PHARMACORESISTANCE IN EPILEPSY:

AN INTEGRATIVE GENETIC & GENOMIC ANALYSIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part, for any degree or other qualification.

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This research was carried out in the Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, the University of Liverpool.

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CONTENTS

Abstract

Pharmacoresistance in Epilepsy: An Integrative Genetic & Genomic Analysis

Nasir Mirza

Epilepsy effects up to 1% of the population, and up to 30% of people with epilepsy are pharmacoresistant—they continue to experience seizures despite treatment with maximal doses of multiple antiepileptic drugs. The causes of drug resistance in epilepsy remain poorly understood. In this work, I have used genetic and genomic analysis techniques to explore the causes of epilepsy pharmacoresistance.

It has been reported that epilepsy pharmacoresistance results from impaired drug penetration into the epileptic focus secondary to a localized dysregulation of drug transporters. Solute carrier (SLC) transporters form the largest superfamily of multidrug transporters. I used novel *in silico* and stringent *ex vivo* strategies for identifying the SLCs that are dysregulated in the pharmacoresistant epileptic human hippocampus. I discovered that the SLCs dysregulated in the pharmacoresistant epileptic human hippocampus are either small metal ion exchangers or transporters of neurotransmitters, not antiepileptic drug transporters, and most likely contribute to pharmacoresistance by enhancing the intrinsic severity of epilepsy. This finding supports the newly-proposed and intuitive 'intrinsic severity hypothesis' of epilepsy pharmacoresistance.

According to the intrinsic severity hypothesis, pharmacoresistance in epilepsy results from the increased dysfunction of the biological pathways which underlie epilepsy. Hence, I proceeded to perform genome-wide genetic and genomic analyses in order to find the most important pathways underlying epilepsy and pharmacoresistance in epilepsy. I performed an integrative analysis of previously published large-scale gene expression profiling studies on brain tissue from epilepsy surgery; the largest and most robust microarray analysis of brain tissue from surgery for pharmacoresistant mesial temporal lobe epilepsy; and the first-ever genome-wide association study (GWAS) of pharmacoresistant focal epilepsy. By integrating the results of the genetic and genomic studies, I was able to show that pharmacoresistance is the result of accumulation of deleterious genetic variants of increasing severity and/or numbers within the genes that constitute the core pathways underlying epilepsy. I also found that the pathways disrupted in pharmacoresistant epilepsy, at both the genetic and genomic levels, belong to many different diverse and disparate functional domains, for example 'axon guidance', 'transmembrane transport of small molecules' and 'cell death signalling via NRAGE, NRIF and NADE'. However, using network analysis techniques, I showed that these seemingly unrelated pathways form a coherent highly interconnected network, and it can be expected that changes in one pathway in this network will have a cascading effect on the rest of the network. The most important pathways in these networks are the central 'hub' pathways, which I identified using betweenness centrality network analysis.

I then performed the first-ever genetical genomics study in epilepsy using hippocampal samples from resective surgery for refractory mesial temporal lobe epilepsy. By integrating genome-wide genetic, genetical genomic and genomic studies, and then performing pathway, network and centrality analysis, I identified the most important putative central *causal* pathways underlying epilepsy pharmacoresistance: 'transmembrane transport of small molecules' and 'Deleted in colorectal cancer (DCC) mediated attractive signalling'.

In conclusion, by performing genome-wide genetic, genetical genomic and genomic studies, followed by integrative analysis, pathway construction and network mapping, I have identified most important putative central causal pathways underlying epilepsy pharmacoresistance.

Publication

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Presentations

- Mirza N., Vasieva O., Marson A.G., Pirmohamed M. (April 2012) Using Contrasting Bioinformatics Approaches to Prioritize Multidrug Transporters Underlying Pharmacoresistance in Epilepsy. Platform presentation at the ILAE Annual Meeting, London, UK.
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Abbreviations

ABC	Adenosine triphosphate-binding cassette
AD	Alzheimer's disease
AED	Antiepileptic drug
BCRP	Breast cancer resistance protein
CCK	Cholecystokinin
CCR1	Chemokine receptor 1
CETA	Concentration equilibrium transport assay
CFG	Convergent functional genomic
CGP	Computational gene prioritization
CLB	Clobazam
CNS	Central nervous system
DAGs	Disease-associated genes
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCC	Deleted in colorectal cancer receptor
DEGs	Differentially-expressed genes
DICER	Differential Correlation in Expression for meta-module Recovery
DSCAM	Down syndrome cell adhesion molecule
EAE	Experimental autoimmune encephalomyelitis
EASE	Expression analysis systematic explorer
EDP	Extreme discordant phenotype
ENCODE	Encyclopedia of DNA Elements
eQTL	Expression quantitative trait loci
FDR	False discovery rate
FGF	Fibroblast growth factor
GABA	δ-Aminobutyric acid
GO	Gene ontology
GPCR	G protein-coupled receptors
GSCA	Gene set co-expression analysis
GWAS	Genome-wide association study
HS	Hippocampal sclerosis
IAC	Inter-array correlation
IBE	International Bureau for Epilepsy
ILAE	International league against epilepsy
ISVA	Independent surrogate variable analysis
JNK	C-JUN N-terminal Kinase
LOOA	Leave-one-out analysis
MAF	Minor allele frequency
MAQC	Microarray Quality Control
MAS5	Microarray suite 5.0
MaTLE	Mass-associated temporal lobe epilepsy
MDT	Multidrug transporter
MIAME	Minimum Information About a Microarray Experiment
MTLE	Mesial temporal lobe epilepsy
NADE	P75ntr-associated Cell Death Executor

NGF	Nerve growth factor
NPC	Normal population controls
NRAGE	Neurotrophin receptor–interacting MAGE homolog
NRIF	Neurotrophin receptor-interacting factor
OC	Overlap coefficient
OHSC	Organotypic hippocampal slice culture
p75NTR	P75 neurotrophin receptor
PC	Principal component
PET	Positron emission tomography
PIP	Polymorphisms-in-probes
PTLE	Paradoxical temporal lobe epilepsy
PwP	Probes-with-polymorphisms
QC	Quality control
QQ	Quantile-quantile
RIF	Regulatory impact factor
RIN	RNA Integrity number
SANAD	Standard and New Antiepileptic Drugs
SLC	Solute carrier
SNPs	Single nucleotide polymorphism
SVA	Surrogate variable analysis
TLE	Temporal lobe epilepsy
TW	Tracy-Widom
VGB	Vigabatrin
VGCC	Voltage-gated calcium channels
vGLUT1	Vesicular glutamate transporter isoform 1
WGCNA	Weighted Gene Co-expression Network Analysis
WTCCC	Wellcome trust case control consortium

CHAPTER ONE

INTRODUCTION

Contents

Chapter 1: Introduction

In this introductory chapter, I will define epilepsy; review its burden on the individual and society; explain its pharmacological treatment; describe the problem of resistance to this treatment; and list the features and prognostic indicators of, and explore possible causes underlying, this drug resistance. I will then define and describe the methodology of integrative genetics and genomic analysis and explain why this approach is needed in order to understand pharmacoresistance in epilepsy.

1.1 What is epilepsy?

In 2005, a Task Force of the International League Against Epilepsy (ILAE) formulated conceptual definitions of 'seizure' and 'epilepsy' (Fisher *et al.* 2005). An epileptic seizure was defined as 'a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain'. Epilepsy was defined as 'a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiologic, cognitive, psychological, and social consequences of this condition'. The definition of epilepsy required the occurrence of at least one epileptic seizure.

More recently, the ILAE commissioned a Task Force to formulate an operational definition of epilepsy for purposes of clinical diagnosis (Fisher *et al.* 2014). In December of 2013, the ILAE Executive Committee adopted the recommendations as a position of the ILAE. According to this definition, epilepsy is a disease of the brain defined by any of the following conditions

1. At least two unprovoked seizures occurring >24 hours apart.
2. One unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years.
3. Diagnosis of an epilepsy syndrome.

Seizure types are divided into two broad categories: (1) generalised or (2) focal (also termed partial or localization-related). Generalized seizures are conceptualized as those that originate at some point within, and rapidly engage bilaterally distributed networks (Berg *et*

al. 2010). Focal seizures originate in networks limited to one cerebral hemisphere (Berg *et al.* 2010).

The behavioural manifestations of seizures are protean. This is especially true among focal seizures where the clinical features vary based on the cortical location of the seizure onset zone. For example, possible features of mesial temporal lobe epilepsy (TLE), which is commonly caused by hippocampal sclerosis (neuronal cell loss and gliosis in the hippocampus), include mnestic, gustatory and olfactory sensations. TLE is the most frequent type of refractory partial epilepsy and considered the prototype of surgically remediable epilepsies (Mayanagi *et al.* 1996).

Table 1.1 Incidences of epileptic syndromes (per 100,000 person years) in three published studies

Type	Syndrome	(Loiseau *et al.* 1990)	(Zarrelli *et al.* 1999)	(Olafsson *et al.* 2005)
Focal epilepsies	Total	15.3	34.9	18.6
	Idiopathic	1.7	0.2	1.6
	Symptomatic	13.6	17.2	8.4
	Cryptogenic	-	17.5	8.6
Generalised epilepsies	Total	7.2	7.7	3.9
	Idiopathic	6.1	3.7	3.1
	Cryptogenic	1.1	1.7	0.7
	Symptomatic	-	2.3	0.1

1.2 Prevalence and burden of epilepsy

Epilepsy is the most common neurological disorder after stroke: 65 million people have epilepsy worldwide (Ngugi *et al.* 2010). The prevalence of epilepsy varies by population. In developed countries, the prevalence is around 700 per 100,000 (Hirtz *et al.* 2007). In low and middle income countries, the prevalence is generally higher. For example, in Ethiopia, a developing country, the prevalence of epilepsy is as high as 29.5 per 1000 (95% confidence interval 20.5 to 40.9 per 1000) (Ngugi *et al.* 2010; Thurman *et al.* 2011). There are 2.6 million people with epilepsy in Europe (Olesen *et al.* 2012), and 400,000 new cases each year (Forsgren *et al.* 2005).

The global disease burden from epilepsy, measured as disability-adjusted life years, is higher than that for Alzheimer's disease and other dementias, multiple sclerosis, and Parkinson's disease combined (Murray *et al.* 2012). The global disease burden from epilepsy is greater than that of breast cancer for women, and nearly four-times greater than that of prostate cancer for men (Murray *et al.* 2012). In Europe, the total cost of epilepsy per year is €13.8 billion (Olesen *et al.* 2012).

At the individual level, the consequences of epilepsy can be severe and include shortened lifespan, physical injury, neuropsychological and psychiatric sequelae, and social and financial disadvantage (Sperling 2004).

1.3 Pharmacological treatment of epilepsy

Currently available antiepileptic drugs (AEDs) aim to achieve symptom control, i.e., suppression of seizures, but have no known impact on the underlying pathophysiology of epilepsy. A seizure is the clinical manifestation of a hyperexcitable neuronal network, in which the electrical balance underlying normal activity is pathologically altered. Effective seizure treatment generally augments inhibitory processes or opposes excitatory processes.

The serendipitous discovery of phenobarbital in 1912 marked the beginning of the modern pharmacotherapy of epilepsy (Yasiry & Shorvon 2012). In the 100 years since, many new AEDs have been discovered (Table 1.2). Their mechanisms of action (Table 1.2) fall into a number of general categories: the main groups include sodium channel blockers, calcium current inhibitors, δ-aminobutyric acid (GABA) enhancers, and glutamate blockers. However, the mode of action of some AEDs falls outside these broad categories. Also, many AEDs possess multiple mechanisms of action. Finally, the primary mode of action of some AEDs remains to be discovered (Guimaraes & Ribeiro 2010).

A proportion of patients with epilepsy are pharmacoresistant (see section 1.4 below): they continue to experience seizures despite treatment with maximal doses of multiple AEDs with different molecular targets and mechanisms of actions. For such patients, surgical treatment options may be considered, including surgical resection of the epileptic focus (for

example the epileptic hippocampus in mesial temporal lobe epilepsy), vagal nerve stimulation (Englot *et al.* 2011), and deep brain stimulation (Lega *et al.* 2010).

Table 1.2 AEDs: table listing year in which the AED was first approved or marketed in the US or Europe, and its mode of action. Data is from (Schmidt & Schachter 2014).

AED	Year	Presumed mechanism of action
Potassium bromide	1857	GABA potentiation?
Phenobarbital	1912	GABA potentiation
Phenytoin	1938	Na^+ channel blocker
Primidone	1954	GABA potentiation
Ethosuximide	1958	T-type Ca^{2+} channel blocker
Diazepam	1963	GABA potentiation
Carbamazepine	1964	Na^+ channel blockade
Valproate	1967	Multiple, including: GABA potentiation, glutamate (NMDA) inhibition, sodium channel and T-type calcium channel blockade
Clonazepam	1968	GABA potentiation
Clobazam	1975	GABA potentiation
Vigabatrin	1989	GABA potentiation
Lamotrigine	1990	Na^+ channel blocker
Oxcarbazepine	1990	Na^+ channel blocker
Gabapentin	1993	Ca^{2+} channel blocker (α2d subunit)
Topiramate	1995	Multiple, including: GABA potentiation, glutamate (AMPA) inhibition, sodium and calcium channel blockade
Levetiracetam	2000	SV2A modulation
Zonisamide	2000	Na^+ channel blocker
Stiripentol	2002	GABA potentiation, Na^+ channel blocker
Pregabalin	2004	Ca^{2+} blocker (α2d subunit)
Rufinamide	2004	Na^+ channel blocker
Lacosamide	2008	Enhanced slow inactivation of voltage gated Na^+ channels
Eslicarbazepine	2009	Na^+ channel blocker
Perampanel	2012	Glutamate (AMPA) antagonist

1.4 Pharmacoresistance in epilepsy

The Task Force of the ILAE Commission on Therapeutic Strategies has defined the minimum criteria for drug resistant epilepsy as the 'failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom' (Kwan *et al.* 2010). To be regarded as an adequate trial, the AEDs must be "appropriate" for the patient's epilepsy and seizure

type, and must be administered at therapeutic doses for sufficient lengths of time. Seizure freedom is defined as freedom from seizures for a minimum of three times the longest pre-intervention inter-seizure interval (determined from seizures occurring within the past 12 months) or 12 months, whichever is longer. This definition is based on observational cohort studies of newly diagnosed epilepsy in adults (Kwan & Brodie 2000; Mohanraj & Brodie 2006) and children (Arts *et al.* 2004) which suggest that once two appropriately trialled AEDs have proven to be inefficacious, the probability of achieving seizure freedom with subsequent AED treatments is minimal. Some recent studies appear to suggest that a proportion of these patients may still become seizure-free with subsequent drug manipulation (Callaghan *et al.* 2007; Luciano & Shorvon 2007), but these studies were retrospective, and did not take into account the reasons for failure which may have included inappropriately chosen AEDs or suboptimal treatment schedules. A recent report from a prospective study in children documented that although many patients who had failed two adequate trials of AEDs had periods of seizure freedom with further drug trials, lasting remission remained elusive (Berg *et al.* 2006).

~30% of epilepsy patients are pharmacoresistant (Shorvon 1996; Tellez-Zenteno *et al.* 2014).

1.5 Prognostic factors for pharmacoresistance in epilepsy

A recent and comprehensive systematic review has summarized the evidence for independent prognostic factors for pharmacoresistant epilepsy derived from eleven published cohort studies (Wassenaar *et al.* 2013). Significant prognostic factors were symptomatic aetiology, focal seizures, younger age at onset, a high initial seizure frequency, epileptic EEG abnormalities, and several clinical items, such as a history of febrile seizures, status epilepticus, and a neurodevelopmental delay

It is important to note that prognostic factors are not necessarily causally related to the outcome (Tripepi *et al.* 2008). Prognostic research is aimed at predicting the risk (that is the probability) of disease without any concern about causality. In contrast, aetiological research aims to investigate the causal relationship between putative determinants and a given disease. In the next section, we consider different theories which have been put forward in order to explain the development of pharmacoresistance in epilepsy.

1.6 Aetiology of pharmacoresistance in epilepsy

Three distinct hypotheses have been put forward to explain the development of pharmacoresistance in epilepsy:

1. The multidrug transporter (MDT) hypothesis.
2. The drug target hypothesis.
3. The intrinsic severity hypothesis.

The merits and demerits of each hypothesis are discussed below. It is important to note, however, that these hypotheses are not mutually exclusive, and may act in an integrated manner to produce pharmacoresistance (Schmidt & Loscher 2009).

1.6.1 The MDT hypothesis

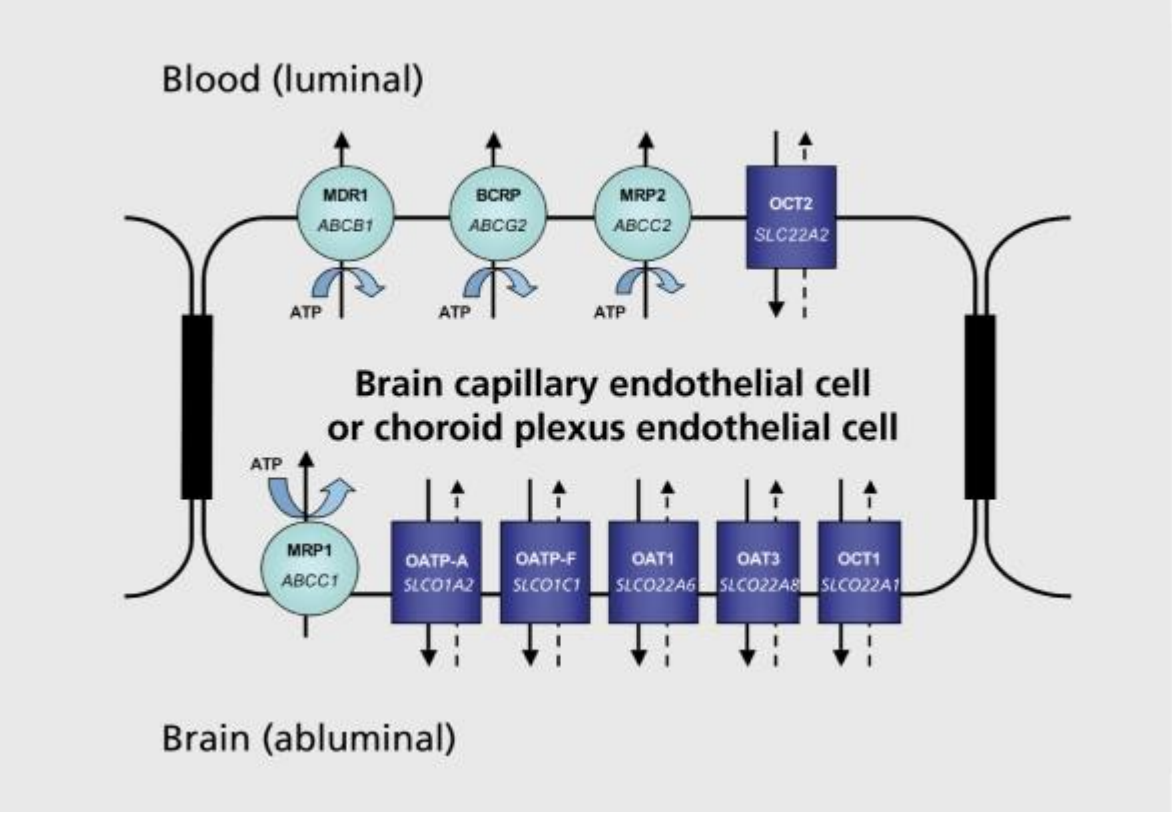


Figure 1.1 Examples of ABC and SLC transporters at the blood-brain barrier (Giardin 2006). ABC transporters require energy from ATP hydrolysis to actively remove compounds from the cell. In contrast, for many (though not all) SLCs the transport mechanism is based upon anion exchange. BCRP: breast cancer resistance protein; MDR1: multidrug resistance protein 1, also known as P-gp; MRP: multidrug resistance protein; OATP: organic anion transporting protein; OCT: organic cation transporter.

Over the last two decades, the most popular and extensively studied hypothesis of epilepsy pharmacoresistance has been the MDT hypothesis (Chayasirisobhon 2009). According to this hypothesis, pharmacoresistance results from impaired drug penetration into the epileptic focus secondary to a localized dysregulation of drug transporters (Chayasirisobhon 2009). MDTs have been implicated in pharmacoresistance in a number of diseases. The MDTs involved in drug resistance in humans are either adenosine triphosphate-binding cassette (ABC) proteins (Tiwari *et al.* 2011) or solute carrier (SLC) proteins (Figure 1.1) (Huang & Sadee 2006). There are over 450 known ABC and SLC proteins in total. However, research on the MDT hypothesis in epilepsy has been focused almost exclusively one ABC transporter: *ABCB1* or P-glycoprotein (P-gp).

1.6.1.1 Studies suggesting increased expression or function of P-gp in epilepsy

P-gp was first suspected of playing a role in pharmacoresistance in 1979 when its expression was found to correlate with resistance to cancer chemotherapy in Chinese hamster ovary cells (Riordan & Ling 1979). In 1995, P-gp expression was shown to be increased (Tishler *et al.* 1995) in epileptic foci resected during brain surgery for intractable epilepsy. Since then, the overexpression of P-gp in epileptogenic lesions resected during brain surgery for refractory epilepsy was shown to be a common feature of different pathologies associated with drug-resistant epilepsy (Aronica *et al.* 2012), for example focal malformations of cortical development, hippocampal sclerosis, and tuberous sclerosis.

Other data from studies on people with drug-resistant epilepsy have also been cited in support of a role for P-gp in epilepsy pharmacoresistance. Post-mortem analysis of tissue from pharmacoresistant temporal lobe epilepsy patients showed a significantly higher percentage area of P-gp-immunopositive labelling in the sclerotic epileptogenic hippocampus than in the contralateral hippocampus (Liu *et al.* 2012). A significant inverse linear correlation was found between the brain-to-plasma concentration ratio of an active metabolite of oxcarbazepine and the level of brain expression of the *ABCB1* mRNA (Marchi *et al.* 2005).

The results of a recent positron emission tomography (PET) imaging study (Feldmann *et al.* 2013) are suggestive of higher P-gp activity in some brain regions for pharmacoresistant patients than for seizure-free temporal lobe epilepsy patients. PET revealed reduced uptake

of the radio-labelled form of verapamil, a P-gp substrate, in pharmacoresistant compared with seizure-free patients. Administration of P-gp inhibitor tariquidar led to an increase in the uptake of the radio-labelled substrate. However, it should be noted that the purported P-gp overactivity was also seen in areas outside the epileptic focus (for example the contralateral hippocampus), which is contrary to the MDT hypothesis, and the response to tariquidar was more pronounced in normal subjects than in the refractory epilepsy patients with purported P-gp overactivity.

The results of some studies on animal models of epilepsy are also consistent with a possible role of P-gp in epilepsy pharmacoresistance. Kainic acid-induced limbic seizures transiently increase P-gp expression in the mouse hippocampus (Rizzi *et al.* 2002). Pharmacoresistant epileptic rats exhibit higher endothelial P-gp expression in limbic regions ipsilateral to the seizure focus than do pharmacosensitive rats (Volk & Loscher 2005). Brain/plasma ratio of phenytoin was significantly lower in brain regions that had P-gp overexpression (temporal hippocampus and parahippocampal cortex) in chronic epileptic rats than in the same brain regions in non-epileptic rats, and administration of P-gp inhibitor tariquidar to chronic epileptic rats significantly increased the phenytoin brain/plasma ratio in these brain regions (van Vliet *et al.* 2007). Also, studies utilising brain microdialysis in rats have shown that carbamazepine (Ma *et al.* 2013) and phenytoin (Potschka & Loscher 2001; Ma *et al.* 2013) levels in cerebral tissue extracellular fluid increase significantly after the administration of P-gp inhibitors. Knock-out mice lacking P-gp protein show significant increase in phenytoin and carbamazepine concentrations in the hippocampus compared with wild-type mice (Rizzi *et al.* 2002). Co-administration of P-gp inhibitor tariquidar to phenobarbital-resistant rats restored the antiepileptic activity of phenobarbital without altering plasma pharmacokinetics of the antiepileptic drug (Brandt *et al.* 2006). Similarly, in the electrical post–status epilepticus rat model for temporal lobe epilepsy, co-administration of tariquidar improved the anticonvulsive action of phenytoin without altering plasma pharmacokinetics of the antiepileptic drug (van Vliet *et al.* 2006).

