashbacks (post-traumatic stress disorder). The various anxiety disorders also share symptoms and high rates of comorbidity between the different disorders are seen (Scott *et al.*, 2006a).

All of the anxiety disorders feature acute (panic attacks) and/or chronic anxiety. A panic attack is an acute period of fear, in the absence of danger, including at least four of the following symptoms (American Psychiatric Association, 1994):

- palpitations/pounding heart/accelerated heart rate
- sweating
- trembling/shaking
- shortness of breath/a sensation of smothering
- choking feeling
- chest pain/discomfort
- nausea/abdominal distress
- feeling dizzy/unsteady/light-headed/faint
- feelings of unreality (derealisation)/being detached from oneself (depersonalisation)

- fear of losing control/going crazy
- fear of dying
- numbness/tingling sensations (paresthesias) or
- chills/hot flushes

Limited symptom panic attacks can also occur, in which less than four of the above symptoms are experienced (American Psychiatric Association, 1994). Chronic anxiety may manifest as persistent "concern" or "worry", or by changes in behaviour intended to reduce the anxiety. Both acute and chronic anxiety can be disabling and contribute to the high levels of disability experienced with mental illness (World Health Organization, 2008).

1.4.1 Fear, Stress and Anxiety

Anxiety is one of the best understood emotions in terms of neurobiology, and anxiety disorders are thought to result from aberrant signalling in fear or stress pathways. Fear, stress and anxiety are closely related emotional responses that most people will experience repeatedly throughout their lives. Anxiety disorders occur when the emotion becomes extreme for the circumstance or is maintained for an unusually prolonged time, such that it interferes with a persons life (Rosen & Schulkin, 1998).

Fear is a normal response to a threatening situation, usually lasting from seconds to hours, that should minimise the individual's risk of harm (Cannon, 1914). The physiological response to fear that accompanies the emotional experience is often referred to as the 'fight or flight' response (Crile, 1912). It includes many of the symptoms of panic attacks, such as palpitations, increased heart rate, sweating and tremor, as well as less obvious changes like increased blood glucose level and cessation of digestion (Cannon *et al.*, 1911; Crile, 1912). Several different regions of the brain contribute to the experience of fear and response to fearful stimuli, with the amygdala thought to be a key region (Etkin, 2010) (Figure 1.16). The amygdala is activated by negative emotional stimuli, such as images of fearful faces (Whalen *et al.*, 1998), amygdala lesions reduce fear-associated behaviour in rodents (Hitchcock & Davis, 1987; Vanderwolf *et al.*, 1988) and electrical stimulation of the right amygdala in epileptic patients increased the sensation of fear (Lanteaume *et al.*, 2007).

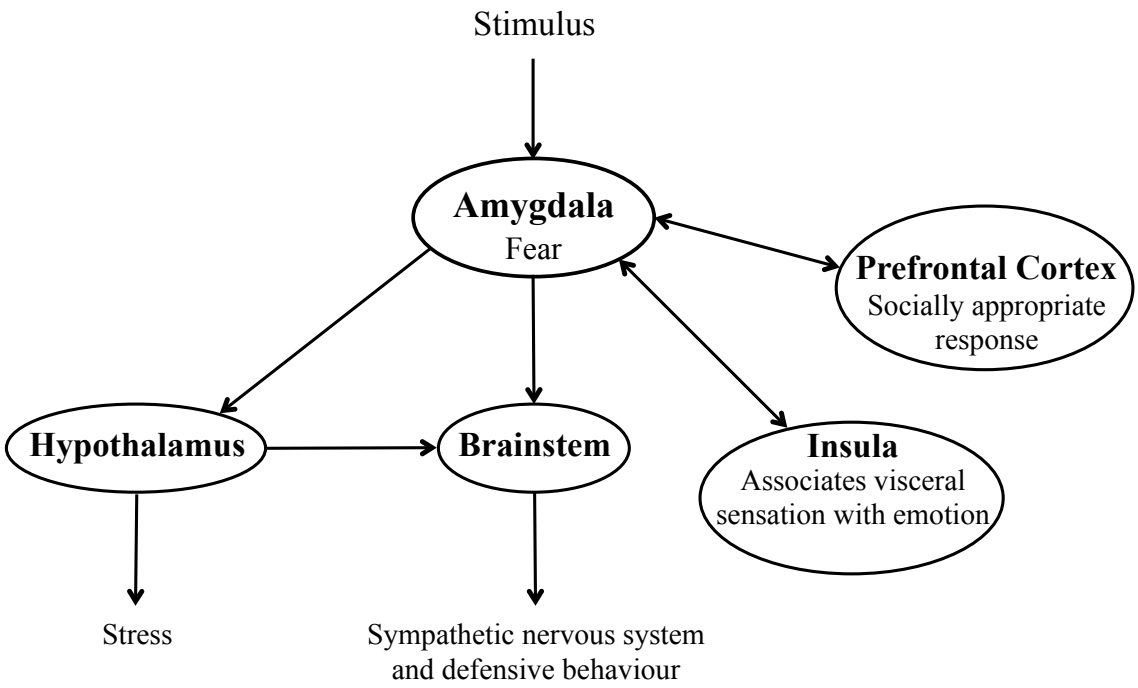


Figure 1.16 Neurobiology of Fear
Some of the key brain regions in the emotional and physical experience of fear are shown.

Cortical regions, including the insula and prefrontal cortex, have also shown associations with fear. Activity in the insula has been associated with negative emotions and this was one of the regions most commonly associated with fear, second only to the amygdala (Phan *et al.*, 2002). The insula processes visceral sensory information (Critchley *et al.*, 2004), recall of emotions (Reiman *et al.*, 1997) and is one of the regions of the brain that are active when making determinations about self (Denny *et al.*, 2012). The integration of this different information in the insula may be important in the association of physiological symptoms with fear and anxiety (Nagai *et al.*, 2007). The prefrontal cortex is involved in decision making and parts of the prefrontal cortex interact with other parts of the brain to determine the appropriate emotional and physiological response to a stimulus (Devinsky *et al.*, 1995). For example, an interaction between amygdala and ventromedial prefrontal cortex was hypothesised to be involved in appropriate compassionate or aggressive responses (King *et al.*, 2006). The prefrontal cortex is involved in a wide variety of emotions (Phan *et al.*, 2002) (Devinsky *et al.*, 1995) and is generally associated with suppression of amygdala activity and the fear response (Etkin, 2010; Quirk *et al.*, 2003).

While the amygdala and cortical regions determine whether there should be a response from the organism, the acute physiological changes associated with fear are predominantly

controlled by brainstem nuclei and the hypothalamus. The sympathetic nervous system is a defensive system that prepares the individual to fight or flee (the fight or flight response) and is often activated by fear. This system is controlled by the medulla, pons and hypothalamus with other regions also influencing the medullary neurons (Morrison, 2001; Sved *et al.*, 2001). Activation of the sympathetic nervous system results in the peripheral release of the neurotransmitter noradrenaline and the hormone adrenaline, both of which activate adrenergic receptors on target tissues, increasing heart rate, respiration and sweating (Rang *et al.*, 1999).

As well as a role in acute fear, the hypothalamus controls the more chronic response to a threatening event – the stress response. The stress response can be triggered by stimuli that cause fear, as described above, or it can be caused by adverse physiological states such as blood loss (Pego *et al.*, 2010). Once initiated, the stress response produces a hormone cascade with corticotropin releasing hormone from the hypothalamus stimulating the release of adrenocorticotropic hormone from the pituitary, which then stimulates the release of cortisol (corticosterone in rodents) from the adrenal glands (Figure 1.17) (Rodrigues *et al.*, 2009).

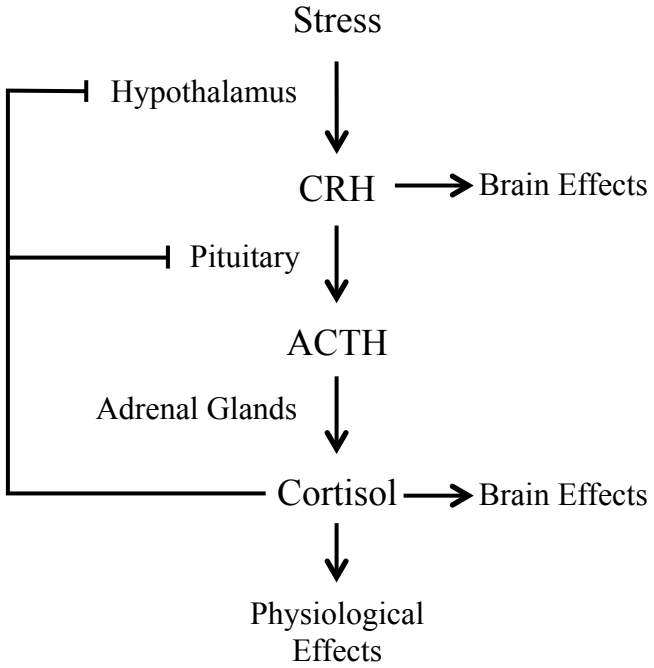


Figure 1.17 Stress Response.
Summary of the hormone cascade produced as part of the response to stress.

The activation of the hormonal cascade described above, resulting in the release of cortisol, is part of the normal response to acute stress. Cortisol stimulates some physiological effects

associated with acute stress, such as mobilising energy stores and increasing cardiac output, while it reduces others, such as immune responses, preventing them from becoming excessive (Sapolsky *et al.*, 2000). Exogenous corticosteroids (cortisol and other related compounds) are used therapeutically for their immunosuppressive effects and a significant proportion of these patients experience mood or cognitive changes, including anxiety (Kenna *et al.*, 2011). These changes in cognitive function are consistent with the presence of glucocorticoid receptors in brain regions such as the hippocampus and amygdala (Sarrieau *et al.*, 1986).

Corticotropin releasing hormone (also known as corticotropin releasing factor), at the start of the stress pathway, acts as both a hormone and a neurotransmitter/neuromodulator. Administration of corticotropin releasing hormone to the brain has been shown to increase anxiety-like behaviour in animal models, including both acute (30 minutes after a single injection) and chronic (seven days) intracereboventricular administration (Buwalda *et al.*, 1997; Spina *et al.*, 2002), as well as injections specifically into the prefrontal cortex (Jaferi & Bhatnagar, 2007) or the extended amygdala (Sahuque *et al.*, 2006). Two receptors for corticotropin releasing hormone have been described in mammals, CRH_1 and CRH_2 receptors, both of which are expressed in the hypothalamus and amygdala (Dautzenberg & Hauger, 2002), presenting a potential role in fear sensitivity. CRH_1 receptor is required for the initial secretion of adrenocorticotropic hormone (Preil *et al.*, 2001), while CRH_2 seems to be involved in the maintenance and termination of the hormonal response (Bale *et al.*, 2000; Coste *et al.*, 2000). Most early evidence suggested that the CRH_1 receptor was responsible for the anxiogenic effects of corticotropin releasing hormone (for example: Heinrichs *et al.*, 1997), however, more recent evidence supports a role for both receptors (Risbrough *et al.*, 2009; Sahuque *et al.*, 2006; Zhao *et al.*, 2007).

Anxiety disorders can occur due to variation in fear or stress systems. Panic attacks, for example, largely result from inappropriate activation of fear pathways and patients with disorders that commonly feature panic attacks, such as phobias and post-traumatic stress disorder, have been found to have increased amygdala activity (Shin & Liberzon, 2010). Acute and chronic stress, on the other hand, can increase a variety of anxiety symptoms by altering activity or structure of brain regions involved in stress or fear (McEwen *et al.*, 2012). This may include the enhancement of amygdala activity by chronic glucocorticoid administration (Skorzewska *et al.*, 2006), or increased corticotropin releasing hormone

expression following stress (Bakshi & Kalin, 2000) and may be involved in the increased risk of anxiety following early life stress (Carpenter *et al.*, 2007; Plotsky & Meaney, 1993).

Successful treatment of anxiety disorders is also associated with correction of many of these fear or stress abnormalities. For example, a neuroimaging study found that patients with a specific phobia of spiders that had been successfully treated with cognitive-behavioural therapy had reduced activity in the insula and part of the prefrontal cortex, towards control values, when viewing a video of a spider (Straube *et al.*, 2006). Similarly, in patients with panic disorder, levels of cortisol were found to increase more rapidly and to higher levels in response to corticotropin releasing hormone than in controls, and this was corrected by twelve weeks treatment with a benzodiazepine (Curtis *et al.*, 1997). Consequently, changes in fear and stress signalling seem likely to be involved in both the pathogenesis and treatment of anxiety disorders.

1.4.1.1 Induction of Panic Attacks

Panic attacks can be induced in experimental animals or human volunteers through the administration of certain agents. These agents are used to predictably produce panic attacks, allowing features of the attacks to be studied in a controlled environment. This has allowed the biochemical properties of panic attacks to be studied in greater detail than could occur with spontaneous attacks, as well as providing an opportunity to test anti-panic agents and to investigate differences between panic disorder patients and other psychiatric patients.

The three most commonly used experimental panicogens are CCK, lactate and carbon dioxide. CCK is administered intravenously in the form of CCK-4 or CCK-5 and is the only one of these panicogens that is also a neurotransmitter (Keck & Strohle, 2005). Lactate is usually administered intravenously in the form of sodium lactate, with sodium chloride (saline) used as the control infusion. Lactate was one of the first substances identified as a panicogen; following the observation that exercise could induce an anxiety-like state in susceptible individuals (Guttmann & Jones, 1940), lactate was found to induce panic attacks in both anxiety patients and, to a lesser extent, healthy controls (Pitts & McClure, 1967). Carbon dioxide challenges come in two forms: moderately increased fraction of inspired carbon dioxide inhaled for an extended length of time, such as 5% carbon dioxide for 15 minutes; or a single deep breath with a large fraction of carbon dioxide, such as 35%. Both of

these paradigms have been shown to induce panic attacks (Gorman *et al.*, 1988; Perna *et al.*, 1995). The panic attacks induced by these compounds are similar to naturally occurring attacks (Pitts & McClure, 1967), with differences related to the specific action of the agent used, such as increased gastrointestinal symptoms with CCK-4 (Koszycki *et al.*, 1991) and increased respiratory symptoms with carbon dioxide (Perna *et al.*, 1994).

These three panicogens all share several features. They all have the capability of inducing panic attacks in both panic disorder patients and healthy controls, with patients showing increased sensitivity (Bradwejn *et al.*, 1991; Perna *et al.*, 1995; Pitts & McClure, 1967; Woods *et al.*, 1988). Additionally, the rate of panic attacks in response to each of these agents can be reduced by treatment with the pharmacological agents used to treat panic disorder. CCK-4 induced panic attacks in panic disorder patients have been shown to respond to chronic treatment with antidepressant medications (Bradwejn & Koszycki, 1994; van Megen *et al.*, 1997) and, in studies of healthy volunteers, to acute benzodiazepine administration (Zwanzger *et al.*, 2003) and seven days treatment with the GABA transaminase inhibitor vigabatrin (Zwanzger *et al.*, 2001). Similarly, both 35% carbon dioxide and lactate-induced anxiety could be reduced in panic disorder patients following treatment with antidepressant medications (Bertani *et al.*, 1997; Yeragani *et al.*, 1988).

Other experimental panicogens include yohimbine and caffeine. Yohimbine is a drug that increases activity of the sympathetic nervous system by blocking the down-regulation that would normally occur via adrenergic autoreceptors. Both noradrenaline and yohimbine administration have been shown to induce anxiety and panic attacks in patients that have a history of panic attacks (Charney *et al.*, 1992). Caffeine is a very commonly used drug globally and, in high doses, can induce anxiety and panic attacks (Vilarim *et al.*, 2011). A number of studies have found higher rates of panic attacks following caffeine administration in panic disorder patients than in controls and this elevated sensitivity to caffeine was also found to extend to healthy first-degree relatives of panic disorder patients (Nardi *et al.*, 2008). Other anxiety disorder patients have also been found to have increased sensitivity to caffeine induced panic attacks, including generalised anxiety disorder (Bruce *et al.*, 1992) and social phobia (Nardi *et al.*, 2009). The anxiogenic properties of caffeine can also be seen in consumption patterns of patients, with panic disorder patients less likely to consume coffee (Boulenger *et al.*, 1984). Similarly in a series of case reports, six patients that had been

experiencing panic attacks improved considerably upon cessation of caffeine consumption (Bruce & Lader, 1989).

1.4.2 Genetics of Anxiety and Panic

Understanding the genetics of anxiety disorders is complicated by gene-environment interactions and by the presence of multiple comorbid conditions. In fact, one of the greatest obstacles to understanding pathogenesis has yet to be overcome for anxiety: a clear definition of the disease. Currently, the most obvious example of this is the debate over whether obsessive-compulsive disorder should continue to be included as an anxiety disorder in the DSM-V (Stein *et al.*, 2010) - although all anxiety disorders have ambiguity regarding their classification. Consequently, advances in molecular and neurobiological understanding of anxiety disorders are also utilised in revising the definitions of these disorders (Leigh, 2009).

Anxiety disorders are undoubtedly caused by a combination of genetic and environmental influences (nature and nurture); however, the estimated genetic contribution varies greatly between disorders and studies. The results of twin studies and modelling of risk factors typically find agoraphobia among the highest for genetic risk factors, while generalised anxiety disorder is among the lowest, with approximately two-fold difference between these (Domschke & Deckert, 2010; Hettema *et al.*, 2005).

Post-traumatic stress disorder represents a unique anxiety disorder in terms of predisposition, as it has an absolute requirement for at least one environmental event. This strong environmental influence is reflected in a lower, but still significant, genetic risk compared to most other anxiety disorders, with estimates of approximately 30% (Kremen *et al.*, 2012). However, even this relatively simple progression quickly becomes complicated as genetic factors, through their influence on daily decisions and life choices, significantly influence the likelihood of an individual being exposed to certain types of trauma (Afifi *et al.*, 2010).

In addition to specific disorders, some individual symptoms or other associated features of anxiety are inherited. A well studied example of this is 35% carbon dioxide induced panic attacks. In addition to panic disorder patients, healthy subjects with a family history of panic disorder also had higher rates of panic attacks in response to inhaling 35% carbon dioxide, compared to subjects with a family history of mood disorder or no family history (Coryell,

1997). Subsequently, twin studies have estimated the heritability of carbon dioxide induced panic attacks to be 50% or more (Battaglia *et al.*, 2007; Roberson-Nay *et al.*, 2013), while the genetic risk for panic disorder is estimated at 48% (Hettema *et al.*, 2001). Furthermore, modelling indicates that panic disorder and panic attacks in response to carbon dioxide share genetic risk factors and this is the major contribution to the high correlation between these phenotypes (Battaglia *et al.*, 2008).

Several researchers have also investigated whether a single genetic factor may predispose individuals to developing a variety of anxiety disorders. A study of over 5000 twins produced a best-fit risk model that included two additive genetic risk factors, as well as shared and unique (to the individual) environmental factors, that predisposed a person to developing several anxiety disorders (Hettema *et al.*, 2005). One of these two genetic factors mainly increased the risk of panic disorder, agoraphobia, generalised anxiety disorder and social phobia, while the other predisposed to animal and situational phobias. Another model from a twin study suggested that there are two genetic factors common to panic disorder, major depression and generalised anxiety disorder: one that predisposes to neuroticism, and one that does not (Hettema *et al.*, 2006b). Additionally, Chantarujikapong *et al.* (2001) found that 20.7% of panic disorder variance was due to genetic factors shared with both generalised anxiety disorder and post-traumatic stress disorder. These results support the hypothesis that certain variants may predispose a person to developing a variety of anxiety disorders.

More detailed investigations into the specific loci involved in anxiety have typically taken a candidate gene approach. These studies have investigated a wide variety of genes, some of which will be discussed in later sections. For example, over 350 genes have been investigated for an association with panic disorder and at least 46 of those have some evidence supporting an association (Maron *et al.*, 2010). However, many of these studies have had small sample sizes (often around 100 cases), included limited variation within the gene(s) and failed to replicate in other populations. Consequently, there is still much to be discovered in anxiety genetics.

1.4.3 Neurotransmitters and Neuromodulators

A large number of different signalling molecules have been implicated in anxiety. This section describes some of these signalling systems and the evidence for their involvement in

anxiety, with a particular focus on systems that include one or more of the Cellular Changes with Antidepressants candidate genes (section 4, page 143). It should also be noted that the monoamine neurotransmitters, serotonin, dopamine and noradrenaline, share many features, including the inhibition of breakdown by monoamine oxidase inhibitors and, for noradrenaline and serotonin, the inhibition of reuptake by tricyclic antidepressants. Although they will not be discussed here, noradrenaline and dopamine also have an association with anxiety.

1.4.3.1 Serotonin

Serotonin (also known as 5-hydroxytryptamine or 5-HT) is a neurotransmitter with a wide range of functions that is found in both the central and peripheral nervous systems (Nestler *et al.*, 2001). Serotonin receptors are clustered into seven different groups, all of which are G-protein coupled except the 5-HT_3 receptors, which are ligand-gated ion channels (Pytliak *et al.*, 2011). The system is controlled via: serotonin synthesis by tryptophan hydroxylase; serotonin reuptake by the serotonin transporter protein; serotonin breakdown by monoamine oxidase type A; and inhibition of serotonin release by 5-HT_1 autoreceptors (Pytliak *et al.*, 2011). The serotonin system is the immediate target of the selective serotonin reuptake inhibitor (SSRI) class of antidepressants and is also altered by several other antidepressant classes (Figure 1.18).

A number of different components in the serotonin system have been found to be associated with anxiety, particularly the serotonin receptor subtype 1A (5-HT_{1A}). This receptor can act as a presynaptic autoreceptor as well as a postsynaptic receptor, meaning that it may either produce serotonergic effects or inhibit serotoninergic effects by reducing further release from the presynaptic cell (Pytliak *et al.*, 2011). Some evidence was found for a negative correlation between 5-HT_{1A} receptor binding and anxiety levels in healthy patients (Tauscher *et al.*, 2001); however, this was not supported in a subsequent study (Rabiner *et al.*, 2002). The strongest support for altered 5-HT_{1A} levels in anxiety has come from studies of patients with anxiety disorders. Panic disorder patients have been found to have lower 5-HT_{1A} binding than controls (Nash *et al.*, 2008; Neumeister *et al.*, 2004), as have patients with social phobia (Lanzenberger *et al.*, 2007). This included changes in key anxiety regions of the brain such as the amygdala and the insula. However, post-traumatic stress disorder patients did not

show these changes (Bonne *et al.*, 2005), suggesting that the association may be disorder specific.

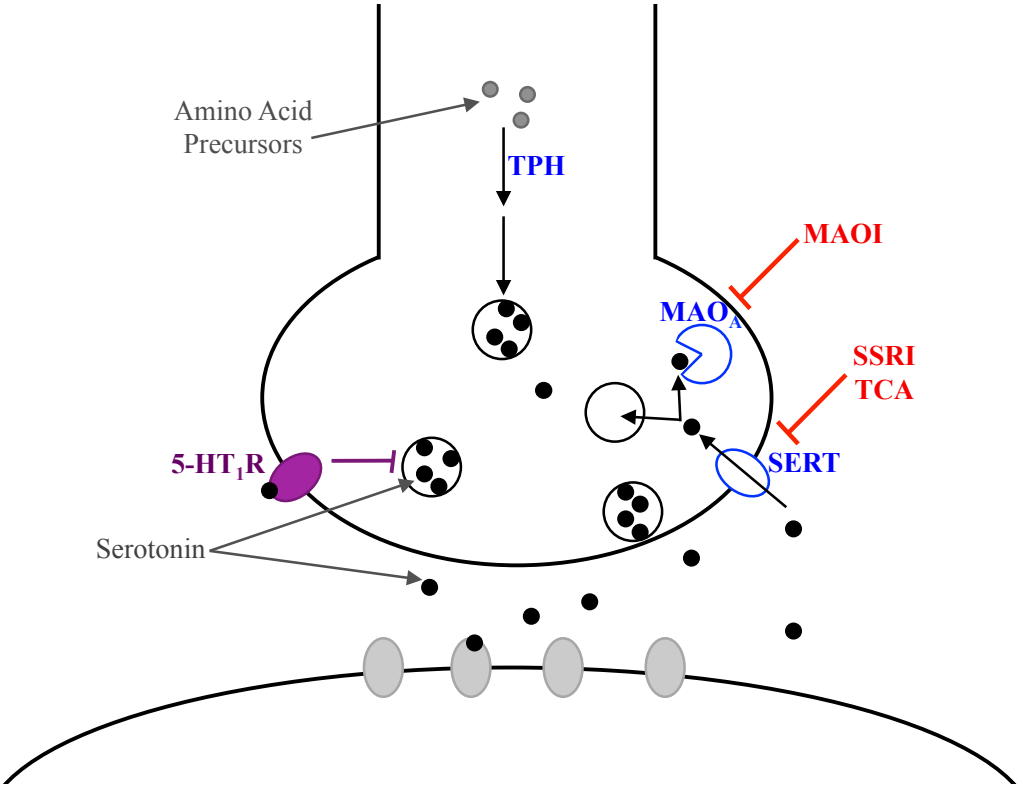


Figure 1.18 Serotonin System.
Serotonin is synthesised from the amino acid tryptophan by enzymes including tryptophan hydroxylase (TPH). Once released, serotonin signalling is terminated by reuptake via the serotonin transporter (SERT) and serotonin is broken down by the enzyme monoamine oxidase type A (MAO_A). Serotonin signalling can also be reduced by activation of autoreceptors ($5\text{-}HT_1R$), which reduce the release of serotonin from the presynaptic cell. The selective serotonin reuptake inhibitors (SSRI) and tricyclic antidepressants (TCA) act by inhibiting SERT, while another antidepressant class, the monoamine oxidase inhibitors (MAOI) reduce the breakdown of serotonin. (See also Figure 1.5, page 6.)

The lack of changes found in post-traumatic stress disorder is also consistent with animal research on the $5\text{-}HT_{1A}$ receptor. Knockout mice that do not express $5\text{-}HT_{1A}$ receptors have been found to have increased levels of anxiety-like behaviour (Ramboz *et al.*, 1998). However, inducing conditional expression of the gene in the hippocampus and cortex of mice during development prevented this phenotype, if induction of expression began 21 days after birth the heightened levels of anxiety seen in knockouts remained (Gross *et al.*, 2002). Hence, it could be hypothesised that $5\text{-}HT_{1A}$ receptor levels during a critical period of development affect predisposition to some anxiety disorders, and post-traumatic stress disorder is not affected as the environmental events occur after this critical time.

Associations with anxiety disorders have also been found for other components of the serotonin system. For example, turnover of serotonin was found to be higher in the brains of panic disorder patients (Esler *et al.*, 2007) and administering a serotonin precursor 30 minutes before CCK-4 reduced panic attacks in healthy female volunteers (Maron *et al.*, 2004). Brain levels of the serotonin transporter protein have also been found to differ between patients and controls, however, the direction of change is not consistent between disorders, with obsessive-compulsive disorder and panic disorder patients showing lower levels, while generalised anxiety disorder patients have higher levels (Nikolaus *et al.*, 2010). Hence, changes in the serotonin system are probably involved in anxiety disorders, but the exact changes are likely to vary between disorders and possibly between different patients with the same disorder.

1.4.3.2 GABA

GABA is the major inhibitory neurotransmitter in the brain. GABA activates at least three receptors: the $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels and the $GABA_B$ receptor is a G-protein coupled receptor that inhibits adenylyl cyclase (Chebib, 2004; Nestler *et al.*, 2001). The relationship between GABA and anxiety is mainly mediated by the $GABA_A$ receptors, which are the target of benzodiazepine anxiolytics.

$GABA_A$ receptors form pentameric ion channels. Potential subunits of the receptors are named α, β, γ, δ, ε, π and θ subunits, most of which can be further divided into subtypes, designated by a subscript number. In most cases, the receptors are made up of two α, two β and a γ subunit (Figure 1.19) (Rudolph & Mohler, 2006). Benzodiazepines bind at the junction between an α and a γ_2 subunit and increase chloride flux upon GABA binding, hence, enhancing GABA signalling (von Blankenfeld *et al.*, 1990). Six different types of α subunit exist, with the different effects of benzodiazepines mediated by different types:

- α_1 subunits mediate the sedative and most of the motor effects of benzodiazepines (Rowlett *et al.*, 2005)
- α_2 subunits are likely to mediate most of the anxiolytic effects as mice with a mutant α_2, that prevents benzodiazepine binding, do not show anxiolytic responses to these drugs (Low *et al.*, 2000; Smith *et al.*, 2012)
- α_3 subunits primarily control the release of dopamine from the midbrain (Yee *et al.*, 2005) and have anxiolytic effects at high receptor occupancy (Dias *et al.*, 2005)
- α_5 subunits are involved in attenuating the startle response (Hauser *et al.*, 2005)

- α_4 and α_6 subunits are benzodiazepine insensitive (Smith, 2001).

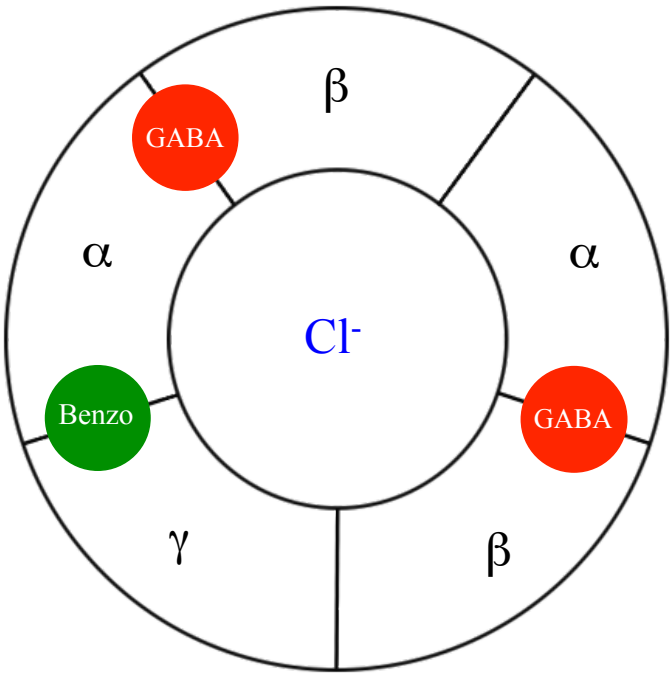


Figure 1.19 $GABA_A$ Receptor.
A schematic of the typical arrangement of $GABA_A$ receptor subunits, with GABA binding sites (red circles) and benzodiazepine binding site (green circle). Note that only $GABA_A$ receptors with particular types of α and γ subunits can bind benzodiazepines.

$GABA_B$ receptors have also been implicated in anxiety, though they have not been as thoroughly studied as $GABA_A$ receptors. Knockout mice that lacked either of the two $GABA_B$ receptor subtypes showed higher anxiety-like responses in the light-dark box than wild type mice, including decreased latency to enter the dark compartment and decreased time in the light compartment (Mombereau *et al.*, 2005). Similarly, a $GABA_B$ receptor modulator, that increases the effects of the receptor when GABA is bound, showed anxiolytic activity in rats tested on the elevated plus maze (a model of anxiety in rodents that utilises innate desire to explore versus preference for dark enclosed spaces), including increased time on open arms and increased entries of open arms (Cryan *et al.*, 2004).

Among patients with anxiety disorders biochemical abnormalities related to the GABA system have been found. Compared to controls, patients with anxiety disorders have been found to have lower levels of $GABA_A$ receptor in their brains and this was one of the most consistent findings across different anxiety disorders (Nikolaus *et al.*, 2010). Patients with post-traumatic stress disorder were found to have lower benzodiazepine binding in their

prefrontal cortex than controls, suggesting that these patients may have less $GABA_A$ receptors in this region (Bremner *et al.*, 2000a). Similarly, panic disorder patients were found to have lower benzodiazepine binding in the hippocampus (Bremner *et al.*, 2000b), prefrontal cortex (Hasler *et al.*, 2008) and brain overall (Malizia *et al.*, 1998), compared to healthy controls. Panic disorder patients have also been found to have other GABA system abnormalities, compared to controls, including reduced GABA in their occipital cortex (Goddard *et al.*, 2001), anterior cingulate cortex (Long *et al.*, 2013) and basal ganglia (Ham *et al.*, 2007). Unexpectedly, higher levels of endogenous positive allosteric regulators of the $GABA_A$ receptor were found in panic disorder patients (Strohle *et al.*, 2002), however, levels of these declined in patients, but not controls, when sodium lactate or cholecystokinin were used to induce panic attacks (Strohle *et al.*, 2003; Zwanzger *et al.*, 2004). While these studies have been small, each including less than 25 patients, collectively these data indicate that GABA system function is altered in anxiety, particularly panic disorder.

Support for a role of the GABA system in the pathogenesis of anxiety can be found in genetic association studies. Candidate gene studies have found associations between *GABBR1* (encoding $GABA_B$ receptor type 1) with obsessive-compulsive disorder (Zai *et al.*, 2005); *GAD1* (glutamic acid decarboxylase 1, an enzyme involved in the synthesis of GABA) with anxiety and depression (Hettema *et al.*, 2006a) and with phobia (Donner *et al.*, 2012); and *SLC6A1* (GABA transporter) with anxiety and severity of panic attacks (Thoeringer *et al.*, 2009). A genome-wide association study found support for the involvement of variants in *GABRA1* ($GABA_A$ receptor α_1 subunit) and *GAD2* in panic disorder (Otowa *et al.*, 2012) and variation in a microRNA that was shown to down-regulate *GABRA6* was also found to be associated with panic disorder (Muinos-Gimeno *et al.*, 2011). Additionally, a SNP in *GABRA6* was associated with a measure of anxiety in 937 normal subjects (Arias *et al.*, 2012) and a repeat length polymorphism in *GABRB3* ($GABA_A$ receptor β_3 subunit) was associated with poorer mental health in patients with post-traumatic stress disorder (Feusner *et al.*, 2001). While replication of most of these results is lacking, they do suggest that inherited variation in the GABA system can alter the severity of anxiety and risk of developing an anxiety disorder.

1.4.3.3 Cholecystokinin

The CCK system has been described in section 1.3 (page 29). Briefly, it consists of the peptide signalling molecule CCK and the G-protein coupled CCK_A and CCK_B receptors. CCK is of interest in panic disorder due to the ability of exogenously administered peptide to induce panic attacks (section 1.4.1.1, page 48). The panicogenic effects of CCK have been attributed to activation of the CCK_B receptor, due to the selectivity of the short peptides (CCK-4 and CCK-5) for this receptor and since pre-treatment with selective CCK_B receptor antagonists can prevent CCK induced panic attacks in panic disorder patients and healthy subjects (Bradwejn *et al.*, 1994; Bradwejn *et al.*, 1995).

Other evidence also supports CCK_B receptor as the main mediator of CCK-associated panic and anxiety. In humans, *CCKBR* expression was found to have increased in the prefrontal and cingulate cortex and cerebellum of suicide victims (Sherrin *et al.*, 2004) and some genetic studies have supported an association for *CCKBR* variation and panic (Muinos-Gimeno *et al.*, 2011; Otowa *et al.*, 2012) (see also section 3.1.1, page 93). Mice that over-express *Cckbr* show higher levels of anxiety than wild-type mice (Chen *et al.*, 2010a; Raud *et al.*, 2003) and CCK_B receptor knockout mice have been found to have reduced anxiety-like behaviour (Abramov *et al.*, 2004; Raud *et al.*, 2005) though this is dependent on gender, strain and environmental variables (Abramov *et al.*, 2008). More consistent effects are found with CCK_B receptor antagonists blocking anxiety-like behaviour in the elevated zero maze (a rodent model of anxiety) (Matto *et al.*, 1997), an animal model of post-traumatic stress disorder (Adamec *et al.*, 1997), maternally deprived rats (Vazquez *et al.*, 2005) and other animals with increased baseline anxiety (Becker *et al.*, 2001; Wang *et al.*, 2003).

Although the CCK_B is the main receptor implicated, there is also evidence that the CCK_A receptor is involved in panic and anxiety. In rats, injection of CCK-8S, but not unsulphated CCK-8, into the nucleus accumbens (part of the brain associated with the caudate/putamen) increased anxiety-like behaviour, and this could be blocked with a CCK_A receptor selective antagonist (Dauge *et al.*, 1989). Other animal studies have also found anxiolytic-like activity from CCK_A receptor antagonists (Ballaz *et al.*, 1997; Hendrie *et al.*, 1993; Hernandez-Gomez *et al.*, 2002). Similarly, rats with a naturally occurring variant that resulted in loss of functional CCK_A receptors, Otsuka Long Evans Tokushima Fatty (OLETF) strain (Nakamura *et al.*, 1998), displayed increased anxiety-like behaviour on the elevated plus maze and

light/dark box (Li *et al.*, 2002; Yamamoto *et al.*, 2000). However, there is conflicting data (Adamec *et al.*, 1997; Farook *et al.*, 2004) and functional studies on anxiety in humans are lacking.

The CCK system is part of, or interacts with fear and stress pathways. Both *CCK* and *CCKBR* expression are high in the human amygdala (see sections 1.3.3.1 and 1.3.3.3, from page 34, http://human.brain-map.org/), and bilateral microinjection of CCK into the amygdala increased anxiety-like behaviour of rats in the elevated plus maze (Perez de la Mora *et al.*, 2007). CCK-4 induced panic attacks in humans have been found to alter cerebral blood flow to the insular cortex (Javanmard *et al.*, 1999), which is closely associated with the amygdala and is thought to be involved in the association of bodily states with emotion. Additionally, both the CCK_B receptor and the CRH_1 receptor have been found to be important in anxiety induced by immobilisation stress in mice; a combination of CCK_B and CRH_1 receptor antagonists were required to completely prevent behavioural changes (Wang *et al.*, 2011). Sherrin *et al.* (2009) also found that repeated low doses of a CRH_1 receptor agonist produced behavioural changes in mice consistent with increased anxiety and significantly increased *CCK* mRNA in several brain regions including the frontal cortex, hippocampus, amygdala and thalamus. CCK_B receptor levels also increased in several brain regions and acute administration of a CCK_B receptor antagonist could reverse the behavioural effects of the chronic administration of the CRH_1 agonist, but a CRH_1 antagonist could not (Sherrin *et al.*, 2009).

Overall, the involvement of CCK and CCK_B receptor in anxiety is strongly supported, and CCK_A receptor may also be involved in some circumstances.

1.4.3.4 Adenosine

Adenosine is a nucleoside signalling molecule that mediates a wide variety of physiological effects. Adenosine activates at least four types of receptor: A_1, A_{2a}, A_{2b} and A_3, all of which are G-protein coupled, with the first two being the main receptors found in the brain (Wei *et al.*, 2011). Antagonism of adenosine receptors is thought to mediate the anxiogenic effects of caffeine (Vilarim *et al.*, 2011).

There are conflicting results regarding whether the A_1 or A_{2a} receptors are most important in anxiety. Studies using knockout animals have provided some support for each receptor having an involvement, with increased anxiety having been found in A_1 (Gimenez-Llort *et al.*, 2002; Johansson *et al.*, 2001) and A_{2a} (Houchi *et al.*, 2008; Ledent *et al.*, 1997) knockout animals by different research groups. However, there have been some conflicting results with one study showing no change in anxiety in A_1 knockouts (Lang *et al.*, 2003), while other signalling systems have also been found to vary in A_{2a} knockout strains (Short *et al.*, 2006; Snell *et al.*, 2000), making it difficult to determine the direct effects of these receptors.

Animal studies using selective agonists and antagonists generally support the A_1 receptor as the main target for caffeine induced anxiety. Blockade of A_1 receptors has been found to reduced anxiety-like behaviour in mice tested on the elevated plus maze (Florio *et al.*, 1998; Prediger *et al.*, 2004) and in zebrafish tested on a similar set up to the light-dark box (Maximino *et al.*, 2011). Similarly, an A_1 selective agonist produced anxiogenic effects in mice (Prediger *et al.*, 2004). In contrast, an A_2 selective agonist had no effect on anxiety-like behaviour in mice (Jain *et al.*, 1995) and A_{2a} selective antagonists did not alter anxiety-like behaviour in mice (Prediger *et al.*, 2004), rats (Thorsell *et al.*, 2007) or zebrafish (Maximino *et al.*, 2011). All of these studies had a short duration between drug administration and behavioural testing, indicating that acute anxiety effects are predominantly mediated by the A_1 receptor.

The A_{2a} receptor, on the other hand, has been most strongly supported in genetic association studies of anxiety. Genetic associations between panic disorder and variants in *ADORA2A* (the gene encoding the A_{2a} receptor) have been found in different cohorts (Deckert *et al.*, 1998; Hamilton *et al.*, 2004; Hohoff *et al.*, 2010). *ADORA2A* variation has also been found to be associated with other anxiety related phenotypes, including generalised anxiety disorder (Donner *et al.*, 2012) and harm avoidant personality features (Hohoff *et al.*, 2010). Additionally, studies with healthy volunteers have shown that the anxiogenic response to caffeine is associated with *ADORA2A* genotype. Normal individuals carrying the homozygous TT genotype at rs5751876 experienced significantly more anxiety with caffeine than placebo, while those with CC or CT genotypes did not show increased anxiety with caffeine (Alsene *et al.*, 2003; Childs *et al.*, 2008; Rogers *et al.*, 2010). In contrast, the two studies of that also investigated *ADORA1* variants and anxiety found no association of that gene with panic disorder (Deckert *et al.*, 1998) or caffeine response (Rogers *et al.*, 2010).

Collectively, the results of studies into the adenosine system support the A_1 receptors being the main receptors mediating acute anxiogenic/anxiolytic response, while A_{2a} receptors modify long-term risk of anxiety.

1.4.4 Pharmacotherapy for Anxiety

Pharmacotherapy for anxiety typically involves treatment of acute attacks with short acting anxiolytics (usually benzodiazepines) and/or long-term prevention of anxiety with antidepressants or other agents (Ninan, 1999). While the actions of benzodiazepines are rapid in onset and show straightforward pharmacodynamics, the antidepressants are much more variable and less well understood. This section discusses the current knowledge of the actions of antidepressants, particularly as they relate to anxiety, as well as a newer class of anxiolytic drugs - gabapentin and pregabalin.

1.4.4.1 Anxiolysis and Anxiogenesis with Antidepressants

The efficacy of antidepressants, particularly SSRIs, for the long-term treatment of anxiety has been well established (Koen & Stein, 2011; Ninan, 1999), however, the short-term effects are more variable. A substantial minority of patients treated with antidepressants experience and initial worsening of anxiety, sometimes referred to as jitteriness syndrome (Sinclair *et al.*, 2009). This is usually transient, declining after a couple of weeks of treatment (Beasley *et al.*, 1992), but tends to be more common in patients being treated for an anxiety disorder (Christmas *et al.*, 2010; Gorman *et al.*, 1987; Ramos *et al.*, 1993; Schneier *et al.*, 1990) than those being treated for depression (Gollan *et al.*, 2012; Li *et al.*, 2011a; Trindade *et al.*, 1998). Despite the transient nature, for some patients the anxiogenic effects are so severe that they discontinue treatment (Schneier *et al.*, 1990).

Evidence from studies conducted with the SSRI citalopram in normal volunteers also supports an initial increase in anxiety risk. Citalopram enhanced recognition of fearful faces (Browning *et al.*, 2007; Harmer *et al.*, 2003) and increased startle responses (Grillon *et al.*, 2007) when administered within a few hours of testing. In contrast, seven days repeated administration reduced attention to fearful faces (Murphy *et al.*, 2009), the accuracy of recognition of negative emotional faces and recall of negative emotional words (Harmer *et al.*, 2004). Furthermore, these changes in cognitive function correlated with changes in

amygdala activity, with increased activity within hours of citalopram administration (Bigos *et al.*, 2008) and reduced activity after seven days of repeated administration (Harmer *et al.*, 2006). This suggests that there is heightened activity in fear pathways following acute administration of SSRIs, followed by a reduction in activity with prolonged treatment.

Chronic antidepressant treatment has been found to be beneficial in a variety of anxiety states. Approximately 50% of patients being treated for major depressive disorder had reduced anxiety levels after two weeks of SSRI treatment (Gollan *et al.*, 2012). Meta-analysis of studies that treated patients afflicted by various anxiety disorders with eight to twelve weeks of paroxetine (an SSRI), compared to placebo, found that remission was significantly more likely in panic disorder, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder and generalised anxiety disorder (Ballenger, 2004). Maintaining patients whose anxiety disorder was effectively treated with an antidepressant drug on that medication also reduces relapse rates, compared to placebo maintenance therapy (Donovan *et al.*, 2010).

In conclusion, antidepressants initially increase risk of anxiety, which results in increased perception of anxiety in some patients. In most of the affected individuals, this increased anxiety reduces after one to three weeks of treatment and the anxiolytic effects of antidepressants become predominant, resulting in clinical improvement with prolonged treatment.

1.4.4.2 Mechanism of Action of Antidepressants

The mechanisms through which antidepressant medications have their acute anxiogenic and chronic anxiolytic effects are poorly understood. In fact, the mechanism for antidepressant action for these drugs is still debated despite having received decades of research. There are two main antidepressant classes used in the treatment of anxiety: the SSRIs and tricyclic antidepressants. In the short-term, both of these drugs increase monoamine neurotransmitter levels in the brain, with SSRIs increasing serotonin levels, while tricyclic antidepressants increase serotonin and noradrenaline to a similar extent and also increase dopamine levels (Rang *et al.*, 1999). The SSRIs are the primary focus of the discussion here as they are the newer class of antidepressants and are the focus of most recent research.

A variety of theories have been postulated about the action of antidepressant medications, with the current leading theory being that they stimulate neurogenesis in the brain.. In animals, neurogenesis has been shown to occur in response to a variety of antidepressant agents, including tricyclic antidepressants, SSRIs (Santarelli *et al.*, 2003), voluntary exercise (Huang *et al.*, 2012) and electroconvulsive shocks (an animal model of electroconvulsive therapy) (Perera *et al.*, 2007). This also fits with anxiety findings from animal models, as mice that lacked adult neurogenesis were found to have a large increase in anxiety-like behaviour, with little change in depressive-like behaviour or general locomotor and exploratory activity (Revest *et al.*, 2009). Similarly, mice selectively bred to have high anxiety-like behaviour had lower levels of neurogenesis than mice bred for normal anxiety-like behaviour following the stress of behavioural testing (Sah *et al.*, 2012). The up to four-week therapeutic lag of anxiolytic effect of antidepressants is also consistent with neurogenesis, since five days administration of fluoxetine to rats did not alter behavioural measures or neurogenesis, but 28 days administration significantly reduced anxiety-like behaviour and increased neurogenesis (Marcussen *et al.*, 2008).

It is only in recent decades that neurogenesis has been shown to occur in the adult human brain (Curtis *et al.*, 2011; Eriksson *et al.*, 1998). There are only a few studies of neurogenesis stimulated by antidepressants that have been conducted in humans, often looking at markers of neurogenesis in post-mortem brain tissue. These studies typically have small sample numbers (approximately 10 cases) and completely matching the cases to controls is often impossible, with potential confounders, such as cause of death or therapeutic interventions, often varying between the groups. Thus it is not surprising that three such studies comparing depressed patients with controls found different results. Two studies found no effect by antidepressants on neurogenesis. However, one of these looked at elderly patients, who would have had reduced rates of neurogenesis regardless of treatment (Lucassen *et al.*, 2010), and the other compared patients treated with antidepressants to a mixture of controls, treated and untreated schizophrenic and bipolar patients and untreated depressed patients (Reif *et al.*, 2006). The third study found a significant increase in dividing cells with antidepressants; however, they did not match their control patients to the cases with the same rigor as the other two studies (Boldrini *et al.*, 2009). None of these three studies reported whether the patients were responding favourably to the administered antidepressant treatment or whether any behavioural therapies were being administered. Thus, the role of neurogenesis in the

antidepressant effect of antidepressant drugs in humans still requires further research, while anxiolytic effects have seen even less study to date.

In animal studies, a necessity for neurogenesis was found by Santarelli *et al.* (2003), who used targeted irradiation to block neurogenesis in mice. When neurogenesis was suppressed, the anxiolytic and antidepressant effects of the SSRI fluoxetine were eliminated. Similar results were found for depressive behaviour in primates, with chronic stress increasing anhedonia, which was blocked by fluoxetine in normal but not irradiated animals (Perera *et al.*, 2011). Although there has been conflicting data, the majority of animal studies, like the two described here, indicated that neurogenesis is needed for SSRI and tricyclic antidepressant action (Petrik *et al.*, 2012). It has been postulated that anxiolytic effects of neurogenesis may be due to improvements in pattern recognition, allowing patients with anxiety disorders to better distinguish safe from dangerous environments (Kheirbek *et al.*, 2012).

Other animal studies have attempted to elucidate the mechanisms through which antidepressants promote neurogenesis. Vascular endothelial growth factor has been implicated, as levels of mRNA and protein increase in rat hippocampus around the time of therapeutic activity and vascular endothelial growth factor receptor inhibitors eliminate both the behavioural effects and increased neurogenesis from antidepressants (Warner-Schmidt & Duman, 2007). Brain-derived neurotrophic factor (BDNF) is also thought to be involved as: expression changes have been found with antidepressant drugs in various models (Tardito *et al.*, 2006); it produced antidepressant-like effects when injected bilaterally into the hippocampi of rats (Shirayama *et al.*, 2002); and transgenic mice lacking its receptor failed to show the behavioural or neurogenesis responses to antidepressants of wild-type litter mates (Li *et al.*, 2008).

While most studies support the importance of neurogenesis in antidepressant action, it is not known whether this is the only change needed to produce antidepressant effects. A variety of other effects have been found with antidepressant drugs some of which have been shown to be necessary, in the specific animal model studied, for certain antidepressant or anxiolytic effects of the drug. For example, β-arrestin 2 was found to be down-regulated by corticosterone (mimicking chronic stress) in mice, which was prevented by fluoxetine administration, while knockout mice that lacked expression of β-arrestin 2 showed a high anxiety phenotype that was not reversed by fluoxetine (David *et al.*, 2009). Similarly, another

study in mice found that fibroblast growth factor 2 signalling in the prefrontal cortex was necessary for the antidepressant effects of fluoxetine and imipramine (Elsayed *et al.*, 2012). Chronic intracerebroventricular fibroblast growth factor 2 infusion had similar behavioural effects to antidepressants and increased the generation of new oligodendrocytes in the prefrontal cortex (Elsayed *et al.*, 2012). Changes in neurotransmitters have also been found in rodents, including reduced GABAergic signalling by fluoxetine and imipramine (Mendez *et al.*, 2012) and increased 5-HT_{1A} receptor levels by fluoxetine (Lin *et al.*, 2012). Additionally, some anxiolytic effects of antidepressants have been reported occur in mice even when neurogenesis is blocked (David *et al.*, 2009), raising doubt over the importance of neurogenesis in the mechanism of action of antidepressants. The combination of findings like this, with findings supporting the importance of neurogenesis have lead to an alternative structural hypothesis, that antidepressants act by enhancing dendritic remodelling in key anxiety brain regions (Gorman & Docherty, 2010).

1.4.4.3 Gabapentin and Pregabalin for Anxiety

Gabapentin and pregabalin are two related drugs primarily used to treat epilepsy and neuropathic pain, which also have anxiolytic effects (Tassone *et al.*, 2007). Pregabalin is the newer of the two compounds and was specifically developed to improve upon gabapentin. Pregabalin is both more potent and has superior pharmacokinetic properties, including a faster peak plasma concentration and almost complete gastrointestinal absorption, providing more consistent effects between patients (Bockbrader *et al.*, 2010). Both drugs are well tolerated, usually only producing mild side effects, including dizziness and drowsiness, and both drugs have similar therapeutic indications, although only pregabalin has been licensed for anxiety treatment (Bockbrader *et al.*, 2010).

The most thoroughly studied anxiety disorder with gabapentin/pregabalin is generalised anxiety disorder. Following initial case presentations showing benefit with gabapentin (Pollack *et al.*, 1998), numerous trials have shown that pregabalin is superior to placebo in the treatment of generalised anxiety disorder (Feltner *et al.*, 2003; Montgomery *et al.*, 2008), with many showing significant benefit over placebo as early as one week after treatment initiation (Montgomery *et al.*, 2006; Pande *et al.*, 2003; Pohl *et al.*, 2005; Rickels *et al.*, 2005). Studies comparing pregabalin to standard treatments for generalised anxiety disorder found a similar therapeutic effect with better tolerability for pregabalin (Feltner *et al.*, 2003; Montgomery *et*

al., 2006; Pande *et al.*, 2003; Rickels *et al.*, 2005). Not surprisingly, meta-analyses have also supported the efficacy of pregabalin in generalised anxiety disorder, showing that it reduces both psychic and somatic anxiety symptoms (Boschen, 2011; Lydiard *et al.*, 2010).

Moderate support has also been found for the use of gabapentin and pregabalin in social phobia. Two randomised controlled trials found that gabapentin or pregabalin were superior to placebo at reducing anxiety symptoms (Pande *et al.*, 1999; Pande *et al.*, 2004). Gabapentin also compared favourably to other pharmacotherapy for social phobia, being amongst the most efficacious treatments (Blanco *et al.*, 2003). Another study of pregabalin found that long-term maintenance in pregabalin responders significantly delayed relapse of social phobia, compared to placebo (Greist *et al.*, 2011). Altogether, these studies suggest that gabapentin/pregabalin may be useful treatments for social phobia, but larger trials are needed to confirm this.

Less research has been conducted into the use of gabapentin or pregabalin for other anxiety disorders. For panic disorder, gabapentin was found to be beneficial for two patients that had failed to respond to other treatments (Pollack *et al.*, 1998), and it reduced the proportion of patients that experienced panic attacks in response to sodium lactate infusion (Layton *et al.*, 2001). However, the largest study in panic disorder, in which 103 patients were randomised to gabapentin or placebo treatment groups, found no significant benefit over placebo for the total sample and only a short-lived benefit for those with more severe baseline symptoms (Pande *et al.*, 2000). This suggests that only a subpopulation of panic disorder patients is likely to benefit from gabapentin or pregabalin. Studies in obsessive-compulsive disorder have typically been conducted in small groups of patients that have failed to respond adequately to other treatments. Gabapentin and pregabalin have been beneficial in this subpopulation (Cora-Locatelli *et al.*, 1998; Di Nicola *et al.*, 2011; Onder *et al.*, 2008; Oulis *et al.*, 2008). Finally, in post-traumatic stress disorder, an animal model and a case report have supported pregabalin use (Paslakis *et al.*, 2011; Zohar *et al.*, 2008), but a retrospective study of injured service members found no difference in post-traumatic stress disorder development in those treated acutely with gabapentin or pregabalin (Fowler *et al.*, 2012).

Thus, gabapentin and pregabalin show clear benefit in the treatment of some anxiety disorders. The use of pregabalin in generalised anxiety disorder is the most researched and best supported, with moderate support for its use in social phobia also. While there is some

support for the use of gabapentin/pregabalin in other anxiety disorders, large randomised controlled trials are generally lacking. Further investigation is warranted as existing studies suggest that pregabalin is well tolerated, has a rapid onset of action and may be efficacious in patients that failed to respond to standard therapy.

1.4.4.4 Mechanism of Action of Gabapentin and Pregabalin

Gabapentin and pregabalin were originally designed as lipophilic GABA analogues, with the expectation that they would be effective in treating disorders where increased GABA signalling was desired, such as epilepsy. Although they have been found to be effective in the treatment of epilepsy and neuropathic pain, as well as psychiatric disorders, most evidence indicates that these compounds do not act on traditional GABA targets, such as GABA receptors (Eutamene *et al.*, 2000; Lanneau *et al.*, 2001; Li *et al.*, 2011b), reuptake proteins (Stringer & Aribi, 2002) or enzymes (Sills *et al.*, 2003).

Following the rejection of traditional GABA targets, the search for specific binding proteins for gabapentin and pregabalin lead to a calcium channel subunit. Gabapentin binding in rat and pig brain suggested that specific binding was occurring with the calcium channel subunits $\alpha_2\delta$ (Gee *et al.*, 1996). This was further supported by a greater than ten fold increase in gabapentin binding following transfection of $\alpha_2\delta$ subunits into COS cells (a fibroblast-like cell line originating from African green monkeys) (Gee *et al.*, 1996). Recent work using *in vitro* binding assays has reinforced these findings since, out of more than 30 central nervous system targets tested, including receptors and neurotransmitter reuptake transporters, $\alpha_2\delta$ was the only protein found to bind pregabalin (Li *et al.*, 2011b).

Four different subtypes of $\alpha_2\delta$ have been identified, each with its own gene (*CACNA2D1* through to *CACNA2D4*). For each of these genes, a single protein product is initially transcribed followed by post-translational modification that cleaves the α_2 subunit from the δ subunit, although they remain associated *in vivo* via disulphide bonds (Taylor *et al.*, 2007). The α_2 subunit has the gabapentin/pregabalin binding site and, of the four different subtypes, only $\alpha_2\delta$-1 and $\alpha_2\delta$-2 bind gabapentin (Marais *et al.*, 2001; Qin *et al.*, 2002). The $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits are both expressed in high to moderate levels in the mouse brain (Marais *et al.*, 2001), further supporting their potential as the targets for the psychiatric and neurological actions of gabapentin.

As $\alpha_2\delta$ forms part of a calcium channel, much research has been conducted to determine the effects of gabapentin/pregabalin binding on the function of calcium channels. Most studies of gabapentin or pregabalin's effects on calcium channels indicate that they reduce voltage-dependent calcium influx at the axon terminus (Bayer *et al.*, 2004; Cunningham *et al.*, 2004; Dooley *et al.*, 2002; Fink *et al.*, 2002; Fink *et al.*, 2000; Stefani *et al.*, 2001; Sutton *et al.*, 2002; van Hooft *et al.*, 2002). However, not all studies support this (for example, see (Schumacher *et al.*, 1998) and those that do often differ in features such as time of onset of gabapentin/pregabalin action. For example, studies that transfected $\alpha_2\delta$-2 into a human kidney cell line showed that gabapentin administration for up to 6 hours had no effect on cell calcium currents, while incubations of over 17 hours significantly reduced currents (Heblich *et al.*, 2008; Hendrich *et al.*, 2008). However, the short-term application of gabapentin inhibited calcium currents in rat dorsal root ganglion neurons within minutes (Martin *et al.*, 2002). One possible explanation for the differences between studies may be cell specific expression of other calcium channel subunits. In rats, expression of the β_2 calcium channel subunit has been found to be higher in cells that show stronger responses to gabapentin (Martin *et al.*, 2002), and excess expression of a human β_4 splice variant in frog oocytes has been found to block the effects of gabapentin (Mich & Horne, 2008). Hence, evidence suggests that gabapentin and pregabalin may alter calcium channel function in a cell or tissue specific manner.

One proposed mechanism for the altered calcium flux produced by gabapentin and pregabalin is reduced trafficking of functional channels to the surface of the cell. Transfection studies of mammalian cells, that used fluorescent binding, showed greatly reduced cell surface expression of calcium channel subunits following 72 hours of gabapentin exposure, compared to control, while total cell levels remained comparable (Heblich *et al.*, 2008). A similar experiment also found reduced calcium currents and reduced calcium channel membrane expression following 40 hours of gabapentin exposure and evidence that gabapentin was acting intracellularly (Hendrich *et al.*, 2008). Furthermore, cell studies have shown that gabapentin reduced cell surface $\alpha_2\delta$-2 containing calcium channels by reducing the recycling of receptors back to the membrane without affecting the rate of internalisation (Tran-Van-Minh & Dolphin, 2010). Hence, it seems that gabapentin can alter calcium currents by reducing the number of calcium channels on the surface of the cell.

Since presynaptic calcium influx is necessary for vesicle fusion, the alteration in calcium channel function may, in turn, alter neurotransmitter release (Figure 1.20). Numerous studies have found that gabapentin and/or pregabalin reduce potassium or voltage induced neurotransmitter release from cell fragments, whole cells and brain slices, in a range of species including rodents and humans. Various different neurotransmitters have been found to be inhibited, including glutamate (Cunningham *et al.*, 2004; Dooley *et al.*, 2000), glycine (Bayer *et al.*, 2004) noradrenaline (Fink *et al.*, 2002), serotonin, acetylcholine (Brawek *et al.*, 2008), dopamine (Reimann, 1983) and GABA (Brawek *et al.*, 2009). Some studies have found negative results, for example Brawek *et al.* (2009) found inhibition of GABA release but no effect on glutamate and, despite finding reductions in several other neurotransmitters, Brawek *et al.* (2008) found no changes in dopamine levels with gabapentin or pregabalin. This is consistent with the calcium channel studies described above and suggests the effects of gabapentin and pregabalin are cell type, not neurotransmitter, specific.

The effects of gabapentin and pregabalin on calcium channel function are still debated and an alternative mechanism of action has been suggested recently – an influence on synaptogenesis via interactions with thrombospondins. Thrombospondins are extracellular proteins with a variety of functions in human health and disease (Adams & Lawler, 2004). Thrombospondins 1 and 2 were identified as the molecules secreted by astrocytes that increase synapse formation in rat glutamatergic neurons (Christopherson *et al.*, 2005). This effect that seems to be more important for some types of neurons as blocking thrombospondins did not reduce synapse formation induced by astrocyte conditioned media in rat GABAergic neurons (Hughes *et al.*, 2010). The $\alpha_2\delta$-1 subunit has been shown to bind thrombospondins and, in rat retinal ganglion cells, this interaction was necessary for the increased synapse formation stimulated by astrocytes and could be blocked by gabapentin (Eroglu *et al.*, 2009). *In vivo* increased synapse formation, specifically excitatory synapses, was seen in animals overexpressing $\alpha_2\delta$-1 (Eroglu *et al.*, 2009). Hence, disruption of this interaction between astrocyte thrombospondins and neuronal $\alpha_2\delta$ may be the mechanism by which gabapentin alters neurobiology.

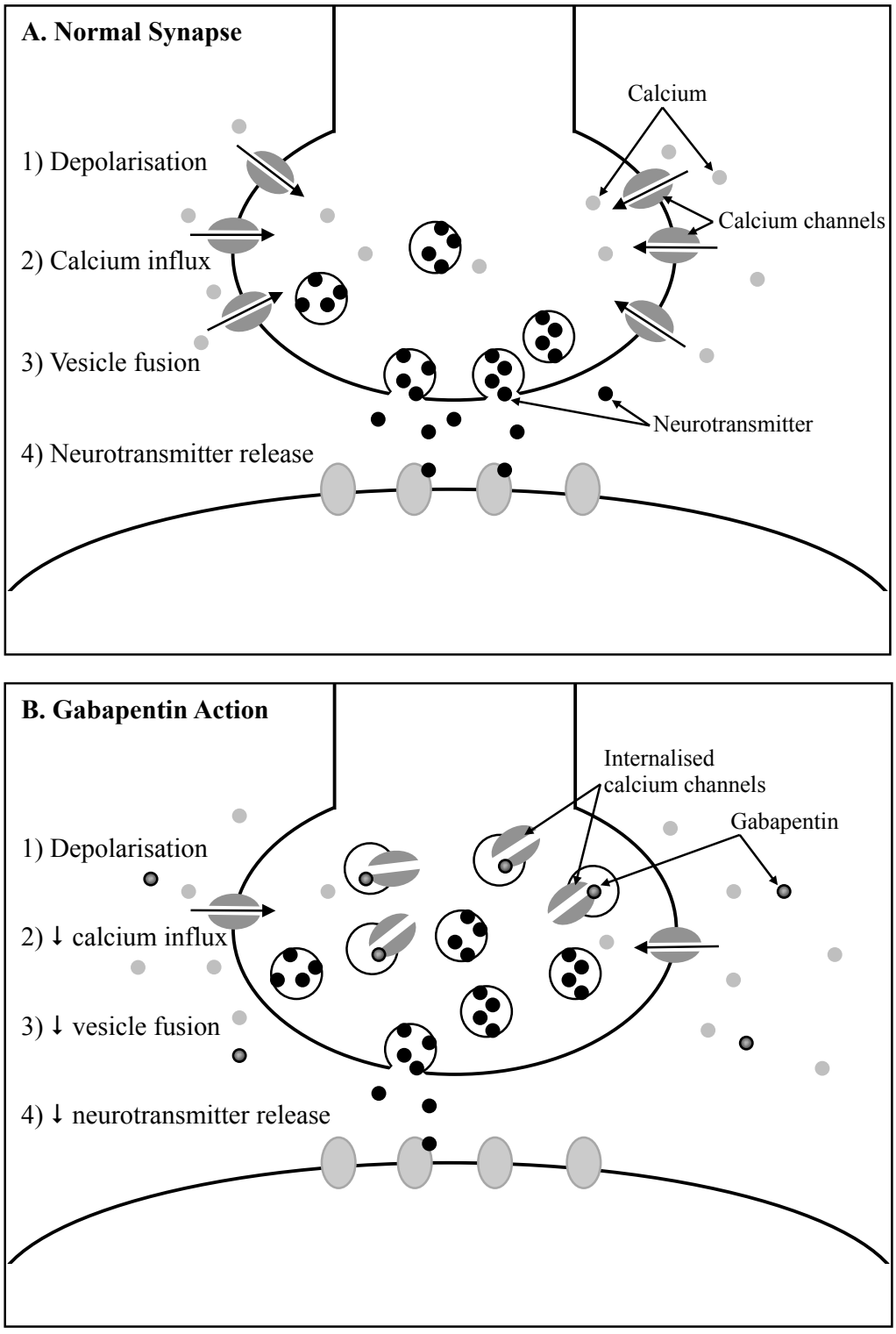


Figure 1.20 Gabapentin Action on Calcium Channels.
A comparison of presynaptic cell function in the absence (A) or presence (B) of gabapentin. Gabapentin causes calcium channels to be internalised, reducing calcium flux and neurotransmitter release following depolarisation.

Most research on gabapentin/pregabalin has focused on their anti-epileptic or analgesic properties and it is not currently clear whether the mechanism of action for the anxiolytic

effects is the same. The anxiolytic response to pregabalin was studied in mice with mutations in either $\alpha_2\delta$-1 or $\alpha_2\delta$-2 that prevent pregabalin binding. In this test $\alpha_2\delta$-2 mutants showed similar results to wild-type, while $\alpha_2\delta$-1 mutants showed no anxiolytic response to pregabalin (Lotarski *et al.*, 2011). Thus, $\alpha_2\delta$-1 seems to be important for anxiety, though the relative involvement of neurotransmitter changes and synaptogenesis have not yet been determined. This is similar to the situation discussed in section 1.4.4.2 (page 61) for the anxiolytic effects of antidepressants, where both structural changes (neurogenesis and dendritic remodelling) and specific signalling systems have been implicated. Both mechanisms could produce the kind of brain changes necessary to correct a mental disorder and it is possible that either or both are necessary. Hence, further research is needed to determine the exact mechanism through which gabapentin and pregabalin reduce anxiety.

1.4.5 Summary

- Anxiety disorders are common and have a significant negative impact on the lives of those afflicted.
- Changes in fear or stress pathways can lead to a person developing acute or chronic anxiety symptoms.
- Acute anxiety in the form of panic attacks can be induced experimentally in both anxiety patients and healthy subjects through the administration of panicogens.
- A mixture of genetic and environmental factors are involved in the pathogenesis of anxiety disorders, including genetic variation that increases the risk of multiple different disorders.
- A variety of neurotransmitters and neuromodulators, including serotonin, GABA, CCK and adenosine, are involved in anxiety pathways within the brain.
- Many of these neurotransmitters/neuromodulators are candidates for the genetic predisposition and the mechanism of action of treatments for anxiety, both of which are still poorly understood.

1.5 Aims

The overall aim of the present study was to gain a better understanding of the pathogenesis and treatment of anxiety disorders, particularly panic disorder. While the pathogenesis and treatment were investigated in separate studies, both used candidate gene approaches, with

some overlap in the candidate genes. This was intended to take advantage of the relationship between pathogenesis and treatment – specifically, that biological changes that cause or increase the risk of disease, should make good targets for the treatment of that disease.

The aims for each part of the project were to:

1. Determine whether common genetic variation in any of the genes in the CCK system is associated with panic in a New Zealand cohort (Genetic Association).
2. Determine whether drugs commonly used to treat anxiety altered the expression of any of the candidate genes, and, if so, what the time course of these changes was (Cellular Changes with Antidepressants).

2 Methodology

2.1 Buffers and Solutions

All reagents were of analytical grade. Most reagents were obtained from Invitrogen (New Zealand), British Drug Houses Ltd (England) or Sigma-Aldrich (New Zealand). Water for all buffers and solutions was distilled and then filtered through a Milli-Q Plus water purification system (Millipore, New Zealand).

Cell Lysis Buffer: 10 mM Tris-HCl pH 8, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 3.5 mM sodium dodecyl sulphate (SDS) and protease inhibitor (one "cOmplete Protease Inhibitor Cocktail Tablet" per 50 ml, Roche, USA). Initially, 10 mg/ml Nonidet P40 was also included in the buffer, but was removed following testing (see section 2.7.1, page 88).

Laemmli's Buffer (protein denaturation and loading dye): 0.2 M dithiothreitol, 140 mM SDS, 48 mM Tris-HCl pH 6.8, 5.4 M glycerol, 0.60 mM bromophenol blue (dissolved in ethanol [Sharlau Chemie, Spain] 2% of final volume), 9.5 mM sodium azide.

Membrane Blocking Buffers: Milk powder block consisted of 5% (w/v) low fat milk powder in PBS. Casein (catalogue number 972-40200) and Odyssey (catalogue number 927-40100) blocks were purchased from LI-COR Biosciences (New Zealand).

PBS (Phosphate Buffered Saline): 1 PBS tablet (Oxoid Ltd, England) dissolved in 100 ml sterile water, as per manufacturers instructions. Final concentrations: 135 mM sodium chloride, 2.7 mM potassium chloride, 8 mM di-sodium hydrogen phosphate, and 1.5 mM potassium di-hydrogen phosphate, pH 7.3.

PBS-T (Phosphate Buffered Saline with Tween): PBS made as above, with 0.1% (v/v) Tween 20.

Polymerase Chain Reaction (PCR) Buffer (final concentration): 50 mM potassium chloride, 10 mM Tris-HCl pH 8.8 and the relevant concentration of magnesium chloride, for example: 1.5 mM.

PCR Loading Dye: 1.2 M sucrose and 0.005% (w/v) Orange G (Harleco, USA) in TBE.

TBE: 89 mM Tris-Borate (pH 8.3) and 2.5 mM EDTA.

TE: 10 mM Tris-HCl pH 8.0 and 1 mM EDTA.

2.2 Gels

2.2.1 Polyacrylamide Gels

Polyacrylamide gels were used in the electrophoresis of proteins. Gels were prepared as described below. Various ratios of Laemmli's buffer to protein lysate were tested with slot-blot and Western blot experiments; a typical dilution was one part Laemmli's buffer to four parts protein lysate.

Separating Gel: 8-12% (w/v) acrylamide (29:1 acrylamide/bis, Bio-Rad, USA), 375 mM Tris-HCl pH 8.8, 3.5 mM SDS, 0.23 M sucrose, 0.025% (v/v) tetramethylethylenediamine, 5.5 mM ammonium persulfate.
Stacking Gel: 2% acrylamide (29:1 acrylamide/bis, Bio-Rad, USA), 63 mM Tris-HCl, 1.7 mM SDS, 0.02% (v/v) tetramethylethylenediamine, 8.8 mM ammonium persulfate.
Preparation: The cassette (Invitrogen, New Zealand) was partially filled with separating gel and allowed to set; then remainder of cassette filled with stacking gel, comb placed at the top and allowed to set. Comb was removed shortly before loading and running gel.

2.2.2 Agarose gels

Agarose gels were used for the electrophoresis of DNA or RNA. Gels were prepared as described below. Five microlitres of PCR Loading Dye was mixed with 5 to 10 µl of amplification product prior to electrophoresis. DNA Molecular Weight Marker V (Roche, New Zealand) was used to determine fragment sizes. Electrophoresis was conducted in TBE at approximately 8 V/cm.

2% agarose gels: 2% (w/v) UltraPure Agarose and 0.01% (w/v) ethidium bromide in TBE.

2.3 Primer Design

All primers designed as part of this study were custom designed and obtained from Invitrogen (New Zealand). Sequence data was obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and used to select sites for design. Primers between 15 and 30 base pairs long were designed and analysed using the DNA calculator provided by Sigma (http://www.sigma-genosys.com/calc/DNACalc.asp) to ensure that:

- Melting point was 50 to 65°C for genotyping primers or 60 to 65°C for quantitative PCR (qPCR) primers, and each primer of the pair was within a couple of degrees of the other.
- G/C content was between 40-60%.
- Secondary structure was weak or absent.

Primers were then further tested and screened for specificity by performing a UCSC In-Silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) prior to purchase.

2.4 Association Studies

2.4.1 Subjects

This study was conducted with the approval of the Multi-region Ethics Committee, New Zealand. DNA and phenotype information that had been collected as part of the South Island Bipolar Study (SIBS) was used in the present study. The probands for the SIBS were identified through the use of the Otago Bipolar Register, outpatient services or advertising. Probands had a diagnosis of bipolar I or bipolar II disorder and had to consent to be interviewed, give blood for DNA extraction, and have biological relatives that the researchers could contact. Family members 16 years of age or older were contacted and invited to participate, unless advised otherwise by probands. Family members also had to consent to be interviewed and give blood for DNA extraction. Family trees were constructed using the Family Interview for Genetic Studies. Further details of the cohort studied are given in sections 3.2 and 3.4.1 (page 98 and 120, respectively).

2.4.2 Selection of SNPs and Haplotypes with Haploview

Genotype data for the CEU population were downloaded (between 14/03/2007 and 21/12/2007) from the HapMap Project website (http://www.hapmap.org/index.html) and analysed using Haploview software (version 3.32, downloaded on 23/06/2006, from http://www.broad.mit.edu/mpg/haploview/). Three analysis methods were used to produce haplotype blocks from this data:

1) The Confidence intervals analysis (Gabriel *et al.*, 2002), the default setting in Haploview, was used to determine the final haplotypes for analysis.
2) The Four-gamete Rule (Wang *et al.*, 2002) was used to select SNPs for genotyping. This method produces the most haplotype blocks and tag SNPs out of the three pre-set analysis methods in Haploview.
3) A custom designed analysis in which one block encompasses the entire gene and any SNPs flanking the gene was also used to select SNPs for genotyping to ensure no relevant SNPs were missed.

For inclusion in the genotyping and analyses, haplotypes had to have a frequency greater than 1% and SNPs had to have a minor allele frequency of at least 3% in the HapMap CEU population.

2.4.3 Genotyping of SNPs

SNPs were genotyped by one of three methods: allele specific primers designed to the SNP; restriction fragment length polymorphism (RFLP) assay; or TaqMan hydrolysis probe assay. If unexpected results were found, such as an allele frequency markedly different to that of the CEU population or if a SNP did not conform to Hardy-Weinberg equilibrium (see section 2.4.4.1, page 80), the genotype of a selection of samples was confirmed by Sanger sequencing.

Some haplotypes could be inferred from one or two SNPs; consequently, SIBS samples were not necessarily genotyped at all tag SNPs (see Appendix A for details). In general, TaqMan hydrolysis probes were genotyped first as these assays quickly produced results that could be easily interpreted. Multiplexes were then run for all samples with an undetermined haplotype. Finally, individual SNPs, including RFLP assays, were conducted to finalise the haplotypes of all individuals. In addition to SIBS samples with undetermined haplotype(s), randomly

selected samples were re-genotyped at each step to confirm the accuracy of the previous haplotype call. Anonymous human DNA samples were used for initial testing of assays and, for many assays, some of these samples were repeated as positive or negative controls.

2.4.3.1 TaqMan Hydrolysis Probes

When possible, SNPs that defined the most common haplotype(s) in each block, as well as any SNPs that would be difficult to design primers to (for example, due to high G/C content surrounding the SNP), were genotyped using TaqMan hydrolysis probes (Applied Biosystems, Australia). This system couples amplification of the region surrounding the SNP with a pair of fluorescently labelled probes. Each of the probes binds specifically to one of the alleles of the SNP and produces a fluorescent signal once incorporated into the DNA. After amplification, the level of fluorescent signal from each probe can be used to determine the genotype (Figure 2.1 C).

Probes were diluted 1:1 with TE to produce 20 times working stock and stored at -20°C in the dark. Amplifications contained 20 ng DNA, 50% (v/v) TaqMan Universal PCR Master Mix with No AmpErase UNG (Applied Biosystems) and 5% (v/v) probe working stock in a total reaction volume of 10 μl. The standard amplification protocol consisted of an initial 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 92°C and 1 minute at 60°C. The alternative amplification protocol had an initial 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 92°C and 90 seconds at 60°C. After amplification, the fluorescent signal was measured on an Applied Biosystems Instrument 7300, which heated samples to 60°C for 30 seconds prior to measuring the fluorescent signal, utilising SDS v1.3.1 or v1.4 software. Genotype calls were usually made automatically by the SDS software, with the threshold for accuracy set at 90%; however, the graph for each plate was also visually inspected and a minority of samples were called or uncalled manually.

2.4.3.2 Primers

Primers were designed as described in section 2.3 (page 75). For allele specific primers, two primers, identical apart from the most 3´ nucleotide, were designed for each SNP, along with a common primer (Figure 2.1 B). Fragment sizes varied from around 100 bp up to 400 bp to

allow multiplexing of primer pairs. For the restriction enzyme digest and sequencing, primers were designed on either side of the SNP, 50 to 200 bp from the SNP (Figure 2.1 A).

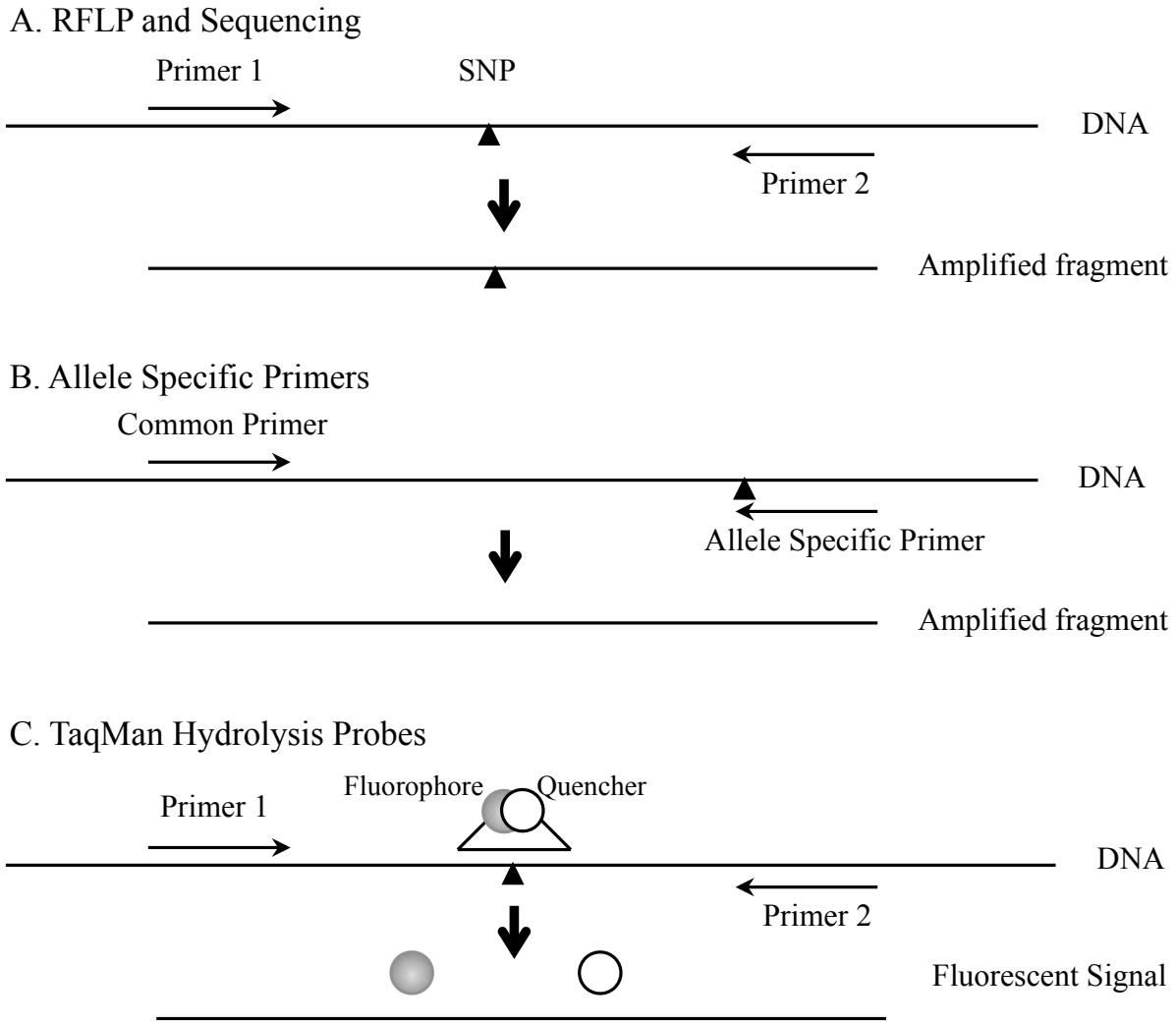


Figure 2.1 Primer Locations.
Locations of primers relative to the SNP (black triangle) are shown for each of the genotyping methods utilised. A. RFLP and Sequencing, primers are located on either side of the SNP 50 to 200 bp away. The alleles present are then determined by sequencing or restriction enzyme digest of the amplified fragment. B. Allele Specific Primers, a common primer is located some distance from the SNP and allele specific primers are located so that they end on the SNP. The alleles present are determined by the presence or absence of a fragment with each of the allele specific primers. C. TaqMan Hydrolysis Probes, both primers are some distance from the SNP. The probe consists of a nucleotide sequence that binds specifically to the SNP, a fluorophore and a quencher. When the DNA fragment is amplified the polymerase hydrolyses the probe, releasing the fluorophore so that the quencher no longer blocks its signal. This fluorescent signal is used to determine the alleles present.

2.4.3.3 Primer Optimisation and Multiplexing

Basic PCR conditions consisted of: PCR buffer (as described in section 2.1, page 73), 100 μM of each deoxynucleoside triphosphate (dNTP), 200 nM of each primer, 1 unit *Taq* polymerase (all from Invitrogen), and 100 ng DNA template, in a total volume of 20 μl. The basic reaction programme was: denature at 94°C for 3 minutes, denature at 94°C for 30 seconds, anneal at 55°C for 30 seconds, extend at 72°C for 45 seconds, repeat last three steps 29 times (30 cycles), extend at 72°C for 5 minutes.

Each set of primers was optimised for magnesium concentration and annealing temperature. To reduce the number of reactions required, primers with similar optimal conditions were multiplexed and the multiplex was further optimised for magnesium concentration, annealing temperature, primer concentration and, if necessary, number of cycles. Multiplexes also had higher concentrations of dNTPs and *Taq* polymerase. Amplified fragments were visualised on 2% agarose gels.

2.4.3.4 RFLP Assay

Primers for *CCKBR* SNP rs2929180 were designed on either side of the SNP so that, when fully digested with the enzyme SacII the following band pattern was seen: individuals homozygous for the minor allele would show two products (186 and 159 bp); homozygous for the major three products (159, 116 and 70 bp); and heterozygous individual four products (186, 159, 116 and 70 bp). Restriction enzyme digest of 15 μl of amplified DNA was conducted using 6 units of SacII in NEBuffer 4 (Biolabs, New Zealand; total volume = 20 μl), incubated at 37°C for a minimum of 6 hours. The resulting fragments were visualised on 2% agarose gels.

2.4.3.5 Sequencing

Samples were amplified using the relevant primers and amplification protocol, then 5 to 9 μl of the product was combined with 0.5 units of shrimp alkaline phosphatase (Affymetrix, USA) and 10 units of exonuclease I (Biolabs, New Zealand) in a 10 μl reaction volume. This was heated to 37°C for 15 minutes, then to 80°C for 15 minutes on a PCR machine. To this, 1 μl of BigDye Terminator v3.1 (Applied Biosystems, Australia) and 0.64 μl (3.2 pmol) of

either forward or reverse primer were added. This was cycled on the following programme: 96°C for 1 minute, 96°C for 10 seconds, 50°C for 15 seconds, 60°C for 4 minutes, the last three steps repeated 24 times (25 cycles).

The resulting product was made up to 20 μl with water, then 2 μl of 3 M sodium acetate, 1 μl of 125 mM EDTA and 50 μl of 100% ethanol (Sharlau Chemie, Spain) were added. This was left at room temperature for 15 minutes, then centrifuged at approximately 10 000 x g for 30 minutes. The supernatant was discarded, 200 μl of 70% (v/v) ethanol added and centrifuged at 10 000 x g for 5 minutes. The supernatant was again discarded and the pellet left to dry. 20 μl of Hi-Di Formamide (Applied Biosystems) was then added, heated to 95°C for 2 minutes then cooled on ice.

Sequences were read using an Applied Biosystems Instrument Prism 310 Genetic Analyzer and 310 Data Collection Software Version 3.1.0. The resulting sequence was viewed using 4peaks software (downloaded on 19/02/09, from http://mekentosj.com/4peaks/).

2.4.4 Statistical Analysis

2.4.4.1 Power Calculations

Power calculations were conducted using on online calculator designed to analyse trios collected for transmission disequilibrium test with discrete traits (http://pngu.mgh.harvard.edu/~purcell/gpc/dtdt.html accessed on 03/02/14). Since this calculator is not designed for the range of family types found the in SIBS cohort, values should be considered approximate estimates only. Multiple analyses were conducted using different marker frequencies and different relative risk scores, to give an indication of the power under different circumstances. For these analyses, the "Genotype relative risk Aa" was defined as the relative risk plus one, the "Genotype relative risk AA" was defined as double the relative risk plus one, to approximate an additive model of inheritance. It was assumed that the marker allele either is the risk allele, or is in very strong disequilibrium with the risk allele, thus "High risk allele frequency (A)" and "Marker allele frequency (B)" were equal, and the "D-prime" was set at 1. The "Prevalence" was set at 0.03, the approximate lifetime prevalence of panic disorder in New Zealand (Oakley Browne *et al.*, 2006b). This is likely to be an underestimate of any panic, however, a subset of analyses with this value set at 0.292,

the prevalence of Any Panic in the SIBS cohort, give identical power results. The "Number of trios" was set at 82, the total number of SIBS families that may be informative on Any Panic. Data presented are for a 5% α value (significance threshold).

2.4.4.2 Hardy-Weinberg

Once genotyping was completed, each tag SNP was tested to ensure it conformed to Hardy-Weinberg equilibrium by Chi-squared test, calculated in Microsoft Excel. For this, the observed/expected formula below was calculated for each genotype for that SNP, and these were added to give the Chi-squared value. If the Chi-squared value was less than 5.991, the 5% threshold for the probability that the results would be found by chance when there are two degrees of freedom, the SNP was considered to be in Hardy-Weinberg equilibrium. Expected was defined based on the allele frequency for that SNP in the SIBS cohort.

Observed/expected formula:

$$\frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

2.4.4.3 FBAT

The programme FBAT (downloaded from http://www.biostat.harvard.edu/~fbat/fbat.htm on 02/09/08) was used to analyse the genotype data. This programme utilises family-based association tests, which originate from the transmission disequilibrium test, to determine whether alleles are under or over transmitted to affected individuals (Laird *et al.*, 2000; Rabinowitz & Laird, 2000). Pedigrees that are informative (can be used in the calculation) typically include an affected individual (the proband) and at least one parent that is heterozygous for the variant being tested (see Figure 2.2 for an example). Additionally, when parental genotype information is missing, FBAT can use genotype information from other relatives to determine the probability of the parent being heterozygous.

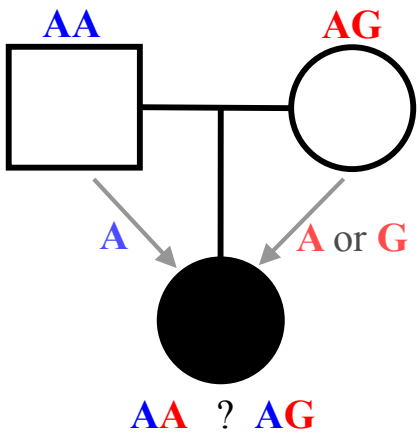


Figure 2.2 Example of an FBAT Informative Pedigree.
A trio is depicted with the father homozygous for the "A" allele and the mother heterozygous at this locus. Thus, in this example, the affected child (proband) must inherit the "A" allele from her father, but could inherit either the "A" or the "G" allele from her father. If the locus is not associated, each allele should be inherited in approximately 50% of these pedigrees. The FBAT programme determines whether this is the case.

Pedigrees were constructed using the format outlined in the FBAT user guide with a separate pedigree for each trait. Unless otherwise stated analyses were conducted using the empirical correction, which takes into account the correlation between genotypes of siblings in extended pedigrees (Lake *et al.*, 2000). Unadjusted *p*-values are presented.

In addition to individual SNPs, haplotypes were also analysed using FBAT. For the purpose of statistical analysis, haplotype blocks were constructed with the CEU data using the Confidence intervals analysis (Gabriel *et al.*, 2002). The resulting tag SNPs were input into FBAT to create the haplotypes within the SIBS cohort.

2.4.4.4 Correction for Multiple Comparisons

A Bonferroni correction for the number of genes analysed was undertaken. This correction consisted of dividing 0.05 (the nominal *p*-value) by 3 (the number of genes tested), resulting in an adjusted significance threshold of 0.017.

Additionally, permutation tests were conducted using Haploview software (version 3.32, downloaded on 23/06/2006, from http://www.broad.mit.edu/mpg/haploview/). For permutation tests, a single pedigree file of SIBS genotype data and a marker information file were loaded into Haploview. All three CCK system genes were included in this file and the phenotype used was 'Any Panic', as this was the primary phenotype of interest in this study.

No other phenotypes were assessed with Haploview. When loading the files the following options were selected "Do association test?", "Family trio data" and "Standard TDT". Following the loading of data, two sets of permutation tests were performed on this data set, both of which included 10 000 permutations. The first set included the SNPs and haplotypes that are presented in the main results (Table 3.6, page 11), while the second set included all SNPs that were analysed (Appendix C). For details on SNP selection, see section 2.4.2 (page 76).

2.5 Cell Culture

2.5.1 Cell lines

Three human neuroblastoma cell lines were used in this study. All were originally obtained from the American Type Culture Collection (ATCC). Full details on each cell line can be obtained from www.atcc.org.

SK-N-DZ (ATCC number CRL-2149): an adherent cell line derived from a bone marrow metastasis of a neuroblastoma tumour in an infant.

SK-N-SH (ATCC number HTB-11): an adherent cell line derived from a bone marrow metastasis of a neuroblastoma tumour in a child.

SH-SY5Y (ATCC number CRL-2266): a subline derived from the SK-N-SH cell line, via SH-SY then SH-SY5. These cells differ from SK-N-SH cells as the SH-SY5Y cells show less morphologic variation, include suspended as well as adherent cells and show different chromosomal abnormalities.

2.5.2 Culture Conditions

The media cells were cultured in consisted of Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemicals Ltd, New Zealand) supplemented with 10% (v/v) Foetal Bovine Serum (Bio International Ltd, New Zealand) and 1% (v/v) Penicillin-Streptomycin (Invitrogen, New Zealand; final concentrations were 50 units penicillin G and 50 μg streptomycin sulphate per millilitre). Cells were stored long-term in liquid nitrogen, then grown at 37°C, in a humidified atmosphere with 10% carbon dioxide. All cell culture work was carried out in a biological safety cabinet class II (Email Westinghouse Pty Ltd, New South Wales, Australia).

For all experiments, cells were cultured in 25 cm^2 (small) or 75 cm^2 (medium) flasks and left in untreated media for at least 12 hours prior to initial drug addition to allow the cells to adhere to the flask. To harvest cells, they were incubated with trypsin (Invitrogen, New Zealand) for approximately 5 minutes. The trypsin was then neutralised with supplemented DMEM and this mix was centrifuged for 5 minutes at 400 x g to produce a cell pellet. Depending on the experiment, the cell pellet was either resuspended in supplemented DMEM and used to seed new flasks, or was immediately placed on ice then stored at -70°C until RNA (section 2.6.1, page 84) or protein extraction (section 2.7.1, page 88) was performed. For long time courses, cells were frozen at multiple time points for a minimum of 28 days; for the short time course, flasks were set up at the same time and harvested at the required time period (6, 24 or 48 hours).

2.5.3 Drug Exposure

All drugs were obtained from Sigma Chemicals Ltd (Auckland, New Zealand). Cells were grown in the presence of 10 μM fluoxetine hydrochloride (fluoxetine), 50 μM gabapentin (gabapentin), 10 μM paroxetine hydrochloride hemihydrate (paroxetine), 10 μM imipramine hydrochloride (imipramine) or no drugs (untreated). All drugs were prepared by dissolving in water, followed by filter sterilisation through a 0.2 μm pore. Solutions were stored for up to one week at 4°C and diluted 1:100 in supplemented DMEM when used.

2.6 Gene Expression Analysis

2.6.1 RNA Extraction

RNA extraction was performed using an RNeasy Mini kit (Qiagen, distributed by Biolab Ltd, New Zealand). The instructions for "isolation of total RNA from animal cells" from the kit were followed, without the addition of β-mercaptoethanol and with the DNase digestion step during washing with buffer RW1. Briefly, frozen cells were thawed at room temperature then homogenised through a 20 gauge needle in the presence of buffer RLT. Ethanol was added and the resulting solution and precipitate were added to an RNeasy Mini column and centrifuged. This was washed with buffer RW1 then incubated with DNase I at room temperature for 15 minutes. This was then washed with buffer RW1 again and then washed

twice with buffer RPE. RNase free water was used to collect the RNA. RNA was stored for a few hours at -20°C before cDNA was produced.

To ensure consistency, RNA was extracted at the same time for all drug groups within a time point/cell line. RNA was quantified using a NanoDrop ND-1000 spectrophotometer prior to cDNA production. The quality of RNA was assessed by running a some of the sample on an agarose gel and visually confirming that two bands of ribosomal RNA were present, indicating that minimal degradation had occurred. If this experiment were to be repeated if the future, additional methods for confirming RNA quality, such as use of a Bioanalyzer, would be conducted to conform to MIQE guidelines (Bustin *et al.*, 2009).

2.6.2 cDNA Production

Superscript III reverse transcriptase (catalogue number 18080) was used to produce cDNA, utilising the reagents and methods supplied by Invitrogen. Within each time point within each cell line, identical quantities of RNA were used as the starting material. For different time points, RNA quantity ranged from 1.5 to 10 μg of RNA per 100 μl reaction, depending on the lowest concentration of RNA at that time point. RNA was primed by incubating at 65°C for 5 minutes with random hexamers and dNTPs. This was then transferred to ice for at least one minute before the rest of the reagents were added. Final concentrations in the 100 μl reaction volume were 5 μg/ml random hexamers, 0.4 mM of each dNTP, 20% (v/v) 5X First-Strand buffer and 5 mM dithiothreitol (DTT), with 80 units of RNase inhibitor (RNaseOUT), and 400 units of reverse transcriptase (Superscript III), made up to 100 μl in sterile, distilled water. The cDNA was then produced by incubating at 25°C for 5 minutes, then 50°C for 60 minutes, and the reaction stopped by heating to 70°C for 15 minutes (all performed on a PCR thermal cycler).

To ensure consistency, cDNA production was conducted at the same time for all drug groups within a time point/cell line. The cDNA produced was quantified using a NanoDrop ND-1000 spectrophotometer and further tested with two sets of primers to ensure the quality of the product prior to running reverse transcription qPCR.

2.6.3 Gene Expression and Normalisation

Gene expression primers were designed as described in section 2.3 (page 75). Primers were designed towards the 3′ end of the gene on either side of a large intron (greater than 500 bp) whenever possible. Primers were then further tested and screened for specificity by testing on a normal PCR cycle with cDNA and genomic DNA to ensure they specifically amplified the cDNA target only.

Gene expression for each time point in the short and long time courses was determined by conducting qPCR reactions, using the SYBR green assay system. Amplifications were conducted using an Applied Biosystems Instrument 7300 Real-Time PCR System and results recorded using SDS v1.3.1 or v1.4.1 software. Reactions were carried out in triplicate and each contained 50% (v/v) Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, New Zealand), 200 nM of each primer and variable amounts of cDNA – depending on the basal gene expression levels, made up to 10 μl with sterile water. The PCR protocol was: 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, 60 seconds at 60°C – the last two steps repeated 39 times (40 cycles), 15 seconds at 95°C, 30 seconds at 60°C and 15 seconds at 95°C. The final three steps comprised the dissociation stage, which gives an indication of the specificity of amplification.

As well as the genes of interest, reference genes that have a stable level of expression under the different conditions also need to be analysed, allowing samples to be normalised so more accurate relative expression levels are obtained. It is not considered sufficient to use one reference gene for normalisation, as was done in the past (Bustin *et al.*, 2009); instead, multiple genes should be used and selected for their stability of expression within the specific drug groups and cell line of the experiment (Vandesompele *et al.*, 2002). In accordance with this methodology, seven reference genes (*PLOR2F, HPRT1, MPRIP, HMBS, UBC, SDHA* and *GAPDH*) were tested for stability of expression in each cell line using geNorm v 3.4 software (downloaded on 31/05/06, from http://medgen.ugent.be/~jvdesomp/genorm/). The set of the four most stably expressed genes was determined for each cell line (listed in Appendix F) and the geometric average of these, as calculated by geNorm for each time/drug, was used as the normalisation factor.

Gene expression was measured by the relative standard curve method (Real-Time PCR Systems Chemistry Guide, Applied Biosystems). This method uses standard curves, produced from cDNA of the same cell line, to convert the quantification cycle (also known as threshold cycle or C_T) values into relative expression levels. This method requires that all genes being analysed, including the reference genes, are expressed in sufficient levels to produce a standard curve and that the quantification cycle values of the experimental samples fall within the standard curve. The standard curve must also be sufficiently straight to ensure estimations are accurate, this is determined by how close the R^2 value is to 1.0 (an ideal fit). Standard curves were produced using Microsoft Excel and the 0.9 was the threshold considered acceptable of the R^2 value, although most curves were above 0.99. Values produced for genes of interest were normalised by dividing by the normalisation factor, described above. Normalised values were then converted to relative expression values by dividing the normalised value for each drug by the normalised value for the untreated cells at the same time point.

2.6.4 Statistical Analysis

In each experiment, technical replicates of samples were set up in triplicate and the quantification cycle values of these three wells were averaged to produce a mean and standard deviation for each sample. These values were normalised and converted to a relative expression as described previously (section 2.6.3, page 86). Each experiment was repeated three times and the median value obtained; these median values form the basis of the results. These analyses were conducted in Microsoft Excel using the formula supplied in the geNorm manual (downloaded on 20/12/07, from http://medgen.ugent.be/~jvdesomp/genorm/).

For the purpose of determining statistical significance of findings in the Long time course experiments a two-way ANOVA was conducted through the website http://vassarstats.net/anova2u.html (accessed 04/02/14). This analysis investigated drug, time and the drug x time interaction for the three antidepressant drugs. The relative expression was used, thus, untreated cells were defined as 1. For the purpose of these analyses, three data points for each time course experiment were utilised to produce an early (4-8 days) mid (12-21 days) and late (28-31 days) time point for each drug. The threshold for statistical significance was $p<0.05$.

2.7 Protein Quantification

2.7.1 Protein Extraction

Frozen cell pellets were thawed and any residual culture media removed. Depending on the size of the pellet, 200 to 500 μl of cell lysis buffer was added and the pellet was homogenised through a 20 gauge needle. During initial testing of protein lysates using the slot-blot and Odyssey Infrared detection system signal was noted in the 700 nm channel of wells containing lysis buffer only (without protein), but not in wells with only PBS. Following further testing, new lysis buffer was made that excluded the nonidet 40. Test lysates were produced with this new lysis buffer and tested on both the slot-blot and in standard Western blots. The altered buffer eliminated the non-specific signal and showed no differences in protein detection; consequently, the new lysis buffer was used for the extraction of proteins for all experimental samples.

2.7.2 Protein Visualisation

Two different methodologies were used to visualise specific proteins within the sample:
1. Slot-blot assays were conducted to initially test for antibody binding and to screen large numbers of samples. This procedure consisted of using a vacuum to pull samples through a nitrocellulose membrane; followed by blocking, primary antibody incubation, secondary antibody incubation and then visualisation.
2. Standard Western blots were conducted with each antibody to ensure a band of the correct size was seen and to confirm the findings from the slot blot experiments. This procedure consisted of gel electrophoresis, then transfer of electrophoresed proteins to a nitrocellulose membrane; followed by blocking, primary antibody incubation, secondary antibody incubation and then visualisation.

2.7.2.1 Slot-Blot

A Bio-Dot SF Microfiltration Apparatus (Bio-Rad, USA), with pre-cut nitrocellulose membranes (0.45 μm) and Bio Dot SF Filter Paper (Bio-Rad, USA), was used for all slot-blot experiments. The following methodology is based on that provided by Bio-Rad, except that

PBS was used in place of Tris buffered saline, to align the methodology with that used for Western blots.

Prior to starting the experiment, the membrane and filter paper were pre-wet with PBS and protein samples were denatured. Various denaturation protocols were trialled including heating to 90°C for 5 minutes (no additional buffers) and adding various amounts of Laemmli's buffer then heating to 70°C for 10 minutes. The best results were seen by denaturing for 10 minutes at 70°C without Laemmli's buffer. PBS was then added to the denatured sample so that the total volume was 200 μl (i.e. 10 μl of denatured sample was mixed with 190 μl of buffer). The dilution in buffer ensured that the sample spread evenly across the well, minimising protein splotches and uneven colouration within the well on the final image.

The Bio-Dot SF was set up by placing the wet filter paper and membrane inside the apparatus and starting the vacuum. The membrane was then re-wet by loading 100 μl of buffer into each of the 48 wells and allowed to run through on gentle vacuum. Following this the denatured, diluted sample was loaded into each well and allowed to run through on gentle vacuum. Finally, a 200 μl wash of PBS was applied and allowed to run through on gentle vacuum. The membrane was then removed and incubated in blocking solution followed by antibodies, as described below.

2.7.2.2 Electrophoresis and Transfer (Western blot)

Polyacrylamide (Bis-Tris) gels were made according to instructions supplied by Invitrogen (see also section 2.2.1, page 74). Protein was denatured by adding Laemmli's buffer (one third of the final volume) and incubating at 70°C for 10 minutes. The resulting samples were loaded onto the gel, along with a running marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen, New Zealand) and Odyssey Two-Colour Protein Molecular Weight Marker (LI-COR Biosciences, New Zealand). Electrophoresis of the samples was conducted at 200 V for 30-60 minutes in an X-Cell SureLock Mini-Cell, with NuPAGE MES running buffer (Invitrogen, New Zealand).

Following electrophoresis, proteins were transferred from the acrylamide gel to nitrocellulose membrane (0.45 Micron; Thermo Scientific, USA). Transfers were conducted using an

X-Cell II Blot Module and NuPAGE Transfer Buffer (Invitrogen, New Zealand). Transfer Buffer was diluted 1 in 20 with distilled water, without the addition of antioxidant or methanol. The nitrocellulose membrane and the filter paper (3MM chromatography paper, Whatman) were pre-wet in Transfer Buffer and then the gel and membrane were placed between two pieces of filter paper. One or two of these sandwiched gels were then placed into the Blot Module, separated from the module with buffer soaked sponges. Transfers were conduced at 30 V for 1 hour.

2.7.2.3 Blocking and Washes

Membranes were blocked for at least four hours to minimise non-specific antibody binding. During initial tests, blocking consisted of incubation with 5% (w/v) low fat milk powder, dissolved in PBS. Once the antibodies for the experiment were confirmed, a comparison was made between milk powder, casein and odyssey blocking buffers. Casein block provided the best signal to noise ratio and was consequently used in all quantification experiments. Following block, membranes were incubated (with shaking) in the relevant primary antibody/antibodies, followed by secondary antibodies. Membranes were washed twice in PBS-T for five minutes between each of the incubations and before imaging.

2.7.2.4 Antibodies

Primary antibodies were obtained from Santa Cruz Biotechnologies Ltd (distributed by Thermo Fisher Scientific New Zealand). The two primary antibodies used were: a rabbit (polyclonal) antibody raised against the adenosine A_{2a} receptor (sc-13937), and a mouse (monoclonal) antibody raised against γ-actin (sc-65638). Following block, membranes were incubated for at least four hours in the relevant primary antibody/antibodies.

Secondary antibodies were infrared labelled, for use with the Odyssey Infrared Imaging System (LI-COR Biosciences, New Zealand). Two secondary antibodies were used: IRDye 800CW Conjugated Goat Anti-Rabbit IgG and IRDye 680 Conjugated Goat Anti-Mouse IgG. Since these two antibodies are conjugated to fluorophores that emit different wavelengths of light, both can be simultaneously measured, allowing two targets to be visualised on the same membrane. Secondary antibodies were diluted 1 in 15 000 in PBS-T. Both antibodies were included together in the dilution (total amount of secondary antibody

was 2 in 15 000). Following primary antibody incubation, membranes were incubated in both secondary antibodies for two hours.

2.7.2.5 Imaging

The Odyssey Infrared Imaging System (LI-COR Biosciences) with Odyssey V3.0 software was used for all protein imaging and analysis. This system has two infrared fluorescent detection channels allowing the two secondary antibodies described above to be detected at once. In accordance with LI-COR's recommended protocols, Tween20 was not used prior to blocking, 0.1% Tween20 was used in wash steps and residual Tween20 was removed prior to imaging by washing with PBS. The following scan settings were used: Resolution = 169 μm; Quality = medium; and Focus offset = 0.0 mm (membrane) or 0.5 mm (gel). The intensity of each channel and scan area varied. All data presented are from membranes that were read while wet with PBS.

2.7.2.6 Quantification

Quantification was conducted using Odyssey V3.0 software. Within an image of a membrane, boxes of equal size were placed around each band and used to generate intensity readings for both the 700 and 800 nm channels. The average intensity, minus the background intensity, of these bands was then used for relative quantification. The intensity of each target protein band (A_{2a} receptor) was normalised to the reference protein (γ-actin) and normalised values were then converted to relative expression values by dividing the value for each drug by the value for the untreated cells at the same time point.

3 Genetic Association Studies

3.1 Background

The genetic predisposition of anxiety disorders can be investigated in a variety of ways, including genetic association studies of candidate genes that are from biological systems thought to be involved in anxiety. Genes from two such systems are investigated here: cholecystokinin (*CCK*, *CCKAR*, and *CCKBR*) and adenosine (*ADORA2A*).

3.1.1 Cholecystokinin and Panic

As discussed in section 1.3 (page 29), cholecystokinin (CCK) is a peptide that acts as a signalling molecule in both the gastrointestinal system and the central nervous system. CCK has two known receptors, CCK_A and CCK_B, which are both G-protein coupled receptors. A variety of evidence supports the involvement of the CCK system in panic attacks and anxiety, including: the neuroanatomical distribution of CCK and its receptors within fear networks (section 1.3.3, page 34); the ability of short CCK peptides to induce panic attacks (section 1.4.1.1, page 48); and molecular studies of the CCK system in humans and animals (section 1.4.3.3, page 57). The association of CCK with panic has led many researchers to the hypothesis that variation in CCK system genes may predispose individuals to developing panic disorder. Table 3.1 provides a summary of past genetic association studies.

Table 3.1 Previous Association Studies of CCK System Genes and Panic

Gene	Variant(s)	Cohort Size(s)	Significant Associations or Trends
CCK	rs1799923	73 to 187 patients	PD+PA, T=risk allele[1] PD p=0.08, T=risk allele[2] PD, C=risk allele[3] Four found no association[4,5,6,7]
	Six other SNPs	73 to 187 patients	PD (1270 C/G), G=risk allele[2] PD (rs754635), C=risk allele[3] Two found no association (four SNPs)[6,8]
	Short tandem repeat (STR), 1.8 to 2.2 kb from cap site	73 patients	Lengths 379 and 383 more frequent in PD[6]
CCKAR	AAA vs AAAAAA in 3'-UTR	99 patients	No association[4]
	At least 9 SNPs	43 to 187 patients	PD (-128G/T and -81A/G), minor=risk allele[9] PD with MDD (246 G/A), A=risk allele[2] Three found no association (up to 9 SNPs)[3,8,10]
CCKBR	Simple sequence repeat	77 parent-child trios	No association[5]
	CT repeat in promoter region	71 to 115 patients	Increased alleles 6 and 7 in PD (allele range: 1 to 16)[4] Increased long alleles in PD[7] No association[11]
	At least 6 SNPs	34 to 187 patients	PD p=0.05 (-215 C/A), C=risk allele; pure PD p=0.08 (109 C/T), C=risk allele[2] PD rs1396860, G=risk allele[8] Three found no association (up to 6 SNPs)[3,12,13]

PD = panic disorder; PD+PA = panic disorder or panic attacks; PD with MDD = patients with both panic disorder and major depression. References: 1. Wang *et al.* (1998), 2. Maron *et al.* (2005), 3. Koefoed *et al.* (2010), 4. Kennedy *et al.* (1999), 5. Hamilton *et al.* (2001), 6. Hattori *et al.* (2001a), 7. Hosing *et al.* (2004), 8. Gratacos *et al.* (2009), 9. Miyasaka *et al.* (2004), 10. Ise *et al.* (2003), 11. Hattori *et al.* (2001b), 12. Kato *et al.* (1996), 13. Yamada *et al.* (2001). Articles were identified by searching PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) using the combination of "panic" with each of "CCK", "CCKAR" and "CCKBR". Bibliographies of included articles were also searched. Candidate gene studies including one or more of the CCK system genes, published up until 2010, were included.