Although the above findings are consistent with a possible role for P-gp in mediating pharmacoresistance in epilepsy, there is also a large body of compelling evidence (discussed below) which suggests that P-gp is unlikely to play a major role in the development of drug resistance in epilepsy.

1.6.1.2 Does P-gp transport AEDs?

Table 1.3 AEDs shown not to be substrates for human P-gp

AED	References
Carbamazepine	(Owen *et al.* 2001; Mahar Doan *et al.* 2002; Weiss *et al.* 2003; Crowe & Teoh 2006; Baltes *et al.* 2007b; Feng *et al.* 2008; Luna-Tortos *et al.* 2008; Zhang *et al.* 2011; Dickens *et al.* 2013)
Diazepam	(Cucullo *et al.* 2007; Feng *et al.* 2008)
Ethosuximide	(Crowe & Teoh 2006; Feng *et al.* 2008; Zhang *et al.* 2010)
Gabapentin	(Weiss *et al.* 2003; Crowe & Teoh 2006)
Lacosamide	(Zhang *et al.* 2013)
Lamotrigine	(Weiss *et al.* 2003; Crowe & Teoh 2006; Feng *et al.* 2008; Dickens *et al.* 2013)
Levetiracetam	(Baltes *et al.* 2007b)
Midazolam	(Feng *et al.* 2008)
Phenobarbitone	(Crowe & Teoh 2006)
Phenytoin	(Weiss *et al.* 2003; Crowe & Teoh 2006; Baltes *et al.* 2007b; Feng *et al.* 2008)
Pregabalin	(Chan *et al.* 2014)
Rufinamide	(Chan *et al.* 2014)
Topiramate	(Weiss *et al.* 2003; Crowe & Teoh 2006)
Valproic acid	(Weiss *et al.* 2003; Baltes *et al.* 2007a)
Vigabatrin	(Crowe & Teoh 2006)
Zonisamide	(Chan *et al.* 2014)

If the overexpression of P-gp truly underlies pharmacoresistance in epilepsy, then many (if not all) AEDs must be substrates for this transporter. Although some studies have demonstrated the transport of a small number of AEDs by P-gp in animal models (Zhang *et al.* 2012), this transport was very weak. For vincristine, a classic P-gp substrate, inhibition of P-gp resulted in a 9-fold increase in brain uptake (Drion *et al.* 1996). In comparison, P-gp inhibition only increased the uptake of phenytoin, carbamazepine, phenobarbital, lamotrigine, and felbamate, 0.5- to 1.1-fold over baseline (Potschka *et al.* 2001; Potschka & Loscher 2001; Potschka *et al.* 2002). In the *ABCB1* knockout mouse model, brain uptake of classic P-gp substrates vinblastine, cyclosporine, and digoxin increased 20- to 50-fold (Schinkel *et al.* 1994; Schinkel *et al.* 1995). In comparison, there was only a 2-fold increase for topiramate, and no increase in brain uptake for phenytoin, phenobarbital, carbamazepine, or lamotrigine (Sills *et al.* 2002). Therefore, the effect of P-gp on the brain uptake of AEDs in animal models is barely measurable (Anderson & Shen 2007). In addition,

there are inter-species differences in the substrate specificity of P-gp (Baltes *et al.* 2007b), and evidence for the lack of P-gp-mediated AED transport is even stronger in human studies. Numerous studies employing many different experimental models have repeatedly demonstrated that the most commonly prescribed AEDs are not substrates for human P-gp (Table 1.3).

Is there any evidence that human P-gp is able to transport AEDs? Cucullo and colleagues (Cucullo *et al.* 2007) demonstrated weak transport of phenytoin by P-gp in an *in vitro* human epileptic blood-brain barrier model. However, other studies using the well-established hCMEC/D3 human blood-brain barrier model (Dickens *et al.* 2013); Caco-2 monolayers (Crowe & Teoh 2006); and transwell systems of polarized MDCKII dog kidney or LLC-PK1 pig kidney cells transfected with human *ABCB1* (Weiss *et al.* 2003; Baltes *et al.* 2007b; Feng *et al.* 2008) have not found evidence to support transport of phenytoin by human P-gp.

Given the lack of evidence for human P-gp-mediated transport of AEDs in studies using conventional widely accepted and well characterized *in vitro* assays, some researchers have recently employed a new *in vitro* system called concentration equilibrium transport assay (CETA) in an attempt to demonstrate transport of AEDs by human P-gp (Luna-Tortos *et al.* 2008). The authors (Luna-Tortos *et al.* 2008) have argued that as AEDs have high permeability across cell barriers and low affinity for P-gp, diffusion across the cell layer may mask directional transport. In CETA, in order to remove the concentration gradient and thus diffusion, the drug is added at identical concentration to both sides of a polarized, P-gp-overexpressing cell monolayer, instead of applying the drug to either the apical or basolateral side as in a conventional bi-directional assay. In the CETA system, P-gp-mediated transport has been demonstrated for phenytoin, phenobarbital, lamotrigine and levetiracetam (Luna-Tortos *et al.* 2008), topiramate (Luna-Tortos *et al.* 2009), oxcarbazepine and eslicarbazepine (Zhang *et al.* 2011), and lacosamide (Zhang *et al.* 2013). However, positive results in the CETA system cannot be taken as proof that P-gp-mediated transport of the AED is relevant in pharmacoresistant epilepsy, for the reasons stated below:

1. The design of the CETA system is not an accurate representation of the conditions *in vivo*: the concentration of an actively transported drug is highly unlikely to be identical on both sides of a biological barrier *in vivo* (Dickens *et al.* 2013).

2. In the CETA system, the transport of even the most potently transported AED (topiramate) was approximately 75% less than for the classic P-gp substrate digoxin (Luna-Tortos *et al.* 2009), further demonstrating that AEDs are not strong substrates for P-gp.
3. Given the high permeability of AEDs across cell barriers and their low affinity for P-gp, the lack of AED transport in traditional bi-directional assays but weak transport in the CETA indicates that the passive diffusion component predominates over the active transport, resulting in the absence of significant efflux (Zhang *et al.* 2013).
4. P-gp inhibitor tariquidar only partially inhibited transport of levetiracetam and phenobarbital (Luna-Tortos *et al.* 2008), demonstrating that P-gp was not solely responsible for the modest transport of these AEDs seen in the CETA system.
5. The AED transport demonstrated by Luna-Tortos and colleagues (Luna-Tortos *et al.* 2008) using the CETA system has not been recreated in an independent study, for at least one AED (Dickens *et al.* 2013).

In summary, after many years of intense research efforts, there remains a lack of convincing evidence that AEDs are high-affinity substrates of human P-gp.

1.6.1.3 Is there an association between ABCB1 genotypes and response to AEDs?

In 2003, Siddiqui and colleagues reported a positive association between the ABCB1 C.3435C>T genotype and poor response to AEDs (Siddiqui *et al.* 2003). Subsequent to this, at least 28 independent genetic association studies (Table 1.3) and at least three meta-analyses (Leschziner *et al.* 2007; Bournissen *et al.* 2009; Haerian *et al.* 2010) have failed to find evidence for this association.

Two other *ABCB1* variants (C.2677C>T and C.1236C>T) have also been the subject of genetic association studies in pharmacoresistant epilepsy. For C.2677C>T, approximately 75% of the genetic association studies have been negative, while all the C.1236C>T genetic association studies have been negative (Table 1.4). Haplotypes of the three aforementioned variants have also been analysed and, again, have produced conflicting results (Table 1.4).

Currently, there is no convincing evidence of a genetic association between *ABCB1* variants and response to AEDs. It should be noted that the vast majority of the genetic association studies are underpowered. There is also a high degree of heterogeneity between the

studies, especially in the definition of drug resistance employed, the controls used, types of epilepsy included, and the range of AEDs utilised. There is a need for a well-designed and adequately powered study in order to resolve the controversial question of whether there is an association between *ABCB1* genetic variants and epilepsy pharmacoresistance.

Table 1.4 Summary of published *ABCB1* genotyping studies.

First author	Year	Country	n - T	n - E	Definition - T	Definition - E	Epilepsy type	AEDs	D	A	Gen
C.3435C>T											
Siddiqui	2003	UK	200	115	≥4 seizures in year	≥1 year seizure free	NS	Various	R	Y	CC
Hajnsek	2004	Croatian	30	30	≥4 seizures in year	≥1 year seizure free	Various	Various	R	Y	TT
Tan	2004	USA	401	208	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Hung	2005	Taiwan	108	223	≥10 seizures in year	≥2 year seizure free	Various	Various	R	Y	CC
Sills	2005	UK	230	170	≥1 seizure in a year	≥1 year seizure free	Various	NS	R	N	-
Kim	2006a	Korea	99	100	≥4 seizures in year	≥1 year seizure free	NS	Various	R	N	-
Kim	2006b	Korea	99	108	≥4 seizures in year	≥1 year seizure free	NS	Various	R	N	-
Seo	2006	Japan	126	84	≥1 seizure in a year	≥1 year seizure free	Various	Various	R	Y	TT
Chen	2007	China	50	164	UTD	UTD	UTD	UTD	R	N	-
Ebid	2007	Egypt	63	37	≥1 seizure in 3 months	3 months seizure free	Various	PHT	P	Y	CC
Hung	2007	Taiwan	114	213	≥10 seizures in year	≥2 year seizure free	Various	Various	R	Y	CC
Kwan	2007	China	221	297	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	Y	CC
Leschziner	2007	UK	73	76	≥4 seizures in year or epilepsy surgery	Any patient not fulfilling DRE criteria	NS	Various	R	N	-
Lu	2007	China	72	62	UTD	UTD	UTD	UTD	R	Y	CC
Shahwan	2007	Ireland	124	242	<50% reduction in seizure frequency in year prior to recruitment	≥50% reduction in seizure frequency in the year before recruitment	Various	Various	R	N	-
Dericioglu	2008	Turkey	89	100	Resective surgery for DRE	Healthy volunteers	Partial	Various	R	N	-
Ozgon	2008	Turkey	44	53	≥4 seizures in year	≥1 year seizure free	Various	CBZ	R	N	-
Gao	2009	China	70	62	UTD	UTD	UTD	UTD	R	N	-

First author	Year	Country	n - T	n - E	Definition - T	Definition - E	Epilepsy type	AEDs	D	A	Gen
Kim	2009	Korea	198	193	≥4 seizures in year	≥1 year seizure free	Partial	Various	R	N	-
Kwan	2009	China	194	270	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	N	-
Lakhan	2009	India	94	231	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Szoeke	2009	Australia, Scotland, Hong Kong	208	334	≥1 seizure in 1 year of initial treatment	No seizures over the first year of treatment	Various	Various	P	N	-
Ufer	2009	Germany	118	103	NS	NS	Various	Various	R	N	-
Vahab	2009	India	113	129	<6 months terminal remission with trials of ≥2 AEDs	≥1 year seizure free	Various	Various	R	N	-
Alpman	2010	Turkey	39	92	≥2 seizures while using ≥2 AEDs within a 2-year period	Healthy individuals	Various	Various	R	N	-
Grover	2010	India	95	133	≥1 seizure in 10 months	≥10 months seizure free	Various	Various	R	N	-
Sanchez*	2010	Spain	111	178	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Dong	2011	China	157	193	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Haerian	2011a	Malaysia	323	362	≥1 seizure in a year	≥1 year seizure free	Various	CBZ or VPA	R	N	-
Haerian	2011b	Malaysia	249	256	≥1 seizure in a year	≥1 year seizure free	Various	VPA	R	N	-
Meng	2011	China	24	60	<50% reduction in seizure frequency in the year prior to last follow-up	≥50% reduction in seizure frequency in the year prior to last follow-up	Various	CBZ	R	N	-
Sayyah	2011	Iran	132	200	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	Y	CC

First author	Year	Country	n - T	n - E	Definition - T	Definition - E	Epilepsy type	AEDs	D	A	Gen
Sporis	2011	Croatian	57	48	≥1 seizure per month over the previous year	≥1 year seizure free	Partial	Various	R	N	-
Qu	2012	China	217	320	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Sterjev	2012	Macedonia	68	94	≥4 seizures in year	≥1 year seizure free	Various	CBZ	R	N	-
Subenthiran	2013	Malaysia	162	152	NS	≥1 year seizure free	Complex partial	CBZ	R	Y	TT
Saygi	2014	Turkey	59	60	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Seven	2014	Turkey	69	83	≥4 seizures over a period of 1 year with three AEDs	NS	Various	Various	R	N	-
C.2677C>T											**-**
Hung	2005	Taiwan	108	223	≥10 seizures in year	≥2 year seizure free	Various	Various	R	N	-
Kim	2006	Korea	99	108	≥4 seizures in year	≥1 year seizure free	NS	Various	R	N	-
Seo	2006	Japan	126	84	≥1 seizure in a year	≥1 year seizure free	Various	Various	R	Y	TT
Kwan**	2009	China	194	270	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	Y	TT
Lakhan	2009	India	94	231	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Vahab	2009	India	113	129	<6 months terminal remission with trials of ≥2 AEDs	≥1 year seizure free	Various	Various	R	N	-
Alpman	2010	Turkey	39	92	≥2 seizures while using ≥2 AEDs within a 2-year period	Healthy individuals	Various	Various	R	N	-
Grover	2010	India	95	133	≥1 seizure in 10 months	≥10 months seizure free	Various	Various	R	N	-
Dong	2011	China	157	193	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Haerian	2011	Malaysia	323	362	≥1 seizure in a year	≥1 year seizure free	Various	CBZ/VPA	R	N	-

First author	Year	Country	n - T	n - E	Definition - T	Definition - E	Epilepsy type	AEDs	D	A	Gen
Haerian	2011	Malaysia	249	256	≥1 seizure in a year	≥1 year seizure free	Various	VPA	R	N	-
Meng	2011	China	24	60	<50% reduction in seizure frequency in the year prior to last follow-up	≥50% reduction in seizure frequency in the year prior to last follow-up	Various	CBZ	R	N	-
Sayyah	2011	Iran	132	200	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	Y	-
Subenthiran	2013	Malaysia	162	152	NS	≥1 year seizure free	Complex partial	CBZ	R	Y	TT
Seven	2014	Turkey	69	83	≥4 seizures over a period of 1 year with three AEDs	NS	Various	Various	R	N	-
C.1236C>T											
Hung	2005	Taiwan	108	223	≥10 seizures in year	≥2 year seizure free	Various	Various	R	N	-
Kim	2006	Korea	99	108	≥4 seizures in year	≥1 year seizure free	NS	Various	R	N	-
Seo	2006	Japan	126	84	≥1 seizure in a year	≥1 year seizure free	Various	Various	R	N	-
Lakhan	2009	India	94	231	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Vahab	2009	India	113	129	<6 months terminal remission with trials of ≥2 AEDs	≥1 year seizure free	Various	Various	R	N	-
Grover	2010	India	95	133	≥1 seizure in 10 months	≥10 months seizure free	Various	Various	R	N	-
Dong	2011	China	157	193	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Haerian	2011	Malaysia	323	362	≥1 seizure in a year	≥1 year seizure free	Various	CBZ or VPA	R	N	-
Haerian	2011	Malaysia	249	256	≥1 seizure in a year	≥1 year seizure free	Various	VPA	R	N	-

First author	Year	Country	n - T	n - E	Definition - T	Definition - E	Epilepsy type	AEDs	D	A	Gen
Meng	2011	China	24	60	<50% reduction in seizure frequency in the year prior to last follow-up	≥50% reduction in seizure frequency in the year prior to last follow-up	Various	CBZ	R	N	-
Haplotype* **											-
Zimprich	2004	Austria	****	****	****	****	TLE	Various	R	Y	CGC
Hung	2005	Taiwan	108	223	≥10 seizures in year	≥2 year seizure free	Various	Various	R	Y	-
Kim	2006	Korea	99	108	≥4 seizures in year	≥1 year seizure free	NS	Various	R	N	-
Seo	2006	Japan	126	84	≥1 seizure in a year	≥1 year seizure free	Various	Various	R	Y	-
Leschziner	2007	UK	73	76	≥4 seizures in year or epilepsy surgery	Any patient not fulfilling DRE criteria	NS	Various	R	N	-
Kwan	2009	China	194	270	≥1 seizure per month over the previous year	≥1 year seizure free	Various	Various	R	Y	-
Lakhan	2009	India	94	231	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Vahab	2009	India	113	129	<6 months terminal remission with trials of ≥2 AEDs	≥1 year seizure free	Various	Various	R	N	-
Alpman	2010	Turkey	39	92	≥2 seizures while using ≥2 AEDs within 2-years	Healthy individuals	Various	Various	R	Y	-
Grover	2010	India	95	133	≥1 seizure in 10 months	≥10 months seizure free	Various	Various	R	N	-
Dong	2011	China	157	193	≥4 seizures in year	≥1 year seizure free	Various	Various	R	N	-
Haerian	2011	Malaysia	323	362	≥1 seizure in a year	≥1 year seizure free	Various	CBZ/VPA	R	N	-
Meng	2011	China	24	60	<50% reduction in seizure frequency in the year prior to last follow-up	≥50% reduction in seizure frequency in the year prior to last follow-up	Various	CBZ	R	N	-

Symbol	Description
T	Drug resistant epilepsy
I	Drug responsive epilepsy
AED	Antiepileptic drug
A	Association found?
Gen	Genotype
D	Design: Prospective or retrospective
UTD	Unable to determine as full article not available or not available in English
TLE	Temporal lobe epilepsy
NS	Not specified
CBZ	Carbamazepine
PHT	Phenytoin
VPA	Sodium valproate
*	In subgroup analysis, CC genotype was significantly associated with DRE in adults or those with symptomatic epilepsy
**	Association found in males and in patients with localization-related epilepsy
***	Haplotypes were of C.1236C>T, C.2677C>T and C.3435C>T in all studies, except for Kwan et al. (2009): rs3789243 and G2677T/A, and Haerian et al. (2011): rs3789243, C.1236C>T, C.2677C>T, rs6949448, C.3435C>T.
****	Cohort was divided into 3 groups according to seizures per month: A ≤2 seizures (n=44), B >2 and and <6 seizures (n=83), C ≥6 seizures (n=66)

1.6.1.4 Transporters other than P-gp

Other transporters studied in epilepsy pharmacoresistance include ABCG2 (breast cancer resistance protein or BCRP), and six ABCC proteins: ABCC1 to ABCC6 (multidrug resistance-associated protein 1 to 6; MRP1 to MRP6).

As is the case for P-gp, the expression of ABCC1-ABBC6 and ABCG2 is increased in epileptic foci resected during brain surgery for intractable epilepsy (Tishler *et al.* 1995; Dombrowski *et al.* 2001; Sisodiya *et al.* 2001; Sisodiya *et al.* 2002; Aronica *et al.* 2003; Aronica *et al.* 2004; Lazarowski *et al.* 2004; Vogelgesang *et al.* 2004; Aronica *et al.* 2005; Kubota *et al.* 2006; Lazarowski *et al.* 2006; Ak *et al.* 2007).

Human ABCC1, ABCC2 and ABCC5 were unable to transport common AEDs (carbamazepine, valproate, levetiracetam, phenytoin, lamotrigine, phenobarbital and topiramate) even in the permissive CETA model (Luna-Tortos *et al.* 2009; Luna-Tortos *et al.* 2010). Similarly, primidone, phenobarbital, phenytoin, carbamazepine, lamotrigine, clonazepam, ethosuximide and valproic acid were not substrates for ABCG2 in transport experiments performed in human BCRP-expressing MDCKII cell lines (Cerveny *et al.* 2006). AED transport assays have not been performed for ABCC3, ABCC4 and ABCC6.

A number of genetic association studies for ABCC2 variants and pharmacoresistant epilepsy have been published; a recent comprehensive meta-analysis of these studies found no significant associations (Grover & Kukreti 2013). Similarly, no association was found between polymorphisms in ABCC2 and response to carbamazepine (Radisch *et al.* 2014). No genetic association has been found between pharmacoresistant epilepsy and variations in ABCC5 or ABCG2 (Kim *et al.* 2009; Kwan *et al.* 2011).

A conspicuous omission in the search for MDTs underlying epilepsy pharmacoresistance is the solute carrier (SLC) superfamily of transporters. The largest superfamily of MDTs is the SLC superfamily (Huang & Sadee 2006). There are more than 400 known SLC proteins. It would be useful to adopt a comprehensive and systematic approach to investigating the role SLC transporters might play in epilepsy drug resistance.

1.6.2 The drug target hypothesis

The second major hypothesis of AED pharmacoresistance is the target hypothesis. According to the target hypothesis, epilepsy pharmacoresistance occurs when changes in drug targets make them less sensitive to AEDs (Schmidt & Loscher 2005; Remy & Beck 2006). Reduced sensitivity to carbamazepine, phenytoin and lamotrigine has been observed in hippocampal neurons from patients with intractable temporal lobe epilepsy (Vreugdenhil *et al.* 1998; Schaub *et al.* 2007). Two forms of the target hypothesis have been proposed: (1) acquired and (2) genetic. In the *acquired form,* the change in the target occurs in conjunction with epileptogenesis, as a result of seizures, or as a consequence of drug treatment. In the *genetic form* there is an inherited, inborn difference in the target that confers resistance.

The following observations have been cited to support the acquired version of this hypothesis. Firstly, in kindled rats, sodium channels were found to exhibit reduced sensitivity to carbamazepine which returned to normal 5 weeks after kindling, indicating that the changes were related to kindling and not the epileptic state per se, which is persistent (Vreugdenhil *et al.* 1998; Vreugdenhil & Wadman 1999). Secondly, there is a loss in benzodiazepine sensitivity in a rat model of temporal lobe epilepsy resulting from alterations in the subunit composition of $GABA_A$receptors (Brooks-Kayal *et al.* 1998). Thirdly, resistance develops to benzodiazepines during prolonged status epilepticus as a result of internalization of synaptic $GABA_A$ receptors (Wasterlain & Chen 2008; Fritsch *et al.* 2010; Joshi & Kapur 2012).

In support of the genetic version of the drug target hypothesis, the following study has been cited. Tate *et al*. (Tate *et al.* 2005) reported that a common functional polymorphism in the *SCN1A* gene, which encodes an isoform of voltage-activated sodium channels, was associated with the maximum doses of phenytoin and carbamazepine used clinically

There are a number of major criticisms of the drug target hypothesis. Firstly, follow-on studies have failed to replicate the association between polymorphism in the *SCN1A* gene and maximum doses of AEDs prescribed (Tate *et al.* 2006; Zimprich *et al.* 2008). Secondly, a comprehensive genetic association study and meta-analysis has failed to identify any

association between SCN1A, SCN2A and SCN3A gene polymorphisms and responsiveness to antiepileptic drugs (Haerian *et al.* 2013). Thirdly, in amygdala-kindled epileptic rats, there is no difference in the inhibitory effect of phenytoin on sodium channels in acutely isolated hippocampal neurons from phenytoin responders and non-responders (Jeub et al., 2002). Fourthly, the validity of the target hypothesis is challenged by the broad range of molecular targets of AEDs, which include subunits of voltage-gated sodium and calcium channels as well $\alpha_2\delta$ proteins that are associated with calcium channels, $GABA_A$receptors, the GAT-1 GABA transporter, the GABA catabolic enzyme GABA transaminase, K_V7/KCNQ/M potassium channels, the synaptic vesicle protein SV2A, and AMPA receptors (Meldrum & Rogawski 2007). Patients with drug-resistant epilepsy are for the most part resistant to all AEDs. Hence, targets of all the AEDs would need to be simultaneously modified to produce true multidrug resistance. Given the diversity of molecular targets it seems improbable that all of the targets would be modified in such a way as to produce pharmacoresistance to all available AEDs. Many of the newer drugs act at novel molecular targets that are entirely distinct from the targets of the older agents. If target-specific mechanisms were a factor in pharmacoresistance, as new, mechanistically novel AEDs were introduced into clinical practice there should have been a substantial reduction in the incidence of pharmacoresistance, but this has not occurred (Rogawski 2013).