Early research into *CCK* found evidence that a SNP in the promoter region may be associated with panic. This polymorphism, rs1799923 – a C to T transition within the promoter region of the main transcript, was found to be associated with panic in a cohort of 98 patients from Iowa, with the minor allele (T) over transmitted to those with panic disorder or panic attacks (Wang *et al.*, 1998). Since then, most other studies investigating *CCK* and panic have included this SNP, although only two out of six have provided any support for involvement. One of these studies found a *p*-value of 0.08 among 127 panic disorder patients with or without comorbidities, with the minor allele over transmitted to patients (Maron *et al.*, 2005).

Although not statistically significant, this result is suggestive of an association and may have been significant in a larger cohort. In contrast, the only other positive study, which investigated 187 Danish Caucasian patients with panic attacks, found that the minor allele was under transmitted to patients (Koefoed *et al.*, 2010), an association in the opposite direction to the previous two studies. Taken together with the four negative studies, it seems unlikely that this SNP is an important direct contributing factor in the pathogenesis of panic, though it may be in linkage disequilibrium with a causative variant in some populations.

Other variants of *CCK*, although less thoroughly studied, have also shown positive results. A study of 127 patients from Estonia found a SNP, described as "1270 C/G", that was significantly associated with panic disorder, as was a haplotype that included this SNP (Maron *et al.*, 2005). The largest genetic association study prior to 2012 that included CCK system candidates, with 187 patients, found that the minor allele of rs754635 was protective against panic attacks (Koefoed *et al.*, 2010). However, one of the other large studies, with 173 patients, found no association with any of the three *CCK* SNPs they analysed (Gratacos *et al.*, 2009). A short tandem repeat was found to vary in length between patients and controls in a study of 73 Japanese panic disorder patients, though the two SNPs investigated showed no association (Hattori *et al.*, 2001a). These mixed findings suggest that genetic variants within the *CCK* gene do increase the risk of developing panic disorder or other related conditions in some groups, but the causative variants have not yet been identified.

While *CCKAR* has been studied less than *CCK,* a wider range of variants have been investigated. Half of all studies of *CCKAR* with panic have found significant effects, although far less than half of the individual variants have shown associations. Two of the three negative studies had less than 100 patients participating and only investigated one variant (Ise *et al.*, 2003; Kennedy *et al.*, 1999), making it unlikely that they would have found any association, regardless of whether one exists. The third negative study investigated three SNPs in 173 patients (Gratacos *et al.*, 2009). Although this is an improvement on one variant, it may not have been sufficient to fully cover common variation within the gene. The largest study of *CCKAR* variants investigated nine SNPs in 187 patients; none of the SNPs showed significant associations with panic attacks, but a haplotype, the AT combination at rs180855 and rs1800857 respectively, was found to be protective (Koefoed *et al.*, 2010). Another positive study only found a significant association with one out of seven SNPs investigated (Maron *et al.*, 2005). Furthermore, the association in this study was not strongest for panic

disorder (p=0.1), but for the subgroup of patients that had panic disorder comorbid with major depressive disorder (p=0.02). Finally, in a study of 109 panic disorder patients, the minor alleles of two *CCKAR* SNPs were found to be over represented among patients, as was the haplotype that combined these (Miyasaka *et al.*, 2004). Hence, there is some support for the involvement of *CCKAR* variation in panic disorder, but the exact nature of this variation and whether it predisposes to panic disorder or comorbid conditions has yet to be determined.

CCKBR has been one of the most popular genes to investigate for association with panic, presumably due to the strong physiological association with this receptor. The first study of this gene in panic disorder sequenced it in 22 patients and identified two variations: one intronic SNP and one missense variant (Kato *et al.*, 1996). They followed up the missense variant (rs1805002) by genotyping 34 panic disorder patients and 112 controls and found no association. A later study also investigated this variant, along with three other SNPs, in 91 panic disorder patients (Yamada *et al.*, 2001); again no significant associations were found. More positive results were found with a CT repeat in the promoter region of *CCKBR* with one German and one Canadian cohort showing significant associations between allele length and panic disorder (Hosing *et al.*, 2004; Kennedy *et al.*, 1999), while studies on a Japanese and a USA cohort showed no association (Hamilton *et al.*, 2001; Hattori *et al.*, 2001b).

Larger, more recent studies of *CCKBR* SNPs have supported a genetic involvement. One of these studies investigated four SNPs in 127 patients and found one SNP with p=0.05 for all panic disorder patients while another SNP had p=0.08 for the 42 patients with pure panic disorder (Maron *et al.*, 2005). Another recent study that also investigated four SNPs found one with nominal significance for panic disorder (Gratacos *et al.*, 2009). Although they found no association with the six SNPs they analysed, Koefoed *et al.* (2010) identified two rare missense variants that were only present in panic disorder patients, suggesting that rare variation of *CCKBR* may be associated with panic. Additional support for *CCKBR* involvement in panic comes from the finding of an association between panic disorder and a SNP within a microRNA that represses *CCKBR* translation (Muinos-Gimeno *et al.*, 2011).

The variable results described above may be due, in part, to the ability of the studies to detect an association. These studies ranged in size from 34 to 187 patients. In contrast, based on average effect sizes identified for complex disease genetic risk variants, it has been estimated that thousands of participants would be required to reliably detect these variants (Ioannidis *et*

al., 2006). Smaller studies increase the likelihood of both false positive results, due to cohort selection problems, and false negative results, as many samples will not be large enough to detect variants with small effect sizes. Additionally, the small number of variants tested by most of these studies would reduce the likelihood of finding an association. Genotyping multiple variants within the gene increases the variation covered and, hence, even if the causative variant was not directly genotyped, it is likely to be in disequilibrium with one of the variants tested. This reduces the likelihood of false negative results, although, if multiple comparisons are not corrected for, it will increase the false positives.

As well as differing on number of patients and variants tested, the population investigated in the studies described above also varied. The two most common geographical groups studied were Japanese (Hattori *et al.*, 2001a; Hattori *et al.*, 2001b; Ise *et al.*, 2003; Miyasaka *et al.*, 2004; Yamada *et al.*, 2001) and European (Gratacos *et al.*, 2009; Hosing *et al.*, 2004; Koefoed *et al.*, 2010; Maron *et al.*, 2005). This, coupled with other groups that were only investigated in one or two studies, represents a wide range of different ethnicities, each of which may have unique genetic and environmental risk factors (Lupski *et al.*, 2011). The psychiatric phenotypes also varied. Many studies allowed psychiatric comorbidities (for example: Koefoed *et al.*, 2010; Maron *et al.*, 2005), though many did not clearly state whether comorbidities were allowed. Some studies, like Wang *et al.* (1998), also analysed patients with panic attacks that did not meet the full criteria for panic disorder. This heterogeneity in the sample populations may have contributed to the variation in the findings (Moonesinghe *et al.*, 2008). Consequently, conclusions regarding the role of CCK system genes in the genetic predisposition to panic disorder cannot yet be made.

3.1.2 *ADORA2A* and Panic

In addition to the CCK system, many genetic association studies have been conducted on other SNPs that may be associated with panic disorder. The adenosine A_{2A} receptor, encoded by the gene *ADORA2A*, has long been of interest in anxiety disorders (section 1.4.3.4, page 58) and was investigated in a recent association study of panic disorder (Hohoff *et al.*, 2010). This investigation looked at eight SNPs from *ADORA2A* in 457 panic disorder cases, compared to 457 blood donor controls. They found significant associations for two SNPs, rs2267076 and rs5751876, in both of which the minor allele (T) was over-represented in cases. Furthermore, when specifically looking at 341 patients with panic disorder and

comorbid agoraphobia, the associations with the previously mentioned SNPs became stronger and a further SNP, rs3761422 was also statistically significant. While this study provides promising evidence for these *ADORA2A* variants, replication is needed to determine their relevance to other populations.

3.1.3 Aims

The above data suggest that the CCK system may be involved in the pathogenesis of panic disorder, though variation in study design and limitations in size prevent clear conclusions being drawn. Hence, this study aimed to clarify if any association exists between common genetic variation in the CCK system genes and panic by genotyping a comprehensive set of SNPs in a New Zealand cohort. Data from the International HapMap project was used to ensure that the maximum genetic information was obtained from the genotyping conducted. Other phenotypes often comorbid with panic were also investigated for association with CCK system gene variation, to determine whether any observed association with panic might be better explained by one of these comorbid conditions. Spurious associations with comorbid conditions may also help explain the variability of past research, as the inclusion and exclusion criteria varied between studies.

Since the results of genetic association studies often vary by cohort, a further aim of this study was to determine the reproducibility of the Hohoff *et al.* (2010) findings of *ADORA2A*. For this part of the research the three SNPs from *ADORA2A,* rs2267076, rs5751876, and rs3761422 were genotyped in our New Zealand cohort. Most findings in anxiety genetics fail to replicate in other cohorts, and replication of these *ADORA2A* findings would support the potential that the SNP(s) identified have a direct association, as opposed to acting via linkage disequilibrium with the causative variant.

3.2 Description of Subjects

The DNA samples and phenotypic information used in the present research had been collected previously, as part of the South Island Bipolar Study (SIBS). The SIBS participants were individuals from Otago, Southland and Canterbury provinces of the South Island of New Zealand, with bipolar I or bipolar II disorder (SIBS probands) and their family members. In total, 786 participants were recruited. The collection of participants DNA and phenotypic

data for the SIBS was completed prior to 2000 and quality control measures were subsequently undertaken by Catherine Sears to ensure the data met current standards (Sears, 2011). Inspection of the data revealed: 82 individuals for which no phenotype or relationship data was available; 59 probands without participating relatives; and 40 individuals that were either too distantly related or had insufficient information to be included in a pedigree. An additional 40 individuals were removed following Mendelian error and microsatellite analysis; these errors may have been due to genetic factors, such as mistaken paternity, or laboratory factors, such as incorrect labelling or degradation of DNA samples. These quality control steps left 565 individuals that were included in pedigrees and had at least some genotype and phenotype information (Sears, 2011). These 565 participants were grouped into 139 pedigrees (200 nuclear families) that varied in size and composition. The smallest pedigrees had three members, at least two of which had genotype/phenotype information, and the largest had twelve members (up to eight with genotype/phenotype information) and spanned three generations.

The majority of participants were New Zealanders of British/Irish/European descent, but the cohort also included 25 participants with one or both parents of other or unknown ethnicity (Table 3.2). Of the 565 participants, 515 were born in New Zealand, 35 in Great Britain or Ireland and the remaining 15 in other or unrecorded countries. The average age of participants at interview was 45 years and ranged from 17 to 94 years.

Table 3.2 Parental Ethnicity of Participants.

	Maternal Ethnicity	Paternal Ethnicity
British/Irish	543	526
European	9	15
Māori	8	9
Polynesian	1	2
Other	0	7
Unknown	4	6

3.3 Technical Results – Genotyping Assays

3.3.1 Selection of SNPs for Analysis

3.3.1.1 CCK System Genes

Genotype information from the CEU population (Phase II) of the International HapMap Project (http://www.hapmap.org/index.html) was downloaded for *CCK*, *CCKAR* and *CCKBR*. The CEU population was the most similar HapMap population to the SIBS cohort and included 30 parent-child trios from Utah (USA) with Northern and Western European ancestry. The CEU population has been found to be similar to Australian cohorts, with tag SNPs selected using the CEU population covering approximately 90% of common variation in two Australian cohorts (Stankovich *et al.*, 2006). Nonetheless, since it has not been studied in New Zealand populations, the selection of tag SNPs was undertaken in such a manner as to maximise the number genotyped, hence, providing an indication as to the genetic similarity of the two populations.

The programme Haploview was used to analyse the HapMap data and to produce haplotype blocks (regions of linkage disequilibrium) and identify tag SNPs (key SNPs, the genotyping of which will allow the haplotype to be determined). For each of the three genes a custom block was created that encompassed the whole gene and any SNPs just outside of the gene (shown in Appendix A). Some genotyping of *CCK* and *CCKAR* had already been conducted as part of earlier projects (Chan, 2007; Lill, 2000; Wilson, 2008), however, these used different methods for determining tag SNPs and different statistical analyses, both to the present study and to each other. Hence these two genes were re-analysed using the same methodology as *CCKBR*, so the final genetic association data could be directly compared. The custom blocks revealed that the SNPs previously genotyped would provide sufficient information for *CCKAR*, however, further SNPs needed to be genotyped from *CCK* to provide the equivalent level of information as the other genes. The additional *CCK* SNPs were rs8192472 and rs4602358. The location of these and all other tag SNPs with a minor allele frequency above 3% from the custom blocks can be seen in Figure 3.1, with further details in Appendix A.

For each of the three genes, haplotypes were also calculated in Haploview. This was conducted using the default setting: the Confidence intervals analysis (Gabriel *et al.*, 2002). Haplotypes had to have a frequency greater than 1%, and SNPs had to have a minor allele frequency of at least 3% in the HapMap CEU population to be included in the final analyses.

CCKBR is the largest of the three genes and has, by far, the most tag SNPs in the custom block. To determine which SNPs to genotype in *CCKBR*, blocks were produced using the Four-gamete Rule (Wang *et al.*, 2002) with five additional SNPs included from the custom block analysis. The Four-gamete Rule tends to produce multiple small blocks within the gene. When combined with the custom block SNPs, this produced four small blocks for *CCKBR*, which made it easier to determine the haplotypes for each block. Once an individual's haplotypes had been determined, they no longer needed to be genotyped at the remaining tag SNPs within the block, reducing the overall amount of genotyping work. The Four-gamete Rule also resulted in a higher number of tag SNPs within the gene than any of the other analysis methods. This was considered useful as the SIBS cohort is not identical to the HapMap CEU population and may, therefore, have different haplotypes and linkage disequilibrium patterns. The additional tag SNPs provide information about the similarity of the two populations and increase confidence that common genetic variation has been sufficiently covered.

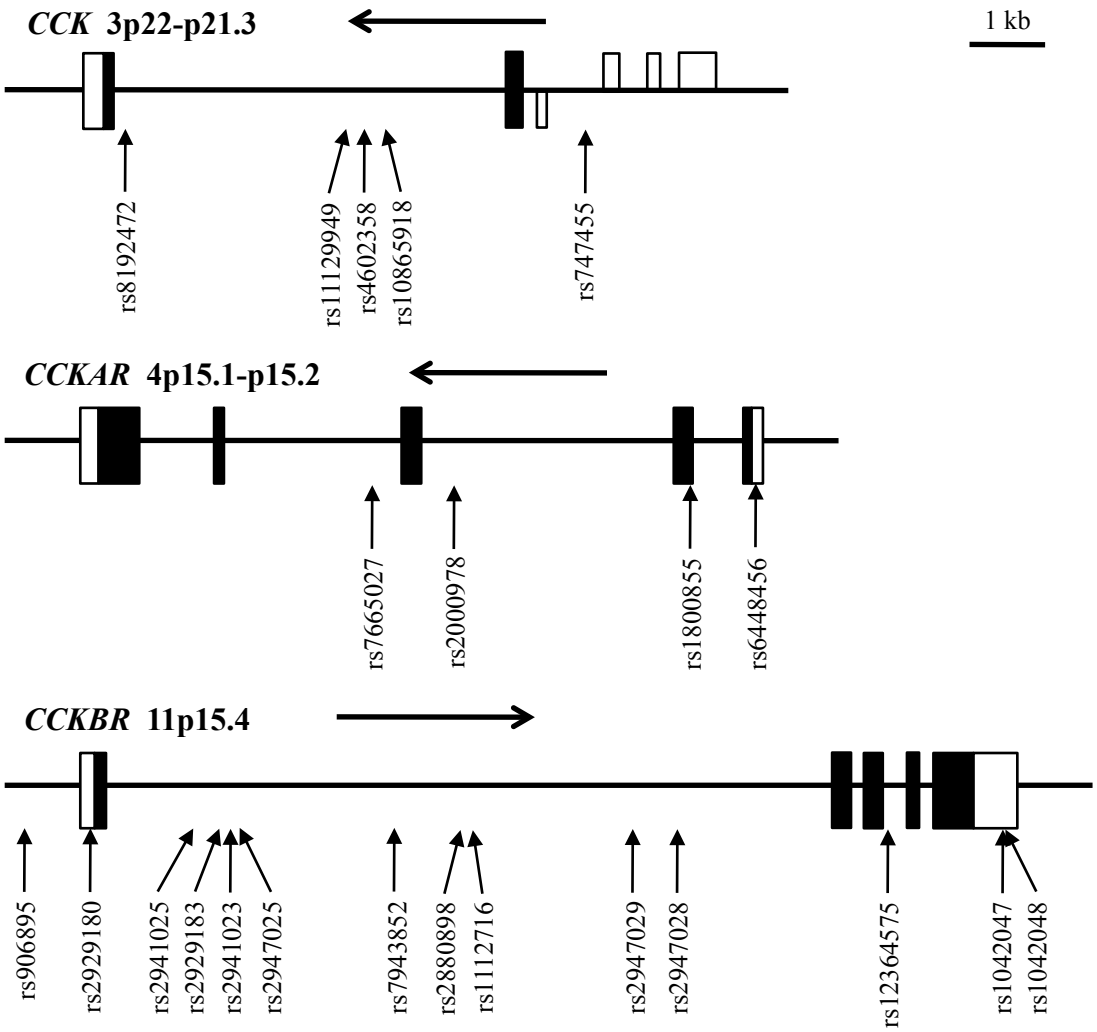


Figure 3.1 CCK System Genes.
Schematic representation of *CCK*, *CCKAR* and *CCKBR* showing the location of tag SNPs (based on custom designed blocks). Boxes represent exons with empty boxes for UTRs. The chromosomal forward strand is shown and arrows next to the gene name indicate direction of gene transcription. *CCK* has two common splice variants as shown by boxes above (variant 1) and below (variant 2) the line. Gene data was obtained from NCBI (http://www.ncbi.nlm.nih.gov/ - downloaded December 2010).

The results of the Four-gamete Rule analysis of the *CCKBR* are presented in Figure 3.2. Tag SNPs were generally determined in Haploview, with one exception: in Block 3 rs2947029 was genotyped instead of rs2880898. The most frequent haplotype in Block 3 was defined by rs2947029, hence, any individuals homozygous for the T allele at this SNP would not need to be genotyped further, reducing the time and reagents required.

Block 1:

rs906895	**rs2929180**	rs11040819	rs11040820	**rs2941025**	**rs2929183**	Freq.
A	**G**	C	A	**C**	**A**	.524
G	**T**	T	A	**T**	**G**	.196
G	**G**	C	A	**C**	**A**	.192
G	**G**	T	A	**T**	**G**	.037
G	**G**	C	A	**C**	**G**	.033

Block 2:

rs2941023	***rs2947025***	rs1396860	**rs4758402**	**rs7943852**	**rs2941029**	rs3793993	Freq.
A	***G***	A	**T**	**C**	**A**	C	.533
G	***C***	A	**T**	**C**	**G**	C	.157
A	***G***	G	**T**	**T**	**G**	G	.192
G	***G***	A	**T**	**C**	**G**	C	.058
G	***C***	A	**C**	**T**	**G**	C	.042
A	***C***	A	**T**	**C**	**G**	C	.018

Block 3:

rs2880898	**rs1112716**	rs12801747	rs11040823	rs11040824	rs11040825	rs11040826	rs11040831	rs11040832	rs11040833	**rs2947029**	**rs2947028**	**rs2947027**	**rs3901667**	rs1805002	Freq.
T	**C**	T	C	G	T	A	G	A	T	**T**	**T**	**A**	**A**	G	.516
T	**C**	T	C	G	T	A	G	A	T	**C**	**C**	**G**	**A**	G	.233
C	**C**	C	T	C	C	G	A	G	C	**C**	**T**	**A**	**A**	G	.144
T	**C**	T	C	G	T	A	G	A	T	**C**	**C**	**A**	**C**	A	.042
T	**C**	T	C	G	T	A	G	A	T	**C**	**C**	**A**	**A**	G	.025
C	**T**	C	T	C	C	G	A	G	C	**C**	**T**	**A**	**A**	G	.022

Block 4:

rs12364575	***rs1042047***	***rs1042048***	Freq.
C	***A***	***A***	.444
C	***C***	***A***	.155
C	***A***	***G***	.103
G	***C***	***G***	.062
G	***A***	***G***	.071
C	***C***	***G***	.081
G	***C***	***A***	.048
G	***A***	***A***	.036

Figure 3.2 *CCKBR* Blocks.
CCKBR haplotype blocks produced using the Four-gamete Rule with additional SNPs from the custom analysis shown in italics. Tag SNPs are in bold.

3.3.1.1.1 Order of Genotyping in *CCKBR*

As can be seen in Figure 3.2, a relatively large number of SNPs, compared to *CCK* and *CCKAR*, needed to be genotyped in *CCKBR*. Rather than genotyping all individuals at all tag

SNPs, blocks were examined visually to identify tag SNPs that defined the most frequent haplotypes. For example, in Block 1, an A allele at rs906895 is seen in the most common haplotype only, therefore, any individual homozygous for the A allele of this SNP should also be homozygous for the common haplotype and have: GG at rs2929180, CC at rs11040819, AA at rs11040820, CC at rs2941025 and AA at rs2929183. Hence, once the two haplotypes of a participant have been determined, the remaining tag SNPs within that block can be inferred without direct genotyping. Some caution needs to be taken with this approach since the SIBS cohort is not identical to the CEU population that the blocks were produced from. To minimise the likelihood of incorrect genotyping, randomly selected inferred samples were also directly genotyped to ensure the genotyping of each SNP was above 90% accuracy. If any sets of sample genotypes did not appear to be at least 90% accurate, the genotyping would be repeated until this threshold was met.

The order in which *CCKBR* SNPs were genotyped was based on whether the SNP defined a more frequent haplotype and the ease of the genotyping method. SNPs rs906895, rs29291023 (Block 1), rs2941029 (Block 2), and rs2927029 (Block 3) were genotyped first in the entire cohort by TaqMan hydrolysis probe (Figure 3.3, see also section 3.3.2.1.1, page 106). Individuals homozygous for the major allele at rs906895 (Block 1), rs2941029 (Block 2) or rs2927029 (Block 3) should be homozygous for the most frequent haplotype from that block and, hence, not require further genotyping at that block. Those with the major allele at rs2929183 (Block 1) have either the most frequent or third most frequent haplotype, thus, when combined with the information from rs906895, these two haplotypes can be determined.

The order and criteria for the genotyping of other SNPs in Blocks 1 to 3 are shown in Figure 3.3. Additionally, all SNPs in Block 4 were genotyped in the full cohort.

3.3.1.2 ADORA2A SNPs

SNPs from the gene *ADORA2A* that were found to be associated with panic were also investigated (Hohoff *et al.*, 2010). The three SNPs included in the present study were: rs2267076, rs5751876 and rs3761422, each of which previously showed an association with panic disorder and agoraphobia.

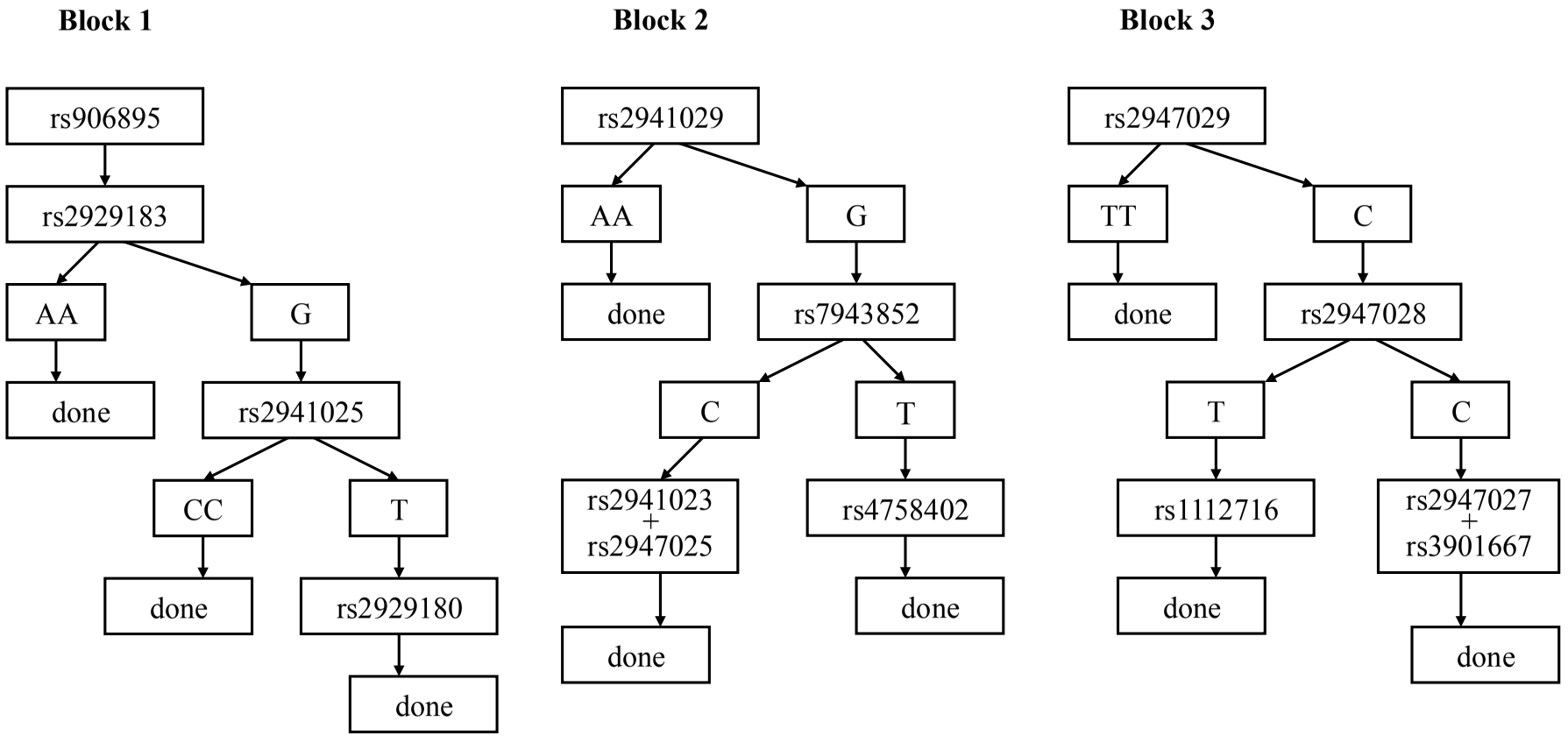


Figure 3.3 Genotyping Order.
The order of genotyping for *CCKBR* SNPs is shown. SNPs at the top of the list were genotyped in all samples, followed by other SNPs as necessary. For example, rs2941029 in Block 2 was genotyped, then any samples with one or two copies of the G allele were further genotyped at rs7943852; the haplotypes of individuals with two A alleles (AA genotype) at rs2941029 were known at Block 2, thus, genotyping of that block was "done" for those individuals.

3.3.2 Optimisation of Assays

A variety of methods were used to genotype SNPs. The basic protocol for each assay is described in section 2.4.3 (page 76); the modifications to those protocols are described below.

3.3.2.1 Genotyping of SNPs

The various methodologies that were used to genotype the different SNPs are described below with further details included in Appendix B. Genotyping of *CCKAR* SNPs had been completed as part of a previous research project and are described elsewhere (Wilson, 2008), as had some of the SNPs in *CCK* (Chan, 2007). Two additional *CCK* SNPs (rs8192472 and rs4602358) were genotyped in all samples and other *CCK* SNPs were re-genotyped as necessary to confirm or complete the previous data.

3.3.2.1.1 TaqMan Hydrolysis Probes

TaqMan hydrolysis probes were the fastest, but most expensive genotyping method used in this study. As a consequence, they were generally used for SNPs that would need to be genotyped in the whole cohort, including *CCKBR* SNPs rs906895, rs29291023 (Block 1), rs2941029 (Block 2) and rs2927029 (Block 3), and the three SNPs genotyped from *ADORA2A* (rs2267076, rs5751876 and rs3761422). In addition, four SNPs from *CCKBR* that would be difficult to determine by any other method were also genotyped using hydrolysis probes. SNPs rs7943852 (Block 2) and rs1042047 (Block 4) were genotyped using hydrolysis probes as the allele specific primers initially designed failed to produce reliable results (see section 3.3.2.1.3, pages 112 and 115). SNP rs1112716 (Block 3) was genotyped using hydrolysis probes as the low G/C content surrounding this SNP prevented the use of allele specific primers. Additionally rs2947025 (Block 2) was genotyped in a subset of the sample using hydrolysis probes.

The clarity of results varied for the different TaqMan hydrolysis probes listed above. An ideal result showed four distinct clusters: one in the lower left corner for the no DNA control samples; one near horizontal cluster of individuals homozygous for “allele X”, utilising the reporter VIC; one near vertical cluster of individuals homozygous for “allele Y”, utilising the reporter FAM; and one cluster on a 45 degree angle of heterozygous individuals (Figure 3.4).

Typically probes produced results sufficiently close to the ideal to allow automatic calling of most samples (90% accuracy threshold) when amplified on one of the recommended programmes (section 2.4.3.1, page 77). All plates were also visually inspected and any questionable automatic calls were manually changed to unknown and the assay repeated for that sample. An example of a plate where manual calls would likely have been used to over-ride automatic calls can be seen in Figure 3.6 (Altered Temperature).

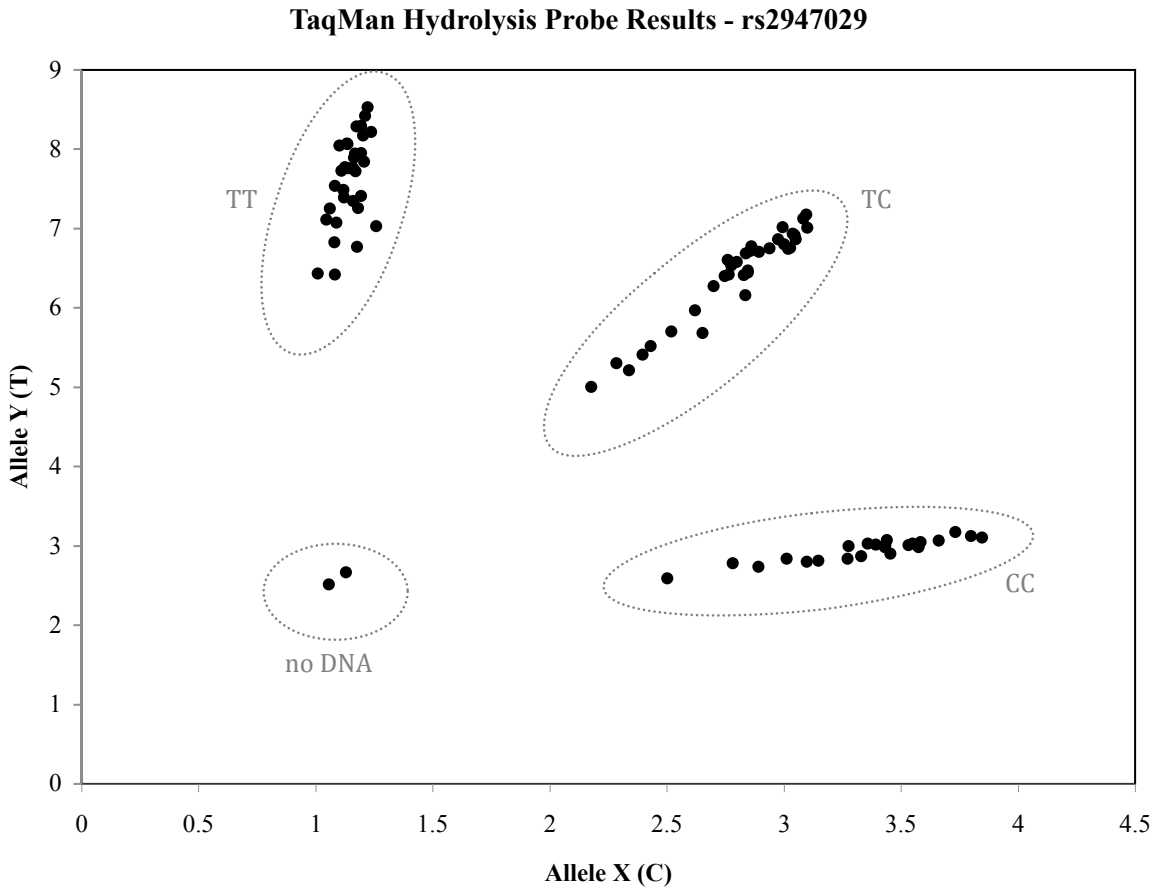


Figure 3.4 Example of an Ideal TaqMan Hydrolysis Probe Result.
The resulting fluorescent signal of a plate of probes designed to genotype rs2947029 is shown. Four distinct clusters are seen, one for the no DNA controls and one for each of the three possible genotypes (TT, TC, CC).

For two of the probe sets amplification with the recommended programmes failed to produce results in which most samples would call automatically. For one of these, rs2941029, a gradient for the annealing/elongation temperature was undertaken (Figure 3.5); based on the results of this, the annealing/elongation temperature was reduced from 60°C to 56°C for the remaining amplifications of this SNP. When the amplification products of the other problematic probe, rs5751876, were visualised on an agarose gel no band was seen, suggesting that the amplification efficiency was poor. This was improved sufficiently to

allow automatic calling of most samples by decreasing the annealing/elongation temperature by one degree to 59°C (Figure 3.6).

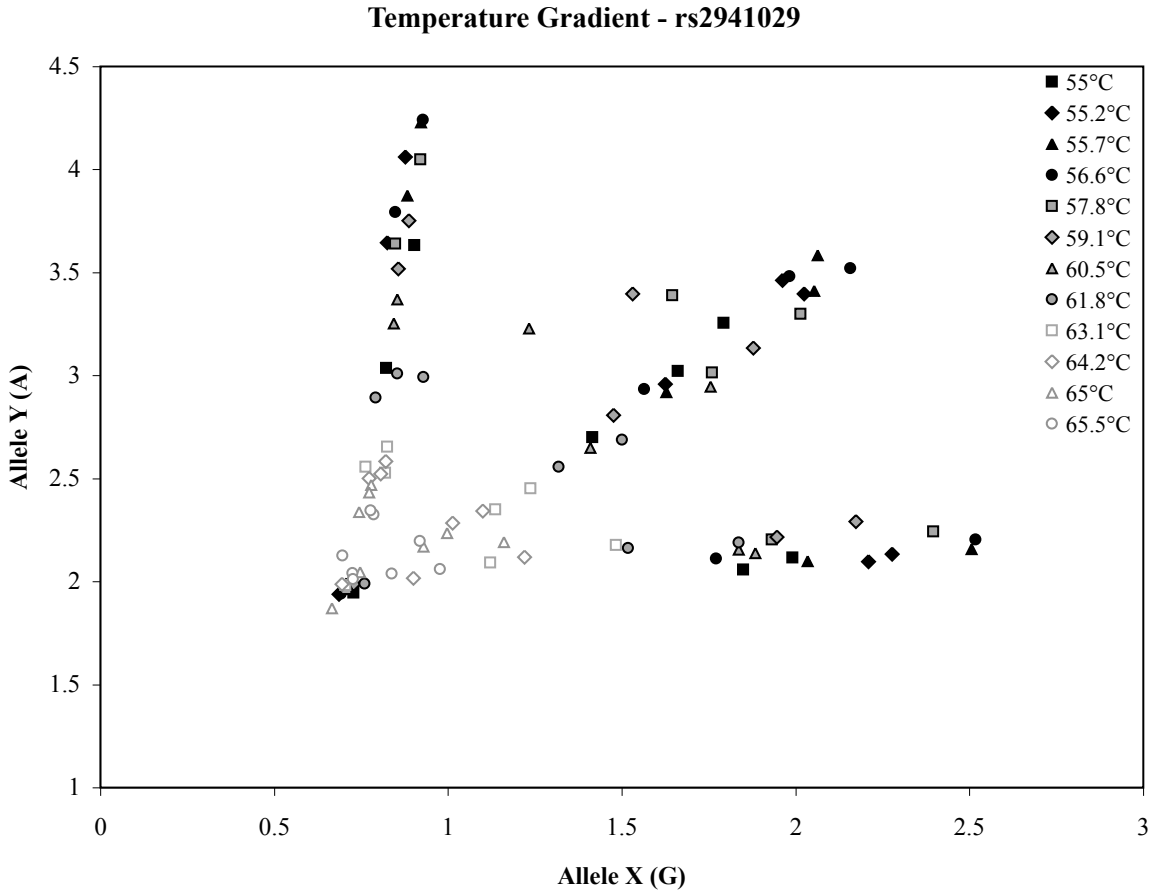


Figure 3.5 Temperature Gradient for rs2941029.
Amplification of the rs2941029 hydrolysis probe was undertaken with the annealing/elongation temperature varied between 55°C and 65.5°C. DNA samples were consistent for all temperatures and included two homozygous AA, two homozygous GG, three heterozygotes and one no DNA control.

Finally, two SNPs genotyped by TaqMan hydrolysis probe showed unexpected allele frequencies or did not fit with Hardy-Weinberg equilibrium. These are detailed in section 3.3.2.2 (page 116).

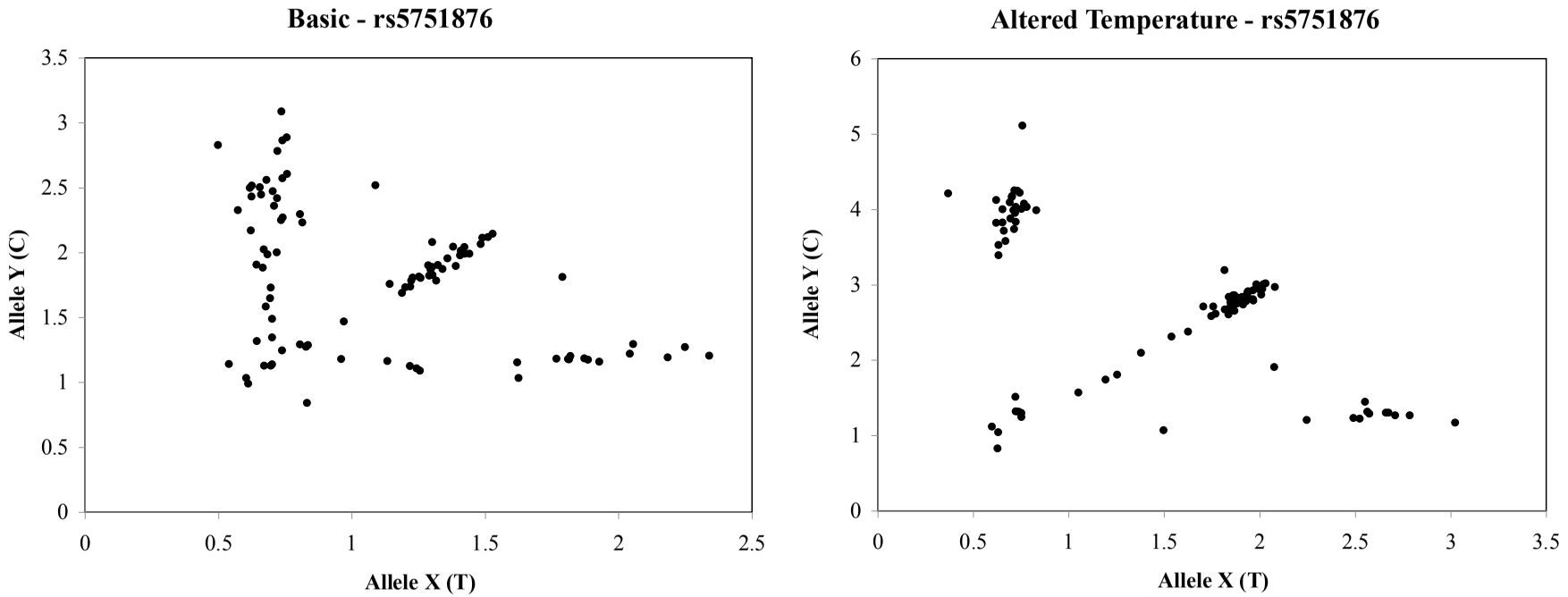


Figure 3.6 Temperature Modification (rs5751876).
Results of TaqMan hydrolysis probe amplifications for rs5751876 using the original annealing/elongation temperature (Basic) and after reducing the temperature by 1°C (Altered Temperature).

3.3.2.1.2 Primer Optimisation

For the tag SNPs that were not genotyped by hydrolysis probe, whenever possible, allele specific primers were designed, with various sized amplification fragments for each SNP to allow multiplexing of assays. For allele specific amplification two primers, identical apart from the 3' nucleotide, were designed to bind to each of the two alleles of the SNP, along with a common primer (see Figure 2.1, page 78, and Appendix B). Additionally, primers for some SNPs were designed on either side of the SNP, so the resulting fragment could be genotyped by RFLP assay or by sequencing. In addition to primers to *CCKBR* SNPs, primers to *CCK* SNPs that had been designed by other members of the laboratory were also tested and re-optimised prior to conducting any new genotyping.

The optimal annealing temperature and magnesium concentration was determined for each primer set (see Figure 3.7 for examples). For amplification of individual SNPs, the highest annealing temperature and the lowest magnesium concentration that produced one clearly visible band on agarose gel were used (optimal values listed in Appendix B). SNP rs2929180 would not amplify under the standard PCR conditions (described in section 2.4.3.3, page 79) and was instead amplified using a different amplification buffer (see section 3.3.2.1.4, page 115, for details).

Since the primer pairs for allele specific primers generally vary from each other by only one base, these sometimes non-specifically amplify the other allele. This is least likely to occur at high annealing temperatures and with low magnesium concentrations, but additional screening is still required to ensure each allele specific primer only amplifies the allele it was designed to. For all allele specific primer sets, DNA samples for a number of different individuals were amplified until at least one individual of each genotype was identified. Non-specific amplification was suspected if homozygotes for one or both alleles were seen at a far lower frequency than expected, or if homozygotes were not seen at all for that allele after screening enough samples that two or more homozygotes would have been expected. This non-specific amplification could potentially be resolved by reducing the number of amplification cycles or by redesigning the primers so that a base near the 3' end did not match the DNA sequence (introducing a wobble), resulting in weaker binding of the primers. For rs4758402 specific amplification required a reduction in the number of cycles from 30 to 28. Additionally, the original primers for the *CCK* SNP rs4602358 (designed by another member

of the lab) were redesigned, substituting an "A" for the original "C" at the fourth base from the 3' end.

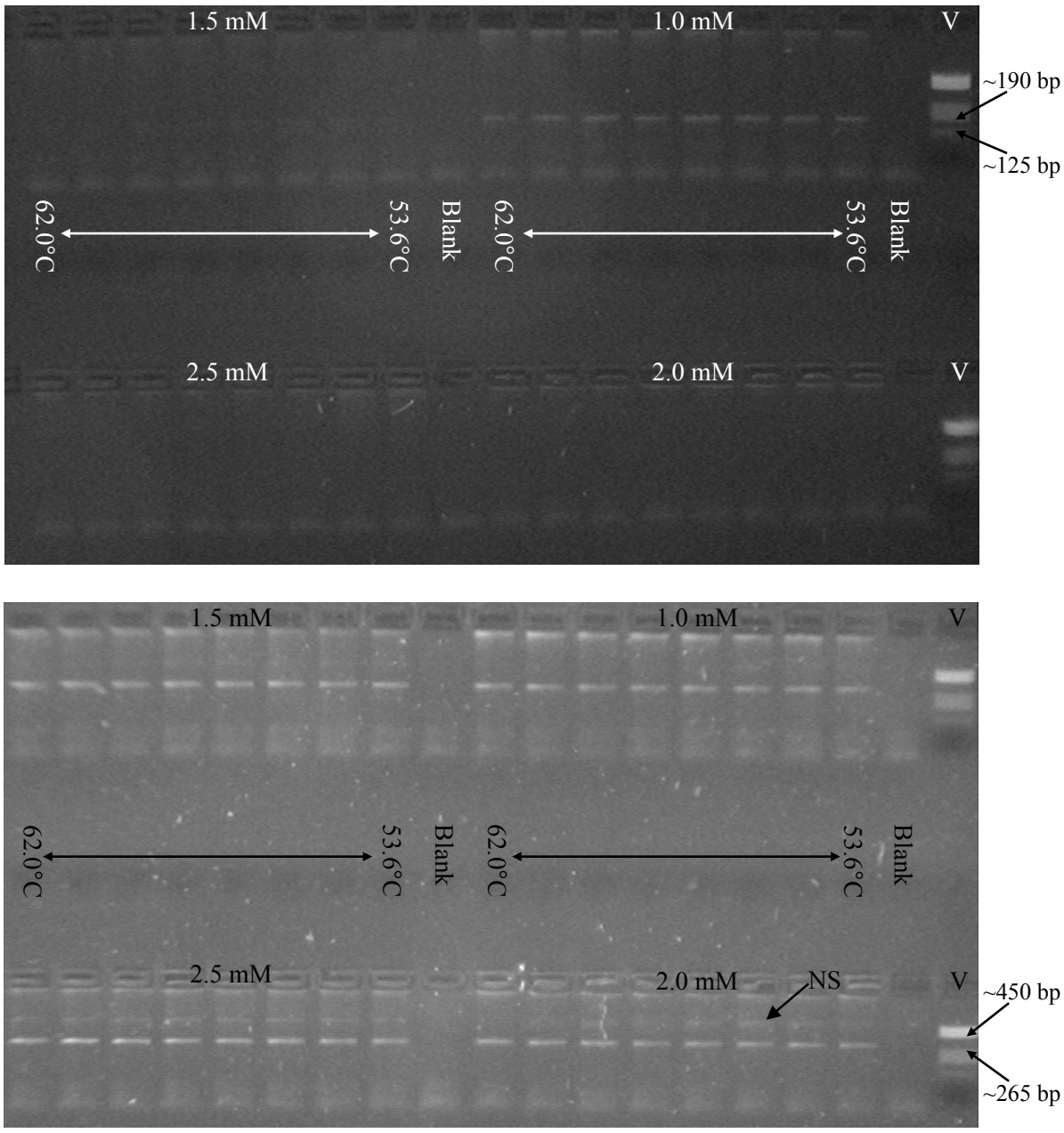


Figure 3.7 Optimisation Examples.
Results of the magnesium and temperature optimisations for rs7943852 (top image, 174 bp fragment) and rs2947027 (bottom image, 301 bp fragment). Amplification failure can be seen at the higher magnesium concentrations for rs7943852 and non-specific amplification (NS) is seen at the higher magnesium concentrations/lower temperatures for rs2947027. V indicates Roche DNA Molecular Weight Marker V; Blank indicates the no DNA control.

3.3.2.1.3 Multiplexing

Multiplexing was conducted to reduce the amount of SIBS DNA used and to reduce the total number of reactions performed. For allele specific primers, all 565 SIBS samples needed to be amplified at least twice (once for each allele), thus requiring a minimum of 12 96-well amplifications for each assay. By multiplexing allele specific primers, when possible, both the total number of assays and the amount of each SIBS DNA sample used was reduced. When designing multiplex assays, the optimal conditions for individual primers were used as an indication of which sets were likely to work well in combination. The resulting multiplex was then optimised in a similar manner to individual primer sets.

Primers to SNPs rs12364575, rs1042047 and rs1042048 were designed so that these SNPs could be included in a multiplex assay with the two *CCK* SNPs (rs8192472 and rs4602358). During the optimisation process, the minor allele of rs1042047 (C) was amplifying non-specifically while, under the same reaction conditions, the major allele (A) was occasionally failing to amplify. This SNP was removed from the multiplex and genotyped by TaqMan hydrolysis probe instead. Removal of this SNP also produced considerable improvements in the amplification of the remaining multiplexed SNPs and eliminated a non-specific band that was larger than any of the expected bands (Figure 3.8).

The final multiplex assay (Multi 4) consisted of: rs1042048 (*CCKBR*), rs8192472 (*CCK*), rs12364575 (*CCKBR*) and rs4602358 (*CCK*). As each SNP had two alleles to be amplified, the multiplex consisted of two reactions for each DNA sample:

	First reaction:	Second reaction:	Size:
rs1042048	minor allele (G)	major allele (A)	324 bp
rs8192472	major allele (G)	minor allele (A)	242 bp
rs12364575	major allele (C)	minor allele (G)	195 bp
rs4602358	minor allele (A)	major allele (G)	131 bp

The multiplex was then optimised for magnesium concentration, dNTP concentration, amount of *Taq* polymerase, number of amplification cycles, and concentration of each primer. The finalised reaction conditions were: 1.5 mM magnesium; double dNTPs (200 μM of each nucleotide); double *Taq* polymerase (2 units); number of cycles reduced to 28; and primer concentrations of rs1042048 and rs8192472 were normal (200 nM each) with increased rs12364575 (250 nM each) and rs4602358 (275 nM each).

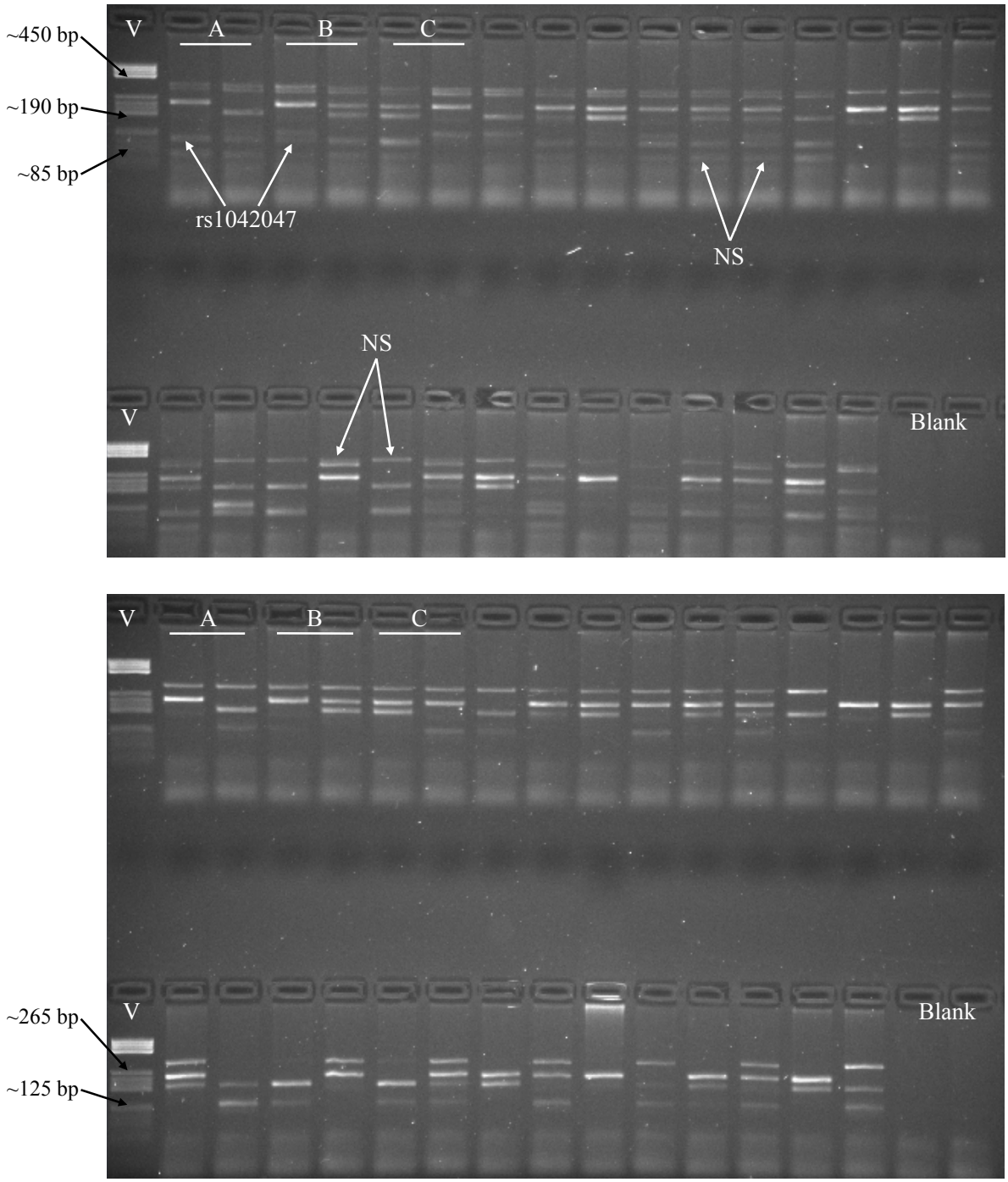


Figure 3.8 Multiplex - Five versus Four SNPs.
Multiplex trial amplifications before (top) and after (bottom) removal of rs1042047 (107 bp amplification fragment). Adjacent lanes contained the two reaction sets for the same DNA sample (the first three of which are labelled as samples 'A', 'B' and 'C'). The 'before' test shows more non-specific fragments (NS) and generally weaker amplification of the targets. V indicates Roche DNA Molecular Weight Marker V; Blank indicates the no DNA control.

Six of the remaining SNPs, all from *CCKBR*, were candidates for multiplexing. Initially two 3-plexes were created based on fragment size and optimal magnesium concentration: Multi 3-1 contained SNPs rs2947027, rs3901667 and rs7943852, which all worked well at

low magnesium concentrations; and Multi 3-2 contained SNPs rs2941025, rs2947028 and rs4758402, which all worked well at moderate magnesium concentrations.

The two reactions for Multi 3-1 included:

	First reaction:	Second reaction:	Size:
rs2947027	major allele (A)	minor allele (G)	301 bp
rs3901667	minor allele (C)	major allele (A)	219/222 bp
rs7943852	major allele (C)	minor allele (T)	174 bp

The two reactions for Multi 3-2 included:

	First reaction:	Second reaction:	Size:
rs2941025	major allele (C)	minor allele (T)	173 bp
rs2947028	major allele (T)	minor allele (C)	117/116 bp
rs4758402	minor allele (C)	major allele (T)	99 bp

These multiplex assays were then optimised in a similar manner to Multi 4 (see Figure 3.9 for an example). The final reaction conditions for Multi 3-1 were: 1.0 mM magnesium; double dNTPs (200 μM of each nucleotide); 1.5 times *Taq* polymerase (1.5 units); number of amplification cycles increased to 35; and normal primer concentration for rs3901667 (200 nM of each primer) with increased rs2947027 (300 nM each) and rs7943852 (325 nM each). The finalised reaction conditions for Multi 3-2 were: 2.25 mM magnesium; double dNTPs (200 μM of each nucleotide); double *Taq* polymerase (2.0 units); number of amplification cycles reduced to 27; and reduced primer concentration of rs4758402 (125 nM each), increased rs2941025 (275 nM each) and rs2947028 (325 nM each).

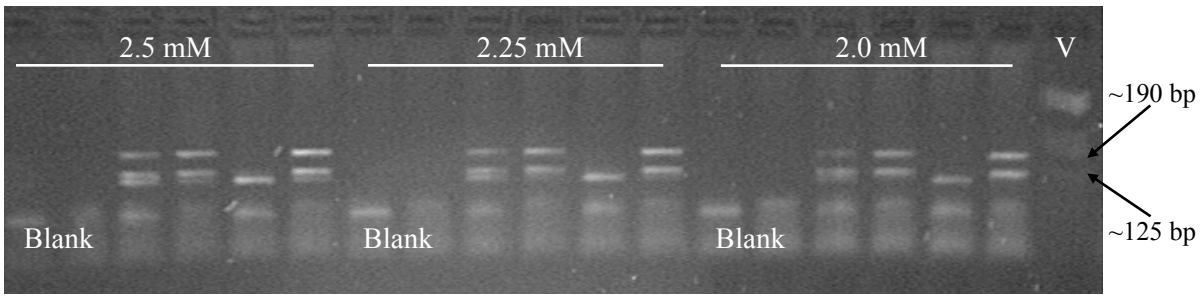


Figure 3.9 Magnesium Gradient for Multi 3-2.
Amplification of two DNA samples at three different magnesium concentrations in a three SNP multiplex containing: rs2941025 (173 bp), rs4758402 (99 bp) and rs2947028 (117/116 bp). V indicates Roche DNA Molecular Weight Marker V; Blank indicates the no DNA control.

Initially, Multi 3-1 was run with any samples that were not fully genotyped at Blocks 2 or 3, followed by Multi 3-2 in any samples still needing to be genotyped at any of the first three blocks. However, problems with the amplification of rs7943852, in Multi 3-1, occurred and, consequently, only around 100 samples were genotyped with this assay (Figure 3.10). The remaining samples were genotyped with Multi 3-2 (if not already fully genotyped at one of the first three blocks), followed by rs7943852 by hydrolysis probe, rs1112716 by hydrolysis probe and/or a 2-plex of rs2947027and rs3901667 (using the same reaction conditions as Multi 3-1), as necessary.

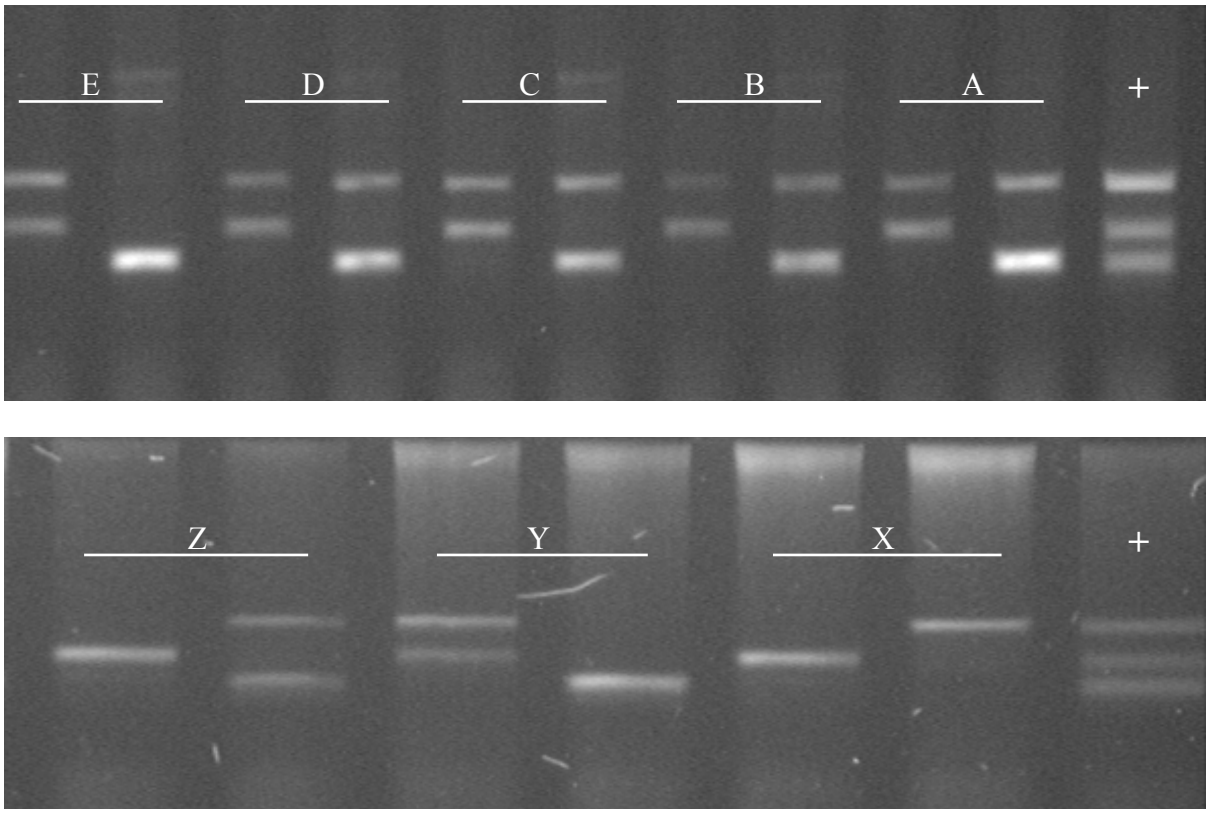


Figure 3.10 Amplification Failure in Multi 3-1.
Top image shows an early amplification of samples with Multi 3-1, between the two lanes, at least one band for each SNP is seen. In the lower image, the smallest band (rs7943852) has failed to amplify in either lane for subject X. "+" indicates a band size marker (created from remaining amplification product in which the amplicon sizes had been previously confirmed).

3.3.2.1.4 RFLP Assay

There were no TaqMan hydrolysis probes available to rs2929180 and allele specific primers could not be designed to the SNP as the surrounding region had high G/C levels. However,

the presence of the minor allele resulted in the loss of a restriction enzyme cut site. Consequently this SNP was genotyped by RFLP assay utilising the restriction enzyme SacII. Primers were designed on either side of the SNP resulting in the amplification of a 345 bp fragment that, when fully digested with SacII, would produce two products in individuals homozygous for the minor allele (159 and 186 bp), three products in those homozygous for the major allele (159, 116 and 70 bp), and four products in heterozygous individuals (159, 186, 116 and 70 bp; Figure 3.11).

Amplification of this 345 bp fragment could not be obtained using the standard reaction buffer (described in section 2.4.3.3, page 79). After trying several different buffers, specific amplification was produced using the Expand Long Template PCR System Buffer 3 (GmbH, Germany). To produce sufficient yield for restriction enzyme digest, additional magnesium was added, resulting in a final magnesium concentration of 3.25 mM.

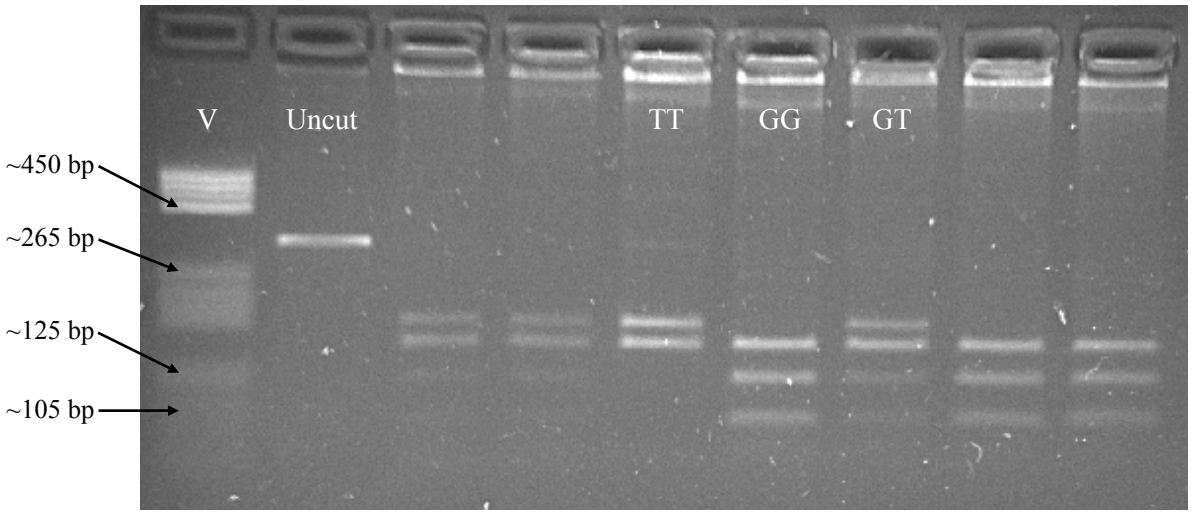


Figure 3.11 Restriction Enzyme Digest.
Example results of restriction enzyme digest of rs2929180 with SacII. At least one sample with each genotype is shown, as well as uncut DNA (345 bp). Fragment sizes of cut DNA are 186, 159, 116 and 70 bp. V indicates Roche DNA Molecular Weight Marker V.

3.3.2.2 Confirmation of Genotyping Accuracy

Several tests were conducted to verify the genotyping of each SNP was at least 90% accurate. If any of these tests suggested genotyping errors DNA sequencing was used to confirm the accuracy of genotyping.

3.3.2.2.1 Haplotypes

For all SNPs that were only genotyped in a subset of the sample, randomly selected individuals whose genotype had been inferred, based on their genotype at other SNPs, were also genotyped. This helped ensure that haplotypes present within our cohort that were not found in the CEU sample were included. If discrepancies occurred, genotyping was repeated at both the originally genotyped and the inferred SNPs.

There were a small number of individuals (<10%) for whom genotyping at different SNPs did not fit the expected pattern based on the haplotypes generated from the CEU data. These were generally haplotypes of low frequency (<1%) seen in a small number of pedigrees. When a rare haplotype like this was found, other members of the pedigree were examined for the presence of this haplotype. The exceptions to this are described below.

SNP rs2929180:
For rs2929180 (genotyped by RFLP), an additional haplotype was identified that subsequent genotyping of 100 individuals indicated had a frequency greater than 1%. As a consequence, this SNP was genotyped in all individuals.

SNP rs1112716:
During genotyping of *CCKBR*, one haplotype was found to have a frequency much higher than that expected based on the HapMap CEU data. The SNP responsible, rs1112716, was genotyped by TaqMan hydrolysis probe in individuals with the minor allele (C) at rs2947029 and the major allele (T) at rs2947028 (CT haplotype); the minor allele (T) of rs1112716 defined the haplotype concerned (Figure 3.12). It was expected that the majority of individuals with the CT haplotype would have the major allele at rs1112716 (C); however, the T allele was found to be the most frequent within this subgroup. The expected frequency of the T allele was 0.022 and the observed frequency was 0.196. Random sampling of individuals without the CT haplotype did not suggest any genotyping problems; only one pedigree was found with a haplotype that varied from the CEU haplotypes: the T allele at rs1112716 was occasionally seen with the G allele at rs2947027, but the frequency of this was below 1%. Nonetheless, sequencing was used to confirm the accuracy of the genotyping of rs1112716.

rs1112716	rs2947029	rs2947028	rs2947027	rs3901667	Expected Frequency	Observed Frequency
C	T	T	A	A	.516	
C	C	C	G	A	.233	
C	C	T	A	A	.144	<.010
C	C	C	A	C	.042	
C	C	C	A	A	.025	
T	C	T	A	A	.022	.196

Figure 3.12 Frequency of Haplotypes Defined by rs1112716.
The tag SNPs defining the haplotypes from *CCKBR* Block 3 (Four-gamete Rule analysis) are shown. The frequency of the haplotype defined by the "T" allele at rs1112716 was much higher in the SIBS cohort (observed frequency) than in the HapMap CEU cohort (expected frequency).

Primers were designed so that a 204 bp fragment would be produced with the SNP in the centre. In the initial testing of the sequencing primers four individuals were genotyped. This set included one individual that was homozygous for the major allele (CC), two heterozygous individuals (CT) and one homozygous for the minor allele (TT), based on the results of the probe assay. All of these four genotypes were confirmed by the sequencing, and an additional SNP (rs1112715) that was co-segregating with rs1112716 was noted six base pairs away. A further ten samples that had been called homozygous for the minor allele by probe were then sequenced. Again, sequencing confirmed the genotype of each of these samples, hence, the genotyping of this SNP by hydrolysis probe was considered to be more than 90% accurate.

CCK SNPs:

As a previous member of the laboratory conducted much of the genotyping of *CCK* some years ago, the accuracy of this data was confirmed by analysing haplotypes. The previous genotyping calls were combined with the two SNPs that were genotyped as part of Multi 4 and these were compared to the predicted haplotypes. Where these did not match, or when genotyping calls were missing, SNPs were repeated individually using the original primers with the re-optimised reaction conditions.

3.3.2.2.2 Hardy-Weinberg Equilibrium

To ensure that both alleles were being accurately amplified, tests were conducted to see if the genotypes for each SNP conformed to Hardy-Weinberg equilibrium (Appendix B). The SNPs with genotype distributions that were significantly outside of Hardy-Weinberg equilibrium are

shown in Table 3.3. This shows an excess of individuals homozygous for the minor allele for each of the five SNPs. As the cohort genotyped is family based, some deviations from Hardy-Weinberg equilibrium were expected, because a family with a high proportion of minor alleles could disrupt the equilibrium of the cohort. For all the *CCKAR* SNPs listed, excluding one family from the Hardy-Weinberg equilibrium analysis was sufficient to eliminate the statistical significance.

Table 3.3 SNPs that were not in Hardy-Weinberg Equilibrium (Observed - Expected).

SNP	Homozygous, Major Allele	Heterozygous	Homozygous, Minor Allele
CCKAR			
rs11726159	477 – 472	69 – 78	8 – 3
rs2725302	375 – 364	150 – 170	30 – 20
rs915889	466 – 460	78 – 89	10 – 4
rs1800855	373 – 363	152 – 172	30 – 20
CCKBR			
rs1042047	288 – 255	162 – 228	84 – 51

Only one *CCKBR* SNP had concerning deviations from Hardy-Weinberg equilibrium: rs1042047. The observed and expected numbers of individuals homozygous for the minor allele differed by more than 30, much more than would be expected from a single family. Additionally, the observed number of heterozygotes was more than 50 individuals less than expected, suggesting that detection of the heterozygote genotype was inadequate. Further investigations of this SNP are detailed below.

3.3.2.2.3 Mendelian Errors

In addition to calculating associations, analysis with FBAT produced a list of family/SNP combinations with Mendelian errors. Individuals from these families were then genotyped again at the relevant SNP(s) to ensure the original call was accurate. For most SNPs, this repetition resulted in few, if any remaining Mendelian errors. In addition, seven individuals were removed from the analysis (recoded as 0/unknown at all SNPs) due to consistent Mendelian errors at multiple SNPs. This may have been due to deteriorating DNA quality or an error in the original labelling of the DNA sample. This final step reduced all but one SNP to less than two Mendelian errors among the 200 nuclear families (Appendix B).

SNP rs1042047 had nine Mendelian errors that were not resolved by re-genotyping the individuals concerned. These results raised questions about the accuracy of genotyping at this SNP, particularly since it also failed to conform to Hardy-Weinberg equilibrium. Consequently, this SNP was sequenced in a subset of the cohort (sequencing conducted by Dr Alison Fitches). Sequencing confirmed that the genotype calls made by TaqMan hydrolysis probe were not accurate, possibly due to the presence of another SNP within the probe binding area. A number of families were genotyped by sequencing and a *p*-value of 0.22 was obtained for Any Panic (18 informative families). As this was far from the significance threshold of $p<0.05$, no further genotyping was conducted and this SNP was excluded from further analysis.

3.4 Associations

3.4.1 Phenotypes of Subjects

Phenotypic information was obtained using the Diagnostic Interview for Genetic Studies (Nurnberger *et al.*, 1994), which was administered by trained interviewers and interpreted by psychiatrists (see also (Edmonds *et al.*, 1998). The Diagnostic Interview for Genetic Studies is specifically designed for psychiatric studies and provides sufficient information for the diagnosis of mood disorders, anxiety disorders and many other psychiatric conditions. Diagnoses were based on DSM-IV criteria (American Psychiatric Association, 1994).

The main phenotypes of interest for this study were lifetime diagnoses of panic, including panic disorder (PD), panic attacks (PA) and limited symptom panic attacks (PS); however, participants often had comorbid conditions that may also interact with the genetic factors studied. The number of subjects with each of the panic phenotypes and comorbidity with other common psychiatric diagnoses is shown in Table 3.4.

Although the primary focus of this research is panic, participants in the cohort utilised were selected based on a family history of bipolar disorder. Within this cohort, panic has been found to frequently be comorbid with bipolar or other mood disorders (Doughty *et al.*, 2004). The majority of these cases developed the mood disorder first (approximately 60%), while 20% experienced panic first and the remainder first experienced both disorders within the same year.

Table 3.4 Number of Participants with each Phenotype

Comorbidity	No Panic	Any Panic	PD	PA	PS
Any Mood	284 (162)	116 (79)	54 (37)	44 (31)	12 (6)
Depression	150 (93)	33 (25)	11 (10)	17 (13)	4 (1)
Any BP	134 (69)	83 (54)	43 (27)	27 (18)	8 (5)
BP I or II	115 (56)	75 (48)	40 (24)	24 (16)	8 (5)
No BP	301 (162)	44 (32)	13 (12)	24 (17)	6 (2)
No Mood	151 (69)	11 (7)	2 (2)	7 (4)	2 (1)
Other Anxiety	90 (46)	65 (46)	41 (30)	17 (11)	4 (3)
Agoraphobia	7 (3)	33 (24)	5 (4)	4 (4)	0 (0)
Social Phobia	44 (16)	34 (25)	29 (20)	7 (4)	2 (2)
Specific Phobia	48 (29)	39 (28)	25 (19)	12 (8)	2 (1)
OCD	5 (3)	8 (6)	5 (4)	1 (0)	2 (2)
No Other Anxiety	345 (185)	62 (40)	15 (9)	34 (24)	10 (4)
Any Substance	94 (22)	44 (21)	23 (13)	15 (6)	4 (1)
Alcohol	79 (15)	33 (16)	16 (10)	11 (4)	4 (1)
No Substance	341 (209)	83 (65)	33 (26)	36 (29)	10 (6)
Total	435 (231)	127 (86)	56 (39)	51 (35)	14 (7)

Values indicate total number of participants (number of females) with a lifetime diagnosis of both of the two phenotypes concerned. Some participants had incomplete or ambiguous data, hence, some numbers do not add up. PD = panic disorder; PA = panic attacks; PS = limited symptom panic attacks; BP = bipolar disorder; OCD = obsessive compulsive disorder; Substance includes any substance abuse or dependence disorder; subsyndromal forms of anxiety and substance disorders were not included.

3.4.2 Associations of CCK System Genes

3.4.2.1 Phenotypes Analysed

The primary phenotype analysed in this study was any panic spectrum disorder (Any Panic), including panic disorder (PD), panic attacks (PA) and limited symptom panic attacks (PS), as well as some subjects that could not be clearly assigned to one of these three panic levels. Since the cohort was selected on the basis of bipolar disorder, a control analysis was undertaken to ensure any associations seen were not with bipolar disorder. For this analysis, individuals with any bipolar disorder diagnosis that did not have a comorbid panic spectrum disorder were coded as affected (BP no Panic).

Secondary analyses were also undertaken to determine whether any SNPs/haplotypes were more strongly associated with phenotypes related to panic and anxiety. Selection of these phenotypes was based on associations found in the New Zealand Mental Health Survey between panic disorder and other psychiatric disorders (Scott *et al.*, 2006a). Diagnostic information and number of affected individuals in our cohort further limited the secondary

analyses. The additional phenotypes analysed were: bipolar disorder I or II (BPD); panic disorder, panic attacks, any phobia or obsessive compulsive disorder (Anxiety); any panic spectrum disorder comorbid with any phobia (Panic+Phobia); any panic spectrum disorder comorbid with any substance abuse or dependence disorder (Panic+Substance); any panic spectrum disorder comorbid with any bipolar disorder (Panic+BP); and subgroups of panic. Subsyndromal forms of disorders were included for "any panic spectrum" and "any bipolar" disorder. No statistical correction was made for these additional phenotypes as they were intended to generate alternative hypotheses for associations with panic, rather than direct hypothesis testing.

3.4.2.2 Power of the Sample

Power calculations were difficult to conduct with the SIBS cohort. Most statistical programmes are not designed for the wide variety of different family types, including probands with one parent, with siblings but without participating parents, discordant parents, and mixtures of affected and unaffected siblings. Additionally, several parameters required for accurate power calculations are not known for panic, such as relative risk conferred by risk variants. To account for this, estimated power for different relative risks and allele frequency combinations were conducted for Any Panic. The programme used to calculate these values requires the number of trios (http://pngu.mgh.harvard.edu/~purcell/gpc/dtdt.html). Since there is a mixture of family types with Any Panic in the SIBS cohort, some that may provide more and some that may provide less information than a trio, the power values should be considered an estimate only. This table shows a low ability to detect a relative risk less than 0.5, or risk alleles with a frequency greater than 0.8 (Table 3.5).

Table 3.5 Estimates of Statistical Power for Any Panic

	Increased Relative Risk						
Allele Freq	0.1	0.2	0.5	1	1.5	2	3
0.1	5.8%	8.0%	20.6%	50.0%	73.7%	87.2%	97.1%
0.2	6.4%	10.0%	29.6%	65.1%	85.1%	93.6%	98.6%
0.3	6.7%	11.3%	33.7%	68.6%	86.1%	93.5%	98.1%
0.4	6.9%	11.8%	34.4%	67.0%	83.3%	90.8%	96.4%
0.5	7.0%	11.7%	32.6%	61.9%	77.5%	85.5%	92.5%
0.6	6.8%	11.1%	29.1%	54.1%	68.5%	76.7%	84.9%
0.7	6.6%	10.1%	24.2%	43.9%	56.1%	63.7%	72.1%
0.8	6.2%	8.7%	18.3%	31.6%	40.4%	46.2%	53.1%
0.9	5.6%	7.0%	11.8%	18.2%	22.5%	25.5%	29.3%

Increased Relative Risk = increased risk conferred by each allele (assuming an additive model). Allele Freq = the frequency of the risk allele.

3.4.2.3 Variants Analysed

The results of most tag SNPs from the custom block analysis and the haplotypes based on the Confidence intervals analysis are presented below (Table 3.6 and Table 3.7). Three SNPs from *CCKBR* were excluded: rs1042047 was excluded due to genotyping errors; and rs4758402 and rs3901667 had minor allele frequencies too low to be analysed for Any Panic. Haplotypes for *CCK* were formed from rs4602358, rs10865918 and rs747455. Haplotypes for *CCKAR* were formed from rs7665027, rs2000978 and rs1800855. Haplotypes for *CCKBR* were formed from rs906895, rs2929180, rs2941025, rs2929183, rs2941023, rs2947025, rs2880898 and rs2947028. Full results of all analyses conducted can be found in Appendix C.

3.4.2.4 Correction for Multiple Comparisons

Correction for multiple comparisons is difficult in family-based cohorts as most statistical programmes are designed for case-control analyses and cannot be used. As a result of this difficulty, two different types of correction were conducted, a permutation analysis and a Bonferroni correction. These may be prone to falsely accepting or falsely rejecting the null hypothesis, respectively, for reasons described below.

A Bonferroni correction for the number of genes analysed was the simplest of the correction methods. The number of genes was chosen since these are independent, whereas the variants tested within each gene are not independent, thus, correcting for total variants tested would be too conservative (Gao *et al.*, 2008). This correction consisted of dividing 0.05 (the nominal *p*-value) by 3 (the number of genes tested), resulting in an adjusted *p*-value threshold of

0.017. This method is likely to result in falsely rejecting the null hypothesis on some occasions, as it does not take into account the individual tests conducted within each gene.

The preferred method for correction of multiple comparisons in genetic association studies is permutation tests (Gao *et al.*, 2010). This method involves repeatedly randomly shuffling phenotypic data and analysing the new data set, thus generating a set of at least hundreds, often tens of thousands, of *p*-values that can then be used to determine the likelihood of a particular test value occurring by chance. Hence, permutation tests give adjustments that are specific to the variants being tested, automatically taking into account the frequency of these alleles in the sample population. Unfortunately, the programme primarily used for the analyses in this study, FBAT, was unable to run permutation tests. The programme Haploview was used for this instead. Since Haploview was not designed to analyse as many family types as FBAT (Purcell *et al.*, 2005; Rabinowitz & Laird, 2000), the use of Haploview programme may have produced inaccuracies in the resulting data.

3.4.2.5 Associations with Any Panic

Results for the primary phenotype, any panic spectrum disorder (Any Panic), and the control phenotype bipolar disorder without comorbid panic (BP no Panic) are shown in Table 3.6. The thresholds for statistical significance were $p<0.05$ (nominal) and $p<0.017$ (adjusted), the later of which uses a Bonferroni correction, taking into account the three genes studied, but not the total number of variants across the genes. Adjusted *p*-values calculated by permutation testing are also shown.

No statistically significant associations were found with *CCK* SNPs or haplotypes and Any Panic (Table 3.6). For *CCKAR*, one SNP and the two most frequent haplotypes were associated with Any Panic at $p<0.05$. Eight of the 13 SNPs analysed in *CCKBR* and one of the three haplotypes showed a significant association with Any Panic, with *p*-values ranging from 0.046 to 0.007. None of these SNPs or haplotypes met the 0.05 threshold after permutation analysis, however, the lowest *p*-values were found for *CCKBR* SNPs, with the lowest of the main variants analysed being rs2941025 ($p=0.118$) and one of the additional SNPs, rs2947027, having a *p*-value of 0.058 (Appendix C).

Table 3.6 CCK System Variant Associations with Any Panic.

	Any Panic				BP no Panic		
Variant	**Families**	**Trans.**	***p*-value**	**Perm. *p***	**Families**	**Trans.**	***p*-value**
***CCK* - SNPs**							
rs8192472	28	-0.2	0.961	0.990	29	-5.7	0.122
rs11129949	25	-1.9	0.508	1	25	-4.1	0.156
rs4602358	33	1.2	0.752	1	34	-1.6	0.638
rs10865918	38	-5.2	0.246	0.999	34	-3.2	0.399
rs747455	30	0.1	0.974	1	31	-3.3	0.294
Haplotypes							
GGG	37	-6.5	0.131	1	37	-3.1	0.408
ATG	31	1.3	0.704	1	33	-3.5	0.264
ATA	27	0.1	0.975	0.995	23	3.1	0.267
GTG	21	4.4	0.097	1	20	3.4	0.280
***CCKAR* - SNPs**							
rs7665027	13	2.3	0.144	1	15	-4.8	**0.044**
rs2000978	21	5.3	**0.043**	1	26	3.5	0.231
rs1800855	21	2.2	0.492	1	20	-2.8	0.337
rs6448456	24	4.9	0.070	1	25	1.3	0.646
Haplotypes							
ATT	35	-7.5	**0.045**	1	34	2.8	0.428
ACT	22	5.4	**0.035**	0.907	25	2.9	0.292
ATA	14	-0.3	0.881	1	13	-0.2	0.913
GTA	12	1.7	0.348	1	16	-5.0	**0.050**
***CCKBR* - SNPs**							
rs906895	33	1.6	0.697	1	38	3.7	0.462
rs2929180	30	7.6	**0.038**	0.243	31	0.7	0.800
rs2941025	32	8.3	**0.021**	0.118	29	0.0	0.987
rs2929183	34	8.9	**0.027**	0.552	31	0.5	0.886
rs2941023	34	8.9	**0.027**	0.552	30	-0.5	0.862
rs2947025	31	6.7	0.077	0.667	27	0.1	0.966
rs7943852	27	-4.3	0.219	0.667	36	1.8	0.720
rs2880898	25	-6.1	**0.032**	0.994	26	1.2	0.800
rs1112716	24	-5.6	**0.046**	0.994	26	1.2	0.800
rs2947029	37	5.3	0.203	1	37	1.4	0.793
rs2947028	36	11.0	**0.009***	0.509	32	0.7	0.844
rs12364575	32	8.8	**0.030**	0.799	32	4.4	0.246
rs1042048	36	-1.9	0.665	1	42	1.0	0.791
Haplotypes							
AGCAAGTT	38	-3.8	0.359	0.995	33	-2.2	0.697
GGCAAGCT	25	-8.4	**0.007***	0.948	22	2.8	0.517
GTTGGCTC	26	5.7	0.088	1	20	-0.9	0.734

Any Panic = individuals with any panic spectrum disorder (panic disorder, panic attacks or limited symptom panic attacks); BP no Panic = individuals with any bipolar disorder and no comorbid panic spectrum disorder (control analysis); Families = number of informative trios for the variant/phenotype combination; Trans. = number of over or under transmissions of the minor allele or haplotype, values are not whole numbers as complete parental genotypes are sometimes unknown; *p*-value = *p*-value generated by FBAT; Perm. *p* = *p*-value generated by Haploview permutation testing. Italicised SNPs were used to form haplotypes. Bold indicates $p<0.05$ (nominal significance), asterix (*) indicates $p<0.017$ (adjusted significance).

No SNPs or haplotypes in *CCK* or *CCKBR* showed a significant association with bipolar disorder without panic (BP no Panic). However, two variants in *CCKAR* were associated with BP no Panic, at $p<0.05$ (Table 3.6).

3.4.2.6 Phenotypes Most Commonly Associated with each Gene.

All variants were also analysed against multiple different phenotypes that have previously been found to be associated with panic (see Appendix C for all results). The phenotype that most frequently exhibited the strongest association with *CCK* variants was Anxiety (Table 3.7). Four *CCK* variants showed the strongest association with Anxiety, though only two had $p<0.05$. Additionally, the bipolar disorder I or II (BPD) phenotype was the most strongly associated for two variants, one of which had $p<0.05$. For *CCKAR*, the strongest association was most frequently seen for a bipolar related phenotype, including panic with comorbid bipolar disorder (Panic+BP) (three, variants all with $p<0.05$), BP no Panic (two variants, both with $p<0.05$) and BPD (one variant with $p=0.013$). For *CCKBR*, the strongest associations were seen with Any Panic or subgroups of this category for eight of the sixteen variants analysed, seven with $p<0.05$. Anxiety and BPD, two of the largest phenotype groups, were the only other strongest associations with $p<0.05$ in *CCKBR*.

Table 3.7 Strongest Associations For CCK System Variants.

Variant	Phenotype	Families	Trans.	*p*-value
***CCK* - SNPs**				
rs8192472	**BPD**	41	-10.6	**0.019**
rs11129949	PA	16	-2.8	0.137
rs4602358	Anxiety	40	7.9	0.103
rs10865918	**Anxiety**	45	-11.2	**0.040**
rs747455	BPD	47	-6.6	0.136
Haplotypes				
GGG	**Anxiety**	41	-11.9	**0.024**
ATG	BP no Panic	33	-3.5	0.264
ATA	Anxiety	32	4.9	0.128
GTG	**Panic+Phobia**	14	4.8	**0.023**
***CCKAR* - SNPs**				
rs7665027	**BP no Panic**	15	-4.8	**0.044**
rs2000978	**BPD**	37	8.8	**0.013***
rs1800855	PA/PS	13	2.1	0.262
rs6448456	**Panic+BP**	17	4.8	**0.048**
Haplotypes				
ATT	**Panic+BP**	27	-7.2	**0.011***
ACT	**Panic+BP**	15	5.1	**0.023**
ATA	Panic+Phobia	11	2.1	0.261
GTA	**BP no Panic**	16	-5.0	**0.050**
***CCKBR* - SNPs**				
rs906895	BP no Panic	38	3.7	0.462
rs2929180	**Anxiety**	34	8.4	**0.026**
rs2941025	**Anxiety**	33	8.4	**0.020**
rs2929183	**Any Panic**	34	8.9	**0.027**
rs2941023	**Any Panic**	34	8.9	**0.027**
rs2947025	Anxiety	33	6.8	0.065
rs7943852	**PA/PS**	16	-5.6	**0.022**
rs2880898	**PA/PS**	15	-6.0	**0.004***
rs1112716	**PA/PS**	14	-5.5	**0.006***
rs2947029	PD/PA	29	5.4	0.165
rs2947028	**Any Panic**	36	11.0	**0.009***
rs12364575	**BPD**	50	11.8	**0.011***
rs1042048	Panic+Substance	16	-3.1	0.224
Haplotypes				
AGCAAGTT	Anxiety	43	-6.1	0.214
GGCAAGCT	**PA/PS**	15	-6.3	**0.006***
GTTGGCTC	Anxiety	29	6.6	0.062

Phenotypes analysed: any panic spectrum disorder (Any Panic); any bipolar disorder without comorbid panic (BP no Panic); bipolar disorder I or II (BPD); panic disorder, panic attacks, any phobia or obsessive compulsive disorder (Anxiety); any panic comorbid with any phobia (Panic+Phobia); any panic comorbid with substance abuse or dependence disorders (Panic+Substance); any panic comorbid with any bipolar disorder (Panic+BP); panic disorder or panic attacks (PD/PA) and panic attacks or limited symptom panic attacks (PA/PS). With the exception of panic attacks and "any bipolar disorder", subsyndromal disorders were not included.

Families = number of informative trios for the variant/phenotype combination; Trans. = number of over or under transmissions of the minor allele or haplotype, values are not whole numbers as complete parental genotypes are sometimes unknown. Italicised SNPs were used to form haplotypes. Bold indicates $p<0.05$ (nominal significance), asterix (*) indicates $p<0.017$ (adjusted significance).

3.4.3 Association with *ADORA2A*

Three SNPs from *ADORA2A* (rs3761422, rs2267076 and rs5751876) were investigated for association with panic disorder or panic attacks (PD/PA) and any panic spectrum disorder comorbid with agoraphobia (Panic with Agoraphobia) in the SIBS cohort. No statistically significant results were found; the lowest *p*-value (0.40) was found for the association between rs2267076 and Panic with Agoraphobia (Table 3.8). As these results are not consistent with those of Hohoff et al. (2010), an additional analysis was undertaken using the optimal offset (-o) setting in FBAT (Lunetta *et al.*, 2000). This analysis includes unaffected individuals in the calculation, increasing the number of informative families. Despite this, there were still no significant associations (lowest p=0.56, rs2267076; full results in Appendix D). Since none of these tests reached nominal statistical significance, no correction for multiple comparisons was undertaken for the *ADORA2A* association tests.

Table 3.8 Associations Between *ADORA2A* and Panic.

SNP	Phenotype	Families	Trans.	*p*-value
rs3761422	PD/PA	33	0.63	0.85
	Panic with Agoraphobia	14	-1.75	0.51
rs2267076	PD/PA	31	1.10	0.73
	Panic with Agoraphobia	14	-2.08	0.40
rs5751876	PD/PA	33	-0.07	0.98
	Panic with Agoraphobia	15	-2.08	0.44

Families = number of informative families in the cohort; Trans. = number of under or over transmissions of the minor allele, values are not whole numbers as complete parental genotypes are sometimes unknown.

3.5 Discussion

The key findings from this study were:

- An association of *CCKBR* variants with panic.
- Some weak support for *CCK* variation being associated with a variety of anxiety phenotypes.
- Preliminary evidence that *CCKAR* variation may be associated with bipolar disorder.
- A lack of association between three previously identified *ADORA2A* SNPs and panic.

3.5.1 Methodological Strengths and Limitations

Strengths of this study, as well as things that could have been improved with different resources are discussed below.

3.5.1.1 Cohort

The SIBS cohort was studied as it provided readily available samples, eliminating the time and resources needed to collect the DNA and phenotypic information. It was not, however, ideal for an anxiety cohort as it was collected on the basis of bipolar disorder and has fewer individuals affected with anxiety than would be required to reliably detect the small genetic effect sizes that may be involved in these disorders (Ioannidis *et al.*, 2006). Additionally, the high rates of bipolar disorders within the families may be associated with different anxiety risk factors than in a cohort where panic was the primary or only diagnosis. Thus, the findings of this study reflect panic in a bipolar cohort, and may not generalise to other anxiety patients.

The SIBS cohort was also family-based, with a variety of different pedigree structures. The family-based cohort meant that no control population was needed, which reduces the likelihood of false-positive findings due to population stratification (Laird *et al.*, 2000). However, the variety of pedigree structures also created problems finding appropriate statistical analysis packages, resulting in different programmes being used for the main analyses, power estimations and multiple correction comparisons. Thus, these different results may not be equally reliable. Similarly, some of the members of the extended pedigrees may have provided little or no additional information to analyses. Any genotyping of such individuals would have been a waste of resources.

If this research were to be repeated, a larger cohort that was selected on the basis of anxiety or panic attacks would be preferable. To have a reasonable ability to replicate the *CCKBR* findings presented here, the study would ideally need to have at least 1000 cases and to include New Zealand participants of predominantly European ancestry. This would be difficult to achieve at any one centre, but, given the high lifetime rates of anxiety in New Zealand, should be attainable if multiple centres were to participate in cohort collection. Indeed, this is how the SIBS cohort was collected, with multiple participating centres around

the South Island of New Zealand (Joyce *et al.*, 2004). If a case-control comparison were to be undertaken, careful consideration should to be given to the selection of the control subjects. These subjects should be representative of the same population as the cases, with particular attention paid to things likely to influence genetics, such as ethnicity. Additionally, careful consideration needs to be given to the inclusion or exclusion of other psychiatric conditions, as these could result in spurious associations. For a family-based study, parent-child trios would probably provide the best ratio of information to genotyping effort. However, it is not always possible to obtain DNA samples from both parents, making it much more difficult to collect a large sample of trios than it would be for case-control subjects. Discordant siblings may also be useful, however, since the first onset of these disorders can be at any age, it would not be possible to be completely certain that the siblings truly are discordant.

3.5.1.2 Candidate Gene vs Genome-wide

The size of the SIBS cohort made a candidate gene approach necessary, as there would not have been sufficient power to find associations after correction for the approximately 500 000 SNPs often included in genome-wide analyses. Genome-wide approaches have the benefit of not requiring an *a priori* hypothesis about what gene or system is involved in the disease. Thus, genome-wide approaches have the potential to identify new biological pathways associated with the disease, as well as elements that do not encode genes, such as microRNAs (Stranger *et al.*, 2011). Fear pathways, however, are moderately well characterised, giving a long list of potential candidate genes to choose from. Nonetheless, a genome-wide approach may still be useful for identifying new components of fear pathways.

In addition to limited power, a genome-wide approach may have increased the difficulty in interpreting the findings of this study. The primary phenotype of interest was panic, however, the cohort was selected through association with bipolar disorder. Selection of candidate genes that have biological plausibility for anxiety or panic attacks increases the likelihood that the associations are truly with panic, rather than spurious associations with bipolar disorder or another disorder found in bipolar pedigrees. It would be difficult to determine if a significant genome-wide association found with this cohort was truly associated with panic.

There are a variety of other methods that could have been used to investigate genetic variation associated with panic. Many of them, including exome sequencing and investigation of

epistatic interactions, would share the problem of limited power that prohibited genome-wide study of the SIBS sample. Other potential investigations, such as gene-environment interactions, were limited by the information available for the subjects. It would have been possible to use sequencing to identify rare variants associated with anxiety in the candidate genes, and this may be a good use of family-based cohorts as multiple carriers in one pedigree may result in enrichment of rare alleles (Zhu & Xiong, 2012). However, it would be more appropriate to focus on rare variants contributing to bipolar disorder in the SIBS cohort, since this is likely to be the main phenotype the families are genetically predisposed to. These alternative methods may be useful in future studies, with larger cohorts selected on the basis of anxiety or panic.

3.5.1.3 SNP Selection from HapMap Data

CCK system SNPs were chosen for analysis based on data from the International HapMap Project CEU population. Analysis methods that maximised the number of tag SNPs were chosen. This was done both to ensure the SIBS cohort was similar to the CEU and to make it less likely that common genetic variation was missed. Most previous studies of CCK system genes did not characterise variation as thoroughly as the present study, and, hence, may have missed important associations. SNPs for *ADORA2A* analyses were restricted to those found significant in the study by Hohoff et al. (2010). This was done as the purpose was to attempt to replicate their findings, however, it may have resulted in too few variants being tested and real associations being missed.

Only genotyping SNPs, however, may also have allowed important associations to be missed. With this methodology copy number variants, insertions, deletions and inversions would only contribute to an association if they were in strong linkage disequilibrium with a SNP or haplotype (International HapMap 3 Consortium, 2010; Stranger *et al.*, 2011). The 1000 Genomes Project was intended to characterise some of these alternative forms of variation, as well as rarer variants than the International HapMap Project captured. However, CCK system genotyping for this study began in 2006, while the 1000 Genome Project began 2008 (1000 Genomes Project Consortium, 2010), hence the 1000 Genomes Project data was not available at the start of genotyping. The combination of tag SNPs and haplotypes genotyped should, nonetheless, represent the majority of common variation in the genes.

One of the ultimate goals of genetic association studies is to identify the causal variant(s). The use of tag SNPs and haplotypes is not well suited to this goal, as these variants are used a proxies for other variation in or near the gene of interest. Direct sequencing would be a means of identifying potential causative variants. This could have been done for each of the three CCK system genes in the SIBS cohort. However, since our results indicated that only *CCKBR* variation was associated with panic, the sequencing efforts for *CCK* and *CCKAR* would have been of little use. Sequencing of *CCKBR* may be useful in future studies into the genetics of panic.

3.5.1.4 Genotyping Methods

The genotyping of this study was conducted between 2008 and 2010, with previous genotyping of *CCK* and *CCKAR* occurring predominantly in 2006 and 2007 respectively (Chan, 2007; Wilson, 2008). A range of different methods for genotyping SNPs were used, depending on what was practical and most efficient (in both cost and time) for the particular SNP. If the experiment was being conducted today, with recent reductions in cost, genotyping arrays and next generation sequencing would be tempting alternatives. However, as discussed above, the additional information these methods would provide is unlikely to be of use in the SIBS cohort, as the necessary corrections for multiple comparisons would greatly reduce the power to find an association.

In a larger cohort, selected for anxiety, newer genotyping methods could be of great advantage. Genotyping arrays could provide the genetic information necessary for not only standard genome-wide association studies, but also gene-gene or gene-environment interaction studies. Sequencing may identify rare variants, not common in the general population, but found in anxiety patients. Other methods, including epigenetic and transcriptome analyses, should also help advance the understanding of anxiety genetics in the future.

3.5.1.5 Analysis with FBAT

FBAT was used to analyse the genotype data and determine whether any of the variants were associated with Any Panic, or any of the other phenotypes used in this study. FBAT was chosen as it was readily available and can obtain information from a wide variety of family

types, allowing the maximum information to be gained from the SIBS cohort. The FBAT analysis settings used for all CCK system SNPs treats unaffected and unknown subjects identically. By changing the settings, FBAT can also perform analyses that incorporate the allele or transmission frequencies to unaffected individuals, increasing the number of informative families and, hence, power. However, given the wide age range of the SIBS cohort (17 to 94 years) and the fact that anxiety disorders can begin at any age, it is likely that some of the participants would have developed disorders after data collection. , If such individuals had been analysed as "unaffected", they would have contributed erroneous data that may have increased false-negative findings. Hence, excluding unaffected individuals should have produced the most reliable results for this cohort.

FBAT also provides feedback on Mendelian errors. Thus, in addition to the main analyses, genotype data was also loaded FBAT into FBAT as soon as initial genotyping of most samples was complete. The outputs of these initial analyses, as well as the identification of haplotypes and determining whether the genotype frequencies were in Hardy-Weinberg equilibrium, were used to identify samples that needed to be re-genotyped. In the case of rs1042047, the Mendelian errors identified by FBAT were one of the reasons for suspecting a problem with the assay for that SNP, which was subsequently confirmed by sequencing. Thus, this additional feature of FBAT proved useful in the present study.

3.5.1.6 Power and Correction for Multiple Comparisons

The power calculations presented are estimates only, due to the difficulty of finding a programme capable of calculating power when multiple different family structures are included. For family-based studies, power varies with different allele frequencies. For high frequency alleles, the power of a family-based study is low, with case-control studies having the advantage (Clerget-Darpoux & Elston, 2007). This was seen in the SIBS sample (Table 3.5), as was limited power to detect an increased relative risk less than 1 (i.e. a total relative risk of up to 2). Thus, the lack of association, for example, with *CCK*, could be due to the risk allele being high frequency or the increased relative risk conferred by the risk allele could have been less than 1. This may be most important in the lack of replication of *ADORA2A* results, as the original study included more participants and would have had greater power to detect an effect than the present study. Power estimates were only calculated for Any Panic, but these values should provide a rough indication for other phenotypes.

Two different methods were used in an attempt to control for the multiple variants tested in the CCK system studies, due to the lack of an ideal correction method. One of these methods was a Bonferroni correction and, since this correction is for independent tests, the number of genes was used as the denominator. This, however, may have resulted in falsely rejecting the null hypothesis since, although they are not independent, the variants within a gene are also not entirely dependent. Although it is often done in genetic association studies, a correction based on the total number of variants tested would be highly conservative and would result in a threshold that was unlikely to be achievable with the size of the SIBS sample.

A permutation analysis was also undertaken using the programme Haploview. Haploview was not designed primarily to conduct association tests and utilised fewer families in its analyses at each variant than FBAT. Differences were also seen with the specific variants that were most strongly associated in *CCKBR*. Thus, the permutation *p*-values give an indication as to the significance of the findings, but may not be completely accurate.

No corrections for multiple comparisons were undertaken for the *ADORA2A* analyses as no significant associations were found. Similarly, the secondary analyses conducted with the CCK system variants included a large number of phenotype/variant combinations with no further correction for multiple comparisons. This increases the likelihood of finding false positive results and, as discussed above, these results should be viewed as hypothesis generating, not testing. Associated with this, the overall pattern and proportion of associations within each gene should be the focus of interpretation, not the *p*-values of individual variants.

3.5.1.7 Selection of Phenotypes

This study used Any Panic, a phenotype focused on panic attacks, as the main phenotype of analysis. This differs from some of the other studies on panic, which have focused on panic disorder. The selection and definition of a phenotype in psychiatric genetics can be difficult because of the limited understanding of psychiatric disorders. This is further compounded by evidence that some genetic risk factors may predispose to multiple different psychiatric disorders (Hettema *et al.*, 2005). Both the present and other studies (for example: Juhasz *et al.*, 2010; van den Oord *et al.*, 2008)) have dealt with this phenotypic uncertainty by focusing on a well-defined feature or symptom that may occur in a variety of mental disorders, rather

than investigating a single disorder. In addition, this study investigated the hypothesis that any detected associations with panic were due to spurious associations with comorbid conditions by identifying the phenotype most strongly associated with each variant. This final set of analyses needs to be interpreted with caution, as no correction for multiple comparisons was undertaken. Hence, findings should not be used to prove an association, but rather, to support or suggest alternative hypotheses for associations with panic.

3.5.2 Genetic Associations with CCK System Genes

The participants of the SIBS were genotyped at multiple SNPs within three genes of the CCK system, *CCK, CCKAR* and *CCKBR*, allowing the determination of haplotypes within each gene. The resulting data suggested that *CCKBR* variants are associated with the development of panic spectrum disorders, while *CCK* variants may be involved in multiple anxiety phenotypes and *CCKAR* variants may be specifically associated with comorbid panic and bipolar disorder.

3.5.2.1 *CCK* Associations

The results for *CCK* were the weakest for all of the CCK system genes. No significant associations were seen for Any Panic and only four of the strongest associations with any phenotype reached nominal significance, with none meeting the criteria for adjusted significance. Given the thorough approach to genotyping and the multiple variants that were analysed in this gene, these results make it unlikely that common genetic variation in *CCK* is involved in the pathogenesis of panic within this population. However, it is still possible that *CCK* variants increase the risk of panic in other populations or that variants our sample size was too small to detect increase panic risk.

Previous research of *CCK* provides only weak evidence of an association with panic. Some of the strongest support comes from previous research in our laboratory, which investigated a smaller number of SNPs and fewer participants from the cohort (Chan, 2007). In that study, the preliminary analysis with panic found one *p*-value of 0.02, but the only other significant associations were found on testing of multiple additional phenotypes. Similarly, most published studies with other cohorts have found no or limited evidence for an association with panic (see Table 3.1 for details).

Although weak, *CCK* variation was usually most strongly associated with anxiety phenotypes. Two of the four significant associations mentioned above were with Anxiety (panic disorder, panic attacks, phobias and obsessive compulsive disorder) and a third was with comorbid panic/phobia. Although no firm conclusions can be drawn from this data, this association is sufficient to warrant further investigation in larger cohorts, particularly cohorts selected on the basis of anxiety. Investigations in large anxiety cohorts may reveal a more robust association with anxiety or with specific types of anxiety disorder other than panic.

3.5.2.2 *CCKAR* Associations

Some weak evidence was found for an association between *CCKAR* variation and panic. Three of the eight variants were nominally associated with Any Panic, though none of these reached the adjusted threshold. The previous study in the SIBS cohort also found no association between *CCKAR* SNPs and panic (Wilson, 2008), and there is little support from other studies for this association. *CCKAR* SNPs have typically shown no significant association with panic (Gratacos *et al.*, 2009; Ise *et al.*, 2003; Kennedy *et al.*, 1999; Koefoed *et al.*, 2010), however, significant associations have been found between *CCKAR* haplotypes and panic (Koefoed *et al.*, 2010; Miyasaka *et al.*, 2004). Nonetheless, it seems unlikely that variation in this gene is of major importance for most individuals with panic.

The strongest associations found for *CCKAR* were for the comorbid panic-bipolar phenotype. Six of the eight variants tested showed associations with *p*-values below the nominal threshold, three of which were with comorbid bipolar/panic and the other three were with one of the other categories of bipolar disorder. Furthermore, two of these associations were below the threshold for adjusted significance, further supporting the importance of these findings.

A comorbid bipolar/panic phenotype has been described in previous epidemiological research. As part of the Otago Familial Bipolar Genetic Study (the predecessor to the SIBS), it was found that rates of panic disorder were much higher than the general population, with 10.9% of bipolar probands and 4.5% of the relatives having panic (Edmonds *et al.*, 1998). They additionally investigated rates of panic spectrum disorders and found 16.4% of probands and 7.5% of relatives had some panic symptoms. A similar analysis was conducted previously as part of the SIBS, with the larger cohort revealing a high prevalence of panic disorder in patients with unipolar depression (6%) and bipolar disorder (16%), with 40% of

patients with bipolar disorder experiencing some panic symptoms (Doughty *et al.*, 2004). In contrast, that study found that, in relatives without affective disorder, there was no panic disorder and few individuals with any panic symptoms (4.6%). Other studies have also found evidence of a specific genetic subtype of bipolar disorder that is defined by comorbid panic. For example, (MacKinnon *et al.*, 1998) found evidence for linkage on chromosome 18q in families with comorbid bipolar disorder and panic disorder, but not for families with bipolar disorder alone.

Involvement of *CCKAR* with this comorbid phenotype has seen little previous investigation. There is some support from a study that followed up on a region associated with bipolar disorder in a genome-wide study, which found *CCKAR* overlapped with a haplotype that was significantly associated with bipolar disorder (Christoforou *et al.*, 2007). Additionally, the panic disorder/major depressive disorder comorbid phenotype has been found to be associated with a variant in *CCKAR* (Maron *et al.*, 2005). Collectively, this indicates that further investigation of *CCKAR* and mood disorders is warranted. Bipolar cohorts have been studied by other research groups (for example: Manolio *et al.*, 2007) and at least some of these are likely to have collected sufficient phenotype and genotype data to allow *CCKAR* and panic analyses. Thus it may be possible to assess the legitimacy of this finding by reanalysing existing data from other cohorts, saving the expense of establishing a new cohort.

3.5.2.3 *CCKBR* Associations

The strongest association found in this study was that for *CCKBR* and panic. In the primary analysis, nine of the 16 variants were significantly associated with Any Panic, two of which were significant below the adjusted threshold. Furthermore, of the ten significant "Strongest Associations", seven of these were with Any Panic or subtypes of panic and four were below the adjusted threshold. The three significant variants that were not associated with panic phenotypes were associated with Anxiety or BPD, which are the two largest groups and, hence, had the highest power to find associations. If these phenotypes were truly associated with *CCKBR* it is expected that they would have shown the highest number of significant strongest associations, hence, these are likely to be false-positives, possibly due to a high overlap with panic phenotypes.

Results of previous studies into *CCKBR* and panic have been variable. While some have found no association (Hamilton *et al.*, 2001; Hattori *et al.*, 2001b; Kato *et al.*, 1996; Koefoed *et al.*, 2010; Yamada *et al.*, 2001), several have found significant associations between *CCKBR* variants and panic (Gratacos *et al.*, 2009; Hosing *et al.*, 2004; Kennedy *et al.*, 1999; Maron *et al.*, 2005). There are a number of possible reasons for this variation, which are addressed in section 3.5.2.4 below.

Since the publication of our findings (Wilson *et al.*, 2012), further support for the involvement of *CCKBR* in panic disorder has come from a genome-wide association study. The study included more than 700 cases with panic disorder and over 1700 controls, making it the largest genome-wide association study that has been conducted on a panic disorder sample. Although no individual variants reached genome-wide significance, *CCKBR* (SNP rs1396860) was found to be amongst 20 genes that were still significant after correction based on genes (Otowa *et al.*, 2012). Other recent studies have also provided support for involvement of *CCKBR* in panic, including the finding of missense variants in the gene in a small number of panic disorder cases (Koefoed *et al.*, 2010) and an association between panic disorder and a variant of a microRNA that represses *CCKBR* translation (Muinos-Gimeno *et al.*, 2011).

Furthermore, the CCK_B receptor is thought to be the target for CCK induced panic attacks as CCK_B receptor selective agonists will induce panic attacks, and selective antagonists block both the psychological and physiological symptoms (Lines *et al.*, 1995). Hence, there is both biological plausibility and genetic evidence supporting the association of *CCKBR* variation with panic.

3.5.2.4 Differences Between Studies

One of the most frequently studied CCK system SNPs is rs1799923 in *CCK*. This SNP was not genotyped directly as part of this research, however, the haplotypes determined allowed the genotype to be inferred at this SNP. The accuracy of this was confirmed by sequencing a subset of our sample (performed by Alison Calder and Alison Fitches); no significant association was seen with Any Panic (Appendix C). Five previous studies also found no significant association (Hamilton *et al.*, 2001; Hattori *et al.*, 2001a; Hattori *et al.*, 2001b; Hosing *et al.*, 2004; Kennedy *et al.*, 1999; Maron *et al.*, 2005). However, two studies found

that the minor allele was over-represented in patients (Maron *et al.*, 2005; Wang *et al.*, 1998), and one found that the minor allele was under-represented in patients (Koefoed *et al.*, 2010).

This is typical of this area of research, with variants that show consistent, reproducible results being unusual. This lack of reproducibility, however, does not necessarily indicate a lack of involvement for the genes. There are several possible reasons for variation between studies. The specific populations studied vary from paper to paper, both in geographical location and inclusion/exclusion criteria. If the variants investigated are not the causal variant(s), but are in linkage disequilibrium with such variants, unpredictable associations between variants for different ethnic groups may have affected the results. Similarly, gene-gene or gene-environment interactions may result in associations with only one study population; for example, if the variant is only causative under environmental conditions common in just one of the study populations. The study populations also vary on phenotype(s) analysed; some studies have looked at pure panic disorder, others panic disorder and panic attacks, and there is variability on the inclusion or exclusion of comorbid conditions. The results of the secondary analyses of the present study suggest that these phenotype differences may have a considerable effect on the study outcome.