In summary, it is unlikely that the drug target hypothesis provides a unifying basis for pharmacoresistance in epilepsy.

1.6.3 The 'intrinsic severity' hypothesis

In 2008, Rogawski and Johnson formulated the 'intrinsic severity hypothesis' of pharmacoresistance in epilepsy (Rogawski & Johnson 2008). They postulated that pharmacoresistance is related to disease severity (Rogawski & Johnson 2008), and severity simply reflects the magnitude of the underlying epileptic process. In addition, separate factors likely regulate severity. Severity factors could be acquired as stochastic events during development or by environmental insults, or they could be genetically determined (Rogawski 2013).

Rogawski and Johnson (Rogawski & Johnson 2008) have argued that the current view of pharmacoresistance in epilepsy conceptualizes resistance as a problem separate from the

disease itself, and this notion has constrained research in the field. This notion has proven equally unfruitful in other human diseases. There has been an enormous effort to define a role for drug transporters including P-gp as a cause of multidrug resistance in cancer and to develop transporter-targeted pharmacological strategies to overcome drug resistance in cancer. However, this line of research has not impacted cancer therapeutics. In contrast, therapies that take advantage of a deep understanding of cancer biology have been remarkably successful (Simon 2006). Similarly in epilepsy, it has been argued (Loscher & Sills 2007) that there is no evidence that pharmacoresistance develops through mechanisms separate from the disease state itself but considerable evidence that the epilepsy in an individual patient has an inherent severity that defines the response to medication.

In order to examine the validity of the intrinsic severity hypothesis, a measure or marker of disease severity must be established. It has been suggested that seizure frequency is an appropriate marker of disease severity in epilepsy.

1.6.3.1 Seizure frequency as a marker of disease severity

The concept that factors related to the occurrence of frequent seizures are associated with refractoriness seems biologically plausible: if the epilepsy is of a nature that seizures are easy to trigger leading to frequent seizures, then the seizures may also be more difficult to suppress. It has been observed in many acute seizure models that suppression of seizures conferred by any given dose of AED can be overcome by increasing the intensity of the pharmacological or electrical seizure stimulus (Barton *et al.* 2001). This suggests that if susceptibility to seizures is sufficiently high, it may not be possible to prevent recurrence of seizures with any nontoxic dose of an AED.

1.6.3.2 Severity predicts treatment response

There have been a number of prospective studies of outcome in newly treated epilepsy (Cockerell *et al.* 1997; MacDonald *et al.* 2000; Sillanpaa & Schmidt 2006) (Musicco *et al.* 1997; van Donselaar *et al.* 1997; Marson *et al.* 2005; Kim *et al.* 2006; Leschziner *et al.* 2006; Mohanraj & Brodie 2006). A consistent finding across these studies is that the single most important factor associated with prognosis is the frequency of seizures in the early phase of epilepsy. Both the number of seizures pre-treatment (Kim *et al.* 2006; Leschziner *et al.* 2006; Mohanraj & Brodie 2006) and in the immediate period after presentation (MacDonald *et al.*

2000) influence the chance of remission. Indeed, the frequency of seizures in the early phase of epilepsy is the dominant risk factor influencing the chance of remission of seizures, outweighing the contribution from other factors associated with prognosis. In the National General Practice Study of Epilepsy (MacDonald *et al.* 2000), having 4 seizures in the 6-month period after diagnosis of epilepsy, compared to having a single seizure, was associated with a halving of the chance of seizure remission. In a hospital-based prospective cohort, patients with 11 or more seizures pre-treatment were more than twice as likely to be uncontrolled than patients with two or less seizures pre-treatment, independent of the time from first seizure to starting treatment (Mohanraj & Brodie 2006). These epidemiological data suggest that there are differences in inherent epilepsy severity reflected in the frequency of seizures in the early phase of the disease that influence an individual patient's response to AEDs, much in the same way that any other disease can vary from mild to severe and show a variable response to treatment. The observation that the occurrence of frequent seizures is associated with poorer outcome suggests that common neurobiological factors may underlie both epilepsy severity and pharmacoresistance.

1.6.3.3 Genetic contribution to intrinsic severity:

It has been suggested that genetic factors contribute to intrinsic severity. A number of observations support this assertion. There are variations in AED sensitivity within outbred rats subjected to the same epileptogenic treatment (Loscher & Rundfeldt 1991; Loscher 2011), even though there is no difference in the severity of the seizure triggering epileptogenesis. These epileptic animals exhibit dramatic individual variability in response to AEDs, strongly suggesting that genetic factors play a key role in determining whether pharmacoresistance does or does not develop. Moreover, several reports have identified specific genetic variants conferring altered epilepsy severity in transgenic mouse models. A subclinical mutation in *Kcnq2* was found to significantly increase epilepsy severity in mice bearing an epilepsy-inducing mutation in *Scn2a (Kearney et al. 2006)*, and, similarly, mutations in *Scn2a* and *Kcnq2* were found to increase epilepsy severity in an *Scn1a* epilepsy mutant (Hawkins *et al.* 2011). Finally, the results of a twin study suggest that genetic determinants of epilepsy susceptibility contribute to response to treatment (Johnson *et al.* 2003). This study observed high correlations for outcome among twins concordant for

epilepsy syndrome, suggesting that epilepsy susceptibility genes have a major influence on outcome.

1.6.3.4 Critiques of the intrinsic severity hypothesis:

The intrinsic severity hypothesis has been criticised for the following reasons:

1. Some previously intractable patients do achieve seizure freedom with new AEDs (Callaghan *et al.* 2007; Luciano & Shorvon 2007; Brodie 2010). This does not affect the potential validity of the intrinsic severity hypothesis, since a new AED that acts by a novel mechanism may be effective in a subset of patients because it is especially suited to the neurobiological abnormality underlying their epilepsy.
2. Some patients with infrequent seizures are drug refractory. It should be noted, however, that there is no absolute scale on which to consider seizures frequent or infrequent. Furthermore, seizure frequency is not the sole measure of severity. Hence, a patient with less frequent seizures may actually have more severe epilepsy in the context of the seizure type and epilepsy type. For example, a person experiencing generalised tonic-clonic seizures with lower frequency may have a more severe epilepsy than another experiencing simple partial seizures with a higher frequency. Finally, if epilepsy is caused by an evolving brain pathology, for example mesial temporal sclerosis worsening over time, epilepsy may become refractory after a period of remission (Berg *et al.* 2003).
3. Where response to treatment is defined as achieving freedom from seizures for a given period of time, it has been suggested that there is a potential for infrequent seizures to be misinterpreted as seizure remission (French 2006). However, if infrequent seizures give an erroneous impression of drug responsiveness because of the long interval of time between seizures, the association of seizure frequency with chance of remission should depend on the duration of the remission period analysed. In fact, the association is the same whether remission of epilepsy is defined as absence of seizures for a period of 1 or 5 years duration (MacDonald *et al.* 2000). In addition, if patients with infrequent seizures and those with frequent seizures were equally drug responsive, then the practice of empirical titration of AED dose according to seizure recurrence should result in patients with frequent seizures achieving more rapid titration and therefore achieving remission of seizures in a

shorter period of time than patients with more widely spaced seizures. In practice, the opposite is observed (Leschziner *et al.* 2006).

4. An alternative interpretation of the epidemiological data is that recurrent seizures render the epilepsy more resistant to treatment later on, leading to an acquired state of drug resistance. However, there is little evidence that 'seizures beget seizures' (Berg & Shinnar 1997; Shorvon & Luciano 2007), and good evidence that the chances of long-term remission of seizures are not dependent on the duration of epilepsy or early drug therapy (Musicco *et al.* 1997; Marson *et al.* 2005; Mohanraj & Brodie 2006).

In summary, the intrinsic severity hypothesis offers an intuitive and appealing explanation of pharmacoresistance in epilepsy, and is supported by a wide range of epidemiological data. At the core of this hypothesis is the idea that pharmacoresistance is caused by increased dysfunction of the neurobiological processes underlying epilepsy. This hypothesis remains untested in the laboratory. As the processes underlying epilepsy are at present poorly understood, it is important that a comprehensive unbiased genome-wide analytical approach is adopted in order to identify the central causal mechanisms behind this condition.

1.7 Analytical strategies for studying complex genetic diseases

1.7.1 The genetic architecture of common epilepsies and response to AEDs

Common or sporadic epilepsies can be defined as epilepsies 'without an elicitable strong family history, and which are not obviously part of a broader condition (e.g. without learning difficulties, somatic malformation or facial dysmorphism)' (Sisodiya & Mefford 2011). Twin and family studies provide persuasive evidence that common epilepsies and response to AEDs are complex genetic phenotypes: they show evidence of hereditability without exhibiting a Mendelian pattern of inheritance (Lennox 1951; Ottman *et al.* 1996; Johnson *et al.* 2003; Vadlamudi *et al.* 2004; Tsai *et al.* 2013).

Complex genetic diseases are thought to be caused by an unknown number of multiple genes, usually interacting with various environmental factors (Davey Smith *et al.* 2005).

There is compelling evidence that common genetic variants contribute to the susceptibility to common disease (Lohmueller *et al.* 2003). Specifically in epilepsy, a number of common variants have been found to increase the risk of developing epilepsy (see section 1.7.2). It has been suggested that epilepsy is caused by a 'heterogeneous but pathogenic subsets of susceptibility alleles drawn from a much larger pool of potential susceptibility genes, meaning variation at no individual susceptibility gene is necessary or sufficient for seizures' (Dibbens *et al.* 2007). However, it should be noted that rare genitive variations have also been found in some cases of common epilepsies (Sisodiya & Mefford 2011). Studies performed to date have found rare genetic variants in only small percentages of people with common epilepsies (Chen *et al.* 2003; Helbig *et al.* 2009; Heinzen *et al.* 2010). For example, Heinzen and colleagues found that the 16p13.11 deletion affects approximately 0.6% of patients in a diverse sporadic epilepsy cohort (Heinzen *et al.* 2010). The role of rare variants in the common epilepsies is at present under exploration by deep-sequencing approaches. No genetic variants have so far been convincingly shown to influence drug response in epilepsy.

Traditional 'candidate gene' approaches have proven unfruitful in uncovering the causes of complex diseases as the number of involved genes is large, the disease genes might differ in different individuals, and each gene has a small effect size (Cho *et al.* 2012). In order to meet the challenge of studying complex genetic diseases, tools have been developed for the simultaneous large-scale genome-wide analysis of genes and gene products. The most widely used of these tools are Genome-Wide Association Studies (GWAS) for the identification of disease-associated genetic variants, and microarray studies for the identification of disease-associated gene transcripts; these tools are described below (sections 1.7.2 and 1.7.3).

The polygenicity underlying a complex disease poses an intriguing translational challenge: how can the many different genes be coherently connected together? A parsimonious hypothesis is that the polygenic basis of a complex disorder is manifested in the dysregulation of one or a number of specific pathways. Genetic variations at many different loci could introduce numerous slight alterations that result in a pathway(s) that is insufficiently robust in response to an environmental insult. Risk for a complex disorder

could be conferred by the dysfunction of the pathway(s) rather than any single gene. Network mapping techniques are needed to understand of the roles of pathways in complex biological traits; these techniques are also discussed below (section 1.7.5).

1.7.2 Studying structural genetic variation:

The structural genetic variants most commonly subjected to whole-genome high throughput analyses are single nucleotide polymorphisms (SNPs). SNPs—common DNA sequence variations in which a single nucleotide differs between individuals—are the most abundant form of human genetic variation (Marth *et al.* 1999). A Genome-Wide Association Study (GWAS) surveys the whole of the genome for disease-associated SNPs. Millions of SNPs are analysed in a typical GWAS. By comparing the allele frequencies of genotyped SNPs between individuals with and without disease, a GWAS can identify putative causal variants or variants that are in strong linkage disequilibrium with putative causal variants. GWAS is a powerful tool in the study of complex diseases. GWAS has an impressive track record of success in complex human diseases and has yielded a plethora of findings: there are now well over 2000 loci that are significantly and robustly associated with one or more complex traits (Visscher *et al.* 2012). GWAS have also led to a number of notable discoveries about response to therapeutics. For example, a SNP close to the IL28B gene is associated with response to treatment for hepatitis C (Ge *et al.* 2009): the good response genotype is associated with a greater than 80% chance of clearance in European- Americans, while the poor response genotype is associated with only about a 30% chance.

Compared to emergent deep-sequencing techniques (see below), GWAS is a mature and inexpensive technology; quality control, imputation, and analysis are readily accomplished; and there are large amounts of data that can be used for comparisons (Klein *et al.* 2010). The SNP content of most commercially-available GWAS arrays is sufficient to capture the majority of common variation in European populations (Klein *et al.* 2010).

To date, there has been only one published GWAS of AED response, which did not find any significant variants (Speed *et al.* 2014). There have been a number of GWAS studies of susceptibility to common epilepsies, and a recent meta-analysis that included 8696 cases and 26 157 controls (2014) identified statistically significant loci at 2q24.3, implicating

SCN1A, and at 4p15.1, harbouring PCDH7. The vast majority of effect sizes for individual variants identified in GWAS are generally small (odds ratio ≤ 1.5) (Stranger *et al.* 2011). It has been suggested by utilizing even larger sample sizes, further susceptibility variants for epilepsy can be identified. The strategy of using very large sample sizes has proven highly effective in other neurological diseases, for example multiple sclerosis (Beecham *et al.* 2013). It should be noted that another technique for increasing the power to detect genetic associations in GWAS is to use network modelling; this method is discussed in further detail below (section 1.7.5).

Next-generation DNA sequencing, which allows a near comprehensive analysis of genetic variants, has to date been applied to only a small cohort of sporadic (idiopathic generalized) epilepsy patients in one study (Heinzen *et al.* 2012) and was restricted to exome sequencing; this limited study did not produce positive results. Larger scale deep-sequencing studies in epilepsy are underway, and are likely to play an important role in finding rare disease-associated genetic variations which cannot be detected through GWAS.

The analytical techniques discussed in this section provide valuable information about genetic structural variations, but do not provide an insight into changes in genetic function associated with epilepsy. High throughput transcriptomic analysis is a useful tool for performing a genome-wide study of changes in genetic function.

1.7.3 Studying genetic function: transcriptomic analysis

Transcriptomic analysis techniques include microarray studies and RNA sequencing (RNA-seq). RNA-seq offers a number of advantages over microarrays, including enhanced sensitivity, but is also more costly (Wang *et al.* 2009b). Currently, microarray analysis is the more commonly used technology. To date, there are no published RNA-seq analyses of epileptic brain tissue, whereas there are a number of such microarray studies, which will be discussed in detail in Chapter 2. The most common form of microarray technology is the cDNA microarray. In this, mRNA extracted from the sample is reverse transcribed, with the simultaneous incorporation of label, and the resulting cDNA provides a quantifiable signal when it binds to complementary DNA spotted, in a grid arrangement, on to a solid support such as a glass slide (Wildsmith & Elcock 2001). The primary output from such studies is a

list of 'differentially expressed' genes: genes with significantly altered levels of expression in disease tissue relative to controls. Traditionally, a few of the most significantly dysregulated genes are chosen for further validation and study.

Microarray studies have been used extensively and successfully to investigate biomarkers and mechanisms of human diseases and drug response (Gupta *et al.* 2010; Mendrick 2011; Aguilar *et al.* 2014; Davies *et al.* 2014; Raddatz *et al.* 2014; Zhu *et al.* 2014).

1.7.4 Limitations of genetic and transcriptomic studies

Although GWAS and microarray studies are powerful investigative techniques, limitations in the way data from these studies are traditionally analysed has constrained their potential impact on the understanding of disease.

Microarray studies, on their own, cannot determine whether identified gene expression changes cause the disease, or are caused by the disease, or are unrelated to the disease process. Furthermore, it has been suggested that many of detected changes in gene expression are specific to laboratory or experimental conditions, and provide limited information about the underlying disease aetiology (Wang *et al.* 2010). Finally, the traditional methodology of choosing a few of the most significantly dysregulated genes for further validation and study underuses the 'depth' inherent in the microarray data set. This piecemeal approach risks missing causally important genes and processes.

GWAS, on their own, cannot determine if any identified associations are due to genetic variations that cause the disease or variations that are unrelated to the disease. Also, individual genetic variations tend to have a small effect size and tests for association may not reach a stringent genome-wide statistical significance threshold. Furthermore, the small effect sizes for these associations do not explain the observed heritability of most traits (Maher 2008). In addition, it is can be challenging to translate a genetic association into a functional connection with the trait: the diseased-associated variant might lie in a genomic region that is yet to be annotated, or it may fall within a gene with multiple potential roles depending on the biological context. Even if the gene is successfully annotated, it might be unsuitable for therapeutic targeting.

In order to overcome the above weakness, integrative data processing techniques have been developed, which are discussed below.

1.7.5 Integrative approaches

The integrative approaches being considered fall into two broad categories: (1) network mapping, and (2) integration of intermediate phenotypes.

1.7.5.1 Network mapping

If a disease is caused by the combined influence of many dysfunctional genes, the individual effect of each gene might be small and thus hard to detect. This difficulty is further aggravated by the fact that, for complex genetic disease, different cases of the same disease might be caused by varying combination of genetic perturbations (Cho *et al.* 2012). Of course, genes do not work in isolation; genes and gene products interact with each other to form complex interaction networks. Thus a perturbation in one gene can be propagated through the interactions, and affect the whole network. The fact that similar disease phenotypes result from the dysfunction of different genes suggests that these different genes are not unrelated but form part of the same molecular network (Schadt 2009). Therefore, in studies of complex diseases, researchers increasingly focus on groups of interconnected genes forming a network.

The network-based analytical approach offers several advantages over single-marker or single-gene analysis. Taking GWAS pathway-based analysis as an example, this method has been shown to boost the power to identify genetic associations (Wang *et al.* 2007). Genetic heterogeneity may cause any one causal variant to exhibit only modest disease risk in the sample as a whole, since different individuals may possess different disease risk polymorphisms at different loci in the same gene, or in different genes. This will reduce power to detect any one variant by traditional association methods. However, if the genes in question are members of the same biological network, then considering the pathway as the unit of analysis may increase power to detect association between the genes and disease. For similar reasons, association of disease with biological pathways may be easier to replicate across different studies than association with individual SNPs. This was clearly

shown in an analysis of Crohn's disease (Wang *et al.* 2009a), where the IL12/IL23 pathway showed evidence of enrichment in four independent GWAS, despite the genes and SNPs involved differing between the studies. In addition, by identifying additional susceptibility genes, pathway-based analysis can be used to fill-in part of the 'missing heritability' described above (Fridley & Biernacka 2011). Furthermore, compared with gene-based or SNP-based analysis, pathway-based analysis may yield more secure insights into disease biology since an associated pathway is likely to implicate function better than a hit in a single gene that may have many functional possibilities. Finally, as the most associated gene in a pathway might not be the best candidate for therapeutic intervention, targeting susceptibility pathways might also have clinical implications for finding additional drug targets.

Numerous network construction techniques have been devised, which can be divided into three broad categories: (1) Physical interaction network construction which is dependent on prior knowledge of protein-protein interactions; (2) Functional interaction network construction which is dependent on prior knowledge of functional interactions; and (3) Co-expression network construction which is independent of prior knowledge. These techniques are described below:

Protein interaction networks (an example is shown in Figure 1.2) can be constructed on the basis of known protein-protein interactions which have been collated, from published literature, in a number of publicly accessible databases (Salwinski *et al.* 2004; Kerrien *et al.* 2012; Chatr-Aryamontri *et al.* 2013). However, it should be noted that the human protein interactome is still far from being fully identified. Current protein-protein interaction data comprise interactions only among a relatively small subset of the proteins known to be present in humans (de Silva *et al.* 2006; Stumpf *et al.* 2008). It has been shown that network inference using incomplete protein-protein interaction data can lead to biased results (de Silva *et al.* 2006).

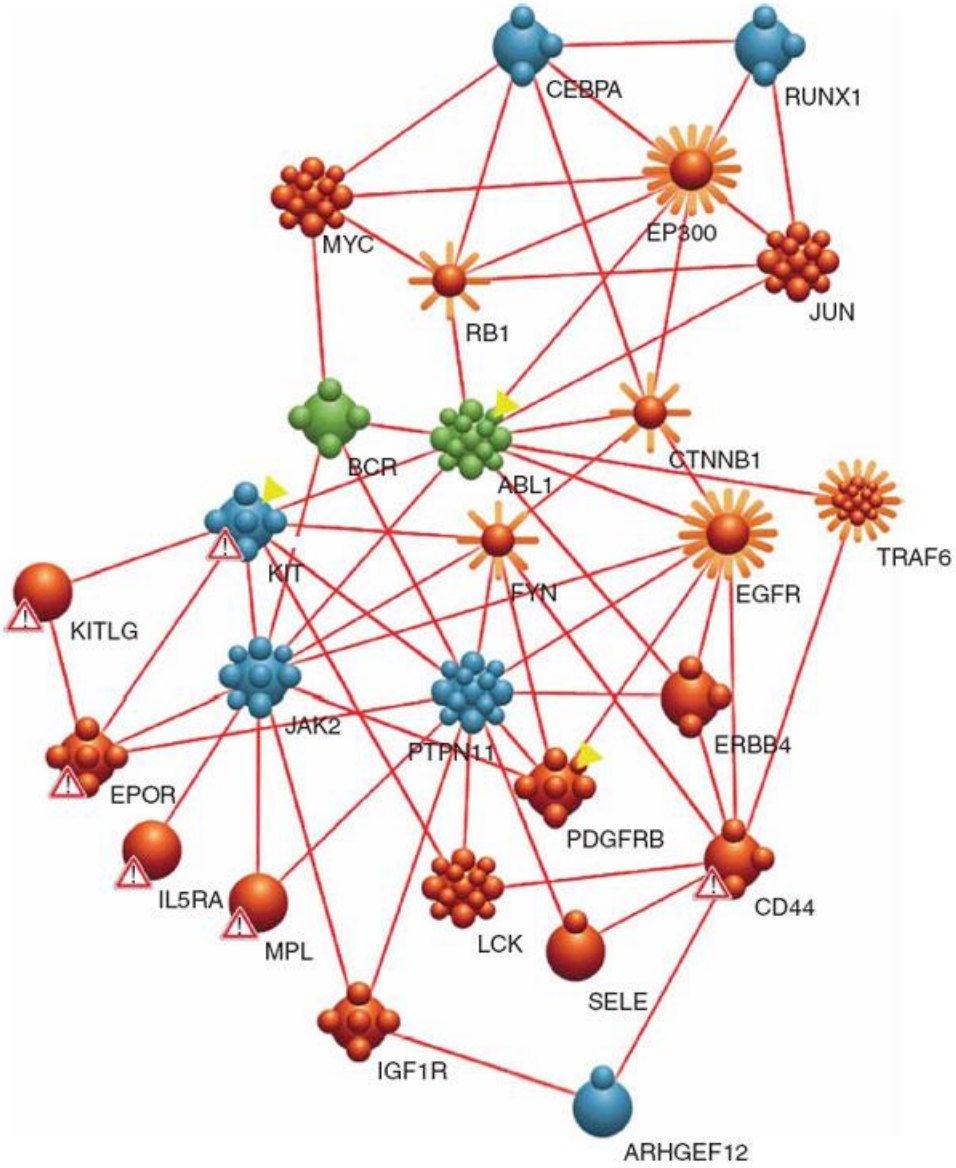


Figure 1.2 An example of a protein-protein interaction network (Russell & Aloy 2008). The disease network is built from known protein-protein interactions around genes associated with the onset of chronic (green) and acute (blue) forms of myeloid leukaemia. Some of the nodes in the disease network are either primary or secondary targets (yellow triangles) of imatinib (Gleevec), a drug used to treat people with chronic myeloid leukaemia. The use of imatinib has several associated adverse effects, the most frequent of which is myelosupression. Imatinib affects a number of proteins in the network directly related to the formation of bone marrow tissue (alert signs). The authors drew the figure with AxPathBuilder (http://www.anaxomics.com/). Nodes have been represented by different shapes depending on the number of interactions they make outside the depicted network, from the 6 interactions of IL5RA to the 347 of TRAF6.