Compared to the present research, most previous studies of CCK system genes have only looked at one to a few polymorphisms per gene. This study used data from the International HapMap Project to select multiple SNPs within each gene and, hence, thoroughly cover common variation in each gene. Therefore, this study may have found associations that other studies missed. Given this, the lack of significant associations with *CCK* SNPs or haplotypes makes it unlikely that common variants at this gene have a clinically significant effect on the risk of developing panic; however, it is not possible to draw conclusions about specific panic phenotypes, such as pure panic disorder, as our cohort was not large enough to investigate these.

3.5.3 Genetic Associations with *ADORA2A*

This study included an attempt at replicating the findings of a recent paper that investigated *ADORA2A*. Unfortunately, no support was found in the SIBS cohort for their associations with panic disorder. There are several possible reasons for this discrepancy. The three cohorts are from different geographical regions and, as such, may have different genetic

backgrounds. This is unlikely to have affected the results for any functional variant analysed, however, if the polymorphisms tested are in linkage disequilibrium with functional variants, the different population genetics may explain the lack of concordance of the results. The study by Hohoff et al. (2010) involved more thorough genotyping than the present study, analysing eight SNPs across the gene. It is possible that, due to different linkage with the functional polymorphism(s), more thorough genotyping of *ADORA2A* in the SIBS cohort would reveal significant associations. The cohorts also differed in size; the SIBS sample size was much smaller than that of Hohoff et al. (2010). Consequently, the present study had less power to detect an association and may have produced false-negative results if the effect size of the contributing variant is small. The SIBS cohort was ascertained based on bipolar disorder, with all participants having at least one family member with bipolar disorder. Consequently, our phenotypes are not representative of individuals with pure anxiety disorders, but rather people with comorbid disorders.

3.5.4 Implications of Findings

The results from this study, coupled with positive findings from other studies, suggest an involvement of *CCKBR* variation in panic. This may ultimately lead to genetic or other tests that will identify those at increased risk of developing panic disorder. There is emerging evidence that cognitive-behavioural therapy can prevent the development of panic disorder in high risk groups (Livermore *et al.*, 2010). Consequently, understanding the genetic predisposition to anxiety has the potential to allow focused preventative interventions, greatly reducing the occurrence of these disorders.

The future identification of a functional variant of *CCKBR*, as well as allowing the identification of at risk individuals, could contribute to a better understanding of brain function at the molecular level. For example, T cells taken from panic disorder patients have been found to have increased CCK_B receptor activity, compared to both controls and patients post-treatment (Akiyoshi *et al.*, 1996). A possible genetic cause of this might be variation in the promoter region, resulting in increased levels of CCK_B receptor, or variation in splice sites resulting in the production of high activity variants such as the intron 4 containing variant or the exon 1b variant (described in section 1.3.3.3, page 37). Indeed, one of the SNPs described in HapMap CEU cohort, rs3793993, is located in the 5′ region near exon 1b and was found to be associated with Any Panic at p=0.020 and PA/PS at p=0.005 in the SIBS cohort

(Appendix C). Such a SNP might only have an effect in specific cellular environments, similar to the suboptimal splicing of intron 4 in *CCKBR* that only occurs when the protein U2AF35 is low (Ding *et al.*, 2002), thus producing a plausible molecular mechanism for a gene-environment interaction.

Similarly, the identification of a specific molecular mechanism for a genetic predisposition may lead to improved treatments. In addition to identifying risk individuals, if the genetic change associated with disorder is known for a particular individual, it may be possible to identify anxiolytic drugs or gene therapy that will prevent or reverse the change. If such a therapy does not exist, there would also be evidence to support its development. This tailored therapy may help improve response rates and reduce unwanted side effects or repeated trials of ineffective medications.

Overall, further research is needed to better understand the genetic factors contributing to panic and anxiety. This study has added to the increasing body of literature supporting the involvement of *CCKBR* variation in panic, but a specific functional variant has yet to be identified. This study has also found preliminary support for involvement of *CCKAR* variation in mood disorders, though this needs to be confirmed in other cohorts.

4 Cellular Changes with Antidepressants

4.1 Background

As discussed in section 1.4.4 (page 60), the mechanism of action of many drugs that reduce anxiety is still poorly understood. It seems likely that structural changes such as neurogenesis or synaptogenesis are involved, though whether this is sufficient to produce the response and how the neurogenesis/synaptogenesis is stimulated are both unanswered questions. Changes in gene expression may be involved in drug-induced changes important for the anxiolytic therapeutic effect.

Candidate genes for the present study were selected based on either genetic location (position) or hypothesised involvement with anxiety or antidepressant mechanism of action (function). Positional candidates were all located in 7q21, a region previously found to be linked to panic within a bipolar cohort (Cheng *et al.*, 2006) and built upon previous research in our laboratory studying genes in this area (Chan, 2007; Wilson, 2008). Linkage studies for panic disorder have found multiple different linkage sites, with little replication between studies (Hamilton, 2009). The selection of 7q21 over the other associated regions was because the phenotype investigated in that study was panic in a bipolar cohort, which echoes the genetic association aspect of the present study. Candidate genes included primarily due to location were: *ADAM22*, *CACNA2D1*, *FZD1*, *GRM3*, *LMTK2*, *SRI*, and *TAC1*. Functional candidates included genes from signalling systems associated with anxiety, including: *ADORA2A*, *CCK*, *CCKAR*, *CCKBR*, *CRHR2*, *NPY*, and *NPY1R*. Other functional candidates were postulated to be involved in antidepressant or benzodiazepine action (*GABRA1*, *GABRA2*, *GABRA3*, *BDNF*, and *TPH2*) or were implicated in recent research (*CDKN1A*). The potential relevance of each of these genes to anxiety is described below.

4.1.1 Positional Candidates

All positional candidates are located in the region 7q21.

4.1.1.1 *ADAM22*

ADAM22 is expressed in various regions of the brain including the hypothalamus and cerebellum of mice (Sagane *et al.*, 2005). While the exact functions of the protein are unknown, ADAM22 (A Disintegrin And Metalloprotease 22) is thought to have an important role in the central nervous system and may be involved in the pathogenesis of epilepsy (Han & Kim, 2008; Sagane *et al.*, 2005). Evidence from mouse hippocampal slices indicates that ADAM22 is a receptor for LGI1 proteins and through this interaction may be involved in alterations of glutamatergic transmission seen with LGI1 (Fukata *et al.*, 2006). This is supported with evidence from chick neurons, showing importance for the growth of dendrites during neuronal cell development (Thomas *et al.*, 2010). ADAM22 has also been shown to reduce cellular proliferation in glial cells and is down-regulated in glioblastomas (D'Abaco *et al.*, 2006). Hence, *ADAM22* changes may be associated with both neuronal structure and gliogenesis changes.

4.1.1.2 *CACNA2D1*

CACNA2D1 is one of four genes that encode the α_2 and δ subunits (produced by proteolytic cleavage) of a five subunit voltage gated calcium channel (Davies *et al.*, 2007). The $\alpha_2\delta$-1 subunits are ubiquitously expressed with high levels in the hippocampus, cerebellum and cerebral cortex (Davies *et al.*, 2007). As discussed in section 1.4.4.4 (page 66), this protein is involved in the anxiolytic actions of gabapentin and pregabalin.

4.1.1.3 *FZD1*

FZD1 encodes a member of the Frizzled family of G-protein coupled receptors (Schulte & Bryja, 2007). It is expressed in a variety of human tissues, with high levels of expression in adult heart, lung, kidney, pancreas, prostate, and ovary (Sagara *et al.*, 1998). Frizzled receptors are activated by WNT lipoglycoprotiens and at least three different signalling pathways can be activated by Frizzled receptors (Schulte & Bryja, 2007). One of these pathways has been shown to be involved in adult neurogenesis in mice (Lie et al., 2005) and research in rat neurons suggested that the interaction between FZD1 receptor and WNT3a might be necessary for synaptogenesis (Varela-Nallar *et al.*, 2009). Hence, *FZD1* is likely to be important for normal brain function.

4.1.1.4 *GRM3*

GRM3 encodes the group II metabotropic glutamate receptor 3 (mGlu3 receptor). This receptor is widely expressed in the brain, including parts of cortex, caudate/putamen and parts of brainstem and is found in neurons, astrocytes and oligodendrocytes (Harrison *et al.*, 2008). About half of all genetic studies investigating *GRM3* variation in schizophrenia have reported a significant association (Cherlyn *et al.*, 2010) and the mood stabiliser lithium significantly reduced expression of *Grm3* in mice (McQuillin *et al.*, 2007). The mGlu3 receptor is also one of three metabotropic glutamate receptors, along with mGlu2 and mGlu5, that have been implicated in anxiety (Durand *et al.*, 2013).

4.1.1.5 *LMTK2*

LMTK2 encodes lemur tyrosine kinase 2, which has been shown in rat brain to be a serine/threonine kinase (Kawa *et al.*, 2004). This protein has several alternative names including brain-enriched kinase, due to its high level of expression in the mouse brain (Kawa *et al.*, 2004). *Lmtk2* was found to be widespread in adult, but not embryonic mouse brain, with expression predominantly found in neurons (Kesavapany *et al.*, 2003). There are few studies investigating the function of *LMTK2* and the only abnormality that was noted in *Lmtk2* knockout mice was defective spermatogenesis (Kawa *et al.*, 2006). There is some evidence that *LMTK2* is involved in the normal trafficking of endosomes within human cells (Inoue *et al.*, 2008) and evidence from mammalian cells indicates that it may be involved in neurite outgrowth stimulated by nerve growth factor (Kawa *et al.*, 2004; Kesavapany *et al.*, 2003). These functions raise the possibility that *LMTK2* is involved in the incorporation or maturation of new neurons produced through adult neurogenesis.

4.1.1.6 *SRI*

SRI codes for the protein sorcin or *so*luble *r*esistance-related *c*alcium-binding prote*in* (Maki *et al.*, 2002). *SRI* is expressed in the brain and has been found in cell bodies, axons and dendrites of neurons in the caudate/putamen of rats; its location and calcium binding function suggest that it may be involved in control of neurotransmitter release (Gracy *et al.*, 1999; Pickel *et al.*, 1997). There is also evidence for involvement of *SRI* in mental disorders as the protein expression was significantly reduced post-mortem in patients with schizophrenia and

major depressive disorder, compared to controls (Beasley *et al.*, 2006) and it was among the 30 most down-regulated genes in lymphoblast lines from patients with panic disorder (Philibert *et al.*, 2007).

4.1.1.7 *TAC1*

TAC1 encodes tachykinin, precursor 1, which is also known as *PPT-A*, *PPT-I*, and *TAC2*. At least four products are produced from the gene: substance P, neurokinin A, neuropeptide K and neuropeptide γ - the later two of which are long variants of neurokinin A (Severini *et al.*, 2002). Of these, substance P and neurokinin A have both shown associations with anxiety-like behaviour in animals, and blockade of their receptors was found to reduce anxiety-like behaviour in mice (Teixeira *et al.*, 1996). Additionally, transgenic mice lacking *Tac1* displayed reduced anxiety-like behaviour in several tests (Bilkei-Gorzo *et al.*, 2002) and the SNP rs10172781 in *TAC1* was found to be associated with panic disorder in humans in a recent genome-wide study (Otowa *et al.*, 2012), supporting a role in the pathogenesis of anxiety. With regards to pharmacotherapy, *Tac1* was one of several genes up-regulated by a single administration of the anxiogenic compound yohimbine to mice, and co-administration of the benzodiazepine diazepam prevented this (Le-Niculescu *et al.*, 2011). Finally, treatment of rats with antidepressants for 40 days was found to reduce substance P levels in various brain regions including the amygdala (Shirayama *et al.*, 1996).

4.1.2 Functional Candidates

4.1.2.1 *ADORA2A*

ADORA2A is located at 22q11.23 and encodes the adenosine A_{2a} receptor. As discussed in section 1.4.3.4 (page 58) the adenosine receptors A_{2a} and A_1 are thought to mediate the effects of caffeine, with the A_1 receptor the main contributor to the acute anxiogenic effects, while genetic variation at *ADORA2A* mediates the long-term risk of anxiety. A role for *ADORA2A* in the pharmacological treatment of anxiety can also be postulated as administration of a single dose of diazepam (an acute anxiolytic drug) increased the expression of this gene in the amygdala of mice (Le-Niculescu *et al.*, 2011).

Further support for a potential role for *ADORA2A* comes from studies showing its involvement in neurodegeneration/neuroprotection. Animals studies have shown that antagonism or genetic knockout of A_{2a} receptors results in a reduction in the functional deficit and neuronal loss from a variety of paradigms, including cerebral ischemia (Chen *et al.*, 1999; Gui *et al.*, 2009) and chemically induced damage (Chen *et al.*, 2001; Popoli *et al.*, 2002). This is consistent with high levels of leaked adenosine, as may occur during tissue damage, contributing to the death of surrounding cells. In contrast, under normal or more mild aversive conditions adenosine signalling via the A_{2a} receptor has been shown to be involved in enhancing neurite outgrowth in rat cells (Cheng *et al.*, 2002; Flajolet *et al.*, 2008) and in BDNF-induced long-term potentiation in rodent brain slices (Diogenes *et al.*, 2004; Tebano *et al.*, 2008). Hence, the adenosine A_{2a} receptor may contribute to either neuronal death or synaptogenesis, depending on the conditions of the signalling. This, coupled with the positive findings from genetic association studies, makes *ADORA2A* a good candidate for the changes produced during chronic anxiolytic therapy.

4.1.2.2 *BDNF*

BDNF (brain derived neurotrophic factor) has been implicated in a wide range of neurological and psychiatric disorders (Autry & Monteggia, 2012; Hartmann *et al.*, 2012). *BDNF* was being studied by other researchers in our laboratory for potential involvement in the actions of mood stabilisers (Sears, 2011) and was added to the present study after changes in *BDNF* expression were found in the human neuroblastoma cell line SH-SY5Y following exposure to some antidepressants (Donnici *et al.*, 2008). The involvement of BDNF signalling in the mechanism of action of antidepressant drugs has been widely studied in various models (Tardito *et al.*, 2006); however, its involvement in anxiolysis has received less research. Correlations have been found between brain BDNF levels and anxiety-like behaviour in animal models (Chen *et al.*, 2006; Maniam & Morris, 2010; Schmidt & Duman, 2010) and some anxiolytic effects of antidepressants were reduced in mice when BDNF signalling was decreased (Li *et al.*, 2008). Some human genetic association studies have also suggested involvement of *BDNF* in anxiety (Colzato *et al.*, 2011; Muinos-Gimeno *et al.*, 2011; Xie *et al.*, 2011).

4.1.2.3 *CCK*, *CCKAR*, and *CCKBR*

The three CCK system genes, *CCK*, *CCKAR* (also known as *CCK1R*) and *CCKBR* (also known as *CCK2R* or gastrin receptor, *GASR*) were included primarily due to their involvement in panic and anxiety (discussed in section 1.4.3.3, page 57). CCK-4 induced panic attacks have been shown to lessen in response to antidepressant and anxiolytic medications (van Megen *et al.*, 1997; Zwanzger *et al.*, 2003) (see also section 1.4.1.1, page 48). Additionally, following administration of the panicogen yohimbine, expression of *Cckbr* was increased in the hippocampus of mice and this was reversed by co-administration of the benzodiazepine diazepam (Le-Niculescu *et al.*, 2011). Diazepam has also been shown to increase CCK receptor levels in some regions in the brains of rats (Harro *et al.*, 1990) and to reduce the responsiveness of rat hippocampal neurons to CCK-8 (Bouthillier & De Montigny, 1988).

Despite strong evidence for involvement in anxiety, only a few studies have investigated CCK system gene involvement in the anxiolytic action of antidepressants. One study found that CCK_B receptor binding affinity was reduced in the hypothalamus of rats treated for 21 days with the tricyclic antidepressants imipramine or amitriptyline, though not by desipramine (another tricyclic antidepressant) or citalopram (an SSRI) (Harro *et al.*, 1997). Research in our own laboratory has also found evidence that antidepressants reduce the expression of *CCKAR* in human neural cell lines (Chan, 2007). These findings suggest that CCK system genes may be involved in antidepressant effects.

4.1.2.4 *CDKN1A*

CDKN1A encodes cyclin-dependent kinase inhibitor 1A, more commonly known as p21. This gene was included as a functional candidate after a study in mice found that 21 days administration of imipramine significantly reduced the levels of both its mRNA and protein, compared to untreated mice (Pechnick *et al.*, 2008). This finding has recently been supported by a study that found three weeks administration of the antidepressants fluoxetine, imipramine or desipramine reduced p21 protein levels and increased neurogenesis in mice (Pechnick *et al.*, 2011). This study also demonstrated a strong inverse association with p21 levels and neurogenesis since: p21 knockout mice had higher levels of neurogenesis; neuronal progenitor cells of wild-type animals rarely expressed both p21 and Ki67 (a marker of

proliferation); and reducing p21 levels in wild-type cells with a silencing RNA increased neurogenesis. Additionally, p21 knockout mice did not show the normal response to imipramine, instead showing reduced neurogenesis and increased immobility on the forced swim test (Pechnick *et al.*, 2011), suggesting that p21 may be involved in the lack of response to therapy in some patients. With regards to anxiety, administration of the anxiogenic compound yohimbine increased *CDKN1A* expression in the hippocampus of mice and this was prevented by co-administration of a benzodiazepine (Le-Niculescu *et al.*, 2011). Hence, it is possible that *CDKN1A* overexpression may have anxiogenic properties.

4.1.2.5 *CRHR2*

CRHR2 encodes coricotropin releasing hormone receptor 2 (CRH_2 receptor, also known as *CRFR2*). This gene was included as corticotropin is a major component of the stress system (see section 1.4.1, page 44) and this gene has been previously investigated in our laboratory (Chan, 2007). Studies of rodents investigating the role of CRH_2 receptor in anxiety have produced conflicting results, with most suggesting that activation of CRH_2 receptor increases anxiety (for example: Bale *et al.*, 2000; Isogawa *et al.*, 2003; Valdez *et al.*, 2003), while others support a decrease in anxiety (Risbrough *et al.*, 2009; Takahashi *et al.*, 2001), or no effect (Coste *et al.*, 2000; Heinrichs *et al.*, 1997). This may be due to CRH_2 receptor having different roles in different brain regions. For example, corticotropin releasing hormone injected into the frontal cortex of rats produced anxiolytic-like effects on the elevated plus maze (Zieba *et al.*, 2008), while injections into the extended amygdala of rats induced conditioned place avoidance (Sahuque *et al.*, 2006), and CRH_2 receptor selective antagonists blocked both of these effects.

Only a small number of studies have investigated CRH_2 receptor associations with antidepressants or anxiolytics. In humans, an association was found between early antidepressant response to the SSRI citalopram and a SNP in *CRHR2*, such that those with the major allele were significantly more likely to show a response after four weeks treatment (Papiol *et al.*, 2007). In rats, maternal deprivation increased *Crhr2* mRNA in some brain regions and decreased it in others; both of these effects were blocked by the tricyclic antidepressant desipramine (Vazquez *et al.*, 2003). These studies support the further investigation of CRH_2 receptor function and expression in anxiolytic treatment.

4.1.2.6 *GABRA1*, *GABRA2* and *GABRA3*

These genes encode the three most common α subunits of the $GABA_A$ receptor. As discussed in section 1.4.3.2 (page 54), these three subunits, along with the α_5 subunit, mediate the different effects of benzodiazepines. The α_2 subunits (encoded by *GABRA2*) are thought to mediate the anxiolytic effects of benzodiazepines (Low *et al.*, 2000; Smith *et al.*, 2012), hence, up regulation of these may also be involved in the response to other anxiolytics. There is also some evidence from mice that some anxiolytic effects may be mediated via α_3 subunits (Dias *et al.*, 2005) and *GABRA1* was associated with panic disorder in a genome-wide study (Otowa *et al.*, 2012). Since most $GABA_A$ receptors contain two α subunits, changes in expression of α_1 or α_3 subunits may affect the proportion of receptors that include an α_2 subunit, or the binding properties of an α_2 containing receptor (del Rio *et al.*, 2001). Although the encoded subunit binds benzodiazepines, *GABRA5* was not included in the present study as it has lower expression than the first three subunits (Whiting *et al.*, 1999).

4.1.2.7 *NPY* and *NPY1R*

NPY encodes neuropeptide Y (also known as neuropeptide tyrosine or PYY4), which, like CCK, is a peptide signalling molecule that is found throughout the brain. There are four neuropeptide Y receptors in humans: Y_1 (encoded by *NPY1R*), Y_2, Y_4 and Y_5 (Lindner *et al.*, 2008). One of the effects of neuropeptide Y is to reduce anxiety, as has been shown in rats injected intracerebroventricularly with neuropeptide Y (Sudakov *et al.*, 2001) and rats transfected with a transgene in the amygdala that increased neuropeptide Y expression (Primeaux *et al.*, 2005). Furthermore, patients with high anxiety have been found to have lower levels of neuropeptide Y (Rasmusson *et al.*, 2000; Sah *et al.*, 2009; Stein *et al.*, 1996), although some contradictory results have also been found (Boulenger *et al.*, 1996; Frisch *et al.*, 2009). Animal studies using Y_1 selective agonists (Sorensen *et al.*, 2004) and antagonists (Cohen *et al.*, 2012; Primeaux *et al.*, 2005) indicated that this receptor is a major contributor to these anxiolytic effects.

Studies have also found that treatments for anxiety alter neuropeptide Y levels. Several studies have found that fluoxetine increased neuropeptide Y levels in specific brain regions in rats (Caberlotto *et al.*, 1998; Zambello *et al.*, 2010) and one study found that voluntary running in rats increased both neuropeptide Y and Y_1 receptor mRNA in the hippocampus

(Bjornebekk *et al.*, 2010). Additionally, neuropeptide Y levels were increased in the CSF of humans being treated with the SSRI citalopram for depression (Nikisch *et al.*, 2005). Finally, previous research from our laboratory found that fluoxetine increased *NPY* expression in SH-SY5Y cells (Chan, 2007), suggesting that further research of these genes is warranted.

4.1.2.8 *TPH2*

TPH2 encodes tryptophan hydroxylase 2, one of the enzymes involved in the synthesis of serotonin (section 1.4.3.1, page 52), and has been studied previously in our laboratory (Chan, 2007). Although tryptophan hydroxylase 1 is also found in some regions, research in mice indicates that tryptophan hydroxylase 2 is the main isoform in most regions of the brain (Gutknecht *et al.*, 2009) and eliminating its expression in mice results in no serotonin production in these areas (Gutknecht *et al.*, 2008). Behavioural studies with *Tph2* knockout mice found reduced anxiety-like behaviour on the elevated plus maze and novelty suppressed feeding test (Mosienko *et al.*, 2012) and several genetic studies have found associations between *TPH2* variants and anxiety in humans (Chi *et al.*, 2013; Kim *et al.*, 2009; Mossner *et al.*, 2006).

TPH2 changes have also been implicated in the mechanism of action of antidepressants. In rats, *TPH2* levels in the midbrain and other parts of the brainstem were reduced after two weeks administration of fluoxetine, with midbrain levels then increasing above control levels after four and eight weeks (Dygalo *et al.*, 2006; Shishkina *et al.*, 2007). These changes mirror the increase followed by decrease in anxiety that has been found with antidepressants. Antidepressant response has been found to correlate with expression of *Tph2* in mice (Kulikov *et al.*, 2011) and variation within the gene in humans (Peters *et al.*, 2004; Tsai *et al.*, 2009; Tzvetkov *et al.*, 2008).

4.1.3 Aims

This study aimed to investigate the mechanism of action of drugs used to treat panic disorder. Preliminary work in the laboratory has identified genes with altered expression in a neural cell line exposed to fluoxetine or gabapentin; the present study includes further biological and positional candidate genes and additional time points. The potential involvement of these genes as part of the anxiolytic mechanism of action has also been further investigated by studying two

more drugs (paroxetine and imipramine) and two further human neural cell lines. It is hypothesised that expression of one or more of the candidate genes will be altered in the neural cell lines by the anxiolytic medications. Furthermore, the two SSRIs, fluoxetine and paroxetine, would be expected to show the most similarities in these changes, followed by the tricyclic antidepressant imipramine. Gabapentin is an anxiolytic that is not an antidepressant medication, so expression changes that are found with both antidepressants and gabapentin would be the most likely to be involved in anxiolysis.

4.2 Technical Results - Cell Culture and Drugs

Cell culture was chosen as the model for this experiment as it allows the study of changes in human brain cells. Neuroblastoma cell lines were cultured in the presence (or absence) of fluoxetine, paroxetine, imipramine or gabapentin. Neuroblastoma are a type of brain cancer, which means they may not be representative of normal human neurons, but can be easily and rapidly grown in culture, a feature that would not be possible with normal human neurons. The fact that these cells are dividing also fits with the hypothesised involvement of neurogenesis in antidepressant action (see section 1.4.4.2, page 61), which would suggest that neuroblasts, not mature neurons, are the targets of antidepressants.

Three different human neuroblastoma cell lines were utilised (SH-SY5Y, SK-N-SH and SK-N-DZ). The fact that these cells are not ideal models for normal neurons was part of the reason for including three cell lines. Additionally, a wide variety of different types of neurons are found in the adult brain and responses to drugs may vary for different cells and different brain regions. Using three cell lines increases the likelihood that relevant effects would have been detected. Moreover, effects seen in all three of the cell lines are more likely to generalise to other neurons. Since drugs may have different effects on different neuronal populations, using multiple cell lines allowed comparisons to be made regarding the uniformity of the any response. Finally, the genes analysed are not necessarily expressed in all neural cells, so including three cell lines allowed a greater number of genes to be analysed.

Cells were grown in the presence of 10 μM fluoxetine hydrochloride (fluoxetine), 50 μM gabapentin (gabapentin), 10 μM paroxetine hydrochloride hemihydrate (paroxetine), 10 μM imipramine hydrochloride (imipramine) or no drugs (untreated). These concentrations were based mainly on two considerations: the likely concentration in the human brain during

therapy, and the concentration tolerated by the cells. The concentration of gabapentin was similar to that found in the plasma of patients treated with gabapentin (Ben-Menachem *et al.*, 1995; Pande *et al.*, 2000). The concentration of fluoxetine was the highest concentration that was not toxic to the cells; this was determined by previous experiments in our laboratory (Chan, 2007) and is consistent with concentrations of fluoxetine found to be tolerated in other cell lines (Schuster *et al.*, 2007). This concentration is lower than that reported in the brain of humans treated with fluoxetine (Henry *et al.*, 2005). Both fluoxetine and gabapentin concentrations were identical to those used in previous research in our laboratory (Chan, 2007; Wilson, 2008).

For paroxetine and imipramine, the concentrations selected based on other research were then tested for a week prior to beginning the experimental protocol to ensure they would not cause toxicity, as toxic effects have been previously described for other cell lines treated with SSRIs (Schuster *et al.*, 2007). Paroxetine concentration was based on levels measured in patients and is similar to the levels used in other cell culture studies (Henry *et al.*, 2000; McHugh *et al.*, 2008). Imipramine concentrations were based on those used in other cell culture studies (Budziszewska *et al.*, 2004; Hwang *et al.*, 2008). Despite testing prior to beginning the experiment, it was observed during the research that the three antidepressant drugs initially slowed the replication rate of the neuroblastoma cell lines studied. This slowed growth was most evident in the first two weeks of exposure and was partially compensated for by seeding higher numbers of cells into the new flasks. In the later weeks of the experiment, however, growth returned to control values, suggesting that resistance to the growth inhibitory effects had occurred.

The cells utilised in this study were cultured on three different occasions. An initial study of gabapentin and fluoxetine was conducted as part of earlier research in the laboratory (Wilson, 2008); the remaining cDNA and some of the results from this study were utilised as part of the first long time course. Additional cells were cultured to expand upon this study, followed by a replicate to confirm the accuracy of the initial results. A short time course was also conducted based on a study by Donnici *et al.* (2008).

4.2.1 Long Time Course

The long time courses were included to mimic the extended period of treatment required for many patients to see the therapeutic effects of antidepressants - approximately one month. For long time courses, cells were cultured in the presence or absence of anxiolytics for a minimum of 28 days. Three flasks were cultured for each drug group, with media and drugs replenished every two to five days. When cells were approximately 70% confluent, the flasks were harvested and pellets from two of the flasks were stored for future RNA or protein extraction, while the third flask (small) was used to reseed three new flasks, continuing the culture of those cells until at least 28 days had passed.

Two separate long time courses were conducted as part of this project. For the first of these (Long A) at least four time points were frozen for each cell line and data from a previous experiment was used for the SH-SY5Y fluoxetine and gabapentin drug groups (Wilson, 2008), hence, for this cell line only paroxetine, imipramine and untreated cells were cultured. The second long time course (Long B) was conducted to replicate the most promising gene expression findings from the first experiment, which were for the antidepressant drugs. For this time course at least three time points were frozen for each cell line, though only the antidepressant drugs were used (gabapentin was not included).

Cells were frozen at the following time points:

Long A – SK-N-DZ (fluoxetine, paroxetine, imipramine and gabapentin): 4, 9, 15, 21, and 28 days.

Long A – SK-N-SH (fluoxetine, paroxetine, imipramine and gabapentin): 5, 12, 18, 26, and 31 days.

Long A – SH-SY5Y (paroxetine and imipramine): 7, 15, 24, and 32 days.

Long B – SK-N-DZ (fluoxetine, paroxetine and imipramine): 7, 11, 20, 25, and 29 days.

Long B – SK-N-SH (fluoxetine, paroxetine and imipramine): 8, 18, and 28 days.

Long B – SH-SY5Y (fluoxetine, paroxetine and imipramine): 9, 19, and 28 days.

The previously conducted extractions from cells (Wilson, 2008) were frozen at the following time points:

Long A – SH-SY5Y (fluoxetine): 4, 11, 16, 23, and 28 days.

Long A – SH-SY5Y (gabapentin): 3, 7, 12, 17, 24, and 29 days.

4.2.2 Short Time Course

To investigate the acute effects of the drugs, multiple medium flasks were set up and incubated in the relevant drug for up to 48 hours. The experiment was conducted with each of the four drugs (as well as a set of untreated flasks), with cells harvested at 6 hours, 24 hours or 48 hours after drugs were added to the media. The short time course was included as a follow up to a study of *BDNF* expression that included the SH-SY5Y cell line. That study cultured cells for 6, 24 or 48 hours in the presence of fluoxetine, reboxetine (a noradrenaline reuptake inhibitor) or desipramine (a tricyclic antidepressant) (Donnici *et al.*, 2008). It found a reduction in *BDNF* mRNA with reboxetine and desipramine at 6 hours, and an increase with the same drugs at 48 hours, but no significant changes were reported for fluoxetine.

4.3 Technical Results - Gene Expression

To determine relative gene expression after exposure to anxiolytic drugs, total RNA was extracted from selected time points for each of the cell culture experiments described above. The extracted RNA was converted to cDNA and utilised in the qPCR experiments described below. Data on the quantity of RNA extracted and cDNA produced for each flask can be found in Appendix E.

4.3.1 Primer Design

As described above, candidate genes were selected based on either location (position) or hypothesised involvement with anxiety or antidepressant action (function). In addition, seven reference genes were also analysed: *PLOR2F, HPRT1, MPRIP, HMBS, UBC, SDHA* and *GAPDH*. These genes encode proteins involved in vital cellular functions, such as nucleotide synthesis, and should theoretically show stable levels of expression in a variety of cellular conditions. Since stable expression cannot be guaranteed, geNorm was used to determine the most appropriate reference genes for each cell line and time course, the results of which are in Appendix F.

Primers for the genes were designed at various times and by several different researchers. Whenever possible, primers were designed to minimise the likelihood of amplifying genomic DNA. The full list of primers, including sizes for both cDNA and genomic DNA fragments,

are listed in Appendix F. Primers designed as part of the present study included: *CDKN1A, SRI* and *TAC1*. For each gene, primers were designed within exons on either side of a large intron to minimise the likelihood of amplifying genomic DNA. Primers were then tested to ensure that the amplification fragment could be produced under qPCR conditions and that only one fragment was seen on gel electrophoresis. For the original *TAC1* primers multiple bands were seen, which may have been due to the multiple splice variants produced from this gene. Consequently, the primers were redesigned within the 3´-UTR.

In addition, to the three primers pairs designed for this study, the previously designed candidate gene primers were tested for amplification specificity. As a result of this testing, one of the primers for *ADORA2A* was redesigned, as were the pairs of primers for *NPY1R* and *GRM3*. The new primers were used for all experiments.

4.3.2 Quality Control – Technical Replicates

Two different sets of technical replicates were conducted for each gene, within each cell line (Table 4.1). Each time point analysed was run in triplicate on the same plate, using the same cDNA/primer/reagent mixture, and the variation in quantification cycle values of these replicates was used as an indication of the intra-assay variability. Variation of less than 5% was considered acceptable for intra-assay variability and data points with greater variation than this were not analysed. This was most common when quantification cycle values were greater than 35 and unusual for quantification cycle values below 30. A dissociation curve was also produced for each run, and these were visually inspected to ensure there was only one peak, consistent with a single PCR product. When multiple dissociation peaks were seen, the affected well(s) was excluded from analysis. The acceptable intra-assay replicates were then averaged and standard deviations were calculated. These values were then normalised and the relative expression of each of the drug treated cells to the untreated cells was calculated. Error bars showing the standard deviation of the intra-assay replicates are shown on Figure 4.3 and Figure 4.5, as examples. Most graphs do not show error bars to avoid confusion between technical and biological replicates.

Each set of samples was also plated in triplicate on three separate occasions and analysed separately each time, giving an indication of the inter-assay variability. The three relative expression values for each drug/time combination were examined to determine whether they

supported the same expression change and the median value was used for the gene expression results presented below.

Table 4.1 Consistency of Technical Replicates

	SK-N-DZ		SK-N-SH		SH-SY5Y	
Gene	**C_q**	**Inter-assay[1]**	**C_q**	**Inter-assay[1]**	**C_q**	**Inter-assay[1]**
Positional Candidates:						
ADAM22	26	okay	25	okay	23	okay
CACNA2D1	25	okay	26	okay	25	okay
FZD1	26	okay	27	okay	28	okay
GRM3	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
LMTK2	27	okay	26	okay	26	okay
SRI	27	okay	26	okay	27	poor
TAC1	N/A	n/a[2]	>35	n/a[2]	28	good[2]
Functional Candidates:						
ADORA2A	29*	okay	28	okay	29	okay
BDNF	25	good	25	okay	29*	poor
CCK	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
CCKAR	29*	okay	>35	n/a[3]	>35	n/a[3]
CCKBR	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
CDKN1A	29*	poor	24	poor	28	poor
CRHR2	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
GABRA1	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
GABRA2	29*	okay	>35	n/a[3]	>35	n/a[3]
GABRA3	26	okay	27	okay	28*	okay
NPY	27	okay	26	okay	25	okay
NPY1R	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]
TPH2	>35	n/a[3]	>35	n/a[3]	>35	n/a[3]

* Approximate value when using increased amounts of cDNA; C_q = quantification cycle value.

1. Inter-assay variability was considered "good" if all sets of three results for each time/drug combination were in the same direction, "okay" if two sets were in the same direction and no results were in opposite directions, and "poor" if one of the three replicates for any set was in the opposite direction to the other two.

2. Only a small number of data points were repeated three times for *TAC1* due to the high variation between time points with no obvious pattern; within the repeated time/drug combinations the consistency was good.

3. Expression levels were too low to allow analysis.

Candidate genes with quantification cycle values above 35 were not analysed because the expression detected was too low to allow confident analysis. This may have been due to low expression levels in the cell lines studied or it may have been due to poor primer binding efficiency. It may have been possible to obtain results for these genes by amplifying the

region of interest in each sample prior to qPCR analysis; however, this adds additional variability to the assay and makes the results unreliable.

4.4 Gene Expression Changes

The gene expression levels presented below are expression relative to the untreated cells from the same time point, after normalisation. Calculating the relative expression of untreated cells ensures all data is on a comparable scale. This relative expression calculation means that for all time points and cell lines, the values for untreated cells equal 1. All time points and different genes have an untreated control, however, since these values are always 1, they are not shown in graphs or tables. Similarly, no zero time point was measured, but, since cells were taken from the same flask(s) immediately prior to drug exposure, all drug groups and cell lines should have been identical at time zero (all equalled 1).

Although a biological replicate was conducted for antidepressant effects in the long time course, since the time points used were not identical, for the purpose of graphical representations, these data were not averaged. Consequently, where relevant, both lines are shown the on graphs, without error bars. For the purpose of determining whether there was evidence of a change or no change, as compared to ambiguous findings, the consistency of the technical replicates was used. If two standard deviations of the normalised intra-assay value did not include 1, a change was considered to have been found. No change was considered to have occurred if two standard deviations did include 1 and was within 0.7 to 1.43. A threshold of two-fold was used for relevance of findings, as this was the change expected based on other studies with antidepressants (Lee *et al.*, 2010; Palotas *et al.*, 2004; Wong *et al.*, 2004).

Expression of the candidate genes was not always detectable in all cell lines. Very low or no expression was detected in any cell line for *GRM3*, *CCK*, *CCKBR*, *CRHR2*, *GABRA1*, *NPY1R* and *TPH2,* hence, these genes were not analysed. Additionally, *CCKAR,* and *GABRA2* only showed acceptable expression in one cell line; consequently, comparisons between cell lines could not be made for these genes.

4.4.1 Short Time Course

The complete short time course results, with all cell lines averaged together, are shown in Table 4.2. An alternative summary, giving an indication of how many time points varied for each cell line and each drug group is shown in Table 4.3. Most genes showed little or no change (see Figure 4.1 and Figure 4.2 for examples) or did not produce sufficiently reliable data to draw conclusions. Genes that showed little (less than two-fold) or no change at all data points in the short time course were: *ADAM22*, *CACNA2D1*, *FZD1*, *LMTK2*, *SRI*, *ADORA2A*, *GABRA2*, and *GABRA3*. Of the genes that demonstrated changes, *TAC1* showed extreme variation, with more than ten-fold change in expression between different time points for the antidepressant drugs (Figure 4.3). The consistency between technical replicates was among the best of all genes studied, indicating that it was unlikely to be due to problems with the primers or qPCR assay. Due to the high variation of this gene and lack of obvious pattern to the changes, *TAC1* was not included in further analyses. Although changes were not as extreme as *TAC1*, *CDKN1A* (Figure 4.4) and *NPY* also showed variation of approximately two-fold, with no obvious pattern by drug or cell line.

Table 4.2 Short Time Course Average Expression for Each Drug

	Fluoxetine (hrs)			Paroxetine (hrs)			Imipramine (hrs)			Gabapentin (hrs)		
Gene	**6**	**24**	**48**	**6**	**24**	**48**	**6**	**24**	**48**	**6**	**24**	**48**
Positional Candidates:												
ADAM22	1.06	0.91	1.00	0.93	1.00	1.00	1.00	0.84	0.96	0.89	1.03	0.98
CACNA2D1	1.06	0.94	0.86	0.85	1.04	0.83	0.92	0.87	0.85	0.88	0.97	0.83
FZD1	0.96	1.02	0.93	0.83	1.19	1.02	1.00	0.93	1.06	0.89	0.95	0.93
LMTK2	0.85	1.03	0.96	0.75	1.04	0.99	0.90	0.85	0.94	0.73	1.08	0.91
SRI	1.06	1.07	0.91	0.92	1.06	1.00	1.11	0.96	0.97	0.92	1.04	0.93
TAC1	4.98	6.39	0.21	6.91	45.0	1.89	79.4	0.36	12.0	0.97	3.73	1.35
Functional Candidates:												
ADORA2A	0.95	0.95	0.81	1.05	0.99	0.96	0.89	1.04	0.85	0.85	1.05	0.89
BDNF	1.09	1.56	0.93	1.07	3.91	1.25	2.17	0.82	1.94	1.03	0.92	1.03
CCKAR	0.44	0.49	0.76	0.79	0.48	0.55	0.59	0.63	0.54	0.86	0.75	0.71
CDKN1A	1.55	1.92	1.40	0.93	1.90	1.45	0.93	1.32	0.90	0.73	0.99	0.75
GABRA2	0.86	1.24	1.09	0.90	1.25	1.17	1.03	0.94	1.17	0.98	0.97	0.88
GABRA3	0.95	0.98	1.17	0.83	1.11	1.24	0.89	0.98	1.01	0.77	0.95	0.96
NPY	1.29	1.11	0.89	1.00	1.17	1.01	0.98	1.15	0.83	0.81	0.94	0.76

Table 4.3 Short Time Course Summary for All Cell Lines

	SK-N-DZ				SK-N-SH				SH-SY5Y			
Gene	**Flu**	**Par**	**Imi**	**Gab**	**Flu**	**Par**	**Imi**	**Gab**	**Flu**	**Par**	**Imi**	**Gab**
Positional Candidates:												
ADAM22	Var	↑	NC	NC	↓	PC	PC	PC	↑	↑	NC	↑
CACNA2D1	PC	↓	PC	NC	PC	PC	PC	PC	NC	↓	NC	NC
FZD1	↓	↓	↓	NC	PC	PC	↓	PC	NC	↑	↑	NC
LMTK2	NC	↓	NC	↓	PC	↓	PC	PC	↓	PC	NC	↓
SRI	NC	NC	↓	↓	PC	PC	PC	PC	NC	NC	NC	NC
TAC1	Not Analysed				Insufficient Expression				Data Extremely Variable			
Functional Candidates:												
ADORA2A	↓	↑	PC	↓	PC	PC	PC	PC	NC	PC	NC	↓
BDNF	Var	NC	↓	↓	PC	PC	PC	PC	PC	↑	↑	PC
CCKAR	↓	↓	↓	↓	Insufficient Expression				Insufficient Expression			
CDKN1A	↑	PC	↓	↓	PC	PC	PC	PC	↑	↑	↑	↓
GABRA2	NC	PC	NC	NC	Insufficient Expression				Insufficient Expression			
GABRA3	Var	↑	↓	NC	PC	PC	PC	PC	NC	↓	NC	↓
NPY	↓	↓	↓	↓	PC	PC	PC	PC	↓	↑	↑	↓

Flu = fluoxetine, Par = paroxetine, Imi = imipramine and Gab = gabapentin. NC = evidence supporting no change (median value[s] close to 1 with good consistency between intra-assay replicates). PC = poor consistency between inter-assay replicates preventing conclusions. Var = evidence variable (some time points increased, others decreased). ↓ = evidence supporting reduced expression at one or more time points. ↑ = evidence supporting increased expression at one or more time points. Symbol size indicates number of time points supporting change: ↓/↑/NC = less than half; ↓/↑/NC = approximately half; and ↓/↑/NC = more than half. The following genes were not included as they had insufficient expression for analysis in all cell lines: *GRM3*, *CCK*, *CCKBR*, *CRHR2*, *GABRA1*, *NPY1R* and *TPH2*.

Other changes in the short time course were only seen in one cell line. *BDNF* showed little to no change in the SK-N-DZ and SK-N-SH cell lines, however, in the SH-SY5Y cell line, fluoxetine and paroxetine treated cells showed a small up-regulation at 6 hours, followed by a much larger up-regulation at 24 hours (Figure 4.5). The other anxiolytic medications and other cell lines did not support this change. *CCKAR* was also only expressed in the SK-N-DZ cell line and was down-regulated 1.5 to 2-fold at most time points with the three antidepressant drugs tested, although gabapentin did not produce the same consistency of changes (Figure 4.6).

Since the majority of genes showed no, small or inconsistent changes, biological replicates were not conducted.

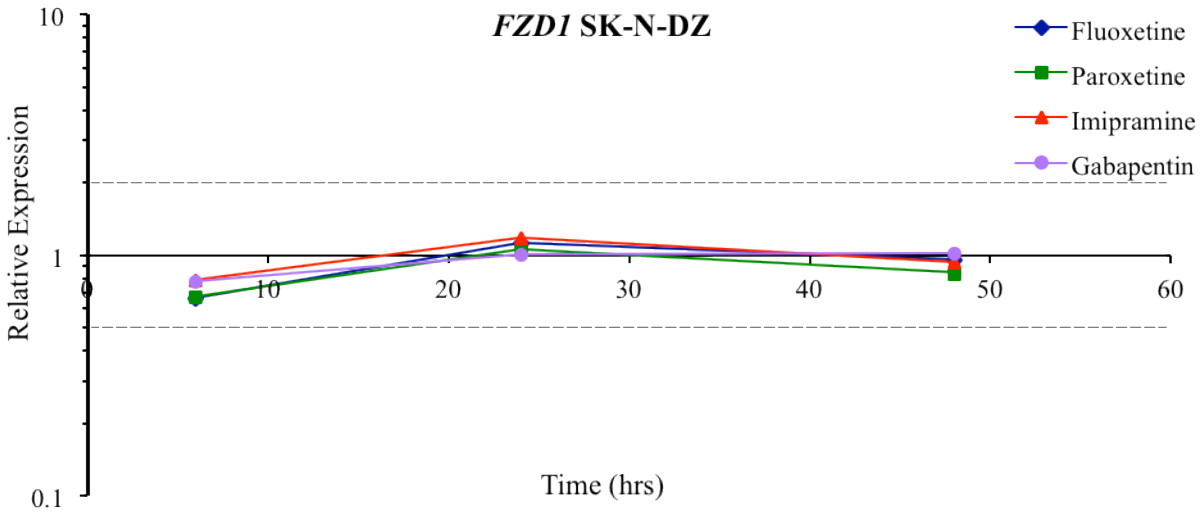


Figure 4.1 Small Changes with *FZD1* at 6 hours.
Down-regulation was found for *FZD1* with the three antidepressants tested at 6 hours; however, this change was consistently less than two-fold. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression.

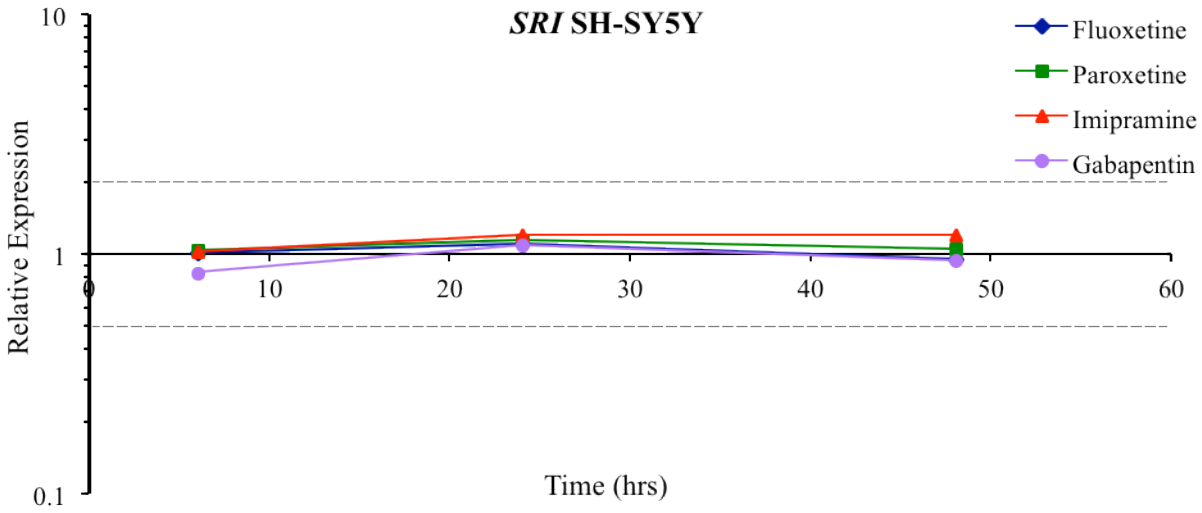


Figure 4.2 No Change with *SRI* over 48 hours.
Data for *SRI* in the SH-SY5Y cell line indicated that the expression of this gene is not altered by any of the anxiolytic drugs tested. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression.

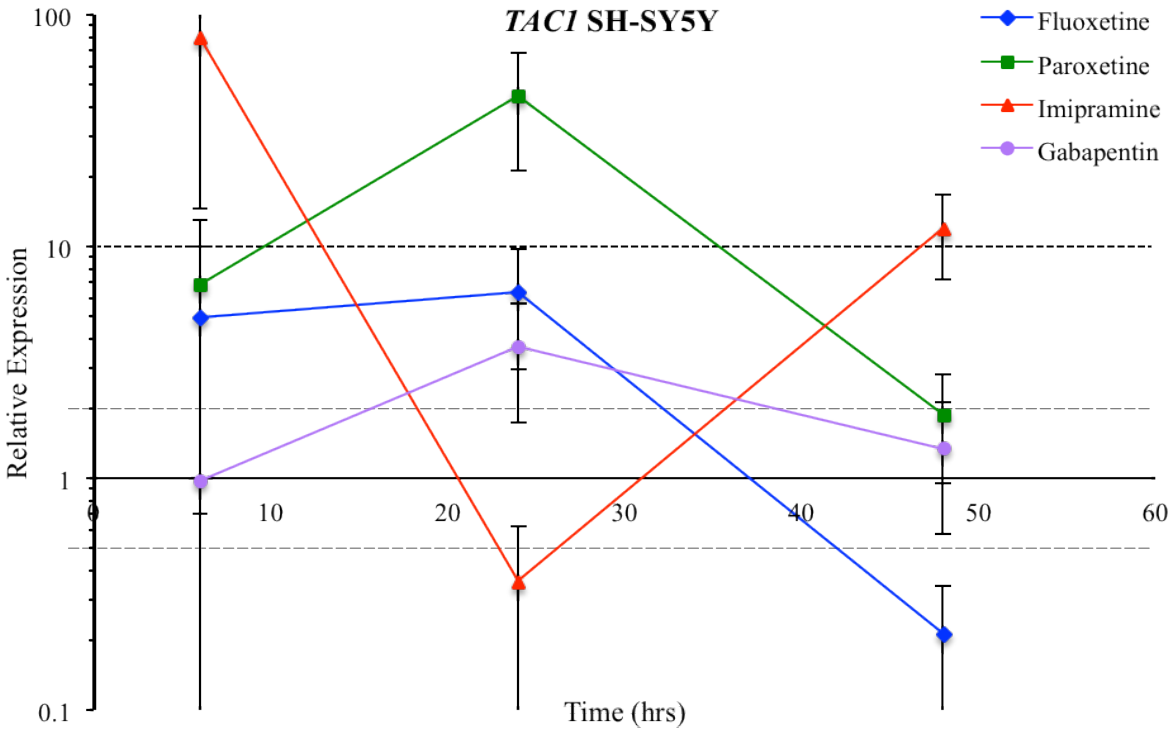


Figure 4.3 Variable Expression of *TAC1* over 48 hours.
Relative expression of *TAC1* after anxiolytic exposure showed high levels of variation over with no obvious pattern by time or drug. Consistency between technical replicates was similar or better than most other genes, making it unlikely that the variation was due to technical issues with the assay. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; grey dashed lines indicate two-fold change in expression; black dotted line indicates ten-fold change in expression; error bars indicate 1 standard deviation for the intra-assay repeats.

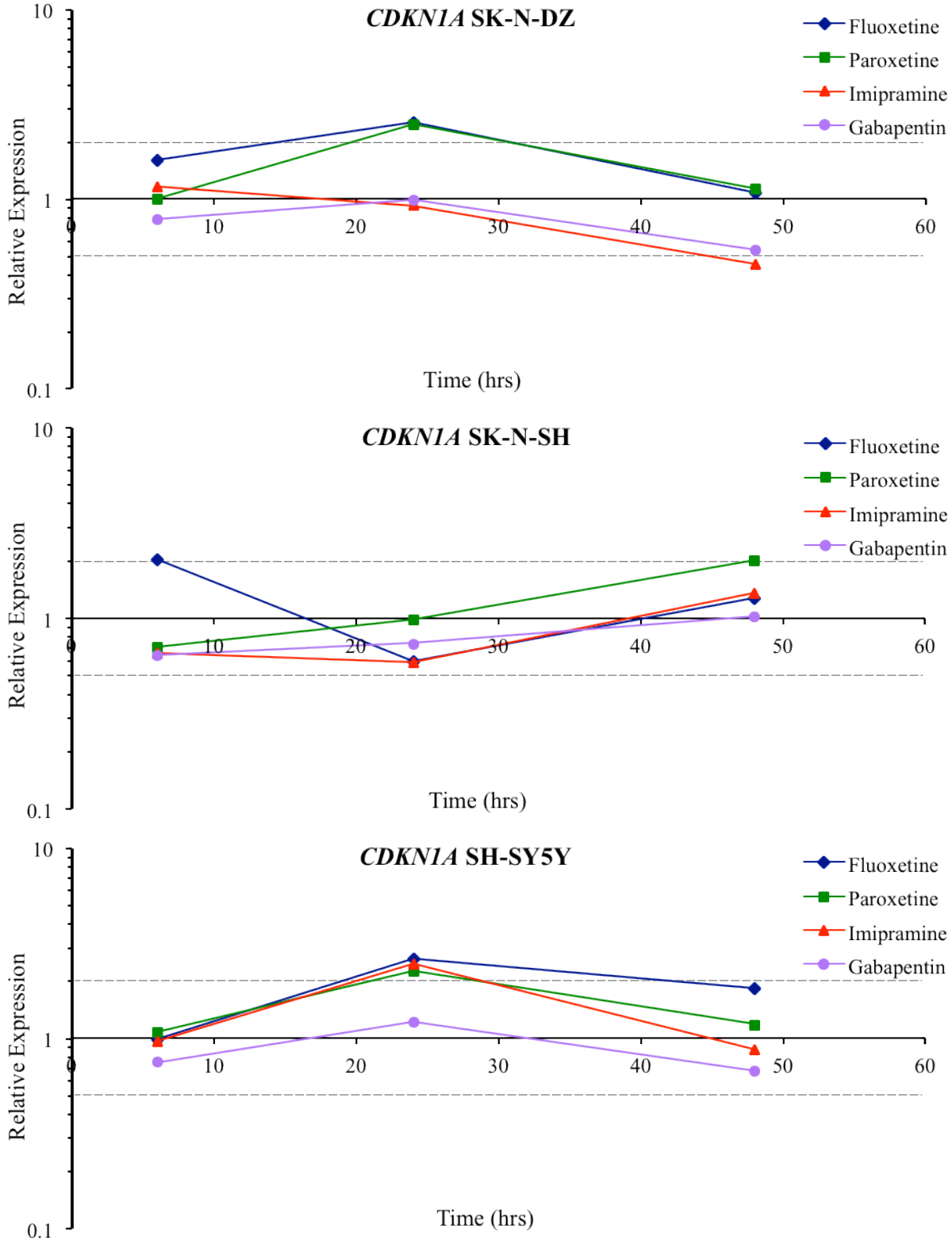


Figure 4.4 Inconsistent Changes with *CDKN1A* over 48 hours.
Although expression changes were seen with *CDKN1A*, these vary by cell line, drug type and duration; for example, at 24 hours antidepressants increased expression in SH-SY5Y (bottom), SSRIs increased expression in SK-N-DZ (top) and antidepressants decreased or did not change expression in SK-N-SH (middle). Graphs show relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression.