While physical interaction networks connect proteins that physically interact with each other, functional interaction networks connect molecules that work together to effect the same function even if they do not necessarily physically interact. These functional units are generally referred to as pathways (see Figure 1.3 for an example). The genes in a pathway work in a tightly regulated and highly coordinated manner to bring about a specific function. A number of high-quality well-annotated manually-curated pathway databases are in existence (Tanabe & Kanehisa 2012; Caspi *et al.* 2014; Croft *et al.* 2014). Typically, each pathway in a database contains not only topological connectivity information but also the roles of molecules such as whether a given molecule is an activator or inhibitor of the activity of another molecule. The pathway databases can be exploited for identifying enriched pathways within a set of results (Curtis *et al.* 2005). Pathway-based analysis has found wide-spread acceptance in microarray analysis, and is now beginning to be applied to GWAS (Yaspan & Veatch 2011). A considerable advantage of identifying significant pathways within the results of an analysis is immediate functional characterisation of the output, as each pathway serves a clearly defined purpose. A limitation of the pathway-enrichment analysis approach, as it has been most widely applied, is that each pathway is considered an isolated entity, and the interactions between them are not explored. However, biological pathways do not work in isolation, and different pathways may interact through either shared components or regulatory mechanisms (Ponzoni *et al.* 2014). With this in view, various methods have recently been devised in order to construct networks of enriched pathways from transcriptomic data. For example, the Enrichment Map tool (Merico *et al.* 2010) displays connections between pathways by analysing the overlap between their annotated genes, whereas other methodologies construct pathway networks based on known physical interactions between proteins belonging to different pathways (Li *et al.* 2008).

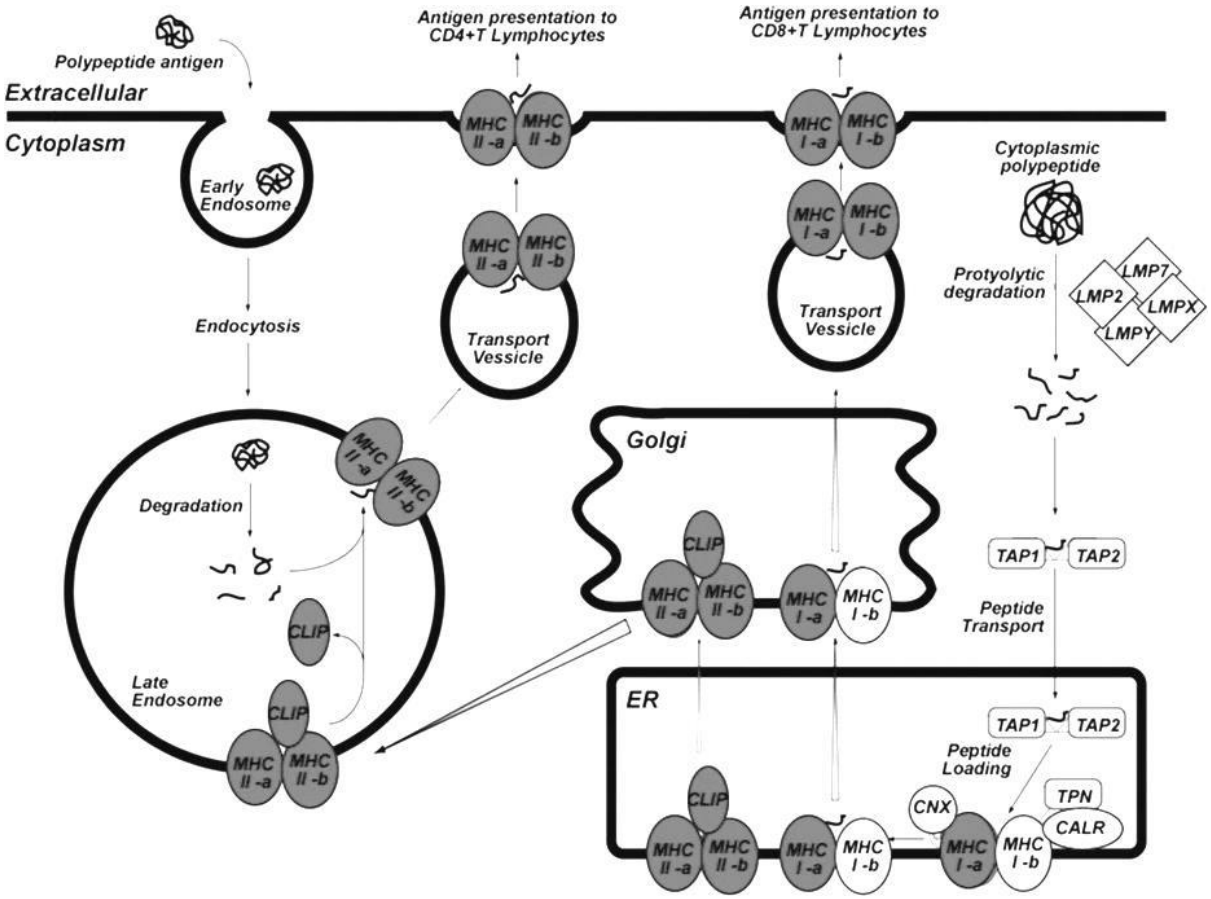


Figure 1.3 Figure of antigen presentation pathway taken from a study by Degenhardt and colleagues (Degenhardt *et al.* 2010). Metastatic and vertical growth phase melanoma cell lines were subjected to microarray-based gene expression analysis. Significantly differentially expressed genes between metastatic and vertical growth phase melanoma cell lines were loaded into and viewed with the Ingenuity pathway analysis software (Jimenez-Marin *et al.* 2009). Red represents decreased expression of components of this pathway in metastatic cells compared to vertical growth phase cells.

Co-expression network construction is based on the premise that functionally related genes are likely to show mutual dependence in their expression patterns (Cho *et al.* 2012). Hence, gene co-expression can be seen as an indication of a functional relationship between the genes. The construction of co-expression networks consists of two steps (Veiga *et al.* 2010). First, a pairwise comparison of all gene expression profiles is performed using a similarity metric. This results in a fully connected network among all genes, with the weight of each

link equal to the similarity metric. Second, the complete set of links is filtered by their weight, using hard or soft thresholding (Langfelder & Horvath 2008). Links that 'survive' thresholding constitute the co-expression network. Dense sub-networks (or modules) are often identified within the larger co-expression network (see Figure 1.4 for an example).

Certain lines of evidence indicate that co-expression networks are biologically meaningful (Veiga *et al.* 2010). The resulting networks have special properties (for example scale-free topology or fat-tailed degree distribution) that are shared by many other biological networks, and are robust to the dataset used for network inference, but disappeared when networks were regenerated using randomised data. Moreover, hubs (genes with many links) in these co-expression networks were enriched in essential genes.

A number of similarity measures can be employed for co-expression network construction, for example correlation or mutual information (MI). It should be noted that correlation is geared towards discovering linear relationships between gene expression profiles, which do not always reflect the biological situation (Lecca & Priami 2013). For example, multiple binding sites and saturation effects can make the relationship between a transcription factor and its target non-linear. Like correlation, MI is a measure that detects statistical dependence between two variables. But unlike correlation, it does not assume linearity, or other specific properties, of the dependence (Faith *et al.* 2007). As such, MI is able to detect regulatory interactions that might be missed by linear metrics such as the correlation coefficient.

Possible biological causes of gene co-expression are direct or indirect regulation of one gene by the other, or co-regulation of both by a third gene. Therefore, gene co-expression can indicate a direct or indirect regulatory relationship. A special from of co-expression network is the gene regulatory network. Gene regulatory network reconstruction algorithms identify regulatory relationships based on the assumption that changes in the expression level of a transcription factor should be mirrored in the expression changes of the genes regulated by the transcription factor. Indirect links in an inferred gene regulatory network are deemed undesirable as they are assumed not to represent physical interactions (Veiga *et al.* 2010). Several filtering techniques have been developed to reduce inference of interactions via intermediaries. For correlation-based networks, vanishing partial correlation (the

correlation between two genes disappears when the effect of a third gene is taken into account) can be used to filter out indirect links (de la Fuente *et al.* 2004). For MI-based network inference methods, an example of an algorithm that filters out indirect links is ARACNe (Margolin *et al.* 2006). ARACNe employs MI to detect regulatory interactions and uses data processing inequality, an information-theoretical property, to test whether a link is indirect, and therefore should be removed from the network. Another example is the context likelihood of relatedness (CLR) algorithm (Faith *et al.* 2007). After computing the MI between regulators and their potential target genes, CLR calculates the statistical significance of each MI value: the algorithm compares the MI between a transcription factor-gene pair to the background distribution of MI scores for all possible transcription factor-gene pairs that include either the transcription factor or its target. The most probable interactions are those whose MI scores stand significantly above the background distribution of MI scores.

The network inference methods described above are unsupervised and rely on gene expression data alone. Supervised and integrative methods of regulatory network inference have also been developed. For example SIRENE (Supervised Inference of Regulatory Networks) (Mordelet & Vert 2008) predicts new gene regulatory relationships by taking as input gene expression data and a list of known regulatory relationships between transcription factors and target genes. In contrast to other methods for regulation inference based on the detection of similarity between expression profiles of transcription factors and their targets, SIRENE is based on the hypothesis that genes regulated by the same transcription factor exhibit similar expression variations.

It has been reported that the overlap between the gene regulatory predictions from different network inference methods can be low (De Smet & Marchal 2010). However, in many biologically meaningful ways, for example enriched biological processes and pathways, the ranks of the degree centrality values and the major hub genes in the inferred networks, there is a high degree of consistency between different methods (de Matos Simoes *et al.* 2013). Furthermore, it has been observed that the results of different network inference methods show a similar degree of overlap with an external validation standard (De Smet & Marchal 2010). This suggests that the discrepancy in predicted interactions is not due to the failure of individual methods to infer biologically relevant interactions, but is

rather due to the complementarity of the different methods. Hence, it is likely that different methods highlight different interaction types, so aggregating the outcomes of complementary methods offers a means of improving the breadth and the accuracy of the predictions. Indeed, it has been shown that an ensemble of the predictions made by the best performing methods ('wisdom of crowds') more closely approximates the true gene regulatory network than the predictions made by each method separately (Marbach *et al.* 2012).

Genome-wide gene network analyses typically produce large networks that involve hundreds of gene interactions. Such networks might have interesting topological properties that are biologically meaningful, but are normally difficult to interpret in terms of cellular functionality. The prediction of the functional role of a network may be possible if the network contains a sufficient number of genes of known functions. If a subset of genes can be assigned a specific functional category, for example a particular biological pathway, then statistical tests can be used to determine if that subset is larger than expected by chance (see Figure 1.4 for an example). A variety of software tools have been developed to perform such an analysis (Tipney & Hunter 2010).

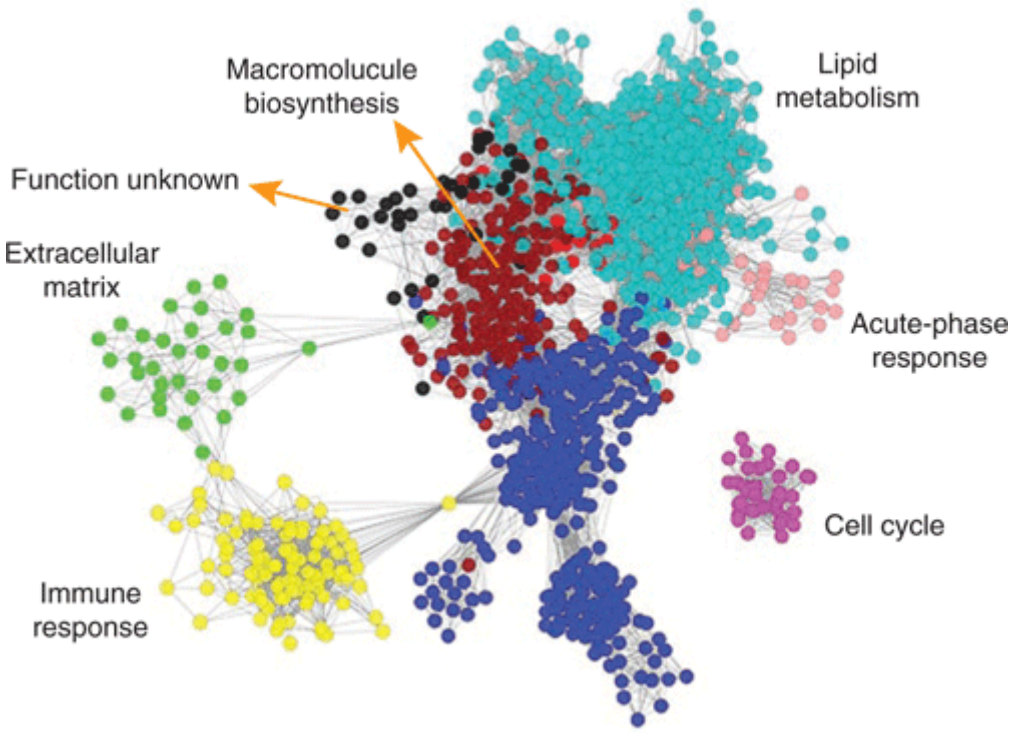


Figure 1.4 Human liver coexpression network (Friend 2010). Gene modules are identified within the network using a hierarchical clustering algorithm. The colours of the nodes represent their module assignments. Pathway enrichment terms are assigned to individual modules.

1.7.5.2 Integration of intermediate phenotypes

The analytical approach of integrating intermediate phenotypes—termed 'systems genetics'—is useful for the identification of genes, pathways and networks that underlie common human diseases. The science of systems genetics has been developed in response to the need to understand how loci identified in GWAS contribute to disease susceptibility. Systems genetics approaches can be integrated with GWAS results to predict causal genes and their functions.

Systems genetics utilizes intermediate molecular phenotypes to bridge DNA variations with the traits of interest. Most commonly, the intermediate molecular phenotype examined is expression of gene transcripts. Transcript levels can be considered intermediate phenotypes as DNA variation contributes to the clinical trait by perturbing gene expression. Studies using mice, rats, and human cells and tissues have revealed that the expression of a high

percentage of genes (≥30%) is substantially influenced by DNA variants (Romanoski *et al.* 2010; van Nas *et al.* 2010; Orozco *et al.* 2012). Most significant SNPs for GWAS map outside protein-coding regions, and >75% of GWAS SNPs map to functional regulatory elements that have been identified in the Encyclopedia of DNA Elements (ENCODE) project (Schaub *et al.* 2012). These results suggest that genetic variants that alter gene expression, rather than variants that alter protein sequences, form the primary basis of natural variation in complex traits.

Genomic sequence variants that correlate with gene-expression levels are termed expression quantitative trait loci (eQTL). eQTL studies (also termed genetical genomics studies) are similar to traditional genetic-association studies, but instead of associating genetic variants with discrete traits such as disease status, eQTL studies correlate genetic variants with quantitative gene-expression levels. It is important that the intermediate phenotype is analysed in the context that is the most relevant to the clinical trait. It has been shown that 69% to 80% of eQTLs operate in a cell-type specific manner (Dimas *et al.* 2009), and 93% of eQTLs within the same tissue show significant evidence of gene-by-environment interactions (Orozco *et al.* 2012). Hence, whole-genome transcriptome-expression analysis should be performed in samples of pathological tissue from affected individuals and whole-genome genotyping must be performed in the same individuals. The transcript levels can then be tested for correlation with genomic sequence variants.

eQTL studies can be used to link genomic sequence variations to transcriptomic changes to the clinical phenotype. An eQTL that maps to a disease locus can be considered a likely causal gene underlying the disease (Thessen Hedreul *et al.* 2013). In addition to identifying the likely causal variants, the eQTL data helps to provide an explanation of its mechanism of action.

Systems genetics techniques with network modelling have been used successfully in a number of human studies. For example, in a recent study employing such techniques, KLF14 was identified as a causal gene for multiple metabolic phenotypes and its mechanism of action was successfully determined (Small *et al.* 2011). In another study, systems genetics techniques revealed that a non-coding polymorphism at the 1p13 locus modulates a

regulatory pathway for lipoprotein metabolism and alters the risk for myocardial infarction in humans (Musunuru *et al.* 2010Musunuru *et al.* 2010).

1.8 Aims of this work

As detailed in the preceding sections, drug resistance in epilepsy is a significant clinical problem, the causes of which remain poorly understood. To date, the MDT hypothesis has been the most widely investigated putative explanation of epilepsy pharmacoresistance, with the vast majority of the research effort being focused on P-gp. However, a review of the evidence indicates that dysregulation of P-gp is unlikely to provide a unifying basis for pharmacoresistance in epilepsy. A glaring omission in the search for MDTs underlying epilepsy pharmacoresistance is the SLC superfamily of transporters—the largest superfamily of MDTs. There is a need to systematically search for evidence of dysregulation of SLCs in refractory epilepsy. The intrinsic severity hypothesis offers an intuitive and appealing explanation of pharmacoresistance in epilepsy, but remains untested in the laboratory. It is important that this possible explanation of drug resistance in epilepsy is objectively investigated. At the core of this hypothesis is the idea that pharmacoresistance is caused by increased dysfunction of the neurobiological pathways underlying epilepsy. Systems genetics techniques with network modelling provide the most suitable tools for trying to decipher the pathways underlying epilepsy and epilepsy pharmacoresistance.

In order to fill the substantial gaps in knowledge described above, the following analyses are proposed in this work:

1. I will Identify the SLC transporters significantly dysregulated in the pharmacoresistant epileptic focus. For this, I will use a robust *in silico* approach that exploits relevant published data and builds upon them using cutting-edge computational tools; I will then verify the output of the *in silico* analysis using a robust *ex vivo* approach.
2. There have been a number of published genome-wide transcriptomic studies on brain tissue from epilepsy surgery, but they have failed to make an impact on our understanding of pharmacoresistance in epilepsy. I will discuss the causes behind this failure and tackle these causes by performing an integrative analysis of previously published large-scale gene expression profiling studies on brain tissue

from epilepsy surgery. Through this analysis, I will identify the genes, pathways and processes most consistently dysregulated in pharmacoresistant epileptic foci, and determine if these results support the MDT hypotheses, the drug target hypothesis or the intrinsic severity hypothesis of pharmacoresistance in epilepsy.

3. In order to overcome some of the shortcomings of previously published transcriptomic studies on brain tissue from epilepsy surgery, I will perform the largest and most robust microarray analysis of resected pharmacoresistant epileptic hippocampal tissue from surgery for refractory mesial temporal lobe epilepsy. I will identify the differentially expressed genes, and differentially expressed and differentially connected pathways. I will then construct a network of differentially regulated pathways and identify the most central pathways in the network.
4. I will perform a GWAS in order to identify genetic variants associated with the pharmacoresistant focal epilepsy phenotype and to identify biological pathways enriched with disease-associated variants. Again, I will construct a network of disease-associated pathways and identify the most central pathways in the network.
5. I will perform a genetical genomics or eQTL study on resected pharmacoresistant epileptic hippocampal tissue from surgery for refractory mesial temporal lobe epilepsy, in order to identify genetic variants that regulate gene expression in the pharmacoresistant epileptic human hippocampus. eQTL analysis is an important tool for identifying causal disease loci.
6. I will integrate the above genetic and genomic studies in order to determine if genetic evidence supports the intrinsic severity hypothesis. I will integrate the aforementioned genetic, genetical genomic and genomic studies in order to identify putative causal genes and pathways. Once again, I will construct a network of causal pathways and identify the most central causal pathways in the network. These are likely to be the most central causal pathways underlying pharmacoresistant epilepsy.

1.9 References

(2014) Genetic determinants of common epilepsies: a meta-analysis of genome-wide association studies. *Lancet Neurol*.

Aguilar H., Urruticoechea A., Halonen P., Kiyotani K., Mushiroda T., Barril X., Serra-Musach J., Islam A., Caizzi L., Di Croce L., Nevedomskaya E., Zwart W., Bostner J., Karlsson E., Tenorio G.P., Fornander T., Sgroi D.C., Garcia-Mata R., Jansen M.P., Garcia N., Bonifaci N., Climent F., Soler M.T., Rodriguez-Vida A., Gil M., Brunet J., Martrat G.,

Gomez-Baldo L., Extremera A.I., Figueras A., Balart J., Clarke R., Burnstein K.L., Carlson K.E., Katzenellenbogen J.A., Vizoso M., Esteller M., Villanueva A., Rodriguez-Pena A.B., Bustelo X.R., Nakamura Y., Zembutsu H., Stal O., Beijersbergen R.L. & Pujana M.A. (2014) VAV3 mediates resistance to breast cancer endocrine therapy. *Breast Cancer Res* **16**, R53.

Ak H., Ay B., Tanriverdi T., Sanus G.Z., Is M., Sar M., Oz B., Ozkara C., Ozyurt E. & Uzan M. (2007) Expression and cellular distribution of multidrug resistance-related proteins in patients with focal cortical dysplasia. *Seizure* **16**, 493-503.

Alpman A., Ozkinay F., Tekgul H., Gokben S., Pehlivan S., Schalling M. & Ozkinay C. (2010) Multidrug resistance 1 (MDR1) gene polymorphisms in childhood drug-resistant epilepsy. *J Child Neurol* **25**, 1485-90.

Anderson G.D. & Shen D.D. (2007) Where is the evidence that p-glycoprotein limits brain uptake of antiepileptic drug and contributes to drug resistance in epilepsy? *Epilepsia* **48**, 2372-4.

Aronica E., Gorter J.A., Jansen G.H., van Veelen C.W., van Rijen P.C., Leenstra S., Ramkema M., Scheffer G.L., Scheper R.J. & Troost D. (2003) Expression and cellular distribution of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors. *Neuroscience* **118**, 417-29.

Aronica E., Gorter J.A., Ramkema M., Redeker S., Ozbas-Gerceker F., van Vliet E.A., Scheffer G.L., Scheper R.J., van der Valk P., Baayen J.C. & Troost D. (2004) Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia* **45**, 441-51.

Aronica E., Gorter J.A., Redeker S., van Vliet E.A., Ramkema M., Scheffer G.L., Scheper R.J., van der Valk P., Leenstra S., Baayen J.C., Spliet W.G. & Troost D. (2005) Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. *Epilepsia* **46**, 849-57.

Aronica E., Sisodiya S.M. & Gorter J.A. (2012) Cerebral expression of drug transporters in epilepsy. *Adv Drug Deliv Rev* **64**, 919-29.

Arts W.F., Brouwer O.F., Peters A.C., Stroink H., Peeters E.A., Schmitz P.I., van Donselaar C.A. & Geerts A.T. (2004) Course and prognosis of childhood epilepsy: 5-year follow-up of the Dutch study of epilepsy in childhood. *Brain* **127**, 1774-84.

Baltes S., Fedrowitz M., Tortos C.L., Potschka H. & Loscher W. (2007a) Valproic acid is not a substrate for P-glycoprotein or multidrug resistance proteins 1 and 2 in a number of in vitro and in vivo transport assays. *J Pharmacol Exp Ther* **320**, 331-43.

Baltes S., Gastens A.M., Fedrowitz M., Potschka H., Kaever V. & Loscher W. (2007b) Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. *Neuropharmacology* **52**, 333-46.

Barton M.E., Klein B.D., Wolf H.H. & White H.S. (2001) Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. *Epilepsy Res* **47**, 217-27.

Beecham A.H., Patsopoulos N.A., Xifara D.K., Davis M.F., Kemppinen A., Cotsapas C., Shah T.S., Spencer C., Booth D., Goris A., Oturai A., Saarela J., Fontaine B., Hemmer B., Martin C., Zipp F., D'Alfonso S., Martinelli-Boneschi F., Taylor B., Harbo H.F., Kockum I., Hillert J., Olsson T., Ban M., Oksenberg J.R., Hintzen R., Barcellos L.F., Agliardi C., Alfredsson L., Alizadeh M., Anderson C., Andrews R., Sondergaard H.B., Baker A., Band G., Baranzini S.E., Barizzone N., Barrett J., Bellenguez C., Bergamaschi L., Bernardinelli L., Berthele A., Biberacher V., Binder T.M., Blackburn H., Bomfim I.L.,

Brambilla P., Broadley S., Brochet B., Brundin L., Buck D., Butzkueven H., Caillier S.J., Camu W., Carpentier W., Cavalla P., Celius E.G., Coman I., Comi G., Corrado L., Cosemans L., Cournu-Rebeix I., Cree B.A., Cusi D., Damotte V., Defer G., Delgado S.R., Deloukas P., di Sapio A., Dilthey A.T., Donnelly P., Dubois B., Duddy M., Edkins S., Elovaara I., Esposito F., Evangelou N., Fiddes B., Field J., Franke A., Freeman C., Frohlich I.Y., Galimberti D., Gieger C., Gourraud P.A., Graetz C., Graham A., Grummel V., Guaschino C., Hadjixenofontos A., Hakonarson H., Halfpenny C., Hall G., Hall P., Hamsten A., Harley J., Harrower T., Hawkins C., Hellenthal G., Hillier C., Hobart J., Hoshi M., Hunt S.E., Jagodic M., Jelcic I., Jochim A., Kendall B., Kermode A., Kilpatrick T., Koivisto K., Konidari I., Korn T., Kronsbein H., Langford C., Larsson M., Lathrop M., Lebrun-Frenay C., Lechner-Scott J., Lee M.H., Leone M.A., Leppa V., Liberatore G., Lie B.A., Lill C.M., Linden M., Link J., Luessi F., Lycke J., Macciardi F., Mannisto S., Manrique C.P., Martin R., Martinelli V., Mason D., Mazibrada G., McCabe C., Mero I.L., Mescheriakova J., Moutsianas L., Myhr K.M., Nagels G., Nicholas R., Nilsson P., Piehl F., Pirinen M., Price S.E., Quach H., Reunanen M., Robberecht W., Robertson N.P., Rodegher M., Rog D., Salvetti M., Schnetz-Boutaud N.C., Sellebjerg F., Selter R.C., Schaefer C., Shaunak S., Shen L., Shields S., Siffrin V., Slee M., Sorensen P.S., Sorosina M., Sospedra M., Spurkland A., Strange A., Sundqvist E., Thijs V., Thorpe J., Ticca A., Tienari P., van Duijn C., Visser E.M., Vucic S., Westerlind H., Wiley J.S., Wilkins A., Wilson J.F., Winkelmann J., Zajicek J., Zindler E., Haines J.L., Pericak-Vance M.A., Ivinson A.J., Stewart G., Hafler D., Hauser S.L., Compston A., McVean G., De Jager P., Sawcer S.J. & McCauley J.L. (2013) Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet* **45**, 1353-60.

Berg A.T. & Shinnar S. (1997) Do seizures beget seizures? An assessment of the clinical evidence in humans. *J Clin Neurophysiol* **14**, 102-10.

Berg A.T., Berkovic S.F., Brodie M.J., Buchhalter J., Cross J.H., van Emde Boas W., Engel J., French J., Glauser T.A., Mathern G.W., Moshe S.L., Nordli D., Plouin P. & Scheffer I.E. (2010) Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. *Epilepsia* **51**, 676-85.

Berg A.T., Langfitt J., Shinnar S., Vickrey B.G., Sperling M.R., Walczak T., Bazil C., Pacia S.V. & Spencer S.S. (2003) How long does it take for partial epilepsy to become intractable? *Neurology* **60**, 186-90.

Berg A.T., Vickrey B.G., Testa F.M., Levy S.R., Shinnar S., DiMario F. & Smith S. (2006) How long does it take for epilepsy to become intractable? A prospective investigation. *Ann Neurol* **60**, 73-9.

Bournissen F.G., Moretti M.E., Juurlink D.N., Koren G., Walker M. & Finkelstein Y. (2009) Polymorphism of the MDR1/ABCB1 C.3435C>T drug-transporter and resistance to anticonvulsant drugs: a meta-analysis. *Epilepsia* **50**, 898-903.

Brandt C., Bethmann K., Gastens A.M. & Loscher W. (2006) The multidrug transporter hypothesis of drug resistance in epilepsy: Proof-of-principle in a rat model of temporal lobe epilepsy. *Neurobiol Dis* **24**, 202-11.

Brodie M.J. (2010) Antiepileptic drug therapy the story so far. *Seizure* **19**, 650-5.

Brooks-Kayal A.R., Shumate M.D., Jin H., Rikhter T.Y. & Coulter D.A. (1998) Selective changes in single cell GABA(A) receptor subunit expression and function in temporal lobe epilepsy. *Nat Med* **4**, 1166-72.

Callaghan B.C., Anand K., Hesdorffer D., Hauser W.A. & French J.A. (2007) Likelihood of seizure remission in an adult population with refractory epilepsy. *Ann Neurol* **62**, 382-9.

Caspi R., Altman T., Billington R., Dreher K., Foerster H., Fulcher C.A., Holland T.A., Keseler I.M., Kothari A., Kubo A., Krummenacker M., Latendresse M., Mueller L.A., Ong Q., Paley S., Subhraveti P., Weaver D.S., Weerasinghe D., Zhang P. & Karp P.D. (2014) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Res* **42**, D459-71.

Cerveny L., Pavek P., Malakova J., Staud F. & Fendrich Z. (2006) Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. *Epilepsia* **47**, 461-8.

Chan P.S., Zhang C., Zuo Z., Kwan P. & Baum L. (2014) In vitro transport assays of rufinamide, pregabalin, and zonisamide by human P-glycoprotein. *Epilepsy Res* **108**, 359-66.

Chatr-Aryamontri A., Breitkreutz B.J., Heinicke S., Boucher L., Winter A., Stark C., Nixon J., Ramage L., Kolas N., O'Donnell L., Reguly T., Breitkreutz A., Sellam A., Chen D., Chang C., Rust J., Livstone M., Oughtred R., Dolinski K. & Tyers M. (2013) The BioGRID interaction database: 2013 update. *Nucleic Acids Res* **41**, D816-23.

Chayasirisobhon S. (2009) The mechanisms of medically refractory temporal lobe epilepsy. *Acta Neurol Taiwan* **18**, 155-60.

Chen L., Liu C.Q., Hu Y., Xiao Z.T., Chen Y. & Liao J.X. (2007) [Association of a polymorphism in MDR1 C.3435C>T with response to antiepileptic drug treatment in ethic Han Chinese children with epilepsy]. *Zhongguo Dang Dai Er Ke Za Zhi* **9**, 11-4.

Chen Y., Lu J., Pan H., Zhang Y., Wu H., Xu K., Liu X., Jiang Y., Bao X., Yao Z., Ding K., Lo W.H., Qiang B., Chan P., Shen Y. & Wu X. (2003) Association between genetic variation of CACNA1H and childhood absence epilepsy. *Ann Neurol* **54**, 239-43.

Cho D.Y., Kim Y.A. & Przytycka T.M. (2012) Chapter 5: Network biology approach to complex diseases. *PLoS Comput Biol* **8**, e1002820.

Cockerell O.C., Johnson A.L., Sander J.W. & Shorvon S.D. (1997) Prognosis of epilepsy: a review and further analysis of the first nine years of the British National General Practice Study of Epilepsy, a prospective population-based study. *Epilepsia* **38**, 31-46.

Croft D., Mundo A.F., Haw R., Milacic M., Weiser J., Wu G., Caudy M., Garapati P., Gillespie M., Kamdar M.R., Jassal B., Jupe S., Matthews L., May B., Palatnik S., Rothfels K., Shamovsky V., Song H., Williams M., Birney E., Hermjakob H., Stein L. & D'Eustachio P. (2014) The Reactome pathway knowledgebase. *Nucleic Acids Res* **42**, D472-7.

Crowe A. & Teoh Y.K. (2006) Limited P-glycoprotein mediated efflux for anti-epileptic drugs. *J Drug Target* **14**, 291-300.

Cucullo L., Hossain M., Rapp E., Manders T., Marchi N. & Janigro D. (2007) Development of a humanized in vitro blood-brain barrier model to screen for brain penetration of antiepileptic drugs. *Epilepsia* **48**, 505-16.

Curtis R.K., Oresic M. & Vidal-Puig A. (2005) Pathways to the analysis of microarray data. *Trends Biotechnol* **23**, 429-35.

Davey Smith G., Ebrahim S., Lewis S., Hansell A.L., Palmer L.J. & Burton P.R. (2005) Genetic epidemiology and public health: hope, hype, and future prospects. *Lancet* **366**, 1484-98.

Davies G.F., Berg A., Postnikoff S.D., Wilson H.L., Arnason T.G., Kusalik A. & Harkness T.A. (2014) TFPI1 mediates resistance to doxorubicin in breast cancer cells by inducing a hypoxic-like response. *PLoS One* **9**, e84611.

de Silva E., Thorne T., Ingram P., Agrafioti I., Swire J., Wiuf C. & Stumpf M.P. (2006) The effects of incomplete protein interaction data on structural and evolutionary inferences. *BMC Biol* **4**, 39.

Degenhardt Y., Huang J., Greshock J., Horiates G., Nathanson K., Yang X., Herlyn M. & Weber B. (2010) Distinct MHC gene expression patterns during progression of melanoma. *Genes Chromosomes Cancer* **49**, 144-54.

Dericioglu N., Babaoglu M.O., Yasar U., Bal I.B., Bozkurt A. & Saygi S. (2008) Multidrug resistance in patients undergoing resective epilepsy surgery is not associated with C.3435C>T polymorphism in the ABCB1 (MDR1) gene. *Epilepsy Res* **80**, 42-6.

Dibbens L.M., Heron S.E. & Mulley J.C. (2007) A polygenic heterogeneity model for common epilepsies with complex genetics. *Genes Brain Behav* **6**, 593-7.

Dickens D., Yusof S.R., Abbott N.J., Weksler B., Romero I.A., Couraud P.O., Alfirevic A., Pirmohamed M. & Owen A. (2013) A multi-system approach assessing the interaction of anticonvulsants with P-gp. *PLoS One* **8**, e64854.

Dimas A.S., Deutsch S., Stranger B.E., Montgomery S.B., Borel C., Attar-Cohen H., Ingle C., Beazley C., Gutierrez Arcelus M., Sekowska M., Gagnebin M., Nisbett J., Deloukas P., Dermitzakis E.T. & Antonarakis S.E. (2009) Common regulatory variation impacts gene expression in a cell type-dependent manner. *Science* **325**, 1246-50.

Dombrowski S.M., Desai S.Y., Marroni M., Cucullo L., Goodrich K., Bingaman W., Mayberg M.R., Bengez L. & Janigro D. (2001) Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42**, 1501-6.

Dong L., Luo R., Tong Y., Cai X., Mao M. & Yu D. (2011) Lack of association between ABCB1 gene polymorphisms and pharmacoresistant epilepsy: an analysis in a western Chinese pediatric population. *Brain Res* **1391**, 114-24.

Drion N., Lemaire M., Lefauconnier J.M. & Scherrmann J.M. (1996) Role of P-glycoprotein in the blood-brain transport of colchicine and vinblastine. *J Neurochem* **67**, 1688-93.

Ebid A.H., Ahmed M.M. & Mohammed S.A. (2007) Therapeutic drug monitoring and clinical outcomes in epileptic Egyptian patients: a gene polymorphism perspective study. *Ther Drug Monit* **29**, 305-12.

Englot D.J., Chang E.F. & Auguste K.I. (2011) Vagus nerve stimulation for epilepsy: a meta-analysis of efficacy and predictors of response. *J Neurosurg* **115**, 1248-55.

Feldmann M., Asselin M.C., Liu J., Wang S., McMahon A., Anton-Rodriguez J., Walker M., Symms M., Brown G., Hinz R., Matthews J., Bauer M., Langer O., Thom M., Jones T., Vollmar C., Duncan J.S., Sisodiya S.M. & Koepp M.J. (2013) P-glycoprotein expression and function in patients with temporal lobe epilepsy: a case-control study. *Lancet Neurol* **12**, 777-85.

Feng B., Mills J.B., Davidson R.E., Mireles R.J., Janiszewski J.S., Troutman M.D. & de Morais S.M. (2008) In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. *Drug Metab Dispos* **36**, 268-75.

Fisher R.S., Acevedo C., Arzimanoglou A., Bogacz A., Cross J.H., Elger C.E., Engel J., Jr., Forsgren L., French J.A., Glynn M., Hesdorffer D.C., Lee B.I., Mathern G.W., Moshe S.L., Perucca E., Scheffer I.E., Tomson T., Watanabe M. & Wiebe S. (2014) ILAE official report: a practical clinical definition of epilepsy. *Epilepsia* **55**, 475-82.

Fisher R.S., van Emde Boas W., Blume W., Elger C., Genton P., Lee P. & Engel J., Jr. (2005) Epileptic seizures and epilepsy: definitions proposed by the International League

Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* **46**, 470-2.

Forsgren L., Beghi E., Oun A. & Sillanpaa M. (2005) The epidemiology of epilepsy in Europe - a systematic review. *Eur J Neurol* **12**, 245-53.

French J.A. (2006) Refractory epilepsy: one size does not fit all. *Epilepsy Curr* **6**, 177-80.

Fridley B.L. & Biernacka J.M. (2011) Gene set analysis of SNP data: benefits, challenges, and future directions. *Eur J Hum Genet* **19**, 837-43.

Friend S.H. (2010) The need for precompetitive integrative bionetwork disease model building. *Clin Pharmacol Ther* **87**, 536-9.

Fritsch B., Stott J.J., Joelle Donofrio J. & Rogawski M.A. (2010) Treatment of early and late kainic acid-induced status epilepticus with the noncompetitive AMPA receptor antagonist GYKI 52466. *Epilepsia* **51**, 108-17.

Gao X., Zhou S., Guo Q. & Sun D. (2009) Polymorphism of multidrug-resistance gene in childhood refractory epilepsy. *J. Clin. Pediatr.* **27**, 1014–8.

Ge D., Fellay J., Thompson A.J., Simon J.S., Shianna K.V., Urban T.J., Heinzen E.L., Qiu P., Bertelsen A.H., Muir A.J., Sulkowski M., McHutchison J.G. & Goldstein D.B. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461**, 399-401.

Girardin F. (2006) Membrane transporter proteins: a challenge for CNS drug development. *Dialogues Clin Neurosci* **8**, 311-21.

Grover S. & Kukreti R. (2013) A systematic review and meta-analysis of the role of ABCC2 variants on drug response in patients with epilepsy. *Epilepsia* **54**, 936-45.

Grover S., Bala K., Sharma S., Gourie-Devi M., Baghel R., Kaur H., Gupta M., Talwar P. & Kukreti R. (2010) Absence of a general association between ABCB1 genetic variants and response to antiepileptic drugs in epilepsy patients. *Biochimie* **92**, 1207-12.

Guimaraes J. & Ribeiro J.A. (2010) Pharmacology of antiepileptic drugs in clinical practice. *Neurologist* **16**, 353-7.

Gupta A.K., Katoch V.M., Chauhan D.S., Sharma R., Singh M., Venkatesan K. & Sharma V.D. (2010) Microarray analysis of efflux pump genes in multidrug-resistant Mycobacterium tuberculosis during stress induced by common anti-tuberculous drugs. *Microb Drug Resist* **16**, 21-8.

Haerian B.S., Baum L., Kwan P., Tan H.J., Raymond A.A. & Mohamed Z. (2013) SCN1A, SCN2A and SCN3A gene polymorphisms and responsiveness to antiepileptic drugs: a multicenter cohort study and meta-analysis. *Pharmacogenomics* **14**, 1153-66.

Haerian B.S., Lim K.S., Mohamed E.H., Tan H.J., Tan C.T., Raymond A.A., Wong C.P., Wong S.W. & Mohamed Z. (2011a) Lack of association of ABCB1 and PXR polymorphisms with response to treatment in epilepsy. *Seizure* **20**, 387-94.

Haerian B.S., Lim K.S., Tan H.J., Mohamed E.H., Tan C.T., Raymond A.A., Wong C.P., Wong S.W., Omar H., Roslan H. & Mohamed Z. (2011b) Association between ABCB1 polymorphism and response to sodium valproate treatment in Malaysian epilepsy patients. *Epileptic Disord* **13**, 65-75.

Haerian B.S., Roslan H., Raymond A.A., Tan C.T., Lim K.S., Zulkifli S.Z., Mohamed E.H., Tan H.J. & Mohamed Z. (2010) ABCB1 C.3435C>T polymorphism and the risk of resistance to antiepileptic drugs in epilepsy: a systematic review and meta-analysis. *Seizure* **19**, 339-46.

Hajnsek S., Basic S., Poljakovic Z. & Bozina N. (2004) Utjecaj C.3435C>T polimorfizma MDR1 gena na ucinkovitost medikamentnog lijecenja epilepsia. *Neurol Croat* **53**, 69–78.

Hawkins N.A., Martin M.S., Frankel W.N., Kearney J.A. & Escayg A. (2011) Neuronal voltage-gated ion channels are genetic modifiers of generalized epilepsy with febrile seizures plus. *Neurobiol Dis* **41**, 655-60.

Heinzen E.L., Depondt C., Cavalleri G.L., Ruzzo E.K., Walley N.M., Need A.C., Ge D., He M., Cirulli E.T., Zhao Q., Cronin K.D., Gumbs C.E., Campbell C.R., Hong L.K., Maia J.M., Shianna K.V., McCormack M., Radtke R.A., O'Conner G.D., Mikati M.A., Gallentine W.B., Husain A.M., Sinha S.R., Chinthapalli K., Puranam R.S., McNamara J.O., Ottman R., Sisodiya S.M., Delanty N. & Goldstein D.B. (2012) Exome sequencing followed by large-scale genotyping fails to identify single rare variants of large effect in idiopathic generalized epilepsy. *Am J Hum Genet* **91**, 293-302.

Heinzen E.L., Radtke R.A., Urban T.J., Cavalleri G.L., Depondt C., Need A.C., Walley N.M., Nicoletti P., Ge D., Catarino C.B., Duncan J.S., Kasperaviciute D., Tate S.K., Caboclo L.O., Sander J.W., Clayton L., Linney K.N., Shianna K.V., Gumbs C.E., Smith J., Cronin K.D., Maia J.M., Doherty C.P., Pandolfo M., Leppert D., Middleton L.T., Gibson R.A., Johnson M.R., Matthews P.M., Hosford D., Kalviainen R., Eriksson K., Kantanen A.M., Dorn T., Hansen J., Kramer G., Steinhoff B.J., Wieser H.G., Zumsteg D., Ortega M., Wood N.W., Huxley-Jones J., Mikati M., Gallentine W.B., Husain A.M., Buckley P.G., Stallings R.L., Podgoreanu M.V., Delanty N., Sisodiya S.M. & Goldstein D.B. (2010) Rare deletions at 16p13.11 predispose to a diverse spectrum of sporadic epilepsy syndromes. *Am J Hum Genet* **86**, 707-18.

Helbig I., Mefford H.C., Sharp A.J., Guipponi M., Fichera M., Franke A., Muhle H., de Kovel C., Baker C., von Spiczak S., Kron K.L., Steinich I., Kleefuss-Lie A.A., Leu C., Gaus V., Schmitz B., Klein K.M., Reif P.S., Rosenow F., Weber Y., Lerche H., Zimprich F., Urak L., Fuchs K., Feucht M., Genton P., Thomas P., Visscher F., de Haan G.J., Moller R.S., Hjalgrim H., Luciano D., Wittig M., Nothnagel M., Elger C.E., Nurnberg P., Romano C., Malafosse A., Koeleman B.P., Lindhout D., Stephani U., Schreiber S., Eichler E.E. & Sander T. (2009) 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. *Nat Genet* **41**, 160-2.

Hirtz D., Thurman D.J., Gwinn-Hardy K., Mohamed M., Chaudhuri A.R. & Zalutsky R. (2007) How common are the "common" neurologic disorders? *Neurology* **68**, 326-37.

Huang Y. & Sadee W. (2006) Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. *Cancer Lett* **239**, 168-82.

Hung C.C., Jen Tai J., Kao P.J., Lin M.S. & Liou H.H. (2007) Association of polymorphisms in NR1I2 and ABCB1 genes with epilepsy treatment responses. *Pharmacogenomics* **8**, 1151-8.

Hung C.C., Tai J.J., Lin C.J., Lee M.J. & Liou H.H. (2005) Complex haplotypic effects of the ABCB1 gene on epilepsy treatment response. *Pharmacogenomics* **6**, 411-7.

Jimenez-Marin A., Collado-Romero M., Ramirez-Boo M., Arce C. & Garrido J.J. (2009) Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. *BMC Proc* **3 Suppl 4**, S6.

Johnson M.R., Milne R.L., Torn-Broers Y., Hopper J.L., Scheffer I.E. & Berkovic S.F. (2003) A twin study of genetic influences on epilepsy outcome. *Twin Res* **6**, 140-6.

Joshi S. & Kapur J. (2012) GABAA Receptor Plasticity During Status Epilepticus. In: *Jasper's Basic Mechanisms of the Epilepsies* (eds. by Noebels JL, Avoli M, Rogawski MA, Olsen RW & Delgado-Escueta AV), Bethesda (MD).

Kearney J.A., Yang Y., Beyer B., Bergren S.K., Claes L., Dejonghe P. & Frankel W.N. (2006) Severe epilepsy resulting from genetic interaction between Scn2a and Kcnq2. *Hum Mol Genet* **15**, 1043-8.

Kerrien S., Aranda B., Breuza L., Bridge A., Broackes-Carter F., Chen C., Duesbury M., Dumousseau M., Feuermann M., Hinz U., Jandrasits C., Jimenez R.C., Khadake J., Mahadevan U., Masson P., Pedruzzi I., Pfeiffenberger E., Porras P., Raghunath A., Roechert B., Orchard S. & Hermjakob H. (2012) The IntAct molecular interaction database in 2012. *Nucleic Acids Res* **40**, D841-6.

Kim D.W., Kim M., Lee S.K., Kang R. & Lee S.Y. (2006a) Lack of association between C.3435C>T nucleotide MDR1 genetic polymorphism and multidrug-resistant epilepsy. *Seizure* **15**, 344-7.

Kim D.W., Lee S.K., Chu K., Jang I.J., Yu K.S., Cho J.Y. & Kim S.J. (2009) Lack of association between ABCB1, ABCG2, and ABCC2 genetic polymorphisms and multidrug resistance in partial epilepsy. *Epilepsy Res* **84**, 86-90.

Kim D.W., Lee S.K., Chu K., Jang I.J., Yu K.S., Cho J.Y. & Kim S.J. (2009) Lack of association between ABCB1, ABCG2, and ABCC2 genetic polymorphisms and multidrug resistance in partial epilepsy. *Epilepsy Res* **84**, 86-90.

Kim L.G., Johnson T.L., Marson A.G. & Chadwick D.W. (2006) Prediction of risk of seizure recurrence after a single seizure and early epilepsy: further results from the MESS trial. *Lancet Neurol* **5**, 317-22.

Kim Y.O., Kim M.K., Woo Y.J., Lee M.C., Kim J.H., Park K.W., Kim E.Y., Roh Y.I. & Kim C.J. (2006b) Single nucleotide polymorphisms in the multidrug resistance 1 gene in Korean epileptics. *Seizure* **15**, 67-72.

Klein R.J., Xu X., Mukherjee S., Willis J. & Hayes J. (2010) Successes of genome-wide association studies. *Cell* **142**, 350-1; author reply 3-5.

Kubota H., Ishihara H., Langmann T., Schmitz G., Stieger B., Wieser H.G., Yonekawa Y. & Frei K. (2006) Distribution and functional activity of P-glycoprotein and multidrug resistance-associated proteins in human brain microvascular endothelial cells in hippocampal sclerosis. *Epilepsy Res* **68**, 213-28.

Kwan P. & Brodie M.J. (2000) Early identification of refractory epilepsy. *N Engl J Med* **342**, 314-9.

Kwan P., Arzimanoglou A., Berg A.T., Brodie M.J., Allen Hauser W., Mathern G., Moshe S.L., Perucca E., Wiebe S. & French J. (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. *Epilepsia* **51**, 1069-77.

Kwan P., Baum L., Wong V., Ng P.W., Lui C.H., Sin N.C., Hui A.C., Yu E. & Wong L.K. (2007) Association between ABCB1 C.3435C>T polymorphism and drug-resistant epilepsy in Han Chinese. *Epilepsy Behav* **11**, 112-7.