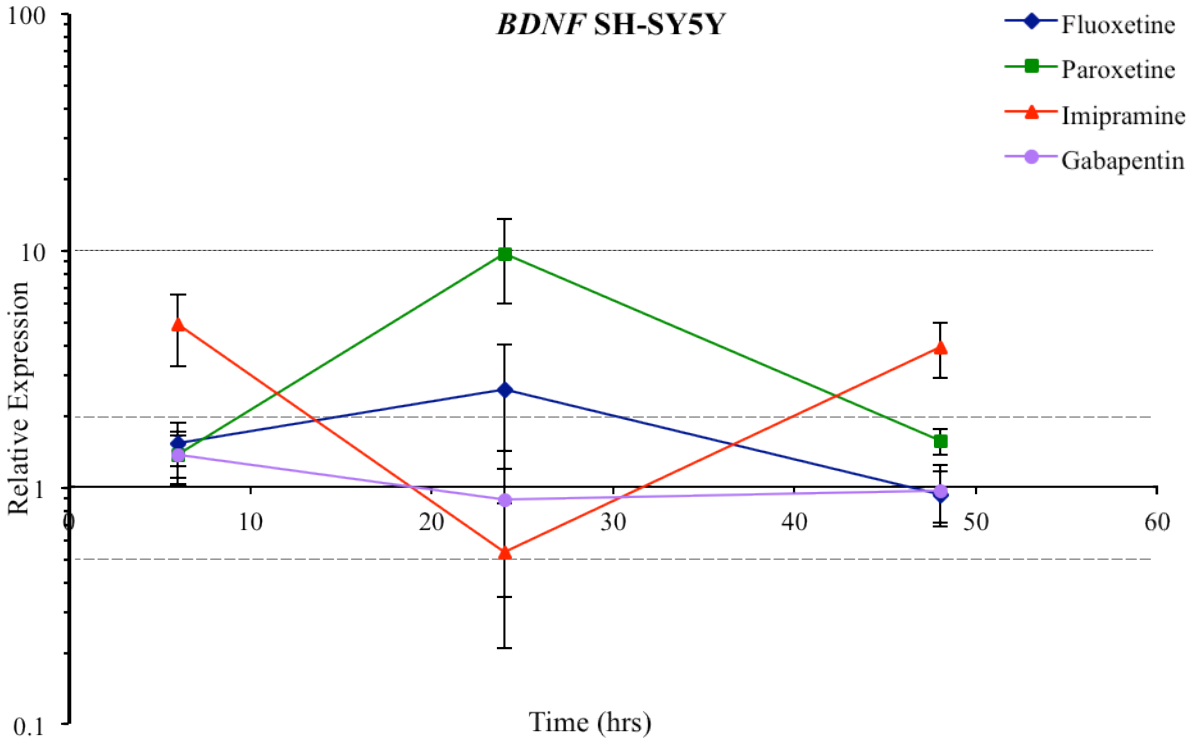


Figure 4.5 *BDNF* Expression in SH-SY5Y Cells over 48 hours.
SSRI antidepressant exposure for 24 hours up-regulated *BDNF* expression in the SH-SY5Y cell line. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; grey dashed lines indicate two-fold change in expression; black dotted line indicates ten-fold change in expression; error bars indicate 1 standard deviation for the intra-assay repeats.

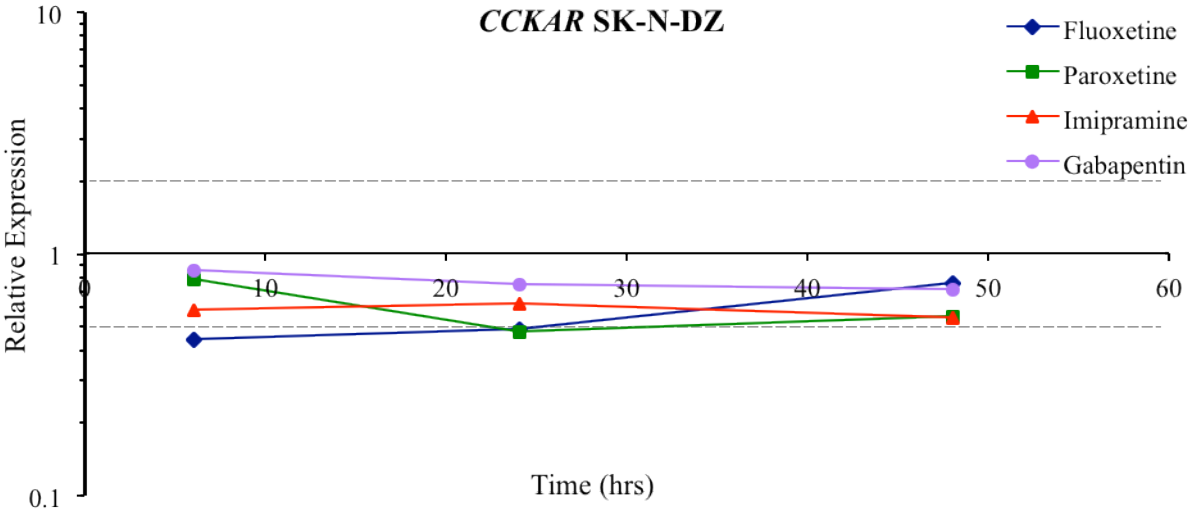


Figure 4.6 Down-regulation of *CCKAR* over 48 hours.
Exposure of the SK-N-DZ cell line with any of the three antidepressants tested resulted in down-regulation of *CCKAR* to approximately 0.5 to 0.75 fold expression of untreated cells. Other cell lines did not have sufficient levels of *CCKAR* expression for analysis. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression.

4.4.2 Long Time Course

A summary of the results for Long A time course is shown in Table 4.4 and the two replications of the antidepressants (Long A and B) shown in Table 4.6. Averages of all cell lines for each drug treatment, with time points grouped into early, mid and late, are shown in Table 4.5. Some genes were not repeated as the results from Long A did not justify the time and resources. This included: *TAC1* and *BDNF*, which showed high levels of variation between different times/drugs (see also short time course data, Figure 4.3 and Figure 4.5); and *GABRA2* usually showed small (less than 1.5 fold) or no change and low expression levels. Of the genes that were analysed in Long B variable or inconsistent data was found for *ADAM22*, *CACNA2D1*, *SRI* and *CDKN1A*. Additionally, small inconsistent changes of less than two-fold or no change was found for *FZD1* and *GABRA3*. The inconsistency of expression of these genes often resulted in the average expression, of all cell lines grouped into three time points, being close to 1 (Table 4.5).

More consistent changes were seen with *LMTK2*. This gene showed a trend towards down-regulation, fluctuating between no change and two-fold down-regulation with all drugs and all cell lines (Figure 4.7). However, when these values were averaged, only very small decreases were seen (Table 4.5). *ADORA2A* also tended to show down-regulated with antidepressant drugs, but not gabapentin (Figure 4.8 and Table 4.5). As in the short time course, *CCKAR* was down-regulated approximately two-fold at most time points with the three antidepressant drugs tested, and some decreases approached ten-fold (Figure 4.9 and Table 4.5). *NPY* also showed a trend towards down-regulation with antidepressants (Figure 4.10). However, the consistency of this between the two time replicates varied by cell line, with both repeats of the SK-N-DZ cells supporting a down-regulation and Long B of the SK-N-SH cells, while the SH-SY5Y cells were equivocal. The averaged values for *NPY*, consequently, predominantly showed no change (Table 4.5).

Since the most consistent results were for *ADORA2A* and *CCKAR*, these were investigated for statistical significance. For this analysis, a two-way ANOVA was conducted for each of the two genes (as described in section 2.6.4, page 87), using the relative expression values for untreated and each of the three different antidepressant drugs at early, mid and late time points. All cell lines were grouped together in a similar way to Table 4.5, however, only one time point was selected to represent early/mid/late for each cell line. For *CCKAR*, the

resulting *p*-values were: 0.17 for the difference between drugs; 0.63 for the difference between time points; and 0.96 for the interaction between drugs and time. For *ADORA2A*, the *p*-values were: 0.12 for the difference between drugs; 0.20 for the difference between times; and 0.62 for the interaction between drugs and time. These values are not statistically significant, however, the lower *p*-values (below 0.20) suggest the possibility of a difference and support further investigation into these two genes.

Table 4.4 Long A Time Course Summary

	SK-N-DZ				SK-N-SH				SH-SY5Y			
Gene	**Flu**	**Par**	**Imi**	**Gab**	**Flu**	**Par**	**Imi**	**Gab**	**Flu**	**Par**	**Imi**	**Gab**
Positional Candidates:												
ADAM22	↑	PC	↓	NC	Var	PC	↑	Var	Var	PC	↓	↓
CACNA2D1	NC	↓	↓	↑	PC	↓	NC	NC	Var	NC	↓	↓
FZD1	NC	↓	Var	NC	NC	PC	NC	NC	↓	NC	NC	Var
LMTK2	NC	NC	↓	↓	↓	NC	↓	↓	Var	↓	↓	↓
SRI	↑	↓	↓	↑	NC	↓	NC	NC	↓	↑	NC	↓
TAC1	Not Analysed				Insufficient Expression				Data Extremely Variable			
Functional Candidates:												
ADORA2A	↓	PC	↓	↓	↓	↓	↓	↑	↓	↓	NC	↓
BDNF	↑	↓	Var	↑	↓	↓	↓	NC	↓	↓	↓	Var
CCKAR	↓	↓	↓	NC	Insufficient Expression				Insufficient Expression			
CDKN1A	↓	PC	↓	↑	↑	NC	NC	↓	Var	↓	Var	Var
GABRA2	↑	↑	↓	Var	Insufficient Expression				Insufficient Expression			
GABRA3	NC	↓	↓	NC	↓	NC	NC	↓	NA	Var	↓	NA
NPY	↓	↓	↓	PC	↑	↑	↑	↑	NA	Var	↑	NA

Flu = fluoxetine, Par = paroxetine, Imi = imipramine and Gab = gabapentin. NC = evidence supporting no change (median value[s] close to 1 with good consistency between intra-assay replicates). PC = poor consistency between inter-assay replicates preventing conclusions. NA = not analysed. Var = evidence variable (some time points increased, others decreased and/or others no change). ↓ = evidence supporting reduced expression at one or more time points. ↑ = evidence supporting increased expression at one or more time points. Symbol size indicates number of time points supporting change: ↓/↑/NC = less than half; ↓/↑/NC = approximately half; and ↓/↑/NC = more than half. The following genes were not included as they had insufficient expression for analysis in all cell lines: *GRM3*, *CCK*, *CCKBR*, *CRHR2*, *GABRA1*, *NPY1R* and *TPH2*.

Table 4.5 Long Time Course Average Expression for Each Drug

	Fluoxetine			Paroxetine			Imipramine			Gabapentin		
Gene	**E**	**M**	**L**	**E**	**M**	**L**	**E**	**M**	**L**	**E**	**M**	**L**
Positional Candidates:												
ADAM22	0.84	0.79	0.80	0.96	1.00	0.93	0.92	0.89	1.07	1.01	0.98	1.30
CACNA2D1	0.83	1.22	1.10	0.91	0.81	0.77	0.93	0.85	0.82	0.92	1.16	0.81
FZD1	1.01	0.91	1.01	0.88	0.89	0.99	0.95	1.09	1.01	1.05	0.99	1.08
LMTK2	0.93	0.88	0.88	0.84	0.89	0.96	0.96	0.84	0.81	0.98	0.92	0.86
SRI	0.93	1.10	0.92	0.87	0.95	0.91	0.97	0.94	0.89	1.03	1.19	1.44
TAC1	1.18	0.67	0.66	1.13	22.38	0.09	1.19	2.55	0.14			
Functional Candidates:												
ADORA2A	0.94	0.66	0.62	0.77	0.64	0.83	0.73	0.76	0.89	0.94	1.08	1.47
BDNF	0.91	0.88	0.95	0.91	1.69	0.96	0.96	1.37	0.90	1.11	1.02	1.07
CCKAR	0.68	0.48	0.66	0.76	0.63	1.04	0.80	0.47	0.61	0.96	1.00	0.85
CDKN1A	1.92	1.06	1.20	1.56	1.23	1.20	1.16	0.86	1.07	1.37	0.98	1.20
GABRA2	1.39	1.36	1.12	0.96	1.24	1.01	0.95	0.99	0.81	1.08	1.08	0.73
GABRA3	1.05	1.11	1.12	0.88	0.95	1.17	0.90	0.90	1.04	1.03	1.08	1.06
NPY	1.02	0.94	2.13	1.04	0.99	1.39	1.00	0.83	1.11	1.00	1.34	1.44

E = early time points (4-11 days); M = mid time points (12-24 days); L = late time points (26-32 days)

Table 4.6 Long A and B Antidepressant Summary

	SK-N-DZ			SK-N-SH			SH-SY5Y		
Gene	**Flu**	**Par**	**Imi**	**Flu**	**Par**	**Imi**	**Flu**	**Par**	**Imi**
Positional Candidates:									
ADAM22	↑/↓	PC/↓	↓/↓	Var/↓	PC/↓	↑/↓	Var/PC	PC/↓	↓/↓
CACNA2D1	NC/↓	↓/↓	↓/↓	PC/↓	↓/PC	NC/PC	Var/↓	NC/Var	↓/↓
FZD1	NC/↓	↓/↓	Var/PC	NC/↓	PC/↓	NC/↓	↓/↑	NC/↓	NC/↓
LMTK2	NC/↓	NC/↓	↓/↓	↓/↓	NC/↓	↓/PC	Var/↓	↓/NC	↓/PC
SRI	↑/↓	↓/↓	↓/↓	NC/↑	↓/NC	NC/NC	↓/PC	↑/Var	NC/NC
Functional Candidates:									
ADORA2A	↓/↓	PC/↓	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓	NC/↓
CCKAR	↓/↓	↓/↓	↓/↓	Insufficient Expression			Insufficient Expression		
CDKN1A	↓/PC	PC/↑	↓/PC	↑/↓	NC/↓	NC/PC	Var/↑	↓/NC	Var/NC
GABRA3	NC/↓	↓/↓	↓/PC	↓/PC	NC/↑	NC/NC	NA/↑	Var/↓	↓/↓
NPY	↓/↓	↓/↓	↓/↓	↑/↓	↑/↓	↑/↓	NA/↑	Var/↑	↑/PC

Flu = fluoxetine, Par = paroxetine, Imi = imipramine and Gab = gabapentin. NC = evidence supporting no change (median value[s] close to 1 with good consistency between intra-assay replicates). PC = poor consistency between within assay replicates preventing conclusions. NA = not analysed. Var = evidence variable (some time points increased, others decreased and/or others no change). ↓ = evidence supporting reduced expression at one or more time points. ↑ = evidence supporting increased expression at one or more time points. Symbol size indicates number of time points supporting change: ↓/↑/NC = less than half; ↓/↑/NC = approximately half; and ↓/↑/NC = more than half. The following genes were not included as they had insufficient expression for analysis in all cell lines: *GRM3*, *CCK*, *CCKBR*, *CRHR2*, *GABRA1*, *NPY1R* and *TPH2*. *TAC1*, *BDNF* and *GABRA2* were not repeated in Long B as changes seen in Long A were small or variable.

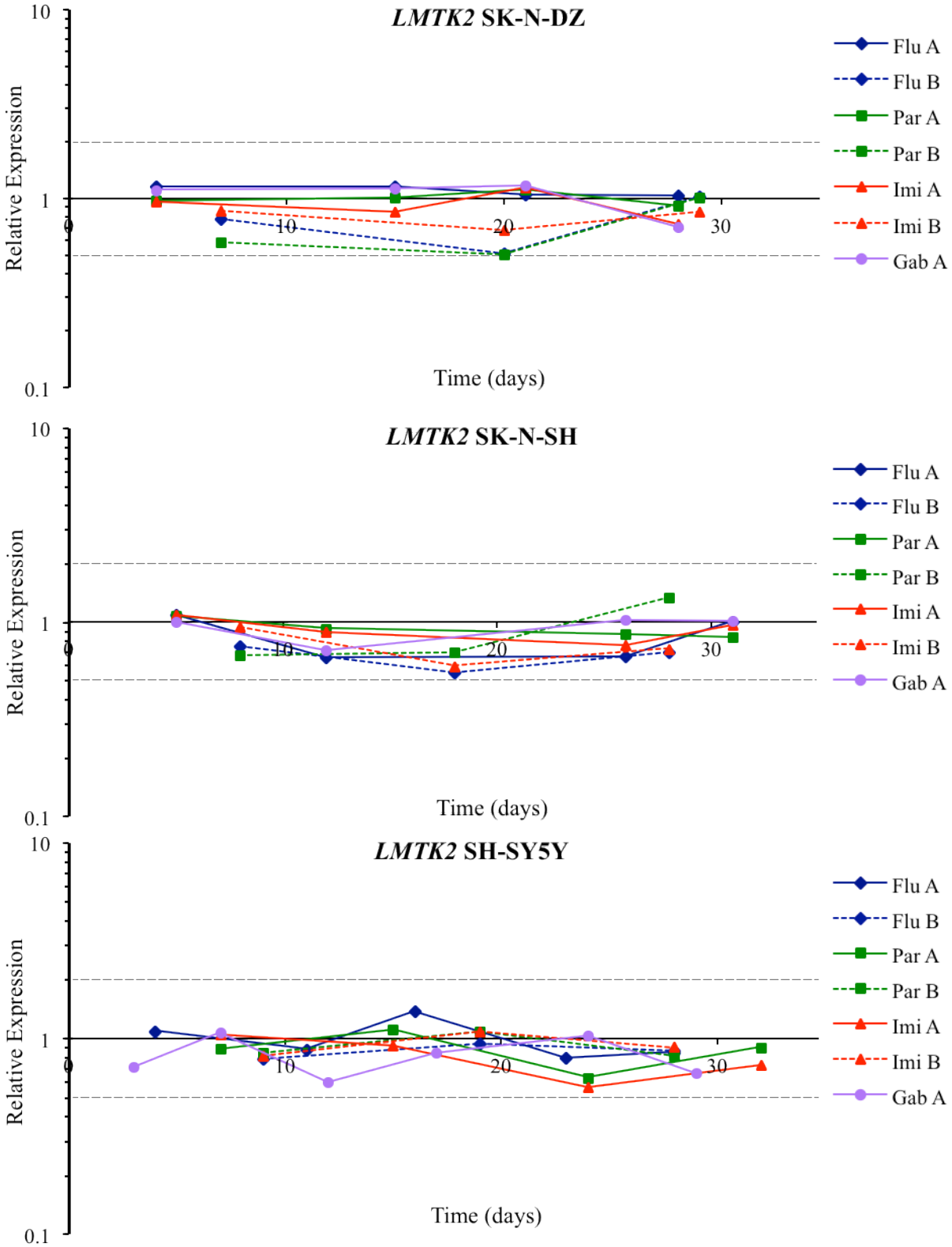


Figure 4.7 Down-regulation of *LMTK2* over 32 days.
Expression of *LMTK2* varied between no change and two-fold down-regulation in all cell lines, with all four drugs. Graphs show relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression; Flu = fluoxetine, Par = paroxetine, Imi = imipramine, Gab = gabapentin; A indicates Long A (the initial 28 day time course) and B indicates Long B (the replication time course).

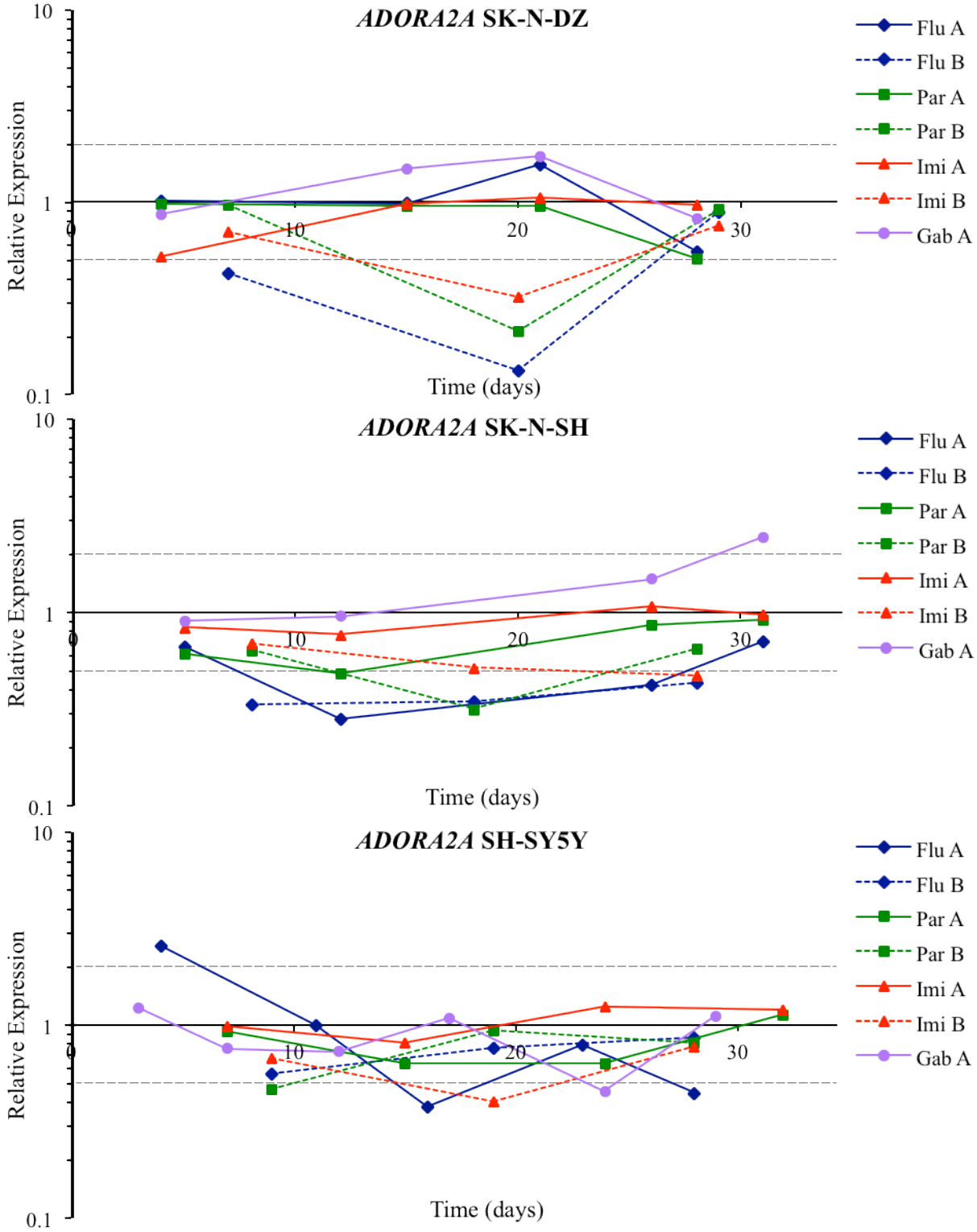


Figure 4.8 Down-regulation of *ADORA2A* over 32 days.
Expression of *ADORA2A* was reduced at most time points, in all cell lines following antidepressant exposure, but not gabapentin. Graphs show relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression; Flu = fluoxetine, Par = paroxetine, Imi = imipramine, Gab = gabapentin; A indicates Long A (the initial 28 day time course) and B indicates Long B (the replication time course).

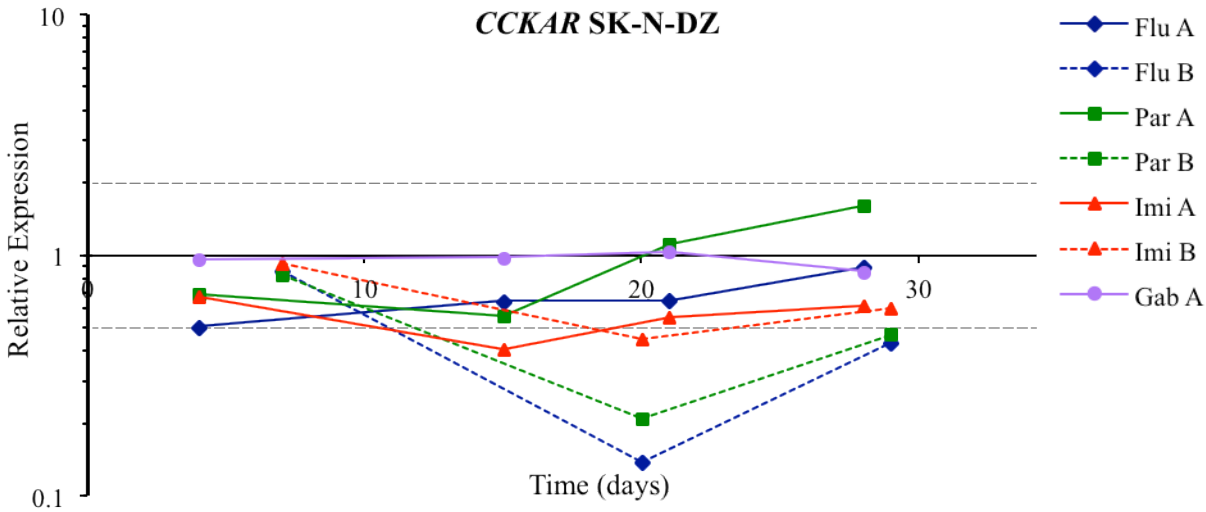


Figure 4.9 Down-regulation of *CCKAR* over 29 days.
Expression of *CCKAR* was reduced at most time points in all cell lines following antidepressant exposure, with no change in gabapentin treated cells. Graph shows relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression; Flu = fluoxetine, Par = paroxetine, Imi = imipramine, Gab = gabapentin; A indicates Long A (the initial 28 day time course) and B indicates Long B (the replication time course).

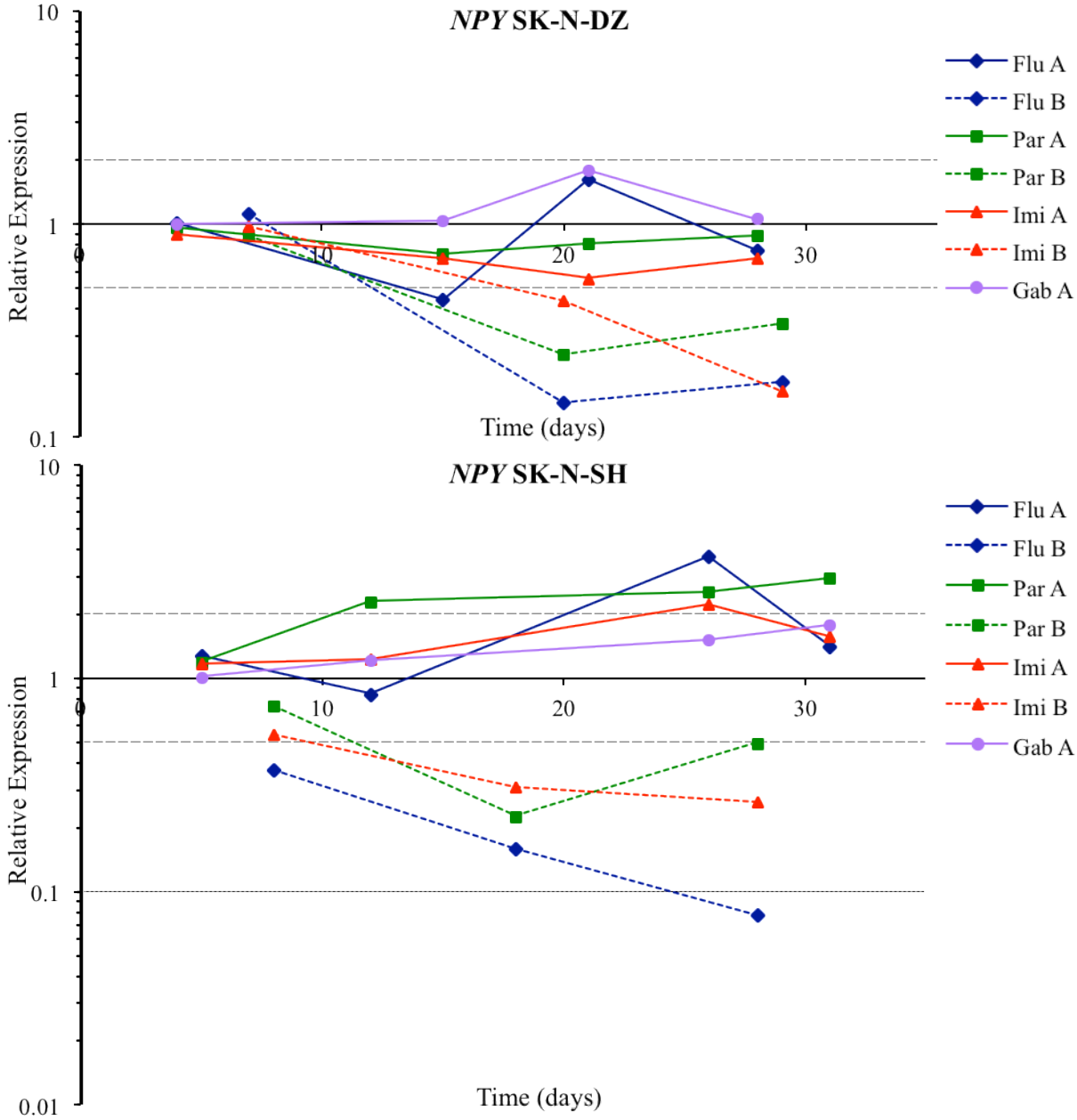


Figure 4.10 Down-regulation of *NPY* over 31 days.
Expression of *NPY* was reduced at most time points following antidepressant exposure in SK-N-DZ cells (top) and an overall downwards trend was seen in SK-N-SH (bottom) though this was not consistent between biological replicates. Graphs show relative expression of anxiolytic treated cells, compared to untreated cells cultured for the same length of time; dashed lines indicate two-fold change in expression; dotted line indicates ten-fold change in expression; Flu = fluoxetine, Par = paroxetine, Imi = imipramine, Gab = gabapentin; A indicates Long A (the initial 28 day time course) and B indicates Long B (the replication time course).

4.5 Technical Results - A_{2a} Protein Levels

Based on the gene expression findings, *ADORA2A* was chosen as the candidate for further analysis; levels of its protein product were assayed in the SK-N-DZ cell line. This gene encodes the adenosine A_{2a} receptor, a membrane bound G-protein coupled receptor, the functions of which are discussed in sections 1.4.3.4 (page 58) and 4.1.2.1 (page 146). An antibody raised against this protein and an antibody raised against γ-actin, which should have stable expression in most conditions, were used to determine the relative expression of the A_{2a} receptor following anxiolytic exposure. Two different methodologies were used: Western blot and slot-blot, both of which involved transfer of protein to a nitrocellulose membrane, followed by immunoblotting and detection of fluorescent signal on the Odyssey Infrared Imaging System.

A slot-blot was used as it allowed a large number of samples to be quantified on one membrane, which would be useful with the multiple drugs, time points and cell lines included in the present study. Initial testing with both antibodies was conducted to determine the optimal dilutions to use and SK-N-DZ test stock serial dilutions were conducted to determine whether the two primary antibodies might compete for binding in the single band. This testing indicated that optimal antibody dilutions would be 1 in 200 for the A_{2a} primary and 1 in 500 for γ-actin, both of which were within the ranges suggested by the manufacturer. The serial dilution showed similar patterns in both the 700 nm (γ-actin) and 800 nm (A_{2a}) channels and no differences when the membrane was incubated in both primary antibodies at the same time.

A potential disadvantage of the slot-blot is that the specificity of the primary antibody binding cannot be determined, since all proteins are deposited in the same band. Consequently, both sets of antibodies were tested by Western blot to determine the specificity of binding before quantification experiments. For this, 20 μl of protein lysate (denatured in 10 μl of Laemmli's buffer, section 2.2.1, page 74) was loaded on the gels; following transfer, membranes were treated identically to those used in the slot-blot, including antibody concentrations. As shown in Figure 4.11, strong signals were seen for both primary antibodies used. The reference protein, γ-actin, gave a single band in the 700 nm channel, which was similar in size to the predicted 42 kDa. The target protein, A_{2a}, gave a strong band that was slightly larger than the expected 45 kDa and several weaker signals, predominantly larger than the main band, all in

the 800 nm channel. This may be due to post-translational modifications, such as glycosylation, which are typical for G-protein coupled receptors, including A_{2a} (Wheatley & Hawtin, 1999). These changes produce small differences in mass, but also alter the charge of the protein, increasing its apparent size on electrophoresis (Schjoldager, 1994). Based on this, the binding of the primary and secondary antibodies was considered to be predominantly specific binding to their target, with negligible non-specific signal.

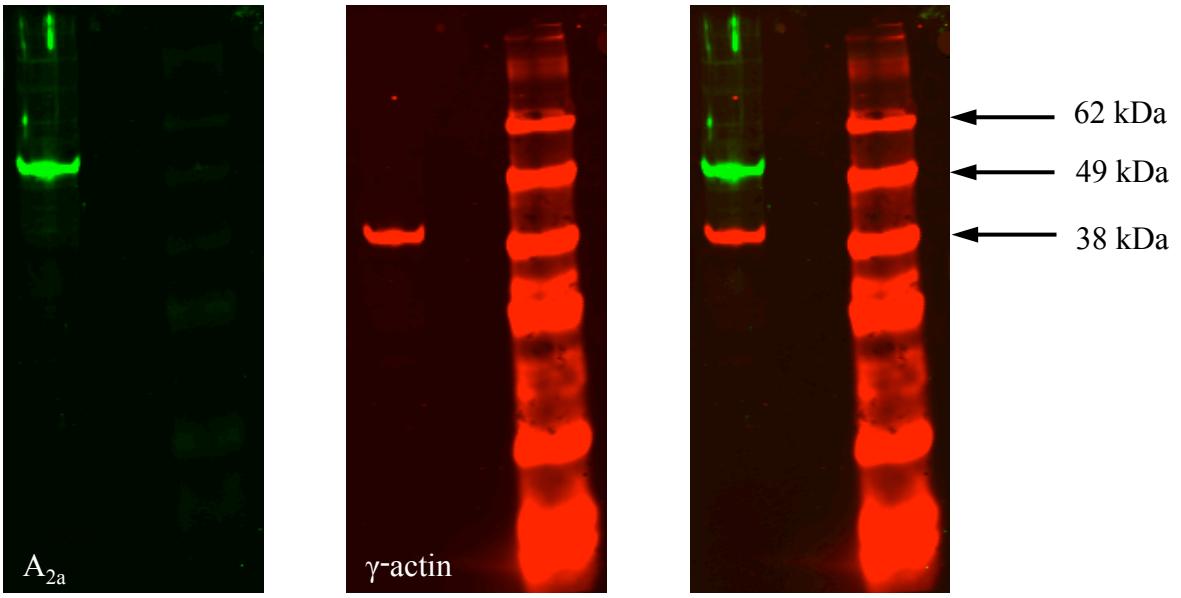
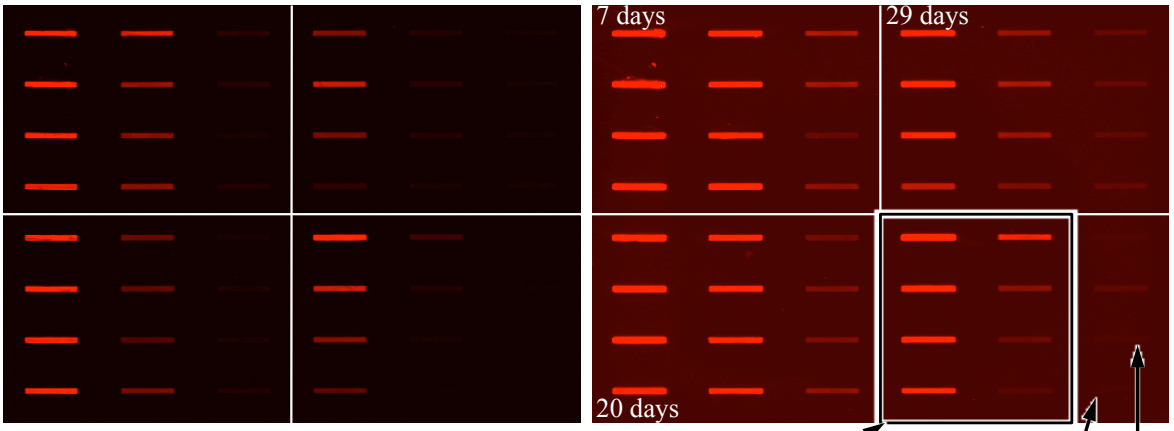


Figure 4.11 A_{2a} and γ-actin Band Patterns.
Images of SK-N-DZ test stock Western blot, using both A_{2a} and γ-actin primary antibodies and Odyssey secondary antibodies. Left image is of A_{2a} (800 nm channel), middle image is of γ-actin (700 nm channel) and right image is the combination of both. SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used as the running marker, the bands of which can be seen in the 700 nm channel.

4.5.1 Slot-blot Quantification

The first test undertaken with the drug treated protein lysates was used to determine approximate relative concentrations of each sample. This test was conducted using the slot-blot, with only the γ-actin primary antibody, with both secondary antibodies to ensure there was no non-specific binding of the 800 nm conjugated secondary (Figure 4.12). Three different dilutions of each of the protein lysates were loaded (1 in 4 serial dilutions, made by dilution with lysis buffer) and the relative intensities of each sample dilution were estimated. Since the samples from the 29 day exposure cells showed the weakest levels of signal, these were not diluted; all other samples were diluted in lysis buffer, as described below, to produce the working stock solutions used in all further assays.

7 day samples:	Untreated	1 in 6
	Fluoxetine	1 in 4
	Imipramine	1 in 3.5
	Paroxetine	1 in 4.5
20 day samples:	Untreated	1 in 3
	Fluoxetine	1 in 3
	Imipramine	1 in 2.5
	Paroxetine	1 in 3.5



Untreated stock	Untreated 1 in 4	Untreated 1 in 16
Fluoxetine stock	Fluoxetine 1 in 4	Fluoxetine 1 in 16
Imipramine stock	Imipramine 1 in 4	Imipramine 1 in 16
Paroxetine stock	Paroxetine 1 in 4	Paroxetine 1 in 16

Figure 4.12 Relative Protein Concentration Determination.
Each of the samples to be quantitated was tested on the slot-blot with the γ-actin primary antibody. The two images are of the same membrane scanned at two different intensities – left image scanned with intensity of 700 nm channel set at 5, right image set at 7. No signal was seen in the 800 nm channel (set at 5 in both images). The 48 wells of the slot-blot were set up as 4 quadrants, with three quadrants containing dilutions of the test samples set up as described in the table, and the 4th quadrant containing positive and negative controls. Serial dilutions (1 in 2) were made from pooled lysate stock by mixing the previous sample with equal volumes of lysis buffer. Two negative controls were included: PBS loading buffer only and lysis buffer.

Once diluted, samples were once again run on the slot-blot and this time the membrane was probed with both primary antibodies (Figure 4.13). Visual inspection of the images indicated that the 7 day samples had low levels of A_{2a} in all samples (relatively red appearance on the combined image) and the 29 day samples had low levels of γ-actin (relatively green), while similar levels of each were in the 20 day samples (yellow). Since this occurred in all samples within a time point, it seemed unlikely that it would affect the relative expression by drug

type, however, it did affect the ability to calculate the relative expression for the 7 and 29 day time points (further detailed below).

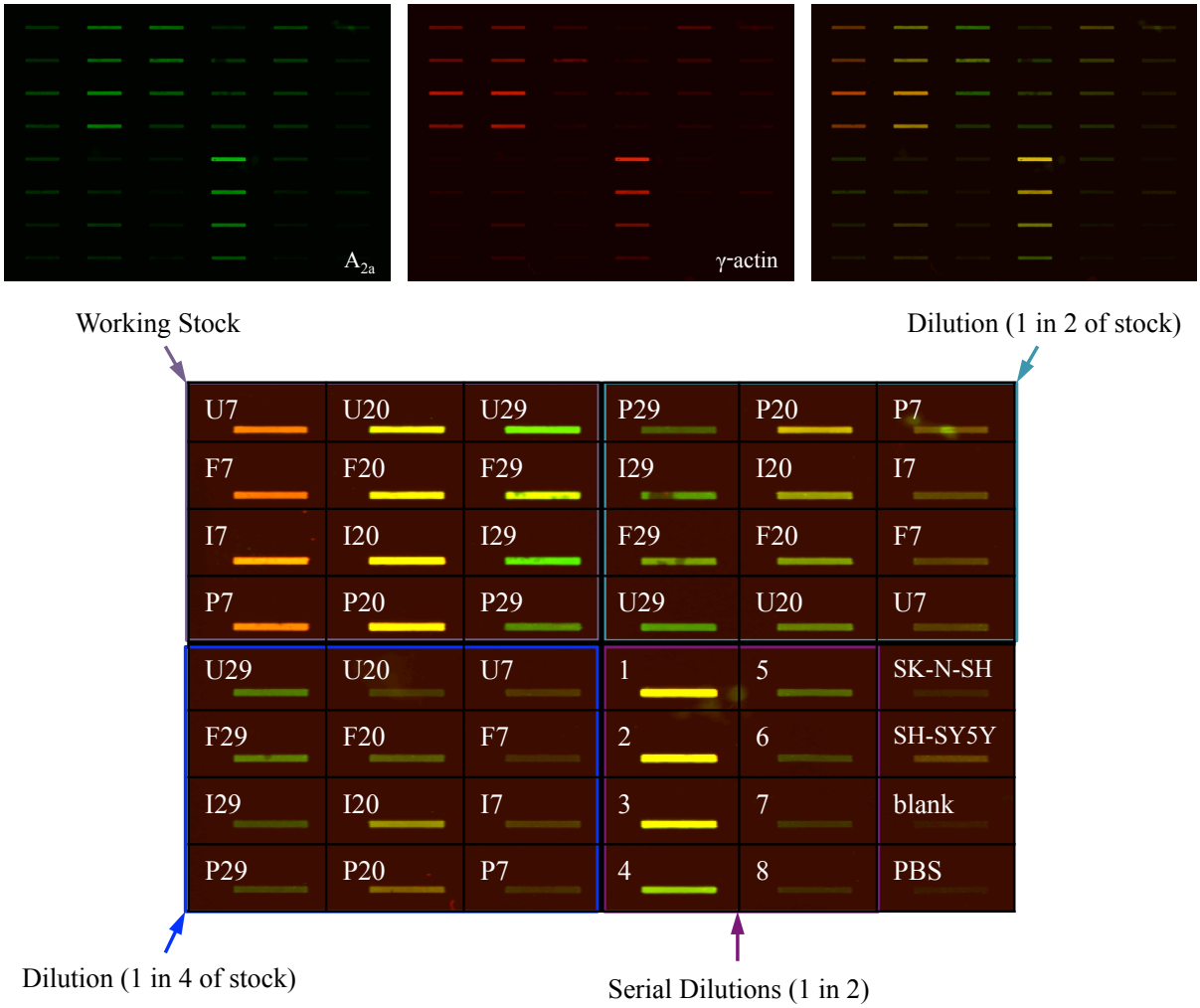


Figure 4.13 Slot-Blot Quantification Pictures.
Images used for analysis of the first slot-blot quantification of the SK-N-DZ cell line. The three different pictures captured using default scanning settings (intensity of 5 for both channels), including the 800 nm channel (left, A_{2a} primary), 700 nm channel (middle, γ-actin primary) and combined images (right), are shown on the top. The layout of the samples, overlain on a scan with intensities set at 6.5, is shown on the bottom. U = untreated; F = fluoxetine; I = imipramine; P = paroxetine; number following the letter is the number of days incubated with drug.

One of the advantages of using the slot-blot system was that the large number of wells meant a serial dilution of pooled lysate could be included (labelled 1-8 in Figure 4.13). These were used to generate a standard curve and, hence, determine the linear range of the fluorescent signal (Figure 4.14). This data indicated that fluorescence counts of at least 200 were required for reliable quantification.

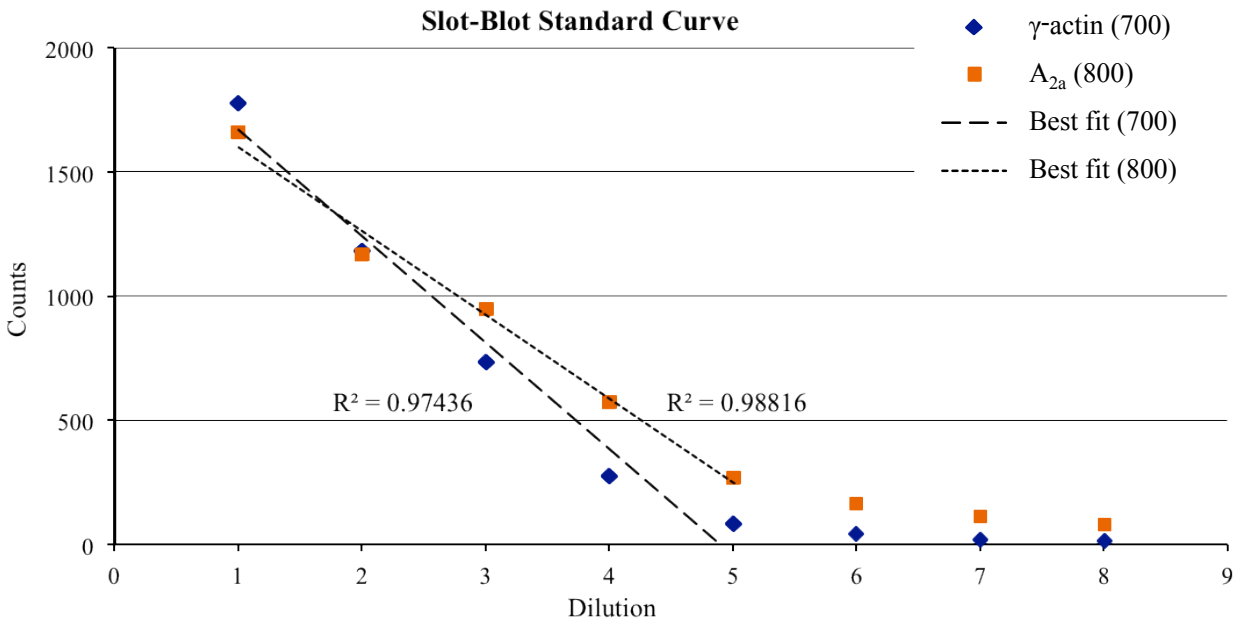


Figure 4.14 Slot-Blot Standard Curve.
Data from 1 in 2 serial dilutions (1-8 in Figure 4.13) are graphed, showing linear relationship between concentration and fluorescence (R^2>0.9) for the top five dilutions.

The data from the slot-blot analyses are shown in Table 4.7. The majority of the counts for the 1 in 2 and 1 in 4 dilutions were below 200; since these are below the linear range of the standard curve, the top concentration used (working stock) should provide the best estimation of relative A_{2a} levels (Figure 4.15).

Table 4.7 A_{2a} Relative Levels – Slot-Blot.

		Working Stock			1 in 2 Dilution			1 in 4 Dilutions		
		Counts		Relative	Counts		Relative	Counts		Relative
Days	Drug	700	800	Level	700	800	Level	700	800	Level
7	Untreated	579	337	1	92	165	1	72	128	1
	Fluoxetine	614	307	0.86	103	162	0.88	40	92	1.27
	Paroxetine	826	389	0.81	180	241	0.75	37	98	1.49
	Imipramine	1257	520	0.71	125	188	0.85	76	135	1.00
20	Untreated	596	793	1	145	357	1	44	136	1
	Fluoxetine	721	802	0.84	220	422	0.78	99	241	0.78
	Paroxetine	1174	1030	0.66	426	474	0.45	190	227	0.38
	Imipramine	1492	1114	0.56	321	475	0.60	270	382	0.45
29	Untreated	219	822	1	101	460	1	74	335	1
	Fluoxetine	516	839	0.43	190	421	0.49	108	329	0.67
	Paroxetine	102	403	1.05	59	241	0.90	39	157	0.89
	Imipramine	153	747	1.30	99	352	0.78	37	193	1.14

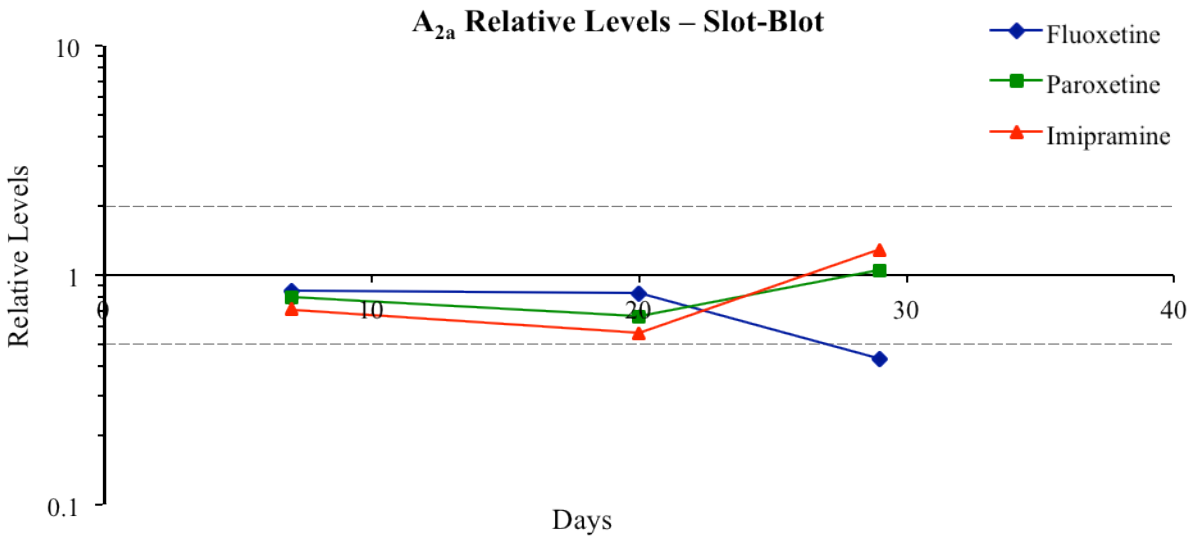


Figure 4.15 A_{2a} Relative Levels – Slot-Blot.
The levels of A_{2a} protein for the three different antidepressant drugs are shown, relative to untreated cells and normalised by γ-actin levels. Data are based on the highest concentration used (working stock). Dashed lines indicate two-fold change in relative levels.

To confirm the accuracy of the slot-blot system, a technical replication with a new aliquot of the same samples was undertaken. As shown in Table 4.8, most samples gave very similar values on repeat quantification.

Table 4.8 Original and Replication Slot-Blot Results.

		Relative A_{2a} Protein Levels	
Days	**Drug**	**Original**	**Repeat**
7	Untreated	1	1
	Fluoxetine	0.86	0.97
	Paroxetine	0.81	1.05
	Imipramine	0.71	0.54
20	Untreated	1	1
	Fluoxetine	0.84	0.79
	Paroxetine	0.66	0.56
	Imipramine	0.56	0.56
29	Untreated	1	1
	Fluoxetine	0.43	0.45
	Paroxetine	1.05	1.27
	Imipramine	1.30	1.34

4.5.2 Western Blot Quantification

For comparison purposes, Western blots were also conducted with the SK-N-DZ lysates. As was seen on the slot-blot, the 7 and 29 day time points failed to show strong specific bands for

either A_{2a} and γ-actin in some wells (Figure 4.16), suggesting that the quantification of these proteins would be unreliable. The values for the 20 and 29 day time points were quantitated and are shown in Table 4.9.

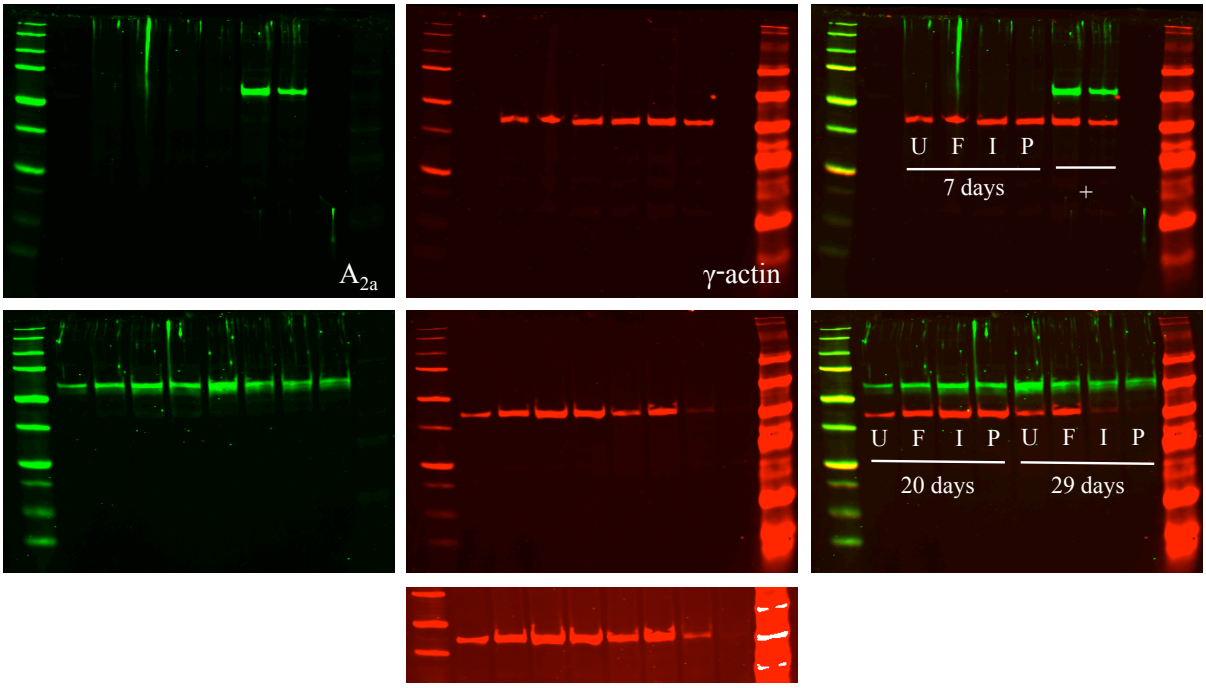


Figure 4.16 Western Blot Quantification Pictures.
Images of two separate Western blots using both A_{2a} and γ-actin primary antibodies, showing all three SK-N-DZ time points. Most images were scanned on the default settings, the bottom image (700 nm channel only) was scanned at intensity 6.5. Each image shows Odyssey Two-Color Protein Molecular Weight Marker on the left and SeeBlue Plus2 Pre-Stained Standard on the right. U = untreated; F = fluoxetine; I = imipramine; P = paroxetine; + = positive control samples.