Kwan P., Wong V., Ng P.W., Lui C.H., Sin N.C., Poon W.S., Ng H.K., Wong K.S. & Baum L. (2009) Gene-wide tagging study of association between ABCB1 polymorphisms and multidrug resistance in epilepsy in Han Chinese. *Pharmacogenomics* **10**, 723-32.

Kwan P., Wong V., Ng P.W., Lui C.H., Sin N.C., Wong K.S. & Baum L. (2011) Gene-wide tagging study of the association between ABCC2, ABCC5 and ABCG2 genetic polymorphisms and multidrug resistance in epilepsy. *Pharmacogenomics* **12**, 319-25.

Lakhan R., Misra U.K., Kalita J., Pradhan S., Gogtay N.J., Singh M.K. & Mittal B. (2009) No association of ABCB1 polymorphisms with drug-refractory epilepsy in a north Indian population. *Epilepsy Behav* **14**, 78-82.

Lazarowski A., Lubieniecki F., Camarero S., Pomata H., Bartuluchi M., Sevlever G. & Taratuto A.L. (2004) Multidrug resistance proteins in tuberous sclerosis and refractory epilepsy. *Pediatr Neurol* **30**, 102-6.

Lazarowski A.J., Lubieniecki F.J., Camarero S.A., Pomata H.H., Bartuluchi M.A., Sevlever G. & Taratuto A.L. (2006) New proteins configure a brain drug resistance map in tuberous sclerosis. *Pediatr Neurol* **34**, 20-4.

Lega B.C., Halpern C.H., Jaggi J.L. & Baltuch G.H. (2010) Deep brain stimulation in the treatment of refractory epilepsy: update on current data and future directions. *Neurobiol Dis* **38**, 354-60.

Lennox W.G. (1951) The heredity of epilepsy as told by relatives and twins. *J Am Med Assoc* **146**, 529-36.

Leschziner G., Jorgensen A.L., Andrew T., Pirmohamed M., Williamson P.R., Marson A.G., Coffey A.J., Middleditch C., Rogers J., Bentley D.R., Chadwick D.W., Balding D.J. & Johnson M.R. (2006) Clinical factors and ABCB1 polymorphisms in prediction of antiepileptic drug response: a prospective cohort study. *Lancet Neurol* **5**, 668-76.

Leschziner G.D., Andrew T., Leach J.P., Chadwick D., Coffey A.J., Balding D.J., Bentley D.R., Pirmohamed M. & Johnson M.R. (2007) Common ABCB1 polymorphisms are not associated with multidrug resistance in epilepsy using a gene-wide tagging approach. *Pharmacogenet Genomics* **17**, 217-20.

Leschziner G.D., Andrew T., Leach J.P., Chadwick D., Coffey A.J., Balding D.J., Bentley D.R., Pirmohamed M. & Johnson M.R. (2007) Common ABCB1 polymorphisms are not associated with multidrug resistance in epilepsy using a gene-wide tagging approach. *Pharmacogenet Genomics* **17**, 217-20.

Liu J.Y., Thom M., Catarino C.B., Martinian L., Figarella-Branger D., Bartolomei F., Koepp M. & Sisodiya S.M. (2012) Neuropathology of the blood-brain barrier and pharmaco-resistance in human epilepsy. *Brain* **135**, 3115-33.

Lohmueller K.E., Pearce C.L., Pike M., Lander E.S. & Hirschhorn J.N. (2003) Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* **33**, 177-82.

Loscher W. & Rundfeldt C. (1991) Kindling as a model of drug-resistant partial epilepsy: selection of phenytoin-resistant and nonresistant rats. *J Pharmacol Exp Ther* **258**, 483-9.

Loscher W. & Sills G.J. (2007) Drug resistance in epilepsy: why is a simple explanation not enough? *Epilepsia* **48**, 2370-2.

Loscher W. (2011) Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure* **20**, 359-68.

Lu J., Ren H., Zhu G., Yu L., Ding D. & Hong Z. (2007) Association of the C.3435C>T polymorphism in the multidrug resistance gene 1 and response to antiepileptic drug treatment in epilepsy patients. *Chinese Journal of Neurology* **40**, 584-7.

Luciano A.L. & Shorvon S.D. (2007) Results of treatment changes in patients with apparently drug-resistant chronic epilepsy. *Ann Neurol* **62**, 375-81.

Luna-Tortos C., Fedrowitz M. & Loscher W. (2008) Several major antiepileptic drugs are substrates for human P-glycoprotein. *Neuropharmacology* **55**, 1364-75.

Luna-Tortos C., Fedrowitz M. & Loscher W. (2010) Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. *Neuropharmacology* **58**, 1019-32.

Luna-Tortos C., Rambeck B., Jurgens U.H. & Loscher W. (2009) The antiepileptic drug topiramate is a substrate for human P-glycoprotein but not multidrug resistance proteins. *Pharm Res* **26**, 2464-70.

Ma A., Wang C., Chen Y. & Yuan W. (2013) P-glycoprotein alters blood-brain barrier penetration of antiepileptic drugs in rats with medically intractable epilepsy. *Drug Des Devel Ther* **7**, 1447-54.

MacDonald B.K., Johnson A.L., Goodridge D.M., Cockerell O.C., Sander J.W. & Shorvon S.D. (2000) Factors predicting prognosis of epilepsy after presentation with seizures. *Ann Neurol* **48**, 833-41.

Mahar Doan K.M., Humphreys J.E., Webster L.O., Wring S.A., Shampine L.J., Serabjit-Singh C.J., Adkison K.K. & Polli J.W. (2002) Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *J Pharmacol Exp Ther* **303**, 1029-37.

Maher B. (2008) Personal genomes: The case of the missing heritability. *Nature* **456**, 18-21.

Marchi N., Guiso G., Rizzi M., Pirker S., Novak K., Czech T., Baumgartner C., Janigro D., Caccia S. & Vezzani A. (2005) A pilot study on brain-to-plasma partition of 10,11-dyhydro-10-hydroxy-5H-dibenzo(b,f)azepine-5-carboxamide and MDR1 brain expression in epilepsy patients not responding to oxcarbazepine. *Epilepsia* **46**, 1613-9.

Marson A., Jacoby A., Johnson A., Kim L., Gamble C. & Chadwick D. (2005) Immediate versus deferred antiepileptic drug treatment for early epilepsy and single seizures: a randomised controlled trial. *Lancet* **365**, 2007-13.

Marth G.T., Korf I., Yandell M.D., Yeh R.T., Gu Z., Zakeri H., Stitziel N.O., Hillier L., Kwok P.Y. & Gish W.R. (1999) A general approach to single-nucleotide polymorphism discovery. *Nat Genet* **23**, 452-6.

Mayanagi Y., Watanabe E. & Kaneko Y. (1996) Mesial temporal lobe epilepsy: clinical features and seizure mechanism. *Epilepsia* **37 Suppl 3**, 57-60.

Meldrum B.S. & Rogawski M.A. (2007) Molecular targets for antiepileptic drug development. *Neurotherapeutics* **4**, 18-61.

Mendrick D.L. (2011) Transcriptional profiling to identify biomarkers of disease and drug response. *Pharmacogenomics* **12**, 235-49.

Meng H., Guo G., Ren J., Zhou H., Ge Y. & Guo Y. (2011) Effects of ABCB1 polymorphisms on plasma carbamazepine concentrations and pharmacoresistance in Chinese patients with epilepsy. *Epilepsy Behav* **21**, 27-30.

Mohanraj R. & Brodie M.J. (2006) Diagnosing refractory epilepsy: response to sequential treatment schedules. *Eur J Neurol* **13**, 277-82.

Murray C.J., Vos T., Lozano R., Naghavi M., Flaxman A.D., Michaud C., Ezzati M., Shibuya K., Salomon J.A., Abdalla S., Aboyans V., Abraham J., Ackerman I., Aggarwal R., Ahn S.Y., Ali M.K., Alvarado M., Anderson H.R., Anderson L.M., Andrews K.G., Atkinson C., Baddour L.M., Bahalim A.N., Barker-Collo S., Barrero L.H., Bartels D.H., Basanez M.G., Baxter A., Bell M.L., Benjamin E.J., Bennett D., Bernabe E., Bhalla K., Bhandari B., Bikbov B., Bin Abdulhak A., Birbeck G., Black J.A., Blencowe H., Blore J.D., Blyth F., Bolliger I., Bonaventure A., Boufous S., Bourne R., Boussinesq M., Braithwaite T., Brayne C., Bridgett L., Brooker S., Brooks P., Brugha T.S., Bryan-Hancock C., Bucello C., Buchbinder R., Buckle G., Budke C.M., Burch M., Burney P., Burstein R., Calabria B., Campbell B., Canter C.E., Carabin H., Carapetis J., Carmona L., Cella C., Charlson F., Chen H., Cheng A.T., Chou D., Chugh S.S., Coffeng L.E., Colan S.D., Colquhoun S., Colson K.E., Condon J., Connor M.D., Cooper L.T., Corriere M., Cortinovis M., de

Vaccaro K.C., Couser W., Cowie B.C., Criqui M.H., Cross M., Dabhadkar K.C., Dahiya M., Dahodwala N., Damsere-Derry J., Danaei G., Davis A., De Leo D., Degenhardt L., Dellavalle R., Delossantos A., Denenberg J., Derrett S., Des Jarlais D.C., Dharmaratne S.D., Dherani M., Diaz-Torne C., Dolk H., Dorsey E.R., Driscoll T., Duber H., Ebel B., Edmond K., Elbaz A., Ali S.E., Erskine H., Erwin P.J., Espindola P., Ewoigbokhan S.E., Farzadfar F., Feigin V., Felson D.T., Ferrari A., Ferri C.P., Fevre E.M., Finucane M.M., Flaxman S., Flood L., Foreman K., Forouzanfar M.H., Fowkes F.G., Fransen M., Freeman M.K., Gabbe B.J., Gabriel S.E., Gakidou E., Ganatra H.A., Garcia B., Gaspari F., Gillum R.F., Gmel G., Gonzalez-Medina D., Gosselin R., Grainger R., Grant B., Groeger J., Guillemin F., Gunnell D., Gupta R., Haagsma J., Hagan H., Halasa Y.A., Hall W., Haring D., Haro J.M., Harrison J.E., Havmoeller R., Hay R.J., Higashi H., Hill C., Hoen B., Hoffman H., Hotez P.J., Hoy D., Huang J.J., Ibeanusi S.E., Jacobsen K.H., James S.L., Jarvis D., Jasrasaria R., Jayaraman S., Johns N., Jonas J.B., Karthikeyan G., Kassebaum N., Kawakami N., Keren A., Khoo J.P., King C.H., Knowlton L.M., Kobusingye O., Koranteng A., Krishnamurthi R., Laden F., Lalloo R., Laslett L.L., Lathlean T., Leasher J.L., Lee Y.Y., Leigh J., Levinson D., Lim S.S., Limb E., Lin J.K., Lipnick M., Lipshultz S.E., Liu W., Loane M., Ohno S.L., Lyons R., Mabweijano J., MacIntyre M.F., Malekzadeh R., Mallinger L., Manivannan S., Marcenes W., March L., Margolis D.J., Marks G.B., Marks R., Matsumori A., Matzopoulos R., Mayosi B.M., McAnulty J.H., McDermott M.M., McGill N., McGrath J., Medina-Mora M.E., Meltzer M., Mensah G.A., Merriman T.R., Meyer A.C., Miglioli V., Miller M., Miller T.R., Mitchell P.B., Mock C., Mocumbi A.O., Moffitt T.E., Mokdad A.A., Monasta L., Montico M., Moradi-Lakeh M., Moran A., Morawska L., Mori R., Murdoch M.E., Mwaniki M.K., Naidoo K., Nair M.N., Naldi L., Narayan K.M., Nelson P.K., Nelson R.G., Nevitt M.C., Newton C.R., Nolte S., Norman P., Norman R., O'Donnell M., O'Hanlon S., Olives C., Omer S.B., Ortblad K., Osborne R., Ozgediz D., Page A., Pahari B., Pandian J.D., Rivero A.P., Patten S.B., Pearce N., Padilla R.P., Perez-Ruiz F., Perico N., Pesudovs K., Phillips D., Phillips M.R., Pierce K., Pion S., Polanczyk G.V., Polinder S., Pope C.A., 3rd, Popova S., Porrini E., Pourmalek F., Prince M., Pullan R.L., Ramaiah K.D., Ranganathan D., Razavi H., Regan M., Rehm J.T., Rein D.B., Remuzzi G., Richardson K., Rivara F.P., Roberts T., Robinson C., De Leon F.R., Ronfani L., Room R., Rosenfeld L.C., Rushton L., Sacco R.L., Saha S., Sampson U., Sanchez-Riera L., Sanman E., Schwebel D.C., Scott J.G., Segui-Gomez M., Shahraz S., Shepard D.S., Shin H., Shivakoti R., Singh D., Singh G.M., Singh J.A., Singleton J., Sleet D.A., Sliwa K., Smith E., Smith J.L., Stapelberg N.J., Steer A., Steiner T., Stolk W.A., Stovner L.J., Sudfeld C., Syed S., Tamburlini G., Tavakkoli M., Taylor H.R., Taylor J.A., Taylor W.J., Thomas B., Thomson W.M., Thurston G.D., Tleyjeh I.M., Tonelli M., Towbin J.A., Truelsen T., Tsilimbaris M.K., Ubeda C., Undurraga E.A., van der Werf M.J., van Os J., Vavilala M.S., Venketasubramanian N., Wang M., Wang W., Watt K., Weatherall D.J., Weinstock M.A., Weintraub R., Weisskopf M.G., Weissman M.M., White R.A., Whiteford H., Wiebe N., Wiersma S.T., Wilkinson J.D., Williams H.C., Williams S.R., Witt E., Wolfe F., Woolf A.D., Wulf S., Yeh P.H., Zaidi A.K., Zheng Z.J., Zonies D., Lopez A.D., AlMazroa M.A. & Memish Z.A. (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2197-223.

Musicco M., Beghi E., Solari A. & Viani F. (1997) Treatment of first tonic-clonic seizure does not improve the prognosis of epilepsy. First Seizure Trial Group (FIRST Group). *Neurology* **49**, 991-8.

Musunuru K., Strong A., Frank-Kamenetsky M., Lee N.E., Ahfeldt T., Sachs K.V., Li X., Li H., Kuperwasser N., Ruda V.M., Pirruccello J.P., Muchmore B., Prokunina-Olsson L., Hall J.L., Schadt E.E., Morales C.R., Lund-Katz S., Phillips M.C., Wong J., Cantley W., Racie T., Ejebe K.G., Orho-Melander M., Melander O., Koteliansky V., Fitzgerald K., Krauss R.M., Cowan C.A., Kathiresan S. & Rader D.J. (2010) From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. *Nature* **466**, 714-9.

Ngugi A.K., Bottomley C., Kleinschmidt I., Sander J.W. & Newton C.R. (2010) Estimation of the burden of active and life-time epilepsy: a meta-analytic approach. *Epilepsia* **51**, 883-90.

Olesen J., Gustavsson A., Svensson M., Wittchen H.U. & Jonsson B. (2012) The economic cost of brain disorders in Europe. *Eur J Neurol* **19**, 155-62.

Orozco L.D., Bennett B.J., Farber C.R., Ghazalpour A., Pan C., Che N., Wen P., Qi H.X., Mutukulu A., Siemers N., Neuhaus I., Yordanova R., Gargalovic P., Pellegrini M., Kirchgessner T. & Lusis A.J. (2012) Unraveling inflammatory responses using systems genetics and gene-environment interactions in macrophages. *Cell* **151**, 658-70.

Ottman R., Annegers J.F., Risch N., Hauser W.A. & Susser M. (1996) Relations of genetic and environmental factors in the etiology of epilepsy. *Ann Neurol* **39**, 442-9.

Owen A., Pirmohamed M., Tettey J.N., Morgan P., Chadwick D. & Park B.K. (2001) Carbamazepine is not a substrate for P-glycoprotein. *Br J Clin Pharmacol* **51**, 345-9.

Ozgon G.O., Bebek N., Gul G. & Cine N. (2008) Association of MDR1 (C.3435C>T) polymorphism and resistance to carbamazepine in epileptic patients from Turkey. *Eur Neurol* **59**, 67-70.

Potschka H. & Loscher W. (2001) In vivo evidence for P-glycoprotein-mediated transport of phenytoin at the blood-brain barrier of rats. *Epilepsia* **42**, 1231-40.

Potschka H., Fedrowitz M. & Loscher W. (2001) P-glycoprotein and multidrug resistance-associated protein are involved in the regulation of extracellular levels of the major antiepileptic drug carbamazepine in the brain. *Neuroreport* **12**, 3557-60.

Potschka H., Fedrowitz M. & Loscher W. (2002) P-Glycoprotein-mediated efflux of phenobarbital, lamotrigine, and felbamate at the blood-brain barrier: evidence from microdialysis experiments in rats. *Neurosci Lett* **327**, 173-6.

Qu J., Zhou B.T., Yin J.Y., Xu X.J., Zhao Y.C., Lei G.H., Tang Q., Zhou H.H. & Liu Z.Q. (2012) ABCC2 polymorphisms and haplotype are associated with drug resistance in Chinese epileptic patients. *CNS Neurosci Ther* **18**, 647-51.

Raddatz B.B., Hansmann F., Spitzbarth I., Kalkuhl A., Deschl U., Baumgartner W. & Ulrich R. (2014) Transcriptomic meta-analysis of multiple sclerosis and its experimental models. *PLoS One* **9**, e86643.

Radisch S., Dickens D., Lang T., Bonnett L., Arlanov R., Johnson M.R., Schwab M., Marson A.G. & Pirmohamed M. (2014) A comprehensive functional and clinical analysis of ABCC2 and its impact on treatment response to carbamazepine. *Pharmacogenomics J*.

Remy S. & Beck H. (2006) Molecular and cellular mechanisms of pharmacoresistance in epilepsy. *Brain* **129**, 18-35.

Riordan J.R. & Ling V. (1979) Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J Biol Chem* **254**, 12701-5.

Rizzi M., Caccia S., Guiso G., Richichi C., Gorter J.A., Aronica E., Aliprandi M., Bagnati R., Fanelli R., D'Incalci M., Samanin R. & Vezzani A. (2002) Limbic seizures induce P-glycoprotein in rodent brain: functional implications for pharmacoresistance. *J Neurosci* **22**, 5833-9.

Rogawski M.A. & Johnson M.R. (2008) Intrinsic severity as a determinant of antiepileptic drug refractoriness. *Epilepsy Curr* **8**, 127-30.

Rogawski M.A. (2013) The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. *Epilepsia* **54 Suppl 2**, 33-40.

Romanoski C.E., Lee S., Kim M.J., Ingram-Drake L., Plaisier C.L., Yordanova R., Tilford C., Guan B., He A., Gargalovic P.S., Kirchgessner T.G., Berliner J.A. & Lusis A.J. (2010) Systems genetics analysis of gene-by-environment interactions in human cells. *Am J Hum Genet* **86**, 399-410.

Salwinski L., Miller C.S., Smith A.J., Pettit F.K., Bowie J.U. & Eisenberg D. (2004) The Database of Interacting Proteins: 2004 update. *Nucleic Acids Res* **32**, D449-51.

Sanchez M.B., Herranz J.L., Leno C., Arteaga R., Oterino A., Valdizan E.M., Nicolas J.M., Adin J. & Armijo J.A. (2010) Genetic factors associated with drug-resistance of epilepsy: relevance of stratification by patient age and aetiology of epilepsy. *Seizure* **19**, 93-101.

Saygi S., Alehan F., Atac F.B., Erol I., Verdi H. & Erdem R. (2014) Multidrug resistance 1 (MDR1) 3435C/T genotyping in childhood drug-resistant epilepsy. *Brain Dev* **36**, 137-42.

Sayyah M., Kamgarpour F., Maleki M., Karimipoor M., Gharagozli K. & Shamshiri A.R. (2011) Association analysis of intractable epilepsy with C.3435C>T and G2677T/A ABCB1 gene polymorphisms in Iranian patients. *Epileptic Disord* **13**, 155-65.

Schadt E.E. (2009) Molecular networks as sensors and drivers of common human diseases. *Nature* **461**, 218-23.

Schaub C., Uebachs M. & Beck H. (2007) Diminished response of CA1 neurons to antiepileptic drugs in chronic epilepsy. *Epilepsia* **48**, 1339-50.

Schaub M.A., Boyle A.P., Kundaje A., Batzoglou S. & Snyder M. (2012) Linking disease associations with regulatory information in the human genome. *Genome Res* **22**, 1748-59.

Schinkel A.H., Smit J.J., van Tellingen O., Beijnen J.H., Wagenaar E., van Deemter L., Mol C.A., van der Valk M.A., Robanus-Maandag E.C., te Riele H.P. & et al. (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.

Schinkel A.H., Wagenaar E., van Deemter L., Mol C.A. & Borst P. (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96**, 1698-705.

Schmidt D. & Loscher W. (2005) Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. *Epilepsia* **46**, 858-77.

Schmidt D. & Loscher W. (2009) New developments in antiepileptic drug resistance: an integrative view. *Epilepsy Curr* **9**, 47-52.

Schmidt D. & Schachter S.C. (2014) Drug treatment of epilepsy in adults. *BMJ* **348**, g254.

Seo T., Ishitsu T., Ueda N., Nakada N., Yurube K., Ueda K. & Nakagawa K. (2006) ABCB1 polymorphisms influence the response to antiepileptic drugs in Japanese epilepsy patients. *Pharmacogenomics* **7**, 551-61.

Seven M., Batar B., Unal S., Yesil G., Yuksel A. & Guven M. (2014) The drug-transporter gene MDR1 C.3435C>T and G2677T/A polymorphisms and the risk of multidrug-resistant epilepsy in Turkish children. *Mol Biol Rep* **41**, 331-6.

Shahwan A., Murphy K., Doherty C., Cavalleri G.L., Muckian C., Dicker P., McCarthy M., Kinirons P., Goldstein D. & Delanty N. (2007) The controversial association of ABCB1 polymorphisms in refractory epilepsy: an analysis of multiple SNPs in an Irish population. *Epilepsy Res* **73**, 192-8.

Shorvon S. & Luciano A.L. (2007) Prognosis of chronic and newly diagnosed epilepsy: revisiting temporal aspects. *Curr Opin Neurol* **20**, 208-12.

Shorvon S.D. (1996) The epidemiology and treatment of chronic and refractory epilepsy. *Epilepsia* **37 Suppl 2**, S1-S3.

Siddiqui A., Kerb R., Weale M.E., Brinkmann U., Smith A., Goldstein D.B., Wood N.W. & Sisodiya S.M. (2003) Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1. *N Engl J Med* **348**, 1442-8.

Siddiqui A., Kerb R., Weale M.E., Brinkmann U., Smith A., Goldstein D.B., Wood N.W. & Sisodiya S.M. (2003) Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1. *N Engl J Med* **348**, 1442-8.

Sillanpaa M. & Schmidt D. (2006) Natural history of treated childhood-onset epilepsy: prospective, long-term population-based study. *Brain* **129**, 617-24.

Sills G.J., Kwan P., Butler E., de Lange E.C., van den Berg D.J. & Brodie M.J. (2002) P-glycoprotein-mediated efflux of antiepileptic drugs: preliminary studies in mdr1a knockout mice. *Epilepsy Behav* **3**, 427-32.

Sills G.J., Mohanraj R., Butler E., McCrindle S., Collier L., Wilson E.A. & Brodie M.J. (2005) Lack of association between the C.3435C>T polymorphism in the human multidrug resistance (MDR1) gene and response to antiepileptic drug treatment. *Epilepsia* **46**, 643-7.

Simko G.I., Gyurko D., Veres D.V., Nanasi T. & Csermely P. (2009) Network strategies to understand the aging process and help age-related drug design. *Genome Med* **1**, 90.

Simon R. (2006) Targets for treatment success. *Nat Clin Pract Oncol* **3**, 1.

Sisodiya S.M. & Mefford H.C. (2011) Genetic contribution to common epilepsies. *Curr Opin Neurol* **24**, 140-5.

Sisodiya S.M., Lin W.R., Harding B.N., Squier M.V. & Thom M. (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* **125**, 22-31.

Sisodiya S.M., Lin W.R., Squier M.V. & Thom M. (2001) Multidrug-resistance protein 1 in focal cortical dysplasia. *Lancet* **357**, 42-3.

Small K.S., Hedman A.K., Grundberg E., Nica A.C., Thorleifsson G., Kong A., Thorsteindottir U., Shin S.Y., Richards H.B., Soranzo N., Ahmadi K.R., Lindgren C.M., Stefansson K., Dermitzakis E.T., Deloukas P., Spector T.D. & McCarthy M.I. (2011) Identification of an imprinted master trans regulator at the KLF14 locus related to multiple metabolic phenotypes. *Nat Genet* **43**, 561-4.

Speed D., Hoggart C., Petrovski S., Tachmazidou I., Coffey A., Jorgensen A., Eleftherohorinou H., De Iorio M., Todaro M., De T., Smith D., Smith P.E., Jackson M., Cooper P., Kellett M., Howell S., Newton M., Yerra R., Tan M., French C., Reuber M., Sills G.E., Chadwick

D., Pirmohamed M., Bentley D., Scheffer I., Berkovic S., Balding D., Palotie A., Marson A., O'Brien T.J. & Johnson M.R. (2014) A genome-wide association study and biological pathway analysis of epilepsy prognosis in a prospective cohort of newly treated epilepsy. *Hum Mol Genet* **23**, 247-58.

Sperling M.R. (2004) The consequences of uncontrolled epilepsy. *CNS Spectr* **9**, 98-101, 6-9.

Sterjev Z., Trencevska G.K., Cvetkovska E., Petrov I., Kuzmanovski I., Ribarska J.T., Nestorovska A.K., Matevska N., Naumovska Z., Jolevska-Trajkovic S., Dimovski A. & Suturkova L. (2012) The association of C.3435C>T single-nucleotide polymorphism, Pgp-glycoprotein gene expression levels and carbamazepine maintenance dose in patients with epilepsy. *Neuropsychiatr Dis Treat* **8**, 191-6.

Stranger B.E., Stahl E.A. & Raj T. (2011) Progress and promise of genome-wide association studies for human complex trait genetics. *Genetics* **187**, 367-83.

Stumpf M.P., Thorne T., de Silva E., Stewart R., An H.J., Lappe M. & Wiuf C. (2008) Estimating the size of the human interactome. *Proc Natl Acad Sci U S A* **105**, 6959-64.

Subenthiran S., Abdullah N.R., Joseph J.P., Muniandy P.K., Mok B.T., Kee C.C., Ismail Z. & Mohamed Z. (2013) Linkage disequilibrium between polymorphisms of ABCB1 and ABCC2 to predict the treatment outcome of Malaysians with complex partial seizures on treatment with carbamazepine mono-therapy at the Kuala Lumpur Hospital. *PLoS One* **8**, e64827.

Szoeke C., Sills G.J., Kwan P., Petrovski S., Newton M., Hitiris N., Baum L., Berkovic S.F., Brodie M.J., Sheffield L.J. & O'Brien T.J. (2009) Multidrug-resistant genotype (ABCB1) and seizure recurrence in newly treated epilepsy: data from international pharmacogenetic cohorts. *Epilepsia* **50**, 1689-96.

Tan N.C., Heron S.E., Scheffer I.E., Pelekanos J.T., McMahon J.M., Vears D.F., Mulley J.C. & Berkovic S.F. (2004) Failure to confirm association of a polymorphism in ABCB1 with multidrug-resistant epilepsy. *Neurology* **63**, 1090-2.

Tanabe M. & Kanehisa M. (2012) Using the KEGG database resource. *Curr Protoc Bioinformatics* **Chapter 1**, Unit1 12.

Tate S.K., Depondt C., Sisodiya S.M., Cavalleri G.L., Schorge S., Soranzo N., Thom M., Sen A., Shorvon S.D., Sander J.W., Wood N.W. & Goldstein D.B. (2005) Genetic predictors of the maximum doses patients receive during clinical use of the anti-epileptic drugs carbamazepine and phenytoin. *Proc Natl Acad Sci U S A* **102**, 5507-12.

Tate S.K., Singh R., Hung C.C., Tai J.J., Depondt C., Cavalleri G.L., Sisodiya S.M., Goldstein D.B. & Liou H.H. (2006) A common polymorphism in the SCN1A gene associates with phenytoin serum levels at maintenance dose. *Pharmacogenet Genomics* **16**, 721-6.

Tellez-Zenteno J.F., Hernandez-Ronquillo L., Buckley S., Zahagun R. & Rizvi S. (2014) A validation of the new definition of drug-resistant epilepsy by the International League Against Epilepsy. *Epilepsia* **55**, 829-34.

Thessen Hedreul M., Moller S., Stridh P., Gupta Y., Gillett A., Daniel Beyeen A., Ockinger J., Flytzani S., Diez M., Olsson T. & Jagodic M. (2013) Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity. *Hum Mol Genet*.

Thurman D.J., Beghi E., Begley C.E., Berg A.T., Buchhalter J.R., Ding D., Hesdorffer D.C., Hauser W.A., Kazis L., Kobau R., Kroner B., Labiner D., Liow K., Logroscino G., Medina M.T., Newton C.R., Parko K., Paschal A., Preux P.M., Sander J.W., Selassie A.,

Theodore W., Tomson T. & Wiebe S. (2011) Standards for epidemiologic studies and surveillance of epilepsy. *Epilepsia* **52 Suppl 7**, 2-26.

Tipney H. & Hunter L. (2010) An introduction to effective use of enrichment analysis software. *Hum Genomics* **4**, 202-6.

Tishler D.M., Weinberg K.I., Hinton D.R., Barbaro N., Annett G.M. & Raffel C. (1995) MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* **36**, 1-6.

Tiwari A.K., Sodani K., Dai C.L., Ashby C.R., Jr. & Chen Z.S. (2011) Revisiting the ABCs of multidrug resistance in cancer chemotherapy. *Curr Pharm Biotechnol* **12**, 570-94.

Tripepi G., Jager K.J., Dekker F.W. & Zoccali C. (2008) Testing for causality and prognosis: etiological and prognostic models. *Kidney Int* **74**, 1512-5.

Tsai M.H., Pardoe H.R., Perchyonok Y., Fitt G.J., Scheffer I.E., Jackson G.D. & Berkovic S.F. (2013) Etiology of hippocampal sclerosis: evidence for a predisposing familial morphologic anomaly. *Neurology* **81**, 144-9.

Ufer M., Mosyagin I., Muhle H., Jacobsen T., Haenisch S., Hasler R., Faltraco F., Remmler C., von Spiczak S., Kroemer H.K., Runge U., Boor R., Stephani U. & Cascorbi I. (2009) Non-response to antiepileptic pharmacotherapy is associated with the ABCC2 -24C>T polymorphism in young and adult patients with epilepsy. *Pharmacogenet Genomics* **19**, 353-62.

Vadlamudi L., Andermann E., Lombroso C.T., Schachter S.C., Milne R.L., Hopper J.L., Andermann F. & Berkovic S.F. (2004) Epilepsy in twins: insights from unique historical data of William Lennox. *Neurology* **62**, 1127-33.

Vahab S.A., Sen S., Ravindran N., Mony S., Mathew A., Vijayan N., Nayak G., Bhaskaranand N., Banerjee M. & Satyamoorthy K. (2009) Analysis of genotype and haplotype effects of ABCB1 (MDR1) polymorphisms in the risk of medically refractory epilepsy in an Indian population. *Drug Metab Pharmacokinet* **24**, 255-60.

van Donselaar C.A., Brouwer O.F., Geerts A.T., Arts W.F., Stroink H. & Peters A.C. (1997) Clinical course of untreated tonic-clonic seizures in childhood: prospective, hospital based study. *BMJ* **314**, 401-4.

van Nas A., Ingram-Drake L., Sinsheimer J.S., Wang S.S., Schadt E.E., Drake T. & Lusis A.J. (2010) Expression quantitative trait loci: replication, tissue- and sex-specificity in mice. *Genetics* **185**, 1059-68.

van Vliet E.A., van Schaik R., Edelbroek P.M., Redeker S., Aronica E., Wadman W.J., Marchi N., Vezzani A. & Gorter J.A. (2006) Inhibition of the multidrug transporter P-glycoprotein improves seizure control in phenytoin-treated chronic epileptic rats. *Epilepsia* **47**, 672-80.

van Vliet E.A., van Schaik R., Edelbroek P.M., Voskuyl R.A., Redeker S., Aronica E., Wadman W.J. & Gorter J.A. (2007) Region-specific overexpression of P-glycoprotein at the blood-brain barrier affects brain uptake of phenytoin in epileptic rats. *J Pharmacol Exp Ther* **322**, 141-7.

Visscher P.M., Brown M.A., McCarthy M.I. & Yang J. (2012) Five years of GWAS discovery. *Am J Hum Genet* **90**, 7-24.

Vogelgesang S., Kunert-Keil C., Cascorbi I., Mosyagin I., Schroder E., Runge U., Jedlitschky G., Kroemer H.K., Oertel J., Gaab M.R., Pahnke J., Walker L.C. & Warzok R.W. (2004) Expression of multidrug transporters in dysembryoplastic neuroepithelial tumors causing intractable epilepsy. *Clin Neuropathol* **23**, 223-31.

Volk H.A. & Loscher W. (2005) Multidrug resistance in epilepsy: rats with drug-resistant seizures exhibit enhanced brain expression of P-glycoprotein compared with rats with drug-responsive seizures. *Brain* **128**, 1358-68.

Vreugdenhil M. & Wadman W.J. (1999) Modulation of sodium currents in rat CA1 neurons by carbamazepine and valproate after kindling epileptogenesis. *Epilepsia* **40**, 1512-22.

Vreugdenhil M., van Veelen C.W., van Rijen P.C., Lopes da Silva F.H. & Wadman W.J. (1998) Effect of valproic acid on sodium currents in cortical neurons from patients with pharmaco-resistant temporal lobe epilepsy. *Epilepsy Res* **32**, 309-20.

Wang K., Li M. & Bucan M. (2007) Pathway-based approaches for analysis of genomewide association studies. *Am J Hum Genet* **81**, 1278-83.

Wang K., Zhang H., Kugathasan S., Annese V., Bradfield J.P., Russell R.K., Sleiman P.M., Imielinski M., Glessner J., Hou C., Wilson D.C., Walters T., Kim C., Frackelton E.C., Lionetti P., Barabino A., Van Limbergen J., Guthery S., Denson L., Piccoli D., Li M., Dubinsky M., Silverberg M., Griffiths A., Grant S.F., Satsangi J., Baldassano R. & Hakonarson H. (2009a) Diverse genome-wide association studies associate the IL12/IL23 pathway with Crohn Disease. *Am J Hum Genet* **84**, 399-405.

Wang Y.Y., Smith P., Murphy M. & Cook M. (2010) Global expression profiling in epileptogenesis: does it add to the confusion? *Brain Pathol* **20**, 1-16.

Wang Z., Gerstein M. & Snyder M. (2009b) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57-63.

Wassenaar M., Leijten F.S., Egberts T.C., Moons K.G. & Uijl S.G. (2013) Prognostic factors for medically intractable epilepsy: a systematic review. *Epilepsy Res* **106**, 301-10.

Wasterlain C.G. & Chen J.W. (2008) Mechanistic and pharmacologic aspects of status epilepticus and its treatment with new antiepileptic drugs. *Epilepsia* **49 Suppl 9**, 63-73.

Weiss J., Kerpen C.J., Lindenmaier H., Dormann S.M. & Haefeli W.E. (2003) Interaction of antiepileptic drugs with human P-glycoprotein in vitro. *J Pharmacol Exp Ther* **307**, 262-7.

Wildsmith S.E. & Elcock F.J. (2001) Microarrays under the microscope. *Mol Pathol* **54**, 8-16.

Yasiry Z. & Shorvon S.D. (2012) How phenobarbital revolutionized epilepsy therapy: the story of phenobarbital therapy in epilepsy in the last 100 years. *Epilepsia* **53 Suppl 8**, 26-39.

Yaspan B.L. & Veatch O.J. (2011) Strategies for pathway analysis from GWAS data. *Curr Protoc Hum Genet* **Chapter 1**, Unit1 20.

Zhang C., Chanteux H., Zuo Z., Kwan P. & Baum L. (2013) Potential role for human P-glycoprotein in the transport of lacosamide. *Epilepsia* **54**, 1154-60.

Zhang C., Kwan P., Zuo Z. & Baum L. (2010) In vitro concentration dependent transport of phenytoin and phenobarbital, but not ethosuximide, by human P-glycoprotein. *Life Sci* **86**, 899-905.

Zhang C., Kwan P., Zuo Z. & Baum L. (2012) The transport of antiepileptic drugs by P-glycoprotein. *Adv Drug Deliv Rev* **64**, 930-42.

Zhang C., Zuo Z., Kwan P. & Baum L. (2011) In vitro transport profile of carbamazepine, oxcarbazepine, eslicarbazepine acetate, and their active metabolites by human P-glycoprotein. *Epilepsia* **52**, 1894-904.

Zhu M.H., Ji S.L., Zhang C.Y., Cui L., Xiong L. & Zheng H.L. (2014) DNA microarray reveals ZNF195 and SBF1 are potential biomarkers for gemcitabine sensitivity in head and neck squamous cell carcinoma cell lines. *Int J Clin Exp Pathol* **7**, 1514-23.

Zimprich F., Stogmann E., Bonelli S., Baumgartner C., Mueller J.C., Meitinger T., Zimprich A. & Strom T.M. (2008) A functional polymorphism in the SCN1A gene is not associated with carbamazepine dosages in Austrian patients with epilepsy. *Epilepsia* **49**, 1108-9.

Zimprich F., Sunder-Plassmann R., Stogmann E., Gleiss A., Dal-Bianco A., Zimprich A., Plumer S., Baumgartner C. & Mannhalter C. (2004) Association of an ABCB1 gene haplotype with pharmacoresistance in temporal lobe epilepsy. *Neurology* **63**, 1087-9.

CHAPTER TWO

AN INTEGRATIVE ANALYSIS OF LARGE-SCALE GENE EXPRESSION PROFILING STUDIES ON BRAIN TISSUE FROM EPILEPSY SURGERY

Contents

Chapter 2: An Integrative Analysis of Large-Scale Gene Expression Profiling Studies on Brain Tissue from Epilepsy Surgery

2.1 Introduction

As detailed in Chapter 1, epilepsies are among the most common neurological disorders, affecting up to 1% of the population (Sander 2003). Most patients with epilepsy become seizure-free with antiepileptic drug (AED) therapy. However, approximately 30% of epilepsy patients have a medically intractable condition even if treated with various AEDs at maximal dosages either alone or in combination (Shorvon 1996). A subset of patients with intractable epilepsy have the potential for a surgical cure, most commonly those with hippocampal sclerosis (Engel 2003). Surgical samples from patients with intractable epilepsy provide a unique opportunity to directly analyse the human epileptic focus in order to determine the causes of pharmacoresistance. Many different causes of pharmacoresistance have been postulated (see Chapter 1). The exact molecular mechanisms underlying pharmacoresistance, however, are still poorly understood. Alterations in the expression of a large number of genes are thought to be responsible, but most of the numerous genes that participate in the development of pharmacoresistance in epilepsy remain unidentified.

To date, the vast majority of studies on samples from epilepsy surgery have focused primarily on a number of selected candidate genes. However, these techniques are not suitable for dissection of multiple interacting molecular pathways or screening potential molecular abnormalities when the list of candidate genes is extensive. In contrast, large-scale microarray studies offer the advantage of assaying gene expression in a comprehensive, unbiased and genome-wide fashion. Analysing the expression profile of many genes simultaneously in large-scale expression studies, without making prior assumptions about candidate genes, allows the identification of new genes and molecular pathways associated with the condition. There have been at least 12 published large-scale gene expression studies on tissue from epilepsy surgery in the last ten years (see below).

However, they have failed to make a significant impact on our understanding of the causes of pharmacoresistance. In our view, this failure is due to several reasons.

First, authors of most microarray studies have focused on selected genes or pathways in their published results. Large-scale gene profiling studies suffer from high numbers of false positive results. Before drawing conclusions about the differential expression of a specific gene, it is thought necessary to demonstrate independent experimental validation (King & Sinha 2001) using techniques such as reverse transcription-polymerase chain reaction. Therefore, it is commonplace to use the microarray as a screening tool, then to validate a few chosen genes for additional investigation. However, this methodology under-uses the 'depth' inherent in the original microarray dataset and is prone to missing potentially important genes and networks. Hence, experts (Rhodes *et al.* 2002) have proposed using multiple microarray datasets that address similar hypotheses to simultaneously cross-validate all of the positive results. This inter-study cross-validation approach has been adopted in the present work.

Second, doubts have been raised about the reproducibility of microarray studies in epilepsy (Lukasiuk & Pitkanen 2004; van Gassen *et al.* 2008; Wang *et al.* 2010). It has been suggested that the majority of changes in gene expression are specific to laboratory or experimental conditions with very few genes demonstrating changes in more than two publications (Wang *et al.* 2010).

Third, the results of different microarray studies have not been integrated to give a coherent picture of the genomic changes involved in epilepsy pharmacoresistance.

We believe that the above problems can be overcome by testing the validity and reproducibility of the individual microarray studies and by performing an integrative analysis of all available microarray results. We are aware of only two previous published reviews which have included microarray studies on non-cancerous brain tissue from epilepsy surgery (Lukasiuk & Pitkanen 2004; Wang *et al.* 2010). However, neither of these reviews included all currently available microarray studies on brain tissue from epilepsy surgery; in fact, the study by Lukasiuk and Pitkänen, (2004) includes only one human microarray study, and the study by Wang et al., (2010) includes only three human large-scale gene expression studies. These reviews found a very small number of genes that showed similar expression profiles

and, hence, found it difficult to draw robust conclusions about the possible mechanism(s) of pharmacoresistance in epilepsy. In the present work, we have performed an integrative analysis of all available microarray studies on non-cancerous brain tissue from epilepsy surgery with the aim of identifying novel and important genes and networks that play a role in pharmacoresistance.

2.2 Methods

2.2.1 Inclusion Criteria

We aimed to include all large-scale gene expression profiling studies fulfilling the following criteria:

1. Recruited patients had clearly defined refractory epilepsy.
2. The epileptic focus was ascertained on the basis of clinical features, electroencephalography, brain imaging, and post-operative tissue histology as necessary.
3. 'Pharmacoresistant' tissue was compared with suitable control (non-epileptic or 'pharmacoresponsive') tissue.
4. Epileptic tissue analysed was not cancerous or neoplastic.
5. At least 500 genes from across the genome were assayed.
6. Minimum Information About a Microarray Experiment (MIAME)-compliant data was provided or, at the very least, a list of significantly regulated genes and the direction of change (up- or down-regulated) was provided.

2.2.2 Search Strategy

We searched records in Medline and Embase, without language restriction, between January 1987 and January 2011. Large-scale microarray technology was first described in 1987 (Kulesh et al., 1987); hence 1987 formed the starting point for our search. We used the following search terms: (1) "gene-expression profiling", or (2) "microarray analysis", or (3) "transcription profiling", or (4) "cluster analysis", or (5) "Affymetrix", (6) or "GeneChip", or (7) "serial analysis of gene expression", or (8) "SAGE", and (1) "epilepsy", or (2) "TLE", or (3) "MTLE". We also hand-searched the reference lists of every primary study, previously published systematic reviews and other review articles.

We also searched public repositories of microarray datasets: Array Express (www.ebi.ac.uk/arrayexpress), GEO (www.ncbi.nlm.nih.gov/geo), Stanford Microarray Database (smd.stanford.edu), University of Pennsylvania RAD (www.cbil.upenn.edu/RAD/php/index.php), UNC Microarray Database (genome.unc.edu), MUSC Microarray Database (proteogenomics.musc.edu/musc_madb.html), Genevestigator (www.genevestigator.com), Princeton University MicroArray database (puma.princeton.edu), University of Tennessee Microarray Database (genome.ws.utk.edu).

2.2.3 Data Gathering: Raw Data

We found 12 published genome-wide gene expression profiling studies on non-neoplastic tissue from epilepsy surgery. Raw data for the study by Ozbas-Gerceker et al., (2006) was available from the GEO database. Minimum Information About a Microarray Experiment (MIAME)-compliant data for the study by van Gassen et al., (2008) was available on the ArrayExpress database. MIAME-compliant data was not publically available for any other study. The corresponding author from each of the remaining ten studies was contacted to request MIAME-compliant microarray results data, but MIAME-compliant data was not provided by any author. As raw data was only available for two studies performed on two entirely different platforms (Serial Analysis of Gene Expression and two-channel oligonucleotide microarray analysis), we judged that a useful integrative analysis of the raw data could not be performed, and raw data was not used in the present study. Instead, we obtained differentially regulated gene lists from individual studies, as detailed below.

2.2.4 Data Gathering: Gene Lists

Six studies published complete lists of all genes which were differentially regulated in epileptic tissue according to each study's individually defined criteria (Becker *et al.* 2002; Becker *et al.* 2003; Arion *et al.* 2006; Jamali *et al.* 2006; Ozbas-Gerceker *et al.* 2006; Lee *et al.* 2007; van Gassen *et al.* 2008). Xi et al., (2009) published a partial list of gene regulated in their study. Xiao et al., (2008) directly provided a list of all genes which were differentially regulated in epileptic tissue according to their individually defined criteria. Three studies did not publish a list of regulated genes and their authors did not provide this on request (Lee *et al.* 2004; Li *et al.* 2006; Liu *et al.* 2007), and hence were excluded from further analysis in this systematic review.

2.2.5 Data Integration

To allow inter-study comparison, gene identifiers from each study were converted to Entrez gene numbers, unless these were provided in the publication. Affymetrix probe numbers were converted to Entrez gene numbers using NetAffx Analysis Center (www.affymetrix.com/analysis). All other gene identifiers were converted to Entrez gene numbers using MatchMiner (build 137, discover.nci.nih.gov/matchminer).

For each gene list, changes in gene expression were changed to a binary score: any gene up-regulated was given a score of +1, while any gene down-regulated was given a score of −1. The nine lists of regulated genes from the nine included studies were integrated in a MS Excel spreadsheet. 42 genes were excluded because of conflicting scores (that is, they were down-regulated in one study but up-regulated in another), leaving 1407 regulated genes in the integrated list incorporating all included studies. The total score was calculated for each gene.

2.2.6 Data Validation

The overlapping probabilities of differentially regulated gene lists were calculated using ConceptGen (conceptgen.ncibi.org/core/conceptGen), a gene set enrichment testing tool. ConceptGen uses the Expression Analysis Systematic Explorer (EASE) score (a modified Fisher's exact test widely employed in published literature) to test pairs of gene lists to determine if there exists a larger number of overlapping genes than is expected by chance (Hosack *et al.* 2003). P-values are then adjusted for multiple testing by calculating the False Discovery Rate (Benjamini & Hochberg 1995), values <0.05 being deemed significant. All of the gene lists from the included studies were tested in pairs in this manner. In addition, to test the validity of the list of genes differentially regulated in only one study, we compared this to a list of all genes from CarpeDB (www.carpedb.ua.edu), a dynamic continuously-updated epilepsy genetics database. We extracted the human genes in the CarpeDB database. In addition, we extracted the rat and mouse genes and mapped them to human homologues using ConceptGen. 278 CarpeDB human, mouse and rat genes in total mapped to ConceptGen.

2.2.7 Gene Ontology Enrichment Analysis

Gene ontology (GO) terms enrichment analysis was performed in the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (david.abcc.ncifcrf.gov). DAVID performs an enrichment test based on the EASE score, and a False Discovery Rate <0.05 is deemed significant. For biological process and cellular component terms, there were a large number of enriched broad GO terms, which made practical interpretation difficult. Hence, for biological process and cellular component domains, 'Go Fat' analysis was performed. Go Fat filters the broadest terms so that they do not overshadow the more specific terms.