Table 4.9 A_{2a} Relative Levels – Western Blot.

		Counts		Relative
Days	**Drug**	**700**	**800**	**Level**
20	Untreated	456	519	1
	Fluoxetine	1119	654	0.51
	Paroxetine	1679	882	0.56
	Imipramine	1853	1187	0.46
29	Untreated	560	1540	1
	Fluoxetine	1198	917	0.28
	Paroxetine	13	756	2.34*
	Imipramine	112	686	21.33*

*These values are likely to be inaccurate due to very low levels of γ-actin.

4.6 A_{2a} Quantification Results

When data from all three quantification experiments (gene expression, and both methods of protein determination) are compared, similar overall trends are seen (Figure 4.17). All three sets of data show down-regulation, most beginning at the 20 day time point. Use of the slot-blot tended to underestimate protein changes, compared to the Western blot, supporting the use of the slot-blot as a method of screening large numbers of samples.

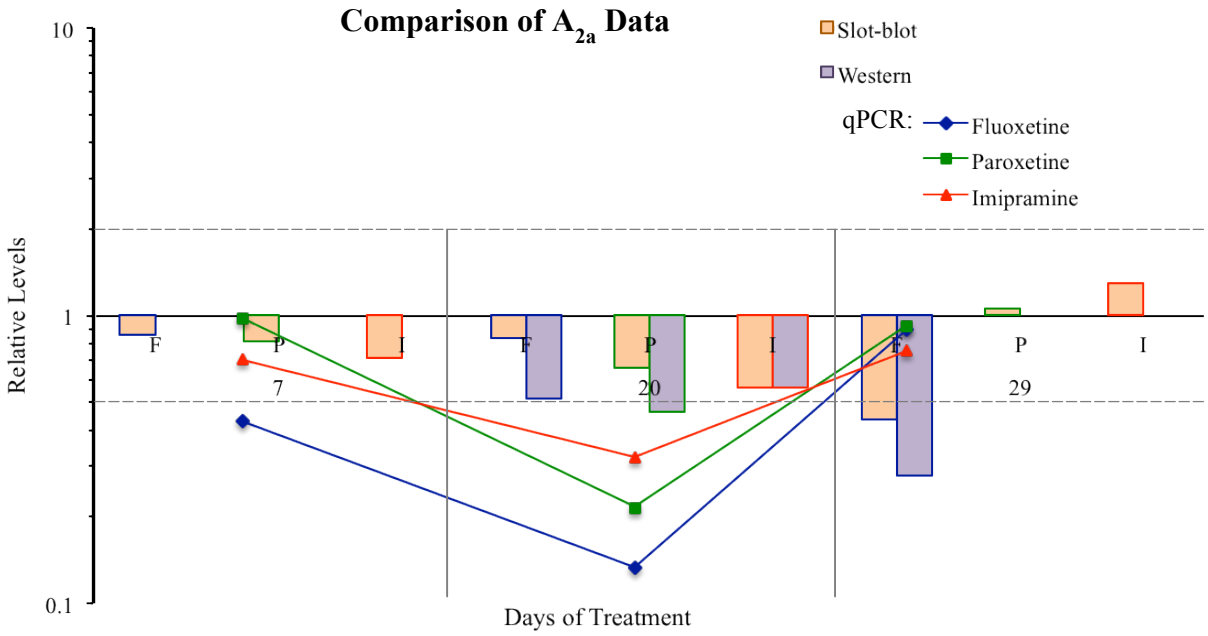


Figure 4.17 Comparison of A_{2a} Data.
Figure shows gene expression (lines), slot-blot protein (orange bars) and Western blot protein (purple bars) levels for the adenosine A_{2a} receptor in SK-N-DZ cells. All data are presented as levels of expression relative to untreated cells. Western blot data for all 7 day samples and the paroxetine and imipramine 29 day samples were not included due to the poor quality of the data. Horizontal dashed lines indicate two-fold change in relative levels; solid vertical lines divide the three incubation times (7, 20 and 29 days). F = fluoxetine; P = paroxetine; I = imipramine.

4.7 Discussion

This study found either negligible or variable changes in most of the candidate genes investigated following exposure of neural cell lines to antidepressant drugs or gabapentin. When changes were seen, these were predominantly reduced expression levels of approximately two-fold. The two most consistent changes in gene expression were seen for *ADORA2A* and *CCKAR*. These changes were not statistically significant, by two-way ANOVA, but *p*-values for treatment effects were less than 0.2. This indicates that there was

less than 20% probability of the results being due to chance, supporting a possible effect that would require further replication to confirm. The lack of statistical significance was not surprising as two-fold changes in expression level are small, making them difficult to reliably detect via real-time PCR, and there were only two (*CCKAR*) or five (*ADORA2A*) biological replicates conducted. Nonetheless, these results indicate that further study of these genes would be useful.

For *ADORA2A*, preliminary further study was undertaken in the form of Western blot and slot-blot experiments. The reductions in *ADORA2A* gene expression were supported by reductions in A_{2a} protein levels in the SK-N-DZ cell line, however, these results also require further replication.

4.7.1 Methodological Strengths and Limitations

Strengths of this study, as well as things that could have been improved with different resources are discussed below.

4.7.1.1 Cell Culture

Cell culture was used in this experiment to model the human brain. The expected site of anxiolytic action is the brain, specifically, neuronal cells. The ideal system to study anxiolytic action in is, thus, human brain. However, brain tissue is only available post-mortem or from patients undergoing surgery for conditions like treatment resistant epilepsy or brain tumours, which may then become confounding factors. These extreme limitations on availability make models necessary. Cell culture was chosen in this study as it allowed the study of human cells. Animal models could also have been used, and would have the advantage of being able to assess whether the drug was producing the desired anxiolytic response at the studied time points.

Animal models would also have provided a complete biological system, with a variety of different cell types, allowing interaction between the drugs and a variety of organs and tissues. A neuronal cell culture system assumes that neurons are the target of the drugs tested. While this is consistent with the neurogenesis hypothesis of antidepressant action (Santarelli *et al.*, 2003), as discussed in section 1.4.4.2 (page 61), it is not currently clear whether

neurogenesis is vital for antidepressant action. Other types of brain cells have also been implicated in anxiety, for example, studies in rodents found changes in microglia in animals that were experimentally induced to have high anxiety levels (Gonzalez-Perez *et al.*, 2001; Wohleb *et al.*, 2013). Thus, it is possible that glial cells are the direct or indirect targets of anxiolytic drugs and that glial interactions with neurons are vital for the effects of the drugs. Such effects would have been missed in the cell culture methods used in the present study, but may be detected in animal models.

As well as glial cells, a variety of different types of neurons are found in the brain. In an attempt to capture this, three different neuronal cell lines were used in this study. Multiple cell lines would have improved the generalisability of the results and increased the likelihood of detecting specific candidates, compared to a single cell line. One example of the importance of this was seen with the changes in *CCKAR*. This gene was only detected in the SK-N-DZ cell line in the present study, and could have been missed had these cells not been included. This could be further improved in future research by studying other cells, such as glial cells. Similarly, by studying co-cultures of neurons and glia, additional candidates may be detected and interactions between cell types could be investigated.

An alternative cell culture system to study would have been to induce differentiation of the neuroblastoma cells, for example, with retinoic acid (Xie *et al.*, 2010). This would have increased the number of features shared with mature neurons (Agholme *et al.*, 2010) and allowed greater control over the time points studied as there would be no need to wait for the cells to reach the desired confluence. However, if neurogenesis is necessary for the anxiolytic effects, inducing differentiation could have prevented the expected changes from being seen. The expression of some genes and proteins would also be altered by the differentiation process, which may be an advantage or disadvantage, depending on the candidate concerned.

Another important feature missing from cell culture was the effect of other organs, particularly liver metabolism. Lack of liver metabolism would likely have prolonged the effects of most of the drugs tested, though it would have also reduced the formation of active metabolites. Norfluoxetine is an active metabolite of fluoxetine that has a longer half-life in both brain and plasma (Qu *et al.*, 2009), resulting in it being more abundant and likely to be the major mediator of the effects of chronic administration. While fluoxetine and norfluoxetine have very similar effects on serotonin levels (Bourdeaux *et al.*, 1998), it is

unknown whether they would have the same effects on the gene expression measured in the present experiments. However, this would have only affected one of the three antidepressant drugs used in this study, making a large impact on the overall results unlikely.

The drugs used in this study were selected to provide a range of classes, as well as allow comparisons with similar classes. However, three of the four drugs studied affected the growth of the neuroblastoma cells. All drugs were tested for one week prior to beginning experimentation to ensure the concentration selected did not produce obvious toxic effects - detected by inspecting the cells under light microscopy to determine whether there was a noticeable increase in detached cells. Despite this testing, growth inhibitory effects, which were most prominent during the first two weeks of exposure, were observed with each of the three antidepressant drugs during the course of the study. These growth inhibitory effects of antidepressants may represent a confounding factor in the analysis, as both the inhibition and recovery from inhibition may have been associated with gene expression changes that would not have been paralleled in the untreated cells. It is unknown whether these effects would occur *in vivo*, thus making the applicability of the results to patients uncertain. Furthermore, this may have particularly affected the positional candidates as 7q21 also contains a multidrug resistance gene and resistance to the drug doxorubicin was associated with increased expression of several genes in this region (Flahaut *et al.*, 2009). If the return to normal growth rates after a couple of weeks of antidepressant exposure was associated with this same multidrug resistance region, up-regulation of the genes in 7q21 would be expected. Since the majority of the findings in the current study were down-regulations, the increase may have cancelled out antidepressant induced decreases that would otherwise have been detected, producing false negative results.

4.7.1.2 Gene Expression Methods

Following cell culture, the expression levels of various candidate genes were measured in each of the cell lines. For this, mRNA was extracted, used to produce cDNA and then individual genes were measured by quantitative real-time PCR with primers specific to that cDNA. This was a multi-step process, with the potential for subtitle differences between drug groups to be introduced at any one of these steps. Care was taken at all of these steps to minimise variation, including completing specific steps for all drug groups from a time point in the same session and with the same reagents, which should have prevented environmental

differences having a large impact. However, small variations may have occurred, and may have contributed to the differences found.

During the course of this study, guidelines were published for quantitative PCR experiments (Bustin *et al.*, 2009). Some of the methods used in the current study did not meet the recommendations in this guideline. For example, no test was conducted to ensure the samples were free from reverse-transcription inhibitors. It was decided not to change methods, so that the different parts of the experiment would be equivalent. However, additional data has been included, to align with the guidelines, and if a new study were to be initiated today, these guidelines would be closely adhered to.

The use of multiple reference genes was a method used in this study that was recommended in the quantitative PCR guidelines (Bustin *et al.*, 2009). In total, seven reference genes were tested for each experimental set, with the five most stably expressed, as determined by geNorm, being used for normalisation. This methodology is considered to produce more reliable results than using a single gene (Bustin *et al.*, 2009; Vandesompele *et al.*, 2002) and is particularly important in an experiment such as this, where gene expression changes induced by the drugs are small. The normalised values were then converted to relative expression of untreated cells, which allowed easier comparison of different genes and different experiments. However, if the expression levels of the untreated cells were inaccurate for any reason, this would have then created biased results in all experimental groups associated with those untreated cells. The replication time course, Long B, had a tendency to show down-regulations, more so than the original long time course. Increased expression in untreated cells is one possible explanation for this.

This study analysed expression levels of candidate genes, rather than using other methods such as gene expression arrays or microarrays. These other methods would have increased the number of genes investigated, reducing the risk of missing a relevant effect, but also increasing the risk of false positive findings. Moreover, given the number of different drugs, time points and cell lines investigated in the present experiment, measuring large numbers of genes would have produced a volume of data that would have been difficult to analyse. Analysing a single cell line and single drug, with one to three different time points, with microarray or gene expression array, may be a useful alternative method for future studies, which would allow a large number of genes to be screened. The time point and genes that

show statistically robust changes could then be further investigated with additional cell lines, additional drugs and, if of interest, additional time points, using quantitative PCR. Reducing the number of drugs, cell lines and time points initially studied would also make it easier to ensure sufficient replications were conducted to detect statistically significant changes.

Compared to candidate gene selection, including gene expression arrays, microarray analysis would present difficulties in interpretation of results related to antidepressant drugs. If the gene with altered expression levels is not know, *a priori*, to be associated with anxiety, it will be impossible to tell, from cell culture alone, whether the change was associated with antidepressant, anxiolytic or other effects of these drugs. Gabapentin was included in the present study to help determine whether changes were associated with anxiety. However, gabapentin rarely showed any gene expression changes, suggesting that it would have little use as an anxiety indicator. Thus, candidate genes currently seem to be the best way to maximise the probability that positive cell culture findings relate to anxiety.

Instead of measuring individual candidate genes, each with their own assay, inclusion of candidate genes on a gene expression array would allow a larger number of candidates to be assessed. These assays use 96 or 384-well plates with each well measuring expression of a different gene. The reference genes, and any other controls desired, are also included on the plate, thus one plate is required for each biological replicate of each treatment type, with multiple biological replicas required for statistical analysis. This would be useful for a screening experiment, as described above, reducing the probability of missing relevant genes and allowing reasonable confidence of association with anxiety for any significant findings. However, this would not be as appropriate for a time course experiment with multiple treatment types, as was conducted in this study, due to the large number of plates that would be required.

4.7.1.3 Protein Level Methods

One of the gene expression findings was followed up with protein assays. This was the last experiment conducted and, similar to the gene expression study, would have been better if more biological replicates were conducted. This was partially limited by time constraints, and was also affected by problems with protein samples. The first cell line to have protein extracted was the SH-SY5Y. These samples were initially assayed on the slot-blot, with

analysable results produced. On Western blot, however, the majority of A_{2a}-associated fluorescence was not located in the expected horizontal band. While it is uncertain why this occurred, it was hypothesised that the sample had degraded during the intervening weeks of storage in refrigeration. When the SK-N-DZ samples were extracted, following an initial test with γ-actin to estimate relative protein levels, the extracted samples were aliquoted into single experiment volumes and kept frozen. The success of the SK-N-DZ assays suggests that this fixed the problem, however, all potential protein data from one cell line had already been lost.

Since multiple drugs and time points were tested for each cell line, a slot-blot system was assessed as a potential method of screening all comparison samples at once, on one membrane. To confirm accuracy, this study compared a slot-blot system to the more frequently used Western blot method for protein quantification. The slot-blot was found to produce results in the same direction, though the degree of change was smaller for most slot-blot data points, than Western blot. This suggests that the slot-blot would be useful to screen large numbers of samples, with subsequent follow up of the most promising candidates.

The use of the Odyssey Infrared Imaging System facilitated the use of the slot-blot. The Odyssey system allows two proteins (the reference protein and the candidate protein) to be visualised and quantified on the same membrane without any need to strip and re-probe the membrane or ensure sufficient separation of the targets on Western blot. This removes any potential for protein loss, which may contribute to inaccuracies, from the experimental protocol. There was, however, potential that the two different fluorescent systems had different sensitivities. Repeating the experiment with primary antibodies against each target protein that had been raised in the other host species may have helped to determine the accuracy of quantification with the Odyssey system. An attempt to do this was conducted with a monoclonal A_{2a} antibody that was raised in mouse (sc-32261, from Santa Cruz), however, this antibody failed to show any binding, even when tested at a 1 in 100 dilution. Due to both time and financial constraints, this line of research was abandoned for this study but may be informative in future research.

Actin, particularly β-actin, is one of the most common reference proteins used in Western blotting. Like β-actin, γ-actin is a cytoskeletal protein (Perrin & Ervasti, 2010) and would be expected to have stable expression under a variety of conditions. This, however, was not seen

in the present experiment as high levels, relative to A_{2a}, were seen at 7 days and low levels at 29 days. A study of rat spinal cord injury found that β-actin, but not β-tubulin, levels increased 7 days post injury compared to sham operated animals (Liu & Xu, 2006). Given the similarities between β- and γ-actin, this may have been responsible for the variation in the present study. Thus, β-tubulin may have been a more appropriate reference protein. While it is not currently common to assay many reference proteins, as is recommended for reference genes, the increasing ability to assay multiple proteins on a single membrane through the use of fluorescent markers may make this a viable option in the future. This would then allow the most stable candidate(s) to be determined for each experiment, allowing more accurate analysis, which is particularly important when changes are small.

4.7.2 Expression Changes

Gene expression changes found in the present study were typically small, approximately two-fold; this is consistent with other gene expression studies of antidepressant drugs, which seldom find greater than three-fold changes (Lee *et al.*, 2010; Palotas *et al.*, 2004; Wong *et al.*, 2004). As a consequence of these small changes, results often vary between studies making it difficult to draw conclusions for specific genes. With this in mind, the results from the present study are discussed below, beginning with genes that have the least evidence for involvement in anxiolytic action. Unless otherwise stated, the results are those of the long time courses, where cells were exposed to drugs for a minimum of 28 days. The short time course is only occasionally discussed as most findings suggested no change; this is also why no replication of the cell culture was conducted for the short time course.

4.7.2.1 Expression Levels Not Analysed

The following genes had expression levels too low to allow analysis in the present study: *GRM3*, *CRHR2*, *NPY1R*, *CCK*, *CCKBR*, *GABRA1* and *TPH2*. All of these have been investigated in other studies of antidepressant or anxiolytic action.

- *GRM3* was found to increase approximately three-fold following 28 days of fluoxetine exposure in SH-SY5Y cells, while the same study found that gabapentin reduced *CRHR2* expression (Chan, 2007). This single study with one cell line and two drugs from different classes is not sufficient to allow conclusions to be drawn on these two genes.

- Similarly, *NPY1R* association had only been reported in one previous study in rats, which found that four weeks of voluntary running, but not the SSRI escitalopram, increased hippocampal mRNA expression of this receptor by approximately 50% (Bjornebekk *et al.*, 2010).
- A study of CCK expression following 21 days antidepressant exposure (imipramine, amitriptyline, desipramine and citalopram) in rats failed to show any changes in CCK protein levels (Harro *et al.*, 1997), however, 14 days fluoxetine exposure decreased *Cck* mRNA in the hypothalamus by 3.5 fold (Conti *et al.*, 2007).
- *CCKBR* was previously shown to increase in the brains of mice following administration of the anxiogenic substance yohimbine and that diazepam reversed this change (Le-Niculescu *et al.*, 2011). Another study, however, failed to show changes in CCK_B receptor levels following 21 days of antidepressant exposure in rats (Harro *et al.*, 1997).
- The $GABA_A$ receptor subunits have been investigated in rodents treated with antidepressant drugs. Following 21 days exposure with imipramine, *Gabra1* was significantly increased in the brainstem (50%), along with most of the other α subunits, with no change in *Gabra2* or *Gabra3* (Tanay *et al.*, 2001). An earlier study also found that imipramine increased *Gabra1* expression 150% above control values (Tanay *et al.*, 1996) and 14 days of the tricyclic antidepressant amitriptyline has also been found to increase *Gabra1* expression (McGeary *et al.*, 2011).
- Although expression levels prevented its investigation in the present study, *TPH2* has been found to be associated with antidepressant action in several other studies. These typically found increased gene expression, three- to twelve-fold, from four weeks in rats (Charoenphandhu *et al.*, 2011; Shishkina *et al.*, 2007), while studies of two weeks or less found reduced expression (approximately half) in cells and rats (Di Lieto *et al.*, 2007; Dygalo *et al.*, 2006). However, there have been some contradictory findings, including different expression changes for different brain regions in rats (Shishkina *et al.*, 2007) and reduced expression after four weeks exposure of cells (Chan, 2007), thus further research is still required.

Some of these low expression candidate genes had been investigated in a previous study in our laboratory that investigated only the SH-SY5Y cell line, including *GRM3*, *CCKAR*, *CRHR2* and *TPH2* (Chan, 2007). Similar differences in detectable expression were found for *GABRA1* between an initial SH-SY5Y study of fluoxetine and gabapentin, in which *GABRA1* levels were more than high enough to analyse (Wilson, 2008), and the present study, where

SH-SY5Y *GABRA1* levels were only just detectable. There are a number of possible reasons why studies of the same cell line may have found different gene expression patterns. There may have been differences in mRNA extraction or cDNA production, though this seems unlikely as the same methods and reagent sources were used. There may also have been changes in gene expression due to subtitle differences in media composition, particularly foetal bovine serum, which was found to alter gene expression in another study (Monstein *et al.*, 1992). The supplier of foetal bovine serum was changed between these different studies, making this a possible contributing factor. Finally, since the neuroblastoma cell lines studied are not homogenous in genotype or phenotype, it is also possible that a subpopulation of cells was inadvertently selected. The fact that only one cell line seems to have been involved gives some support for this, although the cell line that shows the most phenotypic variation (the SK-N-SH) was not problematic. Since all of these changes should have been consistent among the different drug groups, there should not have been any relevant effects on the findings for relative gene expression.

4.7.2.2 Small and Inconsistent Changes

The following genes had small or inconsistent changes with gabapentin and antidepressant exposure in the present study, and little or no evidence from other studies supporting regulation changes in response to anxiolytic drugs: *ADAM22*, *CACNA2D1*, *FZD1* and *SRI*. Thus there is no clear evidence supporting the changes in gene expression of any of these candidates in anxiolysis. In addition, small or inconsistent changes were found in *LMTK2*, *TAC1*, *BDNF, CDKN1A*, *GABRA2*, *GABRA3* and *NPY*, all of which have been studied previously. These seven genes are discussed below.

- *LMTK2* showed small decreases in expression of up to two-fold, though averages were less than 1.5-fold, in the present study (Figure 4.7). Previous research in our laboratory found no change in *LMTK2* expression in the SH-SY5Y cell line following 28 days of fluoxetine or gabapentin. Given this and the small changes seen in the present study, it is unlikely the *LMTK2* is a major contributor to anxiolytic action of these drugs.
- *TAC1* had the largest expression changes of any of the genes included in this study; however, no clear pattern by time or drug was seen (Figure 4.3). A study of mice treated with amitriptyline for 28 days also produced a large change in *Tac1*, with almost 20-fold increased expression (Bohm *et al.*, 2006), suggesting that the variability is a characteristic of the gene and not due to the cell culture methodology. This may have been associated

with the multiple protein products that *TAC1* encodes, or it may have been due to one specific product. Regardless, the high variability makes the results difficult to interpret.

- *BDNF* was included in the present study as an attempt to replicate the findings of another study of antidepressant effects in the SH-SY5Y neuroblastoma cell line. That study measured mRNA levels at 6, 24 and 48 hours (Donnici *et al.*, 2008). No significant change was found for fluoxetine treated cells, while desipramine and reboxetine treated cells had reduced *BDNF* expression at 6 hours (approximately 75% of control), followed by increased expression at 48 hours (approximately 150% of control). The findings for fluoxetine were supported by the present study (Figure 4.5), but desipramine, which is related to imipramine, was not. This may be because desipramine and reboxetine have greater selectivity for noradrenaline than any of the antidepressants included in the present study. BDNF has also been found to be associated with antidepressant action in longer studies, for example, 28 days of fluoxetine exposure was found to increase *Bdnf* expression almost two-fold, in mice (Huang *et al.*, 2012). The changes found in the present study were not consistent enough to support or refute these findings.
- *CDKN1A* showed variable data. Previous research demonstrated a reduction in expression in mice following 21 days of fluoxetine, imipramine or desipramine to approximately half of normal values, and knockout mice supported this being involved in the therapeutic response (Pechnick *et al.*, 2011). While the present study found some support for a down-regulation, particularly in the SK-N-DZ and SH-SY5Y cell lines during the first long time course (Long A), these failed to replicate in the repeat long time course (Long B).
- *GABRA2* and *GABRA3* showed no or very small (less than 1.5-fold) changes at almost all data points, which is consistent with a study in rats that found no significant change in either of these genes after 21 days of imipramine (Tanay *et al.*, 2001). Another study in rats, however, found that 14 days of amitriptyline increased *Gabra2* expression in the amygdala/hippocampus by 1.22-fold (McGeary *et al.*, 2011), raising the possibility of involvement of this gene, which was only analysable in one cell line in the present study.
- *NPY* showed an overall trend of decreased expression in the present study, though some data from the SK-N-SH and SH-SY5Y cell lines also supported an increase (Figure 4.10). Previous research in our laboratory found that *NPY* mRNA levels increased approximately three-fold in the SH-SY5Y cell line following 28 days fluoxetine exposure (Chan, 2007), and CSF levels of neuropeptide Y were increased approximately 1.5-fold by four weeks of citalopram (an SSRI) treatment in depressed patients (Nikisch *et al.*,

2005). Animal studies, including rats, tree shrews and zebrafish have found variable results effects for fluoxetine on *Npy* expression, ranging from a 50% decrease, to 50% increase (Caberlotto *et al.*, 1998; Wong *et al.*, 2013; Zambello *et al.*, 2010). Some of these studies found variable results in different brain regions of the same species, suggesting that effects are region specific.

4.7.2.3 *CCKAR*

Previous research in our laboratory found reduced expression of *CCKAR*, of approximately 2.5-fold, in the SH-SY5Y cell line with 28 days of fluoxetine, but not gabapentin (Chan, 2007). The present study supported and extended that research, finding that *CCKAR* expression was reduced by all antidepressants tested, but not by gabapentin, in the SK-N-DZ cell line (Figure 4.9). Furthermore, this decrease was found to occur very soon after drug exposure, with decreases of approximately two-fold seen within the first 48 hours (Figure 4.6). *Cckar* has also been found to be down-regulated 1.14-fold in rats by 14 days treatment with nerve growth factor, a postulated antidepressant agent, though not by fluoxetine or amitriptyline in the same study (McGeary *et al.*, 2011). However, CCK_A receptor has not been a focus of anxiety research, like CCK or CCK_B receptor, though some evidence for a specific role has been found (see section 1.4.3.3, page 57).

4.7.2.4 Adenosine A_{2a}, Encoded by *ADORA2A*

The strongest finding from this study was the reduced expression of *ADORA2A*, which was seen in all three cell lines, with all antidepressant drugs to varying degrees (Figure 4.8 and Table 4.5). A preliminary study of protein levels in one of the cell lines also supported the gene expression results (Figure 4.17). However, gene expression changes were not supported by previous research in our laboratory, which found no change in *ADORA2A* expression with either fluoxetine or gabapentin (Chan, 2007). That study only investigated the 28 day time point in one cell line, whereas the reduced expression seen in the present study generally began around 7 days and peaked around 15 to 20 days, hence it may have been missed by only studying effects at 28 days.

4.7.3 Implications of Findings

The main findings of this study relate to the actions of antidepressant agents. While the anxiolytic gabapentin was included with the intention of using it to support anxiolytic over antidepressant or side effects of SSRIs and tricyclic antidepressants, changes in gene expression with gabapentin were rare. This causes difficulty in the interpretation of the data as antidepressant drugs have anxiolytic, anxiogenic and antidepressant effects, which cannot clearly be distinguished in a cell culture model.

The effect of decreased CCK_A receptor levels is uncertain. Selective antagonists tend to have anxiolytic effects (Hernandez-Gomez *et al.*, 2002), but complete loss of the receptor is associated with increased anxiety (Li *et al.*, 2002). Given the early, but sustained nature of the down-regulation seen in the present study, the *CCKAR* changes could be associated with either the early anxiogenic or late anxiolytic effects of antidepressants. In addition, the genetic association component of this study found evidence for an association between *CCKAR* variants and bipolar disorder. This suggests that *CCKAR* may be involved in mood regulation (see Table 3.7, page 127) and, therefore, that these changes may be associated with antidepressant effects. Further research is required to determine which of these possibilities is most likely.

Given that *ADORA2A* decreases were greatest towards the middle of the time course and that A_{2a} knockout mice show increased anxiety (Houchi *et al.*, 2008), it is hypothesised that the reduction in *ADORA2A* expression may be involved in the anxiogenic effects some people experience early in their treatment with antidepressants. Consistent with this, the commonly used anxiolytic diazepam has been found to increase *Adora2a* expression, back to normal levels, in the amygdala of mice co-treated with the anxiogenic compound yohimbine (Le-Niculescu *et al.*, 2011).

The potential involvement of A_{2a} receptor expression in the anxiogenic side effect raises the prospect that caffeine use may be a predictor of the anxiogenic effect. Genetic variation in *ADORA2A* has been found to be associated with increased anxiety with caffeine consumption (Alsene *et al.*, 2003; Childs *et al.*, 2008; Rogers *et al.*, 2010). It is possible that these anxiogenic effects of caffeine and antidepressants are related. While this is an extreme extrapolation of the data, it could be tested simply by taking a detailed history of caffeine use

by patients before they start antidepressant therapy, followed by careful monitoring and recording of any anxiogenic response to the antidepressants. If an association were to be proven, the related caffeine use information could be used to identify patients that are at high risk of the adverse reaction.

Although this study intended to find gene expression changes that may be associated with the anxiolytic effects of antidepressant drugs, the major findings may instead be associated with the early anxiogenic effects. The anxiogenic effects of antidepressant is an area that has seen very little research, yet this side effect is severe enough to warrant concurrent therapy with anxiolytic agents and to cause some patients to cease therapy (Schneier *et al.*, 1990). Improved understanding of the mechanism through which these effects occur may allow the identification of those at risk and superior, more targeted therapy that prevents this adverse effect.

5 Conclusions

This study found support for the involvement of *CCKBR* variation in the pathogenesis of panic, which has subsequently received further support from a genome-wide study with over 700 panic disorder cases (Otowa *et al.*, 2012). Additionally, suggestive evidence was found for involvement of *CCKAR* variation in a comorbid panic/bipolar phenotype, as well as *CCKAR* and *ADORA2A* down-regulation with antidepressant drug action. All of these later findings would require replication to be confident that they did not occur by chance.

Collectively, the results from this study support the crossover of candidate systems between pathogenesis and treatment. The main candidate system in the genetic association study was the CCK system, which found support for the involvement of variation in *CCKBR*. *CCKAR*, although only expressed in one cell line, was one of the two leading candidates found in the study of antidepressant effects. Similarly, *ADORA2A* was the strongest candidate for effects of antidepressants, and was also a strong candidate, based on previous research, for genetic association. Thus, the CCK and adenosine systems were found to be candidates for both the pathogenesis and treatment of anxiety.

5.1 Anxiety Genetics in 2013

Despite anxiety disorders being more common, genetic studies into anxiety have lagged behind those of schizophrenia and bipolar disorder. For example, publications with bipolar cohorts of more than 1000 cases were first published more than five years ago (Manolio *et al.*, 2007; The Wellcome Trust Case Control Consortium, 2007), with meta-analyses of over 5000 bipolar cases now occurring (Sklar *et al.*, 2011). Meanwhile, anxiety studies of over 1000 cases are only just being published (Erhardt *et al.*, 2012; Reif *et al.*, 2012), with smaller candidate gene studies still common.

One possible reason for this is the higher estimated genetic contribution to bipolar disorder and schizophrenia, greater than 60% (Gershon *et al.*, 2011), compared to 30-60% for anxiety disorders (Domschke & Deckert, 2010). This lower genetic risk may, however, be beneficial, as environmental factors can be targeted in treatment or prevention. The lack of clinical utility was one of the major arguments presented by Mitchell *et al.* (2010) against the introduction of commercial genetic tests for psychiatric disorders. However, if psychotherapy

or other strategies prove to be useful to prevent the development of a disorder, then information about genetic risk factors could be used to identify the high risk populations most likely to benefit from such interventions.

While genetic risk is lower, the biological pathways for anxiety are better understood than those of many other psychiatric disorders. This may have contributed to the relatively high rates of candidate gene studies of anxiety. The importance of the amygdala, the ability to induce panic attacks with certain substances, and the actions of anxiolytic drugs, such as benzodiazepines, have each contributed to a long list of potential candidates for panic disorder and anxiety. Thus, there has been little need to identify new candidates from linkage studies or, more recently, genome-wide association studies. This may explain why linkage results were rarely been followed up with further study of associated regions (Hamilton, 2009).

There is still much to learn about anxiety, though. While the mechanism of action of benzodiazepines is well understood, there is not yet a consensus as to how antidepressants or pregabalin/gabapentin reduce anxiety. Furthermore, while many studies have been undertaken to show the therapeutic benefit of antidepressants and to compare the different drugs in the long-term control of anxiety (reviewed in Koen & Stein, 2011), few have looked at time points earlier than four-weeks and little molecular research has focused on anxiety, rather than depression. This seems astounding given how common anxiety disorders are, affecting up to a third of the population at some time in their life (Kessler *et al.*, 2007b), and the widespread use of antidepressant medications.

5.2 The Future of Anxiety Research?

Far from being left behind by bipolar and schizophrenia, anxiety genetics can learn from it. The large genome-wide association studies for schizophrenia and bipolar disorder have failed to account for the majority of the genetic risk (Gershon *et al.*, 2011). This does not necessarily mean that large genome-wide studies should be abandoned, but that more consideration may be needed in the collection and analysis phases. The same genetic data that is used for standard genetic association studies could be used to find gene-gene or gene-environment interactions, provided the sample size was large enough and sufficient background information was collected.

Studying genetic interactions is becoming increasing common for complex diseases. Gene-environment interactions have been studied for more than a decade in psychiatry, but the methodology has yet to be perfected (Duncan & Keller, 2011). These studies typically investigate a genetic and an environmental factor that have already been implicated in the disorder, such as, studying the interaction between stressful life events and serotonin transporter variants in depression (Brummett *et al.*, 2008). However, environmental risk factors that interact with genetic risk factors may be more subtitle than the independently associated risk factors. For example, nutritional factors may contribute to the risk for mental disorders in susceptible individuals (Dauncey, 2012). These more subtitle effects could potentially occur years before the disorder, and would be difficult for participants to reliably recall in a retrospective study. Thus, large cohort studies may be necessary to detect these effects. Cohort studies of potential use already exist, such as the Dunedin Multidisciplinary Study. This recruited 1661 babies born at Queen Mary Hospital in 1972/73 (Silva, 1990). Given that the majority of participants are unlikely to develop an anxiety disorder, this study might not be large enough for the analysis of multiple genetic variants. However, combination of data from this and other cohort studies may provide a useful means of investigating gene-environment interactions.

A mixture of genetic and environmental factors may also be involved in drug response. Both successful therapeutic responses and experience of side effects, such as the short-term increased anxiety with antidepressants, vary between patients. Personalising medicine to the patient's genotype has been researched for years, with particular focus on genes encoding drug metabolism proteins (de Leon *et al.*, 2008). Extending this to other genetic factors associated with positive patient outcomes may reduce both side effects and unnecessary trials of multiple drugs that the patient was unlikely to respond to. Thus, a better understanding of associations with drug responses may reduce the suffering of patients.

Studies finding associations with SNPs or haplotypes are unlikely to have identified the causative variant. Further research is then needed to find this variant. Genetic research into *CCKBR* has arguably reached this point. CCK_B receptor has both biological plausibility, due to its involvement in CCK-induced panic attacks, and a range of genetic support, including multiple candidate gene studies and suggestive evidence from a genome-wide study (see section 3.5.2.3, page 137). Rare missense variants have been found in some panic disorder

cases (Koefoed *et al.*, 2010), but questions remain regarding the nature of contributing *CCKBR* variants for most patients.

The identification of a specific molecular mechanism for a genetic predisposition is important as it may lead to improved treatments. This would allow early identification of at risk individuals, with the possibility of offering preventive therapy. Also, if the genetic change associated with disorder is known for a particular individual, it may be possible to identify anxiolytic drugs or gene therapy that will prevent or reverse the change. When such a therapy does not exist, understanding the mechanism causing the disease would provide a basis for the development of new therapies.

Exome sequencing or next-generation sequencing of pooled samples may identify specific risk variants associated with anxiety or with treatment responses. Both of these methods have the advantage of being able to assess all genes in the genome, and both methods have the potential to identify associated rare variants. Focusing on the exome results in a more focused data set, but has the potential to miss relevant interactions occurring outside of coding regions, such as microRNAs. Both of these methods would create difficulties in collecting sufficient numbers of participants and analysing the large amount of data produced.

However, even sequencing the entire genome may still miss relevant changes such as epigenetic effects. Epigenetic refers to changes that occur to DNA that do not alter the nucleotide sequence, but alter gene transcription. Many of these epigenetic changes, like DNA methylation, can be measured directly, providing another potential means of studying patient cohorts. Epigenetic modifications have been found in cancer, neurological disorders such as Alzheimer's disease, and autoimmune diseases (Portela & Esteller, 2010). Epigenetic changes have been postulated to be involved in the mechanism for some environmental contributors to anxiety disorders, such as early life stress (Kofink *et al.*, 2013). Thus, further study of these changes may be of benefit in understanding the pathogenesis of anxiety.

Rather than identifying factors involved in the control of gene expression, an alternative means of identifying genes associated with anxiety disorders is to conduct a transcriptome analysis, giving a snapshot of expression of all genes in a tissue. The ideal tissue to analyse in anxiety disorders is the human brain, particularly the amygdala. Unfortunately, this is only available under very limited circumstances. An alternative option is to use a more easily

accessible tissue, with the potential to confirm any findings in animal or cell culture models. At least one previous study has already done this, comparing lymphoblast cell lines from 16 panic disorder patients to cell lines from 17 controls (Philibert *et al.*, 2007). Unfortunately, the patients were receiving various pharmacological treatments for anxiety, making it unclear as to which changes were associated with panic disorder pathogenesis and which were associated with treatment.

A carefully designed study using transcriptome analysis could provide valuable information on both the pathogenesis and treatment of panic disorder. To identify genes associated with the development of panic disorder, samples could be taken from treatment naïve patients and compared to matched controls. For this purpose, treatment should be kept broad, including psychotherapy as well as pharmacotherapy, as psychotherapy has been found to correlate with changes in the brain (Straube *et al.*, 2006), and these changes may be associated with gene expression changes. Samples could then be taken from the same patients following successful response to therapy. Ideally, a randomised controlled trial would be conducted; however, withholding proven therapy would be an ethical issue. An alternative control for changes over time would be to re-sample the original matched control and compare changes in the controls' values to the changes in the patients' values.

For all of this different research to provide the maximum benefit, it needs to be ensured that high-risk groups are included and considered at all stages. Currently, ethnic minorities are often excluded from psychiatric genetic studies (Oquendo *et al.*, 2010), which may lead to the identification of genetic risk factors for the dominant population only. Similarly, individuals from low socioeconomic groups are also at increased risk of mental disorders. This raises the issue of cost and availability of genetic testing and preventative treatments. These are not simple issues to solve, and will require consideration by the whole of society.

Overall, further research is needed to better understand the genetic factors contributing to panic and anxiety. These are common disorders and improving diagnosis and treatment could benefit many individuals, reducing the impact of these diseases on the whole of society.

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Appendix A – CCK System Custom Blocks

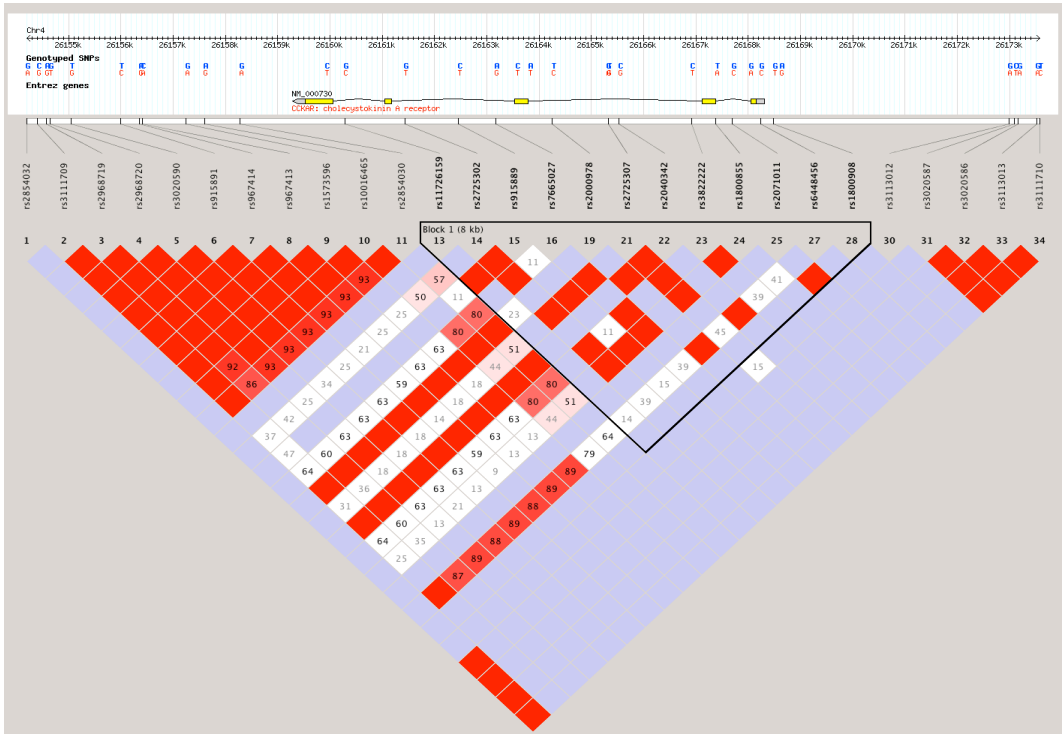
Images below are Haploview generated LD plots, where red indicates strong LD; blocks are custom designed and show all haplotypes with a frequency greater than 0.01 in the HapMap CEU cohort. In the tables bold indicates a tag SNP and "Freq." is the frequency of that haplotype in the HapMap CEU cohort.

CCK:



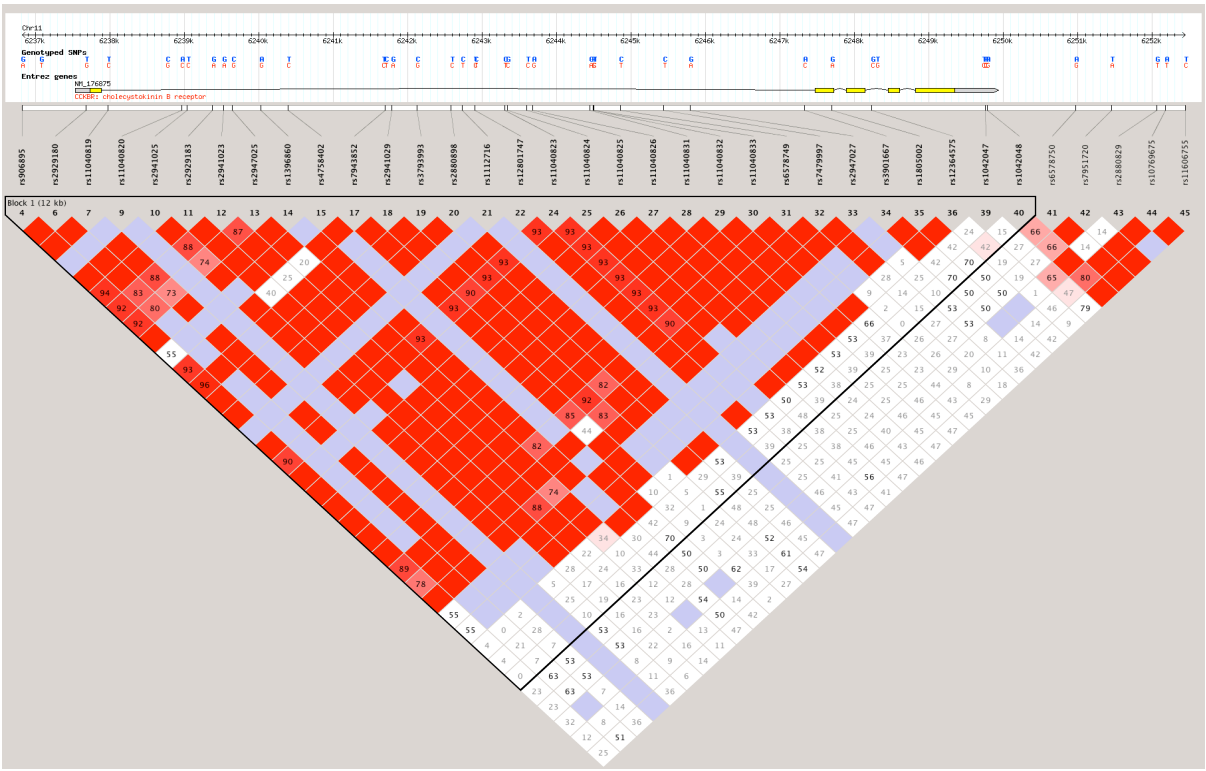
rs8192472	**rs11129949**	**rs4602358**	**rs10865918**	rs11571849	rs1868522	rs754635	rs747456	**rs747455**	rs1799923	rs11571842	rs13069836	Freq.
G	**A**	**G**	**G**	C	A	G	A	**G**	C	G	G	0.246
A	**A**	**A**	**T**	T	G	G	G	**G**	C	A	T	0.153
A	**A**	**A**	**T**	T	G	G	G	**A**	C	A	T	0.138
G	**C**	**G**	**G**	C	A	G	A	**G**	C	G	G	0.132
G	**A**	**A**	**T**	T	G	G	G	**G**	C	A	T	0.072
G	**A**	**A**	**T**	T	G	G	G	**A**	C	A	T	0.062
A	**C**	**G**	**G**	C	A	G	A	**G**	C	G	G	0.035
G	**A**	**G**	**T**	C	A	C	G	**G**	T	G	G	0.029
A	**A**	**G**	**T**	C	A	C	G	**G**	T	G	G	0.029
G	**A**	**G**	**G**	C	A	G	A	**G**	C	G	G	0.025
G	**A**	**A**	**T**	T	G	G	G	**G**	C	A	T	0.025
A	**A**	**G**	**G**	C	A	G	A	**G**	C	G	G	0.021

CCKAR:



rs11726159	rs2725302	**rs915889**	**rs7665027**	**rs2000978**	rs2725307	rs2040342	rs382222	rs1800855	**rs6448456**	Freq.
G	G	**C**	**A**	**T**	T	C	C	T	**G**	0.649
G	G	**C**	**A**	**C**	T	G	C	T	**C**	0.167
G	T	**C**	**G**	**T**	G	C	T	A	**G**	0.100
C	T	**T**	**A**	**T**	G	C	C	A	**G**	0.033
G	T	**C**	**G**	**T**	G	C	T	A	**C**	0.017

CCKBR:



rs906895	**rs2929180**	rs11040819	**rs2941025**	**rs2929183**	**rs2941023**	**rs2947025**	rs1396860	rs4758402	**rs7943852**	rs2941029	rs3793993	rs2880898	**rs1112716**	rs12801747	rs11040823	rs11040824	rs11040825	rs11040826	rs11040831	rs11040832	rs11040833	**rs2947029**	**rs2947028**	rs2947027	rs3901667	rs1805002	**rs12364575**	**rs1042047**	**rs1042048**	Freq.
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**C**	A	A	0.225
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	C	**C**	C	T	C	C	A	G	A	C	**C**	**T**	A	A	G	**C**	A	A	0.082
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**C**	A	**G**	0.058
G	**T**	T	**T**	**G**	**G**	**C**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	A	A	0.056
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**C**	C	A	0.050
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**C**	C	**G**	0.042
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**G**	C	**G**	0.042
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	C	**C**	C	T	C	C	A	G	A	C	**C**	**T**	A	A	G	**C**	A	**G**	0.033
G	**T**	T	**T**	**G**	**G**	**C**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	C	**G**	0.033
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**G**	C	**A**	0.033
G	**T**	T	**T**	**G**	**G**	**C**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**G**	C	**G**	0.024
G	**G**	C	**C**	**G**	**G**	**C**	A	C	**T**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	A	C	A	**G**	A	**G**	0.023
G	**T**	T	**T**	**G**	**A**	**C**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	A	**A**	0.019
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	C	**T**	C	T	C	C	A	G	A	C	**C**	**T**	A	A	G	**C**	A	**A**	0.018
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**G**	A	**G**	0.017
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**C**	C	**A**	0.017
G	**G**	T	**T**	**G**	**G**	**G**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	C	**A**	0.017
G	**T**	T	**T**	**G**	**G**	**G**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	C	**A**	0.017
G	**T**	T	**T**	**G**	**G**	**G**	A	T	**C**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	G	A	G	**C**	A	**A**	0.017
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	A	A	G	**C**	A	**A**	0.017
A	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**G**	A	**A**	0.015
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	C	**C**	C	T	C	C	A	G	A	C	**C**	**T**	A	A	G	**C**	C	**A**	0.012
G	**G**	C	**C**	**A**	**A**	**G**	G	T	**T**	G	G	C	**T**	C	T	C	C	A	G	A	C	**C**	**T**	A	A	G	**C**	C	**A**	0.012
A	**G**	C	**C**	**G**	**G**	**C**	A	C	**T**	G	C	T	**C**	T	C	G	T	G	A	G	T	**C**	**C**	A	C	A	**G**	A	**G**	0.010
G	**G**	C	**C**	**A**	**A**	**G**	A	T	**C**	A	C	T	**C**	T	C	G	T	G	A	G	T	**T**	**T**	A	A	G	**G**	A	**A**	0.010

Appendix B – Genotyping Details

The following pages include all details of final genotyping reactions that are not in the methods/results sections, beginning with *CCK* and *CCKAR*, followed by *CCKBR*, then *ADORA2A*. Grey font colour indicates that the work was primarily conducted as part of another research project.

SNP	Location - GRCh37	Genotyping Method(s)
CCK	Chromosome 3	
rs8192472	Intron; 42299870 bp	Allele specific/Multiplex[A]
rs11129949	Intron; 42302808 bp	Allele specific
rs4602358	Intron; 42303037 bp	Allele specific/Multiplex[A]
rs10865918	Intron; 42303311 bp	Allele specific
rs11571849	Intron; 42304293 bp	Inferred[1]
rs1868522	Intron; 42304784 bp	Inferred[1]
rs754635	Intron; 42305131 bp	Inferred[1]
rs747456	Intron; 42305758 bp	Inferred[1]
rs747455	Intron; 42305964 bp	Allele specific
rs1799923	Main transcript 5' UTR; 42306294 bp	Sequencing/Inferred[1]
rs11571842	Near main transcript 5'; 42306446 bp	Inferred[1]
rs13069836	Near alternative transcript 5'; 42306446 bp	Inferred from rs4602358
CCKAR	Chromosome 4	
rs11726159	Intron; 26484021 bp	RFLP/Inferred from rs915889
rs2725302	Intron; 26485174 bp	Allele specific/Multiplexed with rs2000978
rs915889	Intron; 26486194 bp	Allele specific/Multiplexed with rs1800855
rs7665027	Intron; 26486909 bp	Allele specific/Inferred from rs1800855
rs2000978	Intron; 26487989 bp	Allele specific/Multiplexed with rs2725302
rs2725307	Intron; 26489057 bp	Inferred from rs1800855
rs2040342	Intron; 26489270 bp	Inferred from rs2000978
rs3822222	Intron; 26490652 bp	Inferred from rs7665027
rs1800855	Intron; 26491117 bp	Allele specific/Multiplexed with rs915889
rs6448456	5' UTR; 26491974 bp	Allele specific/Inferred[2]

Multiplexes: A = rs8192472, rs4602358, rs12364575 and rs1042048. Inferred: 1 = rs8192472, rs11129949, rs4602358, rs10865918 and rs747455; 2 = rs7665027 and rs2000978

SNP	Primers	Size (bp)	Reaction Conditions*
CCK			
rs8192472	F=CTCTCAGATTTTGGTCCATCA(G/A) R=GTCCCGGTCACTTATCCTGT	242	1.0 mM 63°C
rs11129949	F=CACAGTTTCTATATGTGCAGTATtT(A/C) R=CATTGAAAGTGCTGCAG	188	2.0 mM 57°C
rs4602358	F=TGGTTCCAAAATTGCTCTAGG R=GAGGGACTAGAAAGAACTTTCaAG(T/C)	131	1.0 mM 61°C
rs10865918	F=TGCCTGAAAATAATTTAGTATCaG(T/G) R=TAGAGCAATTTTGGAACCAAG	211	1.5 mM 60°C
rs11571849	N/A		
rs1868522	N/A		
rs754635	N/A		
rs747456	N/A		
rs747455	F=GGATCCAAGTGTCGTGTGTC R=AGCGAATGGGTAGTTCATaC(C/T)	220	1.0 mM 58°C
rs1799923	F=GGATTAACTCCACCCCACTAGAC R=CAAAGACACACGACACTTGGAT	303	1.0 mM 56°C
rs11571842	N/A		
rs13069836	N/A		
CCKAR			
rs11726159	F=TCCATCAAGAAGTCCAAACC R=TTACCCAGCAAGCAGGAAC	254	2.5 mM 57°C (35)
rs2725302	F=CTGTGCTTGAAAGAAGTCTATGTAT(G/T) R=ATTGTCCCCACCTAAGCAT	404	1.5 mM 62°C
rs915889	F=GCAGCAGCTCCGGTACTTAC R=CTCCTTATAGAGATAAGGAACCTGAC(G/A)	145	1.5 mM 61°C (33)
rs7665027	F=TCACACCATTGCACTCCAG R=CAGAGGCTCTGTGCTTAATCATTA(T/C)	165	1.5 mM 63°C
rs2000978	F=CTGCACCCAGCCAAGAAATA R=TGATGTGAGCCGGAGaC(A/G)	294	1.5 mM 60°C (40)
rs2725307	N/A		
rs2040342	N/A		
rs3822222	N/A		
rs1800855	F=CTCCGTCTTGCTCCTGTT(T/A) R=AGCAACTTACCCATGAAGTAGG	291	1.5 mM 61°C
rs6448456	F=TAATGACCGAAGCGTCTC(G/C) R=AGGTGAACTCAAGTCGCCTT	109	1.5 mM 55°C

*Reaction conditions are magnesium concentration and annealing temperature (number of cycles, if not 30) for amplifications of individual SNPs. Final multiplex reaction conditions can be found in section 3.3.2.1.3 (page 112). F=forward primer(s), R=reverse primer(s); lowercase indicates a wobble.

			HWE		
SNP	**CEU freq**	**SIBS freq**	**χ^2**	***p***	**Additional Notes**
CCK					
rs8192472	0.39	0.303	0.866	>0.5	
rs11129949	0.167	0.116	1.020	>0.5	
rs4602358	0.449	0.436	<0.01	>0.995	
rs10865918	0.467	0.421	<0.01	>0.995	One Mendelian error found
rs11571849	0.441	0.436			
rs1868522	0.45	0.436			
rs754635	0.083	0.146	0.167	>0.9	
rs747456	0.458	0.421			
rs747455	0.2	0.237	0.052	>0.9	One Mendelian error found
rs1799923	0.083	0.146			Many previous studies → accuracy confirmed by sequencing
rs11571842	0.44	0.436			
rs13069836	0.45	0.436			
CCKAR					
rs11726159	0.018	0.077	**8.082**	**<0.025**	HW deviations small
rs2725302	0.169	0.189	**7.866**	**<0.025**	HW deviations small
rs915889	0.05	0.088	**8.915**	**<0.025**	HW deviations small
rs7665027	0.125	0.094	0.869	>0.5	
rs2000978	0.167	0.179	1.948	>0.1	One Mendelian error found
rs2725307	0.194	0.19			
rs2040342	0.175	0.179			
rs3822222	0.125	0.094			
rs1800855	0.172	0.191	**7.182**	**<0.05**	HW deviations small
rs6448456	0.198	0.188	2.368	>0.1	One Mendelian error found

CEU freq and SIBS freq = allele frequencies in the HapMap CEU and SIBS cohorts. HWE: results of Hardy-Weinberg equilibrium tests; χ^2 = Chi squared value (empty spaces indicate a SNP that was not genotyped or analysed directly); *p* = probability that deviation may be found by chance based on Chi square table for two degrees of freedom (Griffiths *et al.*, 2000), <0.05 was considered statistically significant and is in bold.

SNP	Location - GRCh37	Genotyping Method(s)
CCKBR	Chromosome 11	
rs906895	Near 5'; 6280248 bp	TaqMan probe
rs2929180	5' UTR; 6281108 bp	RFLP/Inferred from rs2941025
rs11040819	Intron; 6281400 bp	Inferred from rs2941025
rs2941025	Intron; 6282468 bp	Allele specific/Multiplex[B]/Inferred from rs2929183
rs2929183	Intron; 6282808 bp	TaqMan probe
rs2941023	Intron; 6282955 bp	Allele specific/Inferred[3]
rs2947025	Intron; 6283076 bp	TaqMan probe/Inferred[3]
rs1396860	Intron; 6283452 bp	Inferred[4]
rs4758402	Intron; 6283822 bp	Allele specific/Multiplex[B]/Inferred[5]
rs7943852	Intron; 6285128 bp	Allele specific/Multiplex[C]/TaqMan probe/Inferred[4]
rs2941029	Intron; 6285217 bp	TaqMan probe
rs3793993	Intron; 6285553 bp	Inferred[6]
rs2880898	Intron; 6286010 bp	Inferred[7]
rs1112716	Intron; 6286156 bp	TaqMan probe/Sequencing/Inferred[7]
rs12801747	Intron; 6286326 bp	Inferred[7]
rs11040823	Intron; 6286728 bp	Inferred[7]
rs11040824	Intron; 6286767 bp	Inferred[7]
rs11040825	Intron; 6287029 bp	Inferred[7]
rs11040826	Intron; 6287107 bp	Inferred[7]
rs11040831	Intron; 6287877 bp	Inferred[7]
rs11040832	Intron; 6287918 bp	Inferred[7]
rs11040833	Intron; 6287928 bp	Inferred[7]
rs2947029	Intron; 6288282 bp	TaqMan probe
rs2947028	Intron; 6288867 bp	Allele specific/Multiplex[B]
rs2947027	Intron; 6289221 bp	Allele specific/Multiplex[D]/Inferred from rs2947028
rs3901667	Intron; 6290756 bp	Allele specifics/Multiplex[D]/Inferred from rs2947028
rs1805002	Exon; 6291120 bp; missense – Val->Ile	Inferred from rs3901667
rs12364575	Intron; 6291659 bp	Allele specific/Multiplex[E]
rs1042047	3' UTR; 6293182 bp	TaqMan probe/Sequencing/(Allele specific)
rs1042048	3' UTR; 6293210 bp	Allele specific/Multiplex[E]

Multiplex: B = rs2941025, rs4758402 and rs2947028; C = rs7943852, rs2947027 and rs3901667; D = rs2947027, rs3901667 (and rs7943852); E = rs12364575, rs8192472, rs4602358 and rs1042048. Inferred: 3 = rs4758402, rs7943852 and rs2941029; 5 = rs7943852 and rs2941029; 4 = rs4758402 and rs2941029; 6 = rs4758402 and rs7943852; 7 = rs2947029 and rs2947028

SNP	Primers/Probe Order Number	Size (bp)	Reaction Conditions*
CCKBR			
rs906895	C___9604839_10	N/A	
rs2929180	F=GAAATCTCTAAGAGGAGACG R=CAGCTTTAGCAGCTCCAT	345	3.25 mM 57°C
rs11040819	N/A		
rs2941025	F=GTAGCTCATTTCCTCAAGAGG R=ATTCCTACTTCACAGAGCTCA(G/A)	173	1.5 mM 61°C
rs2929183	C__16165338_10	N/A	
rs2941023	F=TTACCTTAGTCTCAGCTAGCATCA R=CTAGGCCTGACCATGGAA(T/C)	198	1.0 mM 60°C
rs2947025	C__15956573_10	N/A	
rs1396860	N/A		
rs4758402	F=CAGGACAGTCAGAACAGCAT R=CAGTCCAATCTTTAACCTACC(G/A)	99	1.0 mM 62°C
rs7943852	F=ACAGATATAAGCCACCATGTCT R=CATTCAGAGGTCTACTTGTGA(A/G) C__31708456_10	174	1.0 mM 57°C
rs2941029	C__15866600_10	N/A	56°C
rs3793993	N/A		
rs2880898	N/A		
rs1112716	F=CTGTGTGATACTGTAATGATGG R=CACAACCACACATATACACAG C__9604845_10	204	1.5 mM 55°C
rs12801747	N/A		
rs11040823	N/A		
rs11040824	N/A		
rs11040825	N/A		
rs11040826	N/A		
rs11040831	N/A		
rs11040832	N/A		
rs11040833	N/A		
rs2947029	C__26881434_10	N/A	
rs2947028	F=(T)GACTGTGTGACTGGTTGAT(A/G) R=AAGGACCTGCCTGAGTCT	117/116	2.0 mM 65°C
rs2947027	F=TAGCTGGGTGTGGTGATAC(A/G) R=TATGACTGACAGCACAGTAGGTT	301	1.5 mM 57°C
rs3901667	F=CACTGATAGGGCACAGAATATTG R=(GAA)CTGCTCAGTAAATGGTGAAC(T/G)	222/219	1.5 mM 57°C
rs1805002	N/A		
rs12364575	F= CGTGTACACTGTCGTGCAAC R=GAGTTGGCGTGGTAATTCT(C/G)	195	1.0 mM 61°C
rs1042047	F=TCCTAGTTTGACCTCACAGTG(A/C) R=GAAGAACCTTTTCAGTGCAGAAC C___1569887_20 F=GGAACTCTGACAAGGGCTGA R=CCACGGTCCTTAACTGGAAA	107 233	N/A
rs1042048	F= GTAGCTTACCTGACACAAGAGGA R=AGAGGTATGAGATTAGGCCTGA(T/C)	324	1.0 mM 63°C

*Reaction conditions are magnesium concentration and annealing temperature (number of cycles, if not 30) for amplifications of individual SNPs. Final multiplex reaction conditions can be found in section 3.3.2.1.3 (page 112). For rs1042047, allele specific primers could not be optimised and reaction conditions for sequencing are unknown (conducted by another researcher). F=forward primer(s), R=reverse primer(s).

SNP	CEU freq	SIBS freq	HWE χ^2	HWE *p*	Additional Notes
CCKBR					
rs906895	0.458	0.478	0.145	>0.9	One Mendelian error found
rs2929180	0.179	0.196	0.381	>0.5	
rs11040819	0.233	0.199			
rs2941025	0.233	0.199	1.696	>0.1	
rs2929183	0.275	0.255	<0.01	>0.995	
rs2941023	0.236	0.254	0.017	>0.975	
rs2947025	0.217	0.233	0.067	>0.9	
rs1396860	0.198	0.222	2.779	>0.1	
rs4758402	0.042	0.058	0.444	>0.5	Some difficulty genotyping in multiplex → many samples inferred or repeated.
rs7943852	0.233	0.28	2.683	>0.1	Initially part of multiplex - removed due to amplification problems (possible primer degradation).
rs2941029	0.467	0.481	0.538	>0.5	
rs3793993	0.178	0.222			
rs2880898	0.175	0.199	3.751	>0.1	
rs1112716	0.018	0.196	3.410	>0.1	Freq different to HapMap → confirmed by sequencing.
rs12801747	0.175	0.199			
rs11040823	0.175	0.199			
rs11040824	0.175	0.199			
rs11040825	0.175	0.199			
rs11040826	0.175	0.199			
rs11040831	0.175	0.199			
rs11040832	0.175	0.199			
rs11040833	0.175	0.199			
rs2947029	0.492	0.469	2.346	>0.1	
rs2947028	0.3	0.273	0.178	>0.9	
rs2947027	0.233	0.201	1.936	>0.1	
rs3901667	0.042	0.057	0.398	>0.5	
rs1805002	0.042	0.057			
rs12364575	0.217	0.294	1.335	>0.5	
rs1042047	0.345	0.309	**44.78**	**<0.01**	Nine Mendelian errors, sequencing confirmed poor genotyping accuracy.
rs1042048	0.317	0.31	0.612	>0.5	Two Mendelian errors found.

CEU freq and SIBS freq = allele frequencies in the HapMap CEU and SIBS cohorts. HWE: results of Hardy-Weinberg equilibrium tests; χ^2 = Chi squared value (empty spaces indicate a SNP that was not genotyped or analysed directly); *p* = probability that deviation may be found by chance based on Chi square table for two degrees of freedom (Griffiths *et al.*, 2000), <0.05 was considered statistically significant and is in bold.

ADORA2A

SNP	Location - GRCh37	Genotyping Method(s)	Probe Order Number
	Chromosome 22q11.23		
rs3761422	Intron; 24826672 bp	TaqMan probe	C__2446666_20
rs2267076	Intron; 24830595 bp	TaqMan probe	C__2446667_1
rs5751876	Exon 3; 24837301 bp	TaqMan probe	Custom*

* Forward primer = CACCCGCCAGGAGTGT; Reverse primer = GGGCACTCCCTCCACTCA; Reporter sequences = (VIC) CCAGGGCATAGCCAT and (FAM) CAGGGCGTAGCCAT. All identical to those used by Hohoff et al.

			HWE		
SNP	CEU freq	SIBS freq	χ^2	*p*	Additional Notes
rs3761422	0.379	0.412	0.014	>0.975	C to T. Hohoff et al. found T=0.16
rs2267076	0.336	0.369	0.013	>0.975	C to T. Hohoff et al. found T=0.13
rs5751876	-	0.417	0.032	>0.975	Not genotyped in HapMap CEU. C to T. Hohoff et al. found T=0.17

CEU freq and SIBS freq = allele frequencies in the HapMap CEU and SIBS cohorts. HWE: results of Hardy-Weinberg equilibrium tests; χ^2 = Chi squared value (empty spaces indicate a SNP that was not genotyped or analysed directly); *p* = probability that deviation may be found by chance based on Chi square table for two degrees of freedom (Griffiths *et al.*, 2000), <0.05 was considered statistically significant and is in bold.

Appendix C – CCK System Association Results

SNP	SIBS freq.	Based on	Part of Haplotype	Any Panic				
				Fam.	Trans.	Z	*p*-value	Perm. *p*
CCK SNPs								
rs8192472	0.303			28	-0.176	-0.049	0.961	0.994
rs11129949	0.116			25	-1.933	-0.662	0.508	1
rs4602358	0.436		Y	33	1.229	0.317	0.752	1
rs10865918	0.421		Y	38	-5.162	-1.160	0.246	1
rs11571849	0.436	rs4602358		33	1.229	0.317	0.752	1
rs1868522	0.436	rs4602358		33	1.229	0.317	0.752	1
rs754635	0.146	rs1799923		21	4.000	1.419	0.156	1
rs747456	0.421	rs10865918		38	-5.162	-1.160	0.246	1
rs747455	0.237		Y	30	0.117	0.033	0.974	1
rs1799923	0.146			21	4.000	1.419	0.156	1
rs11571842	0.436	rs4602358		33	1.229	0.317	0.752	1
rs13069836	0.436	rs4602358		33	1.229	0.317	0.752	1
CCK haplotypes								
GGG	0.401			37	-6.497	-1.511	0.131	1
ATG	0.240			31	1.330	0.380	0.704	1
ATA	0.204			27	0.089	0.031	0.975	1
GTG	0.150			21	4.441	1.658	0.097	1
CCKAR SNPs								
rs11726159	0.077			12	-0.983	-0.5	0.691	1
rs2725302	0.189			22	2.683	0.833	0.405	1
rs915889	0.088			14	-0.817	-0.397	0.691	1
rs7665027	0.094		Y	13	2.333	1.460	0.144	1
rs2000978	0.179		Y	21	5.250	2.020	0.043	1
rs2725307	0.190	rs2725302		22	2.683	0.833	0.405	1
rs2040342	0.179	rs6448456		24	4.917	1.811	0.070	1
rs3822222	0.094	rs7665027		13	2.333	1.460	0.144	1
rs1800855	0.191		Y	21	2.183	0.686	0.492	1
rs6448456	0.188			24	4.917	1.811	0.070	1
CCKAR haplotypes								
ATT	0.647			35	-7.518	-2.008	0.045	0.963
ACT	0.144			22	5.446	2.106	0.035	1
ATA	0.111			14	-0.341	-0.150	0.881	1
GTA	0.085			12	1.704	0.938	0.348	1

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over- or under-transmissions. Z = Z-score generated by FBAT. Perm. *p* = *p*-value after 10 000 permutations by Haploview.

	BP no Panic				BPD			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCK SNPs								
rs8192472	29	-5.745	-1.546	0.122	41	-10.555	-2.342	0.019
rs11129949	25	-4.083	-1.417	0.156	34	-3.433	-0.927	0.354
rs4602358	34	-1.564	-0.47	0.638	52	-3.119	-0.686	0.493
rs10865918	34	-3.152	-0.843	0.399	55	-3.998	-0.82	0.412
rs11571849	34	-1.564	-0.470	0.638	52	-3.119	-0.686	0.493
rs1868522	34	-1.564	-0.470	0.638	52	-3.119	-0.686	0.493
rs754635	21	2.850	-0.910	0.363	32	6.683	1.577	0.115
rs747456	34	-3.152	-0.843	0.399	55	-3.998	-0.82	0.412
rs747455	31	-3.300	-1.049	0.294	47	-6.55	-1.489	0.136
rs1799923	21	2.850	-0.910	0.363	32	6.683	1.577	0.115
rs11571842	34	-1.564	-0.470	0.638	52	-3.119	-0.686	0.493
rs13069836	34	-1.564	-0.470	0.638	52	-3.119	-0.686	0.493
CCK haplotypes								
GGG	37	-3.114	-0.828	0.408	56	-5.437	-1.132	0.258
ATG	33	-3.464	-1.116	0.264	50	-6.63	-1.539	0.124
ATA	23	3.134	1.109	0.267	40	3.627	0.968	0.333
GTG	20	3.424	1.080	0.280	31	7.788	1.883	0.060
CCKAR SNPs								
rs11726159	14	-1.317	-0.692	0.489	17	-1.633	-0.753	0.452
rs2725302	19	-3.317	-1.147	0.252	27	0.033	0.011	0.991
rs915889	16	-1.150	-0.581	0.561	17	-1.633	-0.753	0.452
rs7665027	15	-4.833	-2.018	0.044	21	-1.167	-0.468	0.640
rs2000978	26	3.483	1.197	0.231	37	8.817	2.485	0.013
rs2725307	19	-3.317	-1.147	0.252	27	0.033	0.011	0.991
rs2040342	25	1.317	0.459	0.646	37	6.317	1.791	0.073
rs3822222	15	-4.833	-2.018	0.044	21	-1.167	-0.468	0.640
rs1800855	20	-2.817	-0.960	0.337	28	-0.467	-0.15	0.881
rs6448456	25	1.317	0.459	0.646	37	6.317	1.791	0.073
CCKAR haplotypes								
ATT	34	2.764	0.792	0.428	55	-4.672	-1.102	0.270
ACT	25	2.903	1.054	0.292	38	7.766	2.26	0.024
ATA	13	-0.212	-0.109	0.913	16	-0.975	-0.473	0.636
GTA	16	-4.951	-1.961	0.050	23	-2.826	-1.083	0.279

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	Anxiety				Panic+Phobia			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCK SNPs								
rs8192472	33	2.945	0.696	0.486	20	-1.010	-0.322	0.748
rs11129949	26	-0.967	-0.278	0.781	17	-1.183	-0.488	0.626
rs4602358	40	7.914	1.632	0.103	20	-2.521	-0.824	0.410
rs10865918	45	-11.164	-2.049	0.040	24	-1.412	-0.408	0.683
rs11571849	40	7.914	1.632	0.103	20	-2.521	-0.824	0.410
rs1868522	40	7.914	1.632	0.103	20	-2.521	-0.824	0.410
rs754635	24	3.667	1.03	0.303	14	5.167	2.343	0.019
rs747456	45	-11.164	-2.049	0.040	24	-1.412	-0.408	0.683
rs747455	41	0.533	0.115	0.909	20	-2.550	-0.900	0.368
rs1799923	24	3.667	1.03	0.303	14	5.167	2.343	0.019
rs11571842	40	7.914	1.632	0.103	20	-2.521	-0.824	0.410
rs13069836	40	7.914	1.632	0.103	20	-2.521	-0.824	0.410
CCK haplotypes								
GGG	41	-11.883	-2.261	0.024	23	-3.170	-0.963	0.336
ATG	40	1.23	0.267	0.789	21	-2.330	-0.839	0.401
ATA	32	4.948	1.522	0.128	15	-0.010	-0.005	0.996
GTG	24	5.054	1.411	0.158	14	4.847	2.277	0.023
CCKAR SNPs								
rs11726159	15	-3.3	-1.583	0.113	<10			
rs2725302	23	1.45	0.465	0.642	14	3.350	1.258	0.208
rs915889	16	-2.633	-1.247	0.212	10	2.350	1.510	0.131
rs7665027	15	2.417	1.216	0.224	<10			
rs2000978	27	5.15	1.726	0.084	12	0.833	0.493	0.622
rs2725307	23	1.45	0.465	0.642	14	3.350	1.258	0.208
rs2040342	29	2.983	0.963	0.336	15	0.500	0.268	0.788
rs3822222	15	2.417	1.216	0.224	<10			
rs1800855	22	1.95	0.633	0.527	14	2.350	0.883	0.377
rs6448456	29	2.983	0.963	0.336	15	0.500	0.268	0.788
CCKAR haplotypes								
ATT	43	-5.249	-1.279	0.201	23	-3.434	-1.137	0.256
ACT	28	5.604	1.879	0.060	13	0.862	0.524	0.600
ATA	17	-2.093	-0.974	0.330	11	2.081	1.125	0.261
GTA	15	1.463	0.74	0.459	<10			

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	Panic+Substance				Panic+BP			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCK SNPs								
rs8192472	13	-0.250	-0.095	0.924	20	-1.476	-0.507	0.612
rs11129949	11	-0.683	-0.461	0.645	19	-0.517	-0.202	0.840
rs4602358	15	-0.317	-0.111	0.912	23	-1.138	-0.359	0.720
rs10865918	15	-1.750	-0.680	0.496	30	-2.595	-0.732	0.464
rs11571849	15	-0.317	-0.111	0.912	23	-1.138	-0.359	0.720
rs1868522	15	-0.317	-0.111	0.912	23	-1.138	-0.359	0.720
rs754635	11	1.667	0.822	0.411	16	3.833	1.527	0.127
rs747456	15	-1.750	-0.680	0.496	30	-2.595	-0.732	0.464
rs747455	14	1.583	0.685	0.493	24	-3.417	-1.147	0.251
rs1799923	11	1.667	0.822	0.411	16	3.833	1.527	0.127
rs11571842	15	-0.317	-0.111	0.912	23	-1.138	-0.359	0.720
rs13069836	15	-0.317	-0.111	0.912	23	-1.138	-0.359	0.720
CCK haplotypes								
GGG	15	-1.924	-0.734	0.463	29	-4.073	-1.192	0.233
ATG	14	1.963	0.824	0.410	26	-2.832	-0.964	0.335
ATA	10	-2.244	-1.265	0.206	22	1.742	0.702	0.482
GTG	11	2.201	1.206	0.228	16	4.531	1.862	0.063
CCKAR SNPs								
rs11726159	<10				<10			
rs2725302	11	1.100	0.603	0.547	16	2.850	1.267	0.205
rs915889	<10				10	-0.233	-0.138	0.890
rs7665027	<10				10	2.417	1.823	0.068
rs2000978	<10				14	5.083	2.235	0.025
rs2725307	11	1.100	0.603	0.547	16	2.850	1.267	0.205
rs2040342	10	1.833	0.836	0.403	17	4.750	1.975	0.048
rs3822222	<10				10	2.417	1.823	0.068
rs1800855	10	0.600	0.642	0.733	16	1.850	0.822	0.411
rs6448456	10	1.833	0.836	0.403	17	4.750	1.975	0.048
CCKAR haplotypes								
ATT	16	-2.990	-1.302	0.193	27	-7.186	-2.556	0.011
ACT	<10				15	5.113	2.281	0.023
ATA	<10				11	-0.263	-0.145	0.885
GTA	<10				<10			

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	PD				PA			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCK SNPs								
rs8192472	11	0.917	0.355	0.723	16	-0.117	-0.045	0.964
rs11129949	11	1.983	0.888	0.374	16	-2.833	-1.485	0.137
rs4602358	14	-0.233	-0.078	0.938	17	1.867	0.773	0.440
rs10865918	17	-0.667	-0.205	0.838	20	-3.400	-1.197	0.231
rs11571849	14	-0.233	-0.078	0.938	17	1.867	0.773	0.440
rs1868522	14	-0.233	-0.078	0.938	17	1.867	0.773	0.440
rs754635	<10				12	2.000	1.200	0.231
rs747456	17	-0.667	-0.205	0.838	20	-3.400	-1.197	0.231
rs747455	12	-1.000	-0.401	0.689	17	0.950	0.403	0.687
rs1799923	<10				12	2.000	1.200	0.231
rs11571842	14	-0.233	-0.078	0.938	17	1.867	0.773	0.440
rs13069836	14	-0.233	-0.078	0.938	17	1.867	0.773	0.440
CCK haplotypes								
GGG	16	-1.753	-0.554	0.579	20	-3.659	-1.346	0.178
ATG	11	0.467	0.201	0.840	19	0.686	0.280	0.779
ATA	10	0.253	0.129	0.897	15	0.418	0.193	0.847
GTG	<10				12	1.907	1.163	0.245
CCKAR SNPs								
rs11726159	<10				<10			
rs2725302	10	1.267	0.636	0.525	10	0.083	0.060	0.952
rs915889	<10				<10			
rs7665027	<10				<10			
rs2000978	<10				15	3.333	1.715	0.086
rs2725307	10	1.267	0.636	0.525	10	0.083	0.060	0.952
rs2040342	<10				16	3.000	1.521	0.128
rs3822222	<10				<10			
rs1800855	<10				10	0.083	0.060	0.952
rs6448456	<10				16	3.000	1.521	0.128
CCKAR haplotypes								
ATT	14	-2.748	-1.291	0.197	18	-2.937	-1.405	0.160
ACT	<10				16	3.337	1.697	0.090
ATA	<10				<10			
GTA	<10				<10			

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	PD/PA				PA/PS			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCK SNPs								
rs8192472	23	0.800	0.238	0.812	18	-0.283	-0.104	0.917
rs11129949	23	-0.850	-0.347	0.728	17	-3.083	-1.475	0.140
rs4602358	28	1.633	0.452	0.651	18	1.200	0.479	0.632
rs10865918	34	-4.067	-0.989	0.322	23	-3.900	-1.306	0.192
rs11571849	28	1.633	0.452	0.651	18	1.200	0.479	0.632
rs1868522	28	1.633	0.452	0.651	18	1.200	0.479	0.632
rs754635	18	2.500	1.115	0.265	14	3.000	1.511	0.131
rs747456	34	-4.067	-0.989	0.322	23	-3.900	-1.306	0.192
rs747455	28	-0.050	-0.014	0.988	17	1.283	0.529	0.597
rs1799923	18	2.500	1.115	0.265	14	3.000	1.511	0.131
rs11571842	28	1.633	0.452	0.651	18	1.200	0.479	0.632
rs13069836	28	1.633	0.452	0.651	18	1.200	0.479	0.632
CCK haplotypes								
GGG	33	-5.412	-1.370	0.171	23	-4.148	-1.446	0.148
ATG	29	1.153	0.339	0.735	20	1.030	0.410	0.682
ATA	23	0.672	0.263	0.793	16	-0.593	-0.254	0.800
GTG	18	2.940	1.442	0.149	14	3.075	1.488	0.137
CCKAR SNPs								
rs11726159	11	-0.733	-0.396	0.692	<10			
rs2725302	19	1.350	0.533	0.594	13	2.083	1.121	0.262
rs915889	13	-0.567	-0.291	0.771	<10			
rs7665027	10	1.417	1.069	0.285	<10			
rs2000978	20	4.583	1.857	0.063	16	4.000	1.897	0.058
rs2725307	19	1.350	0.533	0.594	13	2.083	1.121	0.262
rs2040342	23	4.250	1.642	0.101	17	3.667	1.718	0.086
rs3822222	10	1.417	1.069	0.285	<10			
rs1800855	18	0.850	0.342	0.732	13	2.083	1.121	0.262
rs6448456	23	4.250	1.642	0.101	17	3.667	1.718	0.086
CCKAR haplotypes								
ATT	31	-5.685	-1.879	0.060	22	-5.604	-2.192	0.028
ACT	21	4.779	1.947	0.051	17	4.004	1.881	0.060
ATA	13	-0.374	-0.183	0.855	<10			
GTA	<10				<10			

Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

SNP	SIBS freq.	Based on	Part of Haplotype	Any Panic Fam.	Trans.	Z	*p*-value	Perm. *p*
CCKBR SNPs								
rs906895	0.478		Y	33	1.571	0.389	0.697	1
rs2929180	0.196		Y	30	7.605	2.08	0.038	0.284
rs11040819	0.199	rs2941025		32	8.271	2.311	0.021	0.124
rs2941025	0.199		Y	32	8.271	2.311	0.021	0.124
rs2929183	0.255		Y	34	8.871	2.215	0.027	0.614
rs2941023	0.254		Y	34	8.871	2.215	0.027	0.614
rs2947025	0.233		Y	31	6.705	1.770	0.077	0.770
rs1396860	0.222			28	-7.417	-2.325	0.020	0.958
rs4758402*	0.058			<10				1
rs7943852	0.28			27	-4.333	-1.229	0.219	0.770
rs2941029	0.481			32	2.488	0.637	0.524	1
rs3793993	0.222	rs1396860		28	-7.417	-2.325	0.020	0.958
rs2880898	0.199		Y	25	-6.083	-2.142	0.032	0.997
rs1112716	0.196			24	-5.583	-1.998	0.046	0.997
rs12801747	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040823	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040824	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040825	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040826	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040831	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040832	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs11040833	0.199	rs2880898		25	-6.083	-2.142	0.032	0.997
rs2947029	0.469			37	5.321	1.272	0.203	1
rs2947028	0.273		Y	36	10.955	2.595	0.009	0.572
rs2947027	0.201			30	7.355	2.097	0.036	0.058
rs3901667*	0.057			<10				1
rs1805002*	0.057	rs3901667		<10				1
rs12364575	0.294			32	8.769	2.168	0.030	0.863
rs1042047	0.434			18	3.000	1.225	0.221	
rs1042048	0.31			36	-1.933	-0.432	0.665	1
CCKBR haplotypes								
AGCAAGTT	0.509			38	-3.801	-0.918	0.359	0.997
GGCAAGCT	0.186			25	-8.439	-2.687	0.007	0.963
GTTGGCTC	0.158			26	5.695	1.704	0.088	1

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Removed due to genotyping errors - results presented are those of a subset that were sequenced. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT. Perm. *p* = *p*-value after 10 000 permutations by Haploview.

	BP no Panic				BPD			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCKBR SNPs								
rs906895	38	3.681	0.735	0.462	59	3.836	0.657	0.511
rs2929180	31	0.714	0.253	0.800	46	4.952	1.303	0.193
rs11040819	29	0.048	0.017	0.987	45	3.786	0.985	0.324
rs2941025	29	0.048	0.017	0.987	45	3.786	0.985	0.324
rs2929183	31	0.464	0.143	0.886	50	4.719	1.070	0.285
rs2941023	30	-0.536	-0.174	0.862	49	3.719	0.865	0.387
rs2947025	27	0.131	0.044	0.965	45	4.386	1.068	0.285
rs1396860	28	2.583	0.546	0.585	40	1.083	0.217	0.828
rs4758402*	16	-0.083	-0.045	0.964	19	0.683	0.313	0.755
rs7943852	36	1.750	0.358	0.720	45	0.917	0.182	0.855
rs2941029	35	3.431	0.631	0.528	53	6.086	1.032	0.302
rs3793993	28	2.583	0.546	0.585	40	1.083	0.217	0.828
rs2880898	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs1112716	26	1.167	0.254	0.800	38	0.667	0.141	0.888
rs12801747	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040823	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040824	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040825	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040826	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040831	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040832	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs11040833	26	1.167	0.254	0.800	39	0.167	0.035	0.972
rs2947029	37	1.431	0.263	0.793	57	5.336	0.878	0.380
rs2947028	32	0.714	0.197	0.844	51	5.219	1.115	0.265
rs2947027	34	2.631	0.836	0.403	47	5.452	1.333	0.183
rs3901667*	16	-0.400	-0.218	0.827	18	0.867	0.399	0.690
rs1805002*	16	-0.400	-0.218	0.827	18	0.867	0.399	0.690
rs12364575	32	4.407	1.160	0.246	50	11.81	2.554	0.011
rs1042047	16	1.333	0.483	0.629				
rs1042048	42	1.000	0.264	0.791	58	1.117	0.231	0.817
CCKBR haplotypes								
AGCAAGTT	33	-2.179	-0.390	0.697	53	-5.349	-0.872	0.383
GGCAAGC	22	2.781	0.648	0.517	34	-0.319	-0.067	0.947
GTTGGCTC	20	-0.900	-0.340	0.734	35	3.730	1.046	0.296

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Removed due to genotyping errors - results presented are those of a subset that were sequenced. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	Anxiety				Panic+Phobia			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCKBR SNPs								
rs906895	37	2.669	0.614	0.539	21	-0.679	-0.223	0.824
rs2929180	34	8.402	2.221	0.026	18	2.771	1.273	0.203
rs11040819	33	8.402	2.218	0.020	18	3.271	1.396	0.163
rs2941025	33	8.402	2.218	0.020	18	3.271	1.396	0.163
rs2929183	35	8.486	2.18	0.029	19	3.455	1.340	0.180
rs2941023	35	8.486	2.18	0.029	19	3.455	1.340	0.180
rs2947025	33	6.819	1.842	0.065	19	2.955	1.216	0.224
rs1396860	29	-5.833	-1.701	0.089	13	-4.333	-2.018	0.044
rs4758402*	14	2.000	0.953	0.340				
rs7943852	32	-2.367	-0.525	0.600	15	-2.500	-0.962	0.336
rs2941029	34	2.836	0.669	0.504	19	0.321	0.106	0.915
rs3793993	29	-5.833	-1.701	0.089	13	-4.333	-2.018	0.044
rs2880898	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs1112716	27	-5.667	-1.801	0.072	12	-3.333	-1.754	0.079
rs12801747	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040823	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040824	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040825	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040826	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040831	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040832	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs11040833	28	-6.167	-1.935	0.053	12	-3.333	-1.754	0.079
rs2947029	38	5.002	1.078	0.281	21	1.571	0.501	0.616
rs2947028	36	10.819	2.494	0.013	21	5.205	1.830	0.067
rs2947027	31	7.902	2.26	0.024	17	3.021	1.297	0.195
rs3901667*	12	2.833	1.471	0.141				
rs1805002*	12	2.833	1.471	0.141				
rs12364575	37	6.626	1.635	0.102	20	2.269	0.901	0.367
rs1042048	49	-2.683	-0.497	0.619	25	-1.017	-0.333	0.739
CCKBR haplotypes								
AGCAAGTT	43	-6.051	-1.242	0.214	23	-0.684	-0.212	0.832
GGCAAGC	29	-7.910	-2.185	0.029	13	-4.567	-2.210	0.027
GTTGGCTC	29	6.575	1.866	0.062	16	3.049	1.279	0.201

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	Panic+Substance				Panic+BP			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCKBR SNPs								
rs906895	15	1.475	0.629	0.529	25	0.905	0.290	0.772
rs2929180	<10				22	3.405	1.460	0.144
rs11040819	<10				21	2.905	1.275	0.202
rs2941025	<10				21	2.905	1.275	0.202
rs2929183	12	3.267	1.545	0.122	24	2.838	0.972	0.331
rs2941023	12	3.267	1.545	0.122	24	2.838	0.972	0.331
rs2947025	12	3.267	1.545	0.122	22	1.838	0.648	0.517
rs1396860	10	-1.667	-0.828	0.408	16	-1.167	-0.510	0.610
rs4758402*								
rs7943852	11	-0.917	-0.415	0.678	16	-1.333	-0.575	0.565
rs2941029	14	1.750	0.675	0.500	23	1.655	0.561	0.575
rs3793993	10	-1.667	-0.828	0.408	16	-1.167	-0.51	0.610
rs2880898	<10				15	-1.917	-0.911	0.362
rs1112716	<10				14	-1.417	-0.693	0.488
rs12801747	<10				15	-1.917	-0.911	0.362
rs11040823	<10				15	-1.917	-0.911	0.362
rs11040824	<10				15	-1.917	-0.911	0.362
rs11040825	<10				15	-1.917	-0.911	0.362
rs11040826	<10				15	-1.917	-0.911	0.362
rs11040831	<10				15	-1.917	-0.911	0.362
rs11040832	<10				15	-1.917	-0.911	0.362
rs11040833	<10				15	-1.917	-0.911	0.362
rs2947029	16	3.000	1.208	0.227	27	3.155	0.943	0.346
rs2947028	14	4.267	1.914	0.056	25	4.421	1.394	0.163
rs2947027	<10				20	2.655	1.173	0.241
rs3901667*								
rs1805002*								
rs12364575	10	4.200	1.959	0.050	25	5.569	1.635	0.102
rs1042048	16	-3.133	-1.215	0.224	28	-0.633	-0.188	0.851
CCKBR haplotypes								
AGCAAGTT	16	-2.444	-0.921	0.357	27	-2.880	-0.870	0.384
GGCAAGC	11	-2.471	-1.129	0.259	17	-3.473	-1.535	0.125
GTTGGCTC	<10				19	2.808	1.207	0.228

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	PD				PA			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCKBR SNPs								
rs906895	14	1.167	0.417	0.677	19	0.500	0.188	0.851
rs2929180	<10				19	3.533	1.522	0.128
rs11040819	10	2.167	1.237	0.216	20	4.700	1.832	0.067
rs2941025	10	2.167	1.237	0.216	20	4.700	1.832	0.067
rs2929183	14	3.350	1.400	0.162	19	4.117	1.563	0.118
rs2941023	14	3.350	1.400	0.162	19	4.117	1.563	0.118
rs2947025	13	2.683	1.167	0.243	18	3.117	1.279	0.201
rs1396860	13	-0.833	-0.398	0.690	13	-4.333	-2.082	0.037
rs4758402*								
rs7943852	14	0.750	0.295	0.768	13	-3.583	-1.704	0.088
rs2941029	13	1.667	0.591	0.554	17	0.750	0.305	0.760
rs3793993	13	-0.833	-0.398	0.690	13	-4.333	-2.082	0.037
rs2880898	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs1112716	11	-0.583	-0.289	0.772	11	-3.333	-2.820	0.029
rs12801747	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040823	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040824	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040825	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040826	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040831	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040832	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs11040833	11	-0.583	-0.289	0.772	12	-3.833	-2.385	0.017
rs2947029	14	3.167	1.106	0.269	18	2.250	0.848	0.396
rs2947028	15	3.850	1.575	0.115	19	5.533	1.972	0.049
rs2947027	<10				19	3.283	1.306	0.192
rs3901667*								
rs1805002*								
rs12364575	13	4.200	1.709	0.087	17	1.283	0.479	0.632
rs1042048	18	-1.967	-0.700	0.484	20	-1.300	-0.492	0.622
CCKBR haplotypes								
AGCAAGTT	15	-1.870	-0.668	0.504	19	-2.054	-0.760	0.447
GGCAAGC	13	-2.173	-0.971	0.331	12	-4.294	-2.186	0.029
GTTGGCTC	<10				16	2.945	1.309	0.191

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

	PD/PA				PA/PS			
SNP	**Fam.**	**Trans.**	**Z**	***p*-value**	**Fam.**	**Trans.**	**Z**	***p*-value**
CCKBR SNPs								
rs906895	29	1.667	0.430	0.667	21	-0.667	-0.239	0.811
rs2929180	26	6.200	1.793	0.073	21	3.867	1.550	0.121
rs11040819	28	6.867	2.034	0.042	22	5.033	1.848	0.065
rs2941025	28	6.867	2.034	0.042	22	5.033	1.848	0.065
rs2929183	30	7.467	1.952	0.051	21	4.450	1.597	0.110
rs2941023	30	7.467	1.952	0.051	21	4.450	1.597	0.110
rs2947025	28	5.800	1.597	0.110	20	3.450	1.326	0.185
rs1396860	24	-5.167	-1.806	0.071	17	-7.083	-2.789	0.005
rs4758402*								
rs7943852	23	-2.833	-0.869	0.385	16	-5.583	-2.283	0.022
rs2941029	27	2.417	0.646	0.518	19	-0.750	-0.294	0.769
rs3793993	24	-5.167	-1.806	0.071	17	-7.083	-2.789	0.005
rs2880898	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs1112716	20	-3.917	-1.617	0.106	14	-5.500	-2.741	0.006
rs12801747	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040823	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040824	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040825	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040826	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040831	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040832	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs11040833	21	-4.417	-1.786	0.074	15	-6.000	-2.901	0.004
rs2947029	29	5.417	1.388	0.165	22	0.083	0.029	0.977
rs2947028	31	9.383	2.359	0.018	21	5.533	1.840	0.066
rs2947027	26	5.950	1.803	0.071	21	3.617	1.352	0.176
rs3901667*								
rs1805002*								
rs12364575	27	5.483	1.508	0.132	20	4.783	1.513	0.130
rs1042048	35	-3.267	-0.783	0.434	20	-0.300	-0.110	0.912
CCKBR haplotypes								
AGCAAGTT	31	-3.924	-1.001	0.317	23	-0.360	-0.125	0.900
GGCAAGC	22	-6.467	-2.225	0.026	15	-6.266	-2.728	0.006
GTTGGCTC	23	4.823	1.520	0.128	18	3.245	1.344	0.179

*Freq too low to analyse at Any Panic. Blue indicates SNP was not analysed directly and data was inferred from another SNP. Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

Appendix D – *ADORA2A* Association Results

SNP	SIBS freq.	Phenotype	Empirical Correction			
			Fam.	Trans.	Z	*p*-value
rs3761422	0.412	PD/PA	33	0.63	0.18	0.85
		Panic with Agoraphobia	14	-1.75	-0.66	0.51
rs2267076	0.369	PD/PA	31	1.10	0.35	0.73
		Panic with Agoraphobia	14	-2.08	-0.84	0.4
rs5751876	0.417	PD/PA	33	-0.07	-0.02	0.98
		Panic with Agoraphobia	15	-2.08	-0.78	0.44

SNP	SIBS freq.	Phenotype	Maximum Families			
			Fam.	Trans.	Z	*p*-value
rs3761422	0.412	PD/PA	47	2.41	0.74	0.46
		Panic with Agoraphobia	42	2.82	1.05	0.29
rs2267076	0.369	PD/PA	41	2.98	1.13	0.26
		Panic with Agoraphobia	37	0.75	0.37	0.71
rs5751876	0.417	PD/PA	36	-0.34	-0.14	0.89
		Panic with Agoraphobia	52	1.81	0.56	0.57

Fam. = number of informative families. Trans. = number of over or undertransmissions. Z = Z-score generated by FBAT.

Appendix E – RNA and cDNA Measurements

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Long A – SK-N-DZ					
4 days	Untreated	1.90	483	1.61	623
	Fluoxetine	1.99	319	1.63	630
	Paroxetine	1.96	428	1.63	608
	Imipramine	1.96	353	1.63	644
	Gabapentin	1.94	449	1.64	615
15 days	Untreated	2.09	764	1.59	667
	Fluoxetine	1.78	568	1.57	669
	Paroxetine	1.94	465	1.58	659
	Imipramine	1.98	104	1.71	744
	Gabapentin	1.91	576	1.59	660
21 days	Untreated	2.07	713	1.56	703
	Fluoxetine	2.02	834	1.52	656
	Paroxetine	1.92	462	1.52	628
	Imipramine	2.05	745	1.59	701
	Gabapentin	1.99	1011	1.57	709
28 days	Untreated	2.00	1370	1.42	712
	Fluoxetine	2.03	1175	1.60	871
	Paroxetine	2.09	942	1.52	910
	Imipramine	2.06	1335	1.42	702
	Gabapentin	2.01	1284	1.41	710

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl).

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Long A – SK-N-SH					
5 days	Untreated	1.92	443	1.83	980
	Fluoxetine	2.01	260	1.83	989
	Paroxetine	2.00	267	1.82	1031
	Imipramine	1.92	423	1.83	977
	Gabapentin	2.01	205	1.83	1017
12 days	Untreated	1.95	445	1.60	687
	Fluoxetine	2.10	193	1.58	693
	Paroxetine	2.10	200	1.58	687
	Imipramine	1.94	456	1.60	679
	Gabapentin	2.09	493	1.58	687
26 days	Untreated	1.95	474	1.57	695
	Fluoxetine	2.04	283	1.58	691
	Paroxetine	1.99	410	1.56	697
	Imipramine	2.08	845	1.58	689
	Gabapentin	1.87	518	1.57	695
31 days	Untreated	2.04	214	1.57	696
	Fluoxetine	2.05	172	1.57	692
	Paroxetine	2.04	252	1.57	686
	Imipramine	2.03	179	1.56	692
	Gabapentin	2.04	219	1.57	685

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl).

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Long A – SH-SY5Y					
4 days	Untreated*	2.11	365	1.77	938
	Fluoxetine*	2.11	299	1.77	920
11 days	Untreated*	2.01	309	1.78	931
	Fluoxetine*	2.03	276	1.77	927
16 days	Untreated*	1.96	336	1.83	1028
	Fluoxetine*	1.97	300	1.83	1088
23 days	Untreated*	1.97	323	1.65	575
	Fluoxetine*	1.99	327	1.67	554
28 days	Untreated*	2.04	462	1.77	945
	Fluoxetine*	2.03	369	1.77	1126
7 days	Untreated	2.02	311	1.59	689
	Paroxetine	2.04	254	1.58	746
	Imipramine	2.00	307	1.59	682
15 days	Untreated	1.83	545	1.59	690
	Paroxetine	2.08	216	1.59	671
	Imipramine	2.03	330	1.59	655
24 days	Untreated	2.02	326	1.63	708
	Paroxetine	1.88	525	1.63	694
	Imipramine	2.00	416	1.62	718
32 days	Untreated	2.04	366	1.64	665
	Paroxetine	2.05	229	1.64	696
	Imipramine	2.05	311	1.62	689
3 days	Untreated*	2.05	713	1.74	451
	Gabapentin*	2.01	491	1.70	426
7 days	Untreated*	2.05	643	1.76	466
	Gabapentin*	2.00	404	1.76	569
12 days	Untreated*	1.90	208	1.81	459
	Gabapentin*	2.06	272	1.71	602
17 days	Untreated*	2.11	365	1.77	938
	Gabapentin*	2.10	325	1.78	913
24 days	Untreated*	2.01	309	1.78	931
	Gabapentin*	1.99	229	1.78	903
29 days	Untreated*	1.96	336	1.83	1028
	Gabapentin*	1.96	172	1.83	1079

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl); *Long A cells that were grown as part of a previous experiment.

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Long B – SK-N-DZ					
7 days	Untreated	1.89	467	1.55	703
	Fluoxetine	1.94	354	1.55	701
	Paroxetine	1.85	489	1.75	857
	Imipramine	1.86	483	1.76	872
20 days	Untreated	1.89	487	1.60	685
	Fluoxetine	2.01	221	1.55	699
	Paroxetine	2.00	281	1.58	684
	Imipramine	1.99	323	1.57	697
29 days	Untreated	1.95	409	1.60	678
	Fluoxetine	2.01	197	1.56	697
	Paroxetine	2.01	244	1.58	681
	Imipramine	2.00	227	1.56	697
Long B – SK-N-SH					
8 days	Untreated	2.09	114	1.59	677
	Fluoxetine	2.07	81	1.57	675
	Paroxetine	2.11	90	1.58	676
	Imipramine	2.14	99	1.58	690
18 days	Untreated	2.08	95	1.59	666
	Fluoxetine	2.08	40	1.58	669
	Paroxetine	2.03	80	1.59	631
	Imipramine	2.05	96	1.58	643
28 days	Untreated	2.05	151	1.62	633
	Fluoxetine	2.07	58	1.61	634
	Paroxetine	2.05	109	1.62	679
	Imipramine	2.05	138	1.60	648

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl).

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Long B – SH-SY5Y					
9 days	Untreated	1.96	329	1.61	677
	Fluoxetine	1.99	235	1.60	678
	Paroxetine	1.96	304	1.62	667
	Imipramine	1.98	334	1.60	678
19 days	Untreated	1.96	322	1.61	657
	Fluoxetine	1.99	149	1.65	667
	Paroxetine	1.97	318	1.61	671
	Imipramine	1.95	326	1.61	664
28 days	Untreated	1.97	274	1.62	674
	Fluoxetine	1.95	70	1.62	662
	Paroxetine	1.96	220	1.61	648
	Imipramine	1.98	172	1.60	676
Short – SK-N-DZ					
6 hours	Untreated	2.09	1175	1.84	905
	Fluoxetine	2.09	753	1.85	912
	Paroxetine	2.10	847	1.86	895
	Imipramine	2.18	903	1.87	923
	Gabapentin	2.09	831	1.84	916
24 hours	Untreated	1.89	509	1.89	905
	Fluoxetine	2.10	751	1.87	890
	Paroxetine	1.95	484	1.89	908
	Imipramine	1.94	477	1.87	923
	Gabapentin	1.86	541	1.91	921
48 hours	Untreated	1.97	433	1.58	725
	Fluoxetine	2.01	371	1.56	725
	Paroxetine	1.96	460	1.56	732
	Imipramine	1.91	504	1.56	725
	Gabapentin	1.89	522	1.55	729

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl).

Duration	Treatment	RNA Rat.	RNA Quan.	cDNA Rat.	cDNA Quan.
Short – SK-N-SH					
6 hours	Untreated	1.90	502	1.75	927
	Fluoxetine	1.97	449	1.76	921
	Paroxetine	1.97	447	1.75	915
	Imipramine	1.96	472	1.75	928
	Gabapentin	1.94	472	1.75	927
24 hours	Untreated	1.90	495	1.86	899
	Fluoxetine	1.95	452	1.76	895
	Paroxetine	1.91	493	1.75	895
	Imipramine	1.86	535	1.81	882
	Gabapentin	1.90	506	1.78	893
48 hours	Untreated	1.94	473	1.76	904
	Fluoxetine	1.95	465	1.77	887
	Paroxetine	1.97	433	1.77	884
	Imipramine	1.95	460	1.77	886
	Gabapentin	1.88	511	1.75	894
Short – SH-SY5Y					
6 hours	Untreated	2.00	774	1.82	756
	Fluoxetine	2.06	721	1.81	893
	Paroxetine	2.07	715	1.81	900
	Imipramine	1.84	569	1.83	940
	Gabapentin	2.04	1088	1.59	718
24 hours	Untreated	2.07	901	1.86	893
	Fluoxetine	2.06	862	1.81	800
	Paroxetine	1.83	566	1.83	920
	Imipramine	2.08	753	1.81	893
	Gabapentin	2.07	981	1.82	885
48 hours	Untreated	2.07	874	1.82	888
	Fluoxetine	1.88	534	1.82	926
	Paroxetine	2.07	734	1.82	860
	Imipramine	2.06	734	1.82	894
	Gabapentin	2.06	824	1.82	894

RNA Rat. = 260/280 nm ratio (optimum is 2.0); RNA Quan. = quantity of RNA (ng/µl); cDNA Rat. = 260/280 nm ratio (optimum is 1.8); cDNA Quan. = quantity of cDNA (ng/µl).

Appendix F – qPCR Primers

Grey font colour indicates design conducted by another researcher or as part of another project.

Gene	Full Name	Gene Location	Selection Criteria
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	12p13	Ref.
HMBS	hydroxymethylbilane synthase	11q23.3	Ref.
HPRT1	hypoxanthine phosphoribosyltransferase 1	Xq26.1	Ref.
MPRIP	myosin phosphatase Rho interacting protein	17p11.2	Ref.
POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	22q13.1	Ref.
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	5p15	Ref.
UBC	ubiquitin C	12q24.3	Ref.
ADAM22	ADAM metallopeptidase domain 22	7q21	7q21
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	7q21	7q21
FZD1	frizzled homolog 1 (Drosophila)	7q21	7q21
GRM3	glutamate receptor, metabotropic 3	7q21	7q21
LMTK2	lemur tyrosine kinase 2	7q21	7q21
SRI	sorcin	7q21	7q21
TAC1	tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma)	7q21	7q21
ADORA2A	adenosine A2a receptor	22q11.23	Function
BDNF	brain-derived neurotrophic factor	11p13	Function
CCK	cholecystokinin	3p22-p21.3	Function
CCKAR	cholecystokinin A receptor	4p15.1-p15.2	Function
CCKBR	cholecystokinin B receptor	11p15.4	Function
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	6p21.2	Function
CRHR2	corticotropin releasing hormone receptor 2	7p14.3	Function
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	5q34	Function
GABRA2	gamma-aminobutyric acid (GABA) A receptor, alpha 2	4p12	Function
GABRA3	gamma-aminobutyric acid (GABA) A receptor, alpha 3	Xq28	Function
NPY	neuropeptide Y	7p15.1	Function
NPY1R	neuropeptide Y receptor Y1	4q31.3-q32	Function
TPH2	tryptophan hydroxylase 2	12q21.1	Function

Gene	Forward Primer	Reverse Primer
GAPDH	GTGAAGGTCGGAGTCAACGG	CCATTGATGACAAGCTTCaCG
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
MPRIP	ATCTCAGCCATCGAAGCCAT	TGGCTCTTCTCCAGCTCCC
POLR2F	CCCGAAAGATCCCCATCAT	CACCCCCCAGTCTTCATAGC
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
UBC	ATTTGGGTCGCgGTTCTTG	TGCCTTGACATTCTCGATGGT
ADAM22	TTGCAACACTTACTTCCCTCAC	GTGCCAGCAATTATGCCT
CACNA2D1	TGACGGTCCAAATCCTTG	CACAGGGAGGGATTTAATCC
FZD1	ATACGGATGCCTGGCAAC	GCTGTCTTGTTAAGGAAAGCAA
GRM3	AGACTGCACCTCAACAGGT	TCACAGAGATGAGGTGGTG
LMTK2	GACACTCCCTCTGTTCAGTCC	GGGCAACTTGAGACTTCAATC
SRI	AGCTTTCGAAGACGGGATACT	GCTCCAGTTGGAATGTTGATTAC
TAC1	TGGCTTATGAAAGGAGTGC	ACCTGTCATTGCCCATTG
ADORA2A	TGACCGCTACATTGCCATC	CTGACCGCAGTTGTTCCA
BDNF	AGCGTGTGTGACAGTATTAGTG	AGAAGTATTGCTTCAGTTGGCC
CCK	CTGGCAAGATACATCCAGCAG	tCCGGTCACTTATCCTGTGG
CCKAR	ATGGATGTGGTTGACAGCCTT	TACAAGAGAATCTGCACCGCT
CCKBR	GCCAGACCTGGTCCGTACT	TCACTGTCGCCGTCAAAG
CDKN1A	GGTGGACCTGGAGACTCTCA	GATTAGGGCTTCCTCTTGGAGA
CRHR2	TACTGCAACACGACCTTGGA	TCCAAGCATTCTCGATAGGC
GABRA1	GTGGTTGTAGCAGAAGATGGAT	TCAAGTGGAAATGAGTGGTC
GABRA2	GCCATGGACTGGTTTATTG	GCATAAGCGTTGTTCTGTATCA
GABRA3	GCACTGATTGAATTTGCCAC	ACGATGTTGAAGGTAGTGCTG
NPY	CGACACTACATCAACCTCATCAC	GGGAACATTTTCTGTGCTTTC
NPY1R	TCCAAAGAGGATTGTTCAGTTC	AGCAGGTCAACTGTTCAGCTT
TPH2	GACTCCTTTCCTCCATTGGA	GCATTCCTGTAAGCAAGTTG

Lowercase indicates a wobble.

Gene	Genomic Size (bp)	cDNA Size (bp)	cDNA Fragment Location
GAPDH	1925	203	Between exons 2+4.
HMBS	3252	64	Between exons 1+2.
HPRT1	-	94	Between exons 6+7 (third and fourth to last exons).
MPRIP	68	68	Multiple splice variants. Fragment in middle of long variants, absent in shortest variants, F primer spans two exons.
POLR2F	65	65	In coding region of 3' exon.
SDHA	877	86	Between exons 2+3.
UBC	2769	133, 361, etc	Primers in different exons. Multiple splice variants.
ADAM22	2295	105	Between exons 19+20 (towards 3' end).
CACNA2D1	-	132	Between last two exons.
FZD1	138	138	(Only one exon in gene.)
GRM3	-	129	Between last two exons.
LMTK2	-	118	Between exons 3+4 (middle of gene).
SRI	2131	129	Between last two exons.
TAC1	103	103	Within last exon.
ADORA2A	-	145	Between last two exons.
BDNF	130	130	Within last exon.
CCK	-	103	Between last two exons. Amplifies both splice variants.
CCKAR	814	143	Between exons 1+2.
CCKBR	-	124	Between exons 3+4 (towards 3' end).
CDKN1A	1308	104	Between last two exons. Multiple splice variants amplified.
CRHR2	-	140	Between exons 2+3.
GABRA1	-	124	Between exons 7+8 (second and third to last exons).
GABRA2	-	170, 350	Between exons 8+9 (last two).
GABRA3	-	143	Between exons 9+10 (last two).
NPY	-	105	Between exons 2+3 (middle of gene).
NPY1R	-	103	Between exons 1+2.
TPH2	-	95	Between last two exons. Amplifies both splice variants.

Reference Genes Used for Each Set of cDNA:

	SK-N-DZ		SK-N-SH		SH-SY5Y	
	Long A + Short	**Long B**	**Long A + Short**	**Long B**	**Long A + Short**	**Long B**
GAPDH	✓		✓	✓	✓	✓
HMBS	✓	✓	✓			✓
HPRT1		✓	✓	✓	✓	✓
MPRIP						
POLR2F		✓		✓		✓
SDHA	✓	✓			✓	
UBC	✓		✓	✓	✓	