2.2.8 Pathway Analysis

Ingenuity Systems (www.ingenuity.com) Core Analysis was used to determine significantly enriched canonical pathways and to build significantly enriched networks from the full list of differentially regulated genes. The significance of the association between our data set genes and the canonical pathways was measured in two ways: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed, and (2) a Fischer's exact test was used to calculate a p-value, determining the probability that the association between the genes in the data set and the canonical pathway was explained by chance alone. Networks were ranked by a score: the higher the score, the lower the probability of finding the observed data set genes in a given network by chance. The score takes into account the number of data set genes and the size of the network, and is the negative log of the p-value.

2.2.9 Comparison with an Alzheimer's Disease Brain Microarray Dataset

To counter arguments that the genetic changes highlighted by this analysis are non-specific and could be found in any brain pathology, we compared our results with the results of a large-scale gene expression profiling study on brain samples from 34 individuals with Alzheimer's disease (AD) (Liang *et al.* 2008). From this study, we extracted the list of genes exhibiting differential expression between AD-affected and normal hippocampi—an AD hippocampus dataset was used as most of the included epilepsy studies utilized hippocampal tissue. 3738 unique genes could be mapped to Entrez gene IDs. Ingenuity Systems Pathway Analysis was used to determine significantly enriched canonical pathways

from this gene list. The top ten canonical pathways from the epilepsy and AD datasets were then compared.

2.3 Results

2.3.1 Gene lists

We found 12 published large-scale genome-wide gene expression profiling studies on non-neoplastic tissue from epilepsy surgery (Becker *et al.* 2002; Becker *et al.* 2003; Arion *et al.* 2006; Jamali *et al.* 2006; Ozbas-Gerceker *et al.* 2006; Lee *et al.* 2007; van Gassen *et al.* 2008; Xiao *et al.* 2008; Xi *et al.* 2009). Gene lists were available for nine genome-wide gene expression studies, and these were included in the present review. Seven studies published one differentially regulated gene list each. However, Lee et al., (2007) and van Gassen et al., (2008) used more than one type of control tissue and published more than one list of differentially regulated genes each. Lee et al., (2007) used two types of controls: (1) histologically normal CA1 from patients with 'paradoxical temporal lobe epilepsy (PTLE)', citing the evidence that the poor seizure free outcome (44%) in PTLE following hippocampectomy suggests that the hippocampus is unlikely to be epileptogenic in this group, and (2) histologically normal CA1 tissue from 'mass-associated temporal lobe epilepsy (MaTLE)' where the mass lesion was outside the hippocampus, citing the evidence that mesial temporal lobe epilepsy (MTLE) granule cells are hyperexcitable while those in MaTLE are not. The authors presented two lists of differentially regulated genes: (1) genes regulated by 1.5 fold or more for both MTLE vs. PTLE and MTLE vs. MaTLE, and (2) genes regulated 1.5 fold or more when MaTLE and PTLE were considered replicates of non-sclerotic hippocampi. Regulated genes from both lists were integrated into the current systematic review, giving a list of 758 unique genes that could be mapped to Entrez gene numbers. Similarly, van Gassen et al., (2008), compared slices of sclerosed hippocampus with histologically normal hippocampal slices from patients with MTLE but no hippocampal sclerosis (HS). However, van Gassen et al., (2008) also compared normal hippocampal autopsy samples with histologically normal hippocampal slices from patients with MTLE but no HS. The non-HS MTLE and HS groups were found to share a large group of differentially expressed genes when compared to the autopsy group, and there was a significant overlap between functional gene classes affected in non-HS MTLE and HS groups when compared to autopsy samples. Hence, the autopsy vs non-HS MTLE gene list was also deemed

appropriate for inclusion in the current systematic review. In total, van Gassen et al., (2008) presented three differentially regulated gene lists: (1) autopsy vs HS, (2) autopsy vs non-HS, and (3) non-HS vs HS. Regulated genes from all three lists were integrated into the current systematic review. 24 genes had conflicting changes in expression (downregulated in one list but upregulated in another) and were excluded, leaving 521 unique genes.

2.3.2 Study characteristics

The nine included studies had sample sizes ranging from six to 60. All studies used test tissue from the temporal lobe, and all but three studies (Jamali *et al.* 2006; Xiao *et al.* 2008; Xi *et al.* 2009) used test tissue from the hippocampus. Gene expression profiling platforms used included one-channel microarrays, two-channel microarrays and Serial Analysis of Gene Expression (Table 2.1). The smallest microarray chip used was able to assay up to 588 unique genes (Clontech's Atlas Human Neurobiology array used by Becker et al. 2002), while the largest was able to assay up to 21,329 unique genes (Operon's Human Array-Ready oligo set version 2.0 used by van Gassen et al. 2008).

We assessed the methodological quality of the included studies. Each study had specific methodological strengths and weaknesses. For example, van Gassen et al. (2008) performed a like-for-like tissue comparison, detailed probe- and array-level quality control procedures, corrected p-values for multiple testing, and deposited MIAME-compliant data in a publically-accessible repository, but used a modest sample size and applied no fold change criterion to differentially regulated gene lists. On the other hand, Xiao et al. (2008) used a large sample size and applied appropriate fold change criterion, but did not perform a like-for-like tissue comparison, or detail probe- and array-level quality control procedures, or perform correction for multiple testing, or deposit their data in a public database. Table 2.2 lists the criteria used to assess methodological quality, and the quality assessment outcomes for the individual studies.

Table 2.1 Included large-scale gene expression profiling studies on brain tissue from epilepsy surgery

Name	Test tissue	Control tissue	Chip	Number of regulated genes
Lee *et al.* 2007	Sclerosed CA1 from MTLE (n=8)	Histologically normal CA1 from MaTLE (n=6) or PTLE (n=6)	Affymetrix U133A	674 (MTLE vs PTLE *and* MTLE vs MaTLE) 947 (MTLE vs others)
van Gassen *et al.* 2008	Sclerosed hippocampus from MTLE patients (n=4)	Hippocampus from MTLE patients without HS (n=4) and autopsy (n=4)	Human Array-Ready oligo set version 2.0, Operon Biotechnologies	Autopsy vs non-HS: 322 Autopsy vs HS: 322 non-HS vs HS: 206
Xi *et al.* 2009	Anterior temporal neocortex epileptogenic zone (n=40)	Anterior temporal neocortex removed for raised ICP (n=20)	ns	143
Ozbas-Gerceker *et al.* 2006	Anterior hippocampus (n=6)	Anterior hippocampus tissue from autopsy (n=1)	SAGE	143
Xiao *et al.* 2008	Temporal lobe (n=40): HS, FCD, etc	Temporal neocortex, hippocampus, parietal cortex or frontal cortex from brain trauma patients (n=20)	Biostar H-40s	142
Arion *et al.* 2006	'Spiking areas' from anterolateral temporal cortical samples (n=6)	'Non-spiking' from anterolateral temporal cortical samples (n=6)	Affymetrix U133A	76
Becker *et al.*2003	Sclerosed CA1 from MTLE (n=5)	Dentate gyrus from same patients (n=5)	Affymetrix U133A	25
Becker *et al.* 2002	Sclerosed hippocampus (n=3)	Normal hippocampus from tumor patients (n=2) or epilepsy patients (n=1)	Atlas, Clontech	21
Jamali *et al.* 2006	Entorhinal cortex (n=5)	Lateral temporal neocortex (n=5)	Micromax	16

FCD=focal cortical dysplasia; HS=hippocampal sclerosis; ICP=intracranial pressure; IE=intractable epilepsy; MaTLE=mass-associated temporal lobe epilepsy; MTLE=mesial temporal lobe epilepsy; NIE=non-intractable epilepsy; ns=not specified; PTLE=paradoxical temporal lobe epilepsy; SAGE=serial analysis of gene expression.

Table 2.2 Critical appraisal of included studies. FDR: false discovery rate; FWER: Family-wise error correction.

Study	Sample size	Like-for-like test & control tissues used	Data QC procedures detailed	Correction for multiple testing	Effect size criterion applied	Raw data provided
Lee et al. 2007	20	✓	×	FDR < 0.05	Fold change ≥ 1.5	×
van Gassen et al. 2008	12	✓	✓	FWER < 0.05	×	✓
Arion et al. 2006	12	✓	✓	FDR=0.057	Fold change ≥ 1.2	×
Jamali et al. 2006	10	×§	✓	na§	Fold change ≥ 2.56	×
Xi et al. 2009	60	✓	×	×∞	Fold change ≥ 2	×
Ozbas-Gerceker et al. 2006	7	✓*	×	×	×	✓
Xiao et al. 2008	60	×	×	×	Fold change ≥ 2	×
Becker et al. 2003	10	×	✓	×	Fold change ≥ 1.5	×
Becker et al. 2002	6	✓	×	×∞	×∞	×

§In the study by Jamali et al. 2006, tissue from the entorhinal cortex (test) was compared with tissue from the lateral temporal neocortex (control). However, genes were only considered regulated in the epileptic focus if quantitative RT–PCR confirmed no significant difference in expression between the entorhinal cortex and lateral temporal neocortex of non-epileptic autopsy brain samples. The statistical test employed was the 'z-score'.

∞Did not specify what, if any, statistical test used.

*Autopsy control used.

2.3.3 Study heterogeneity

There was heterogeneity within the studies and the number of genes found to be regulated in the individual studies varied widely (Table 2.1). The largest parts of this variation can be explained by:

1. The number of genes that can be assayed by the different arrays (see above).
2. The widely differing criteria used to define statistically significant change in gene expression (Table 2.2).

Other sources of heterogeneity were:

1. Differences in sample handling and RNA extraction techniques.
2. Different gene expression profiling platforms used.
3. Varying microarray data normalization procedures.
4. Test tissue used: Sclerosed hippocampus was used by most studies, but other anatomical regions and other pathologies were also employed by some studies (Table 2.1).
5. The use of at least four different types of control tissue: (1) non-epileptic brain tissue removed from the same patients as part of the surgical procedure, (2) normal hippocampal tissue from autopsy, (3) normal brain tissue removed from other patients for various indications, (4) histologically normal hippocampal tissue from patients with MTLE.

While acknowledging the heterogeneity within the studies, it is important to note that there is also an underlying commonality in the design of the studies: comparing gene expression between pharmacoresistant epileptic brain samples and non-epileptic or pharmacoresponsive brain samples. Although some of the differentially expressed genes identified in individual studies will be specific to the histological characteristics of the tissues being compared and to laboratory conditions, a significant subset of genes in each list represents the universal genetic changes linked with pharmacoresistance. As validation of this concept, we determined the size of the overlap between pairs of gene lists and calculated if this size was greater than would be expected by chance alone (see 'Data validation' section below).

2.3.4 Data validation

The overlapping probabilities of differentially regulated gene lists were calculated—all of the gene lists from the included studies were tested in pairs. The size and statistical significance of the overlaps are shown in Appendix 1. Our results show that there was a statistically significant overlap between gene lists from studies done on different types of brain tissue using different array platforms (elaborated further below).

To test the validity of the list of genes differentially regulated in only one study, we compared this to a list of all human genes and human homologues of mouse and rat genes from CarpeDB (www.carpedb.ua.edu), a dynamic continuously-updated epilepsy genetics database. Of the 278 genes thus extracted from CarpeDB, 54 overlapped with our list of genes differentially regulated in only one study. This overlap was statistically significant (FDR $P=1.2x10^{-24}$).

2.3.5 Overlapping gene lists

Genes differentially expressed in three or four studies are listed in Table 2.3. Prominently represented in these two lists are genes involved in neuroinflammation, in the control of synaptic transmission and in the restructuring of neuronal networks—specific examples are discussed below (see Discussion section). Genes differentially regulated in one study or two studies are shown in Appendix 2.

2.3.6 Gene Ontology Enrichment Analysis

The top 25 significantly enriched GO terms are listed in Table 2.4. The cellular component ontology term enrichment suggests that the most important processes in the development of pharmacoresistance are occurring in neuronal projections, in the growth cone, at the pre- and post-synaptic terminal, in the cytoskeleton and in membrane-bound vesicles. The most important molecular functions are calcium transport and signalling, cytoskeletal function, and transporter activity. The most significant biological processes are synaptic transmission and synaptic plasticity, regulation of the action potential, cellular cation homeostasis, axonal and dendritic morphogenesis, and cytoskeletal organization.

2.3.7 Pathway Analysis

The top 25 significantly enriched canonical pathways and networks are shown in Table 2.5. Details of all enriched pathways and networks are provided in Appendix 3. An explanation of

how some of the most significant pathways and networks might contribute to the development of pharmacoresistance is presented in the Discussion section.

2.3.8 Comparison with an Alzheimer's disease brain microarray dataset

To counter arguments that the genetic changes highlighted by this analysis are non-specific and could be found in any brain pathology, we compared our results with the results of a microarray study on AD-affected hippocampi. From the AD study, we extracted 3738 unique genes which could be mapped to Entrez IDs. Ingenuity Systems Pathway Analysis was used to determine significantly enriched canonical pathways from this gene list. In Table 2.6, we compare the top ten canonical pathways from the AD and the epilepsy datasets which convincingly demonstrated that there was no overlap between the identified top canonical pathways for the two datasets. In addition, the processes performed by the canonical pathways were also very different. For example, the top five canonical pathways in the pharmacoresistant epilepsy dataset are related to restructuring of neuronal networks (cholecystokinin signalling), modulation of synaptic transmission (synaptic long term potentiation, neuropathic pain signalling), and neuroinflammation (semaphorin signalling, chemokine signalling), while the top five pathways in the AD dataset are related to protein degradation (protein ubiquitination pathway), energy metabolism (mitochondrial dysfunction, oxidative phosphorylation), and translational regulation (regulation of eIF4 and p70S6K signalling, eIF2 signalling).

Table 2.3 Genes differentially expressed in three or four studies

Symbol	Description	Molecular function	Up or down regulated	Number of studies
GABRA5	GABA-A receptor, alpha 5	GABA-A receptor activity	Down	4
NRGN	Neurogranin	calmodulin binding	Down	4
CCL2	Chemokine (C-C motif) ligand 2	CCR2 chemokine receptor binding	Up	4
GFAP	Glial fibrillary acidic protein	protein binding	Up	4
CPLX2	Complexin 2	syntaxin binding	Down	3
ENC1	Ectodermal-neural cortex	actin binding	Down	3
HPCAL4	Hippocalcin like 4	calcium channel regulator activity	Down	3
INHBA	Inhibin, beta A	cytokine activity	Down	3
PLCB1	Phospholipase C, beta 1	calcium ion binding	Down	3
PSD	Pleckstrin and Sec7 domain containing	ARF guanyl-nucleotide exchange factor activity	Down	3
SNAP25	Synaptosomal-associated protein, 25kDa	SNARE binding	Down	3
STMN2	Stathmin-like 2	protein binding	Down	3
CAPN3	Calpain 3, (p94)	calcium ion binding	Up	3
CD99	CD99 molecule	protein binding	Up	3
CDK2AP1	CDK2-associated protein 1	DNA binding	Up	3
DYNLT1	Dynein, light chain, Tctex-type 1	motor activity	Up	3
OGG1	8-oxoguanine DNA glycosylase	damaged DNA binding	Up	3
PABPC4	Poly(A) binding protein, cytoplasmic 4	RNA binding	Up	3
RDX	Radixin	actin binding	Up	3
SPARC	Secreted protein, acidic, cysteine-rich	calcium ion binding	Up	3
TF	Transferrin	ferric iron binding	Up	3
ZFP36L1	Zinc finger protein 36, C3H type-like 1	AU-rich element binding	Up	3

Table 2.4 Gene ontology term enrichment

Molecular Function	Biological Process	Cellular Component
protein binding	transmission of nerve impulse	neuron projection
Binding	synaptic transmission	Synapse
calmodulin binding	cell projection organization	Vesicle
cytoskeletal protein binding	neuron development	cytoplasmic vesicle
kinase activity	cell-cell signalling	cell fraction
protein kinase activity	Behaviour	Cytosol
actin binding	neuron differentiation	cell projection
nucleotide binding	neuron projection development	vesicle membrane
protein serine/threonine kinase activity	learning or memory	membrane-bounded vesicle
ribonucleotide binding	cell projection morphogenesis	plasma membrane part
purine ribonucleotide binding	response to organic substance	Axon
transferase activity, transferring phosphorus-containing groups	cell morphogenesis	cytoplasmic membrane-bounded vesicle
phosphotransferase activity, alcohol group as	cellular component morphogenesis	synapse part
purine nucleotide binding	cell part morphogenesis	cell soma
structural molecule activity	cell motion	insoluble fraction
transporter activity	neuron projection morphogenesis	cytoplasmic vesicle part
lipid binding	regulation of synaptic plasticity	cytoplasmic vesicle membrane
adenyl ribonucleotide binding	regulation of neuron projection development	membrane fraction
cation-transporting ATPase activity	response to endogenous stimulus	coated vesicle
protein complex binding	regulation of cell projection organization	clathrin-coated vesicle
ATP binding	intracellular signalling cascade	plasma membrane
protein tyrosine kinase activity	glial cell development	site of polarized growth
di-, tri-valent inorganic cation transmembrane transporter activity	cellular ion homeostasis	synaptic vesicle
#24 not significant	regulation of cell death	clathrin coated vesicle membrane
#25 not significant	regulation of axonogenesis	growth cone

Table 2.5 Top 25 canonical pathways

Canonical Pathways
Cholecystokinin/Gastrin-mediated Signaling
Synaptic Long Term Potentiation
Semaphorin Signaling in Neurons
Neuropathic Pain Signaling In Dorsal Horn Neurons
Chemokine Signaling
GNRH Signaling
RhoA Signaling
Renin-Angiotensin Signaling
Role of NFAT in Cardiac Hypertrophy
Glutamate Receptor Signaling
Axonal Guidance Signaling
CDK5 Signaling
Integrin Signaling
CXCR4 Signaling
Ephrin Receptor Signaling
Molecular Mechanisms of Cancer
Melatonin Signaling
Thrombin Signaling
CREB Signaling in Neurons
Protein Kinase A Signaling
IGF-1 Signaling
Regulation of Actin-based Motility by Rho
Calcium Signaling
ERK/MAPK Signaling
Corticotropin Releasing Hormone Signaling

Table 2.6 Top 10 canonical pathways

Pharmacoresistant epilepsy	**Alzheimer's disease**
Cholecystokinin/Gastrin-mediated Signalling	Protein Ubiquitination Pathway
Synaptic Long Term Potentiation	Mitochondrial Dysfunction
Semaphorin Signalling in Neurons	Regulation of eIF4 and p70S6K Signalling
Neuropathic Pain Signalling In Dorsal Horn Neurons	eIF2 Signalling
Chemokine Signalling	Oxidative Phosphorylation
GNRH Signalling	Purine Metabolism
RhoA Signalling	Breast Cancer Regulation by Stathmin1
Renin-Angiotensin Signalling	NRF2-mediated Oxidative Stress Response
Role of NFAT in Cardiac Hypertrophy	Huntington's Disease Signalling
Glutamate Receptor Signalling	Estrogen Receptor Signalling

2.4 Discussion

2.4.1 Study heterogeneity and validation

While there is heterogeneity within the studies included in this review, there also is an underlying commonality in their design: comparing the gene expression between pharmacoresistant epileptic brain samples and non-epileptic or pharmacoresponsive brain samples. Although some of the differentially expressed genes identified in individual studies will be specific to the histological characteristics of the tissues being compared and to laboratory conditions, a significant subset of genes in each list represents the universal genetic changes linked with pharmacoresistance. As validation of this concept, our pairwise comparisons showed that there was a statistically significant overlap between gene lists from studies done on different types of brain tissue using different types of array technologies. For example, a study comparing various epileptic focus pathologies (focal cortical dysplasia, temporal lobe malacia, hippocampus sclerosis, neuron loss, neuron degeneration, gliosis, astrocytosis) with normal tissue from various brain regions of brain trauma patients using a Biostar H-40s microarray (Xiao et al., 2008) had a statistically significant overlap (FDR=1.2×10^{-2}) with a study comparing sclerosed CA1 with histologically normal CA1 from MTLE patients using an Affymetrix U133A microarray (Lee et al., 2007).

In addition, a significant number of the 1174 genes which were differentially expressed in only one study (a 'non-corroborated gene set') are also likely to be relevant to epileptogenesis and pharmacoresistance. To demonstrate this we compared the non-corroborated gene set with CarpeDB, a dynamic continuously-updated epilepsy genetics database. Of the 278 genes extracted from CarpeDB, 54 genes overlap with our non-corroborated gene set. This overlap is highly significant (FDR=1.2×10^{-24}). This suggests that the non-corroborated gene list is also an important data source which should be appropriately studied and interpreted (see below).

It is of course possible that some of the gene expression changes identified in this analysis are the consequence, rather than the cause, of refractory seizures; this limitation is inherent in large-scale gene expression profiling studies and, hence, also in this analysis. The causative role of candidate genes identified as potentially playing a part in the development of pharmacoresistance will need to be established with further investigation, such as

electrophysiological studies in hippocampal neuronal cultures or studies in rodent epilepsy models where the expression of the relevant gene has been appropriately altered. Another limitation which applies to all transcriptomic studies, and also to this analysis, is that mRNA concentration may not be a predictor of protein abundance or activity because of the many processes downstream of mRNA synthesis (for example, alternative mRNA splicing and microRNA regulation) that may affect the total amount and activity of protein in tissue.

2.4.2 Data integration

Microarray technology has become an important tool for biological research, but it can suffer from both low sensitivty and high false positive rates (Rhodes *et al.* 2002). One way of overcoming these limitations is to use meta-analysis methods that integrate the results of separate microarray studies. Meta-analysis is a classical statistical methodology for combining results from different studies addressing the same scientific questions, and has recently been applied to the analysis of microarray data, increasing both the sensitivity and reliability of measurements of gene expression changes (Rhodes *et al.* 2002). Two basic meta-analysis methods have been applied to microarray studies: (1) combining p-values for each gene from the individual studies to estimate an overall p-value for each gene across all studies (Rhodes *et al.* 2002), and (2) integrating effect size estimates to obtain an overall estimate of the average effect size (Choi *et al.* 2003). However, the majority of studies included in this review did not provide the basic information that would allow either meta-analysis method to be applied. This being the case, we integrated the gene lists provided by each study to generate lists of genes consistently regulated in two, three or four studies. This integrative technique successfully improved the reliability of the regulated gene lists, without enhancement of the sensitivity. Hence, the 233 genes shown in this review to overlap between two or more studies are a 'corroborated gene set' unlikely to be specific to the histological characteristics of the tissues being compared or to laboratory conditions, and most likely to represent universal genetic changes linked with epileptogenesis and pharmacoresistance. For GO term enrichment analysis, canonical pathway analysis and network analysis, we included both the corroborated and non-corroborated gene sets. This is because a significant number of the non-corroborated genes are also likely to be relevant to pharmacoresistance, as demonstrated by the significant overlap with the CarpeDB database. Further, in terms of genes found to be regulated in single studies, we took the

view that different genes validate each other if they are significantly enriched together in a GO term category, canonical pathway or an interaction network.

2.4.3 What does this analysis tell us about the mechanism of pharmacoresistance?

Current theories on pharmacoresistance in epilepsy include the multidrug transporter (MDT) hypothesis, the drug target hypothesis, and the inherent severity hypothesis (Schmidt & Loscher 2009). It has recently been suggested that these processes are likely to act not in a mutually exclusive manner, but rather an integrated manner, to cause pharmacoresistance (Schmidt & Loscher 2009). The current analysis supports this view, suggesting that all three hypotheses may be important. However, much of the integrated evidence generated by this analysis is weighted towards the intrinsic severity hypothesis.

2.4.3.1 MDT hypothesis

According to the MDT hypothesis, pharmacoresistance results from impaired drug penetration into the epileptic focus secondary to dysregulation of drug transporters (Chayasirisobhon 2009). Studies on the role of MDTs in pharmacoresistant epilepsy have, hence far, been limited to a small number of selected adenosine triphosphate-binding cassette (ABC) transporters, with a predominant focus on *ABCB1* (Lazarowski *et al.* 2007). It is interesting to note that *ABCB1*, which has been the focus of the majority of research on the role of MDTs in epilepsy pharmacoresistance (Hughes 2008), was not shown to be dysregulated in any of the included studies, even though a probe for *ABCB1* was present on the arrays of at least four of the nine studies. The role that solute carrier (SLC) transporters may play in epilepsy pharmacoresistance is much neglected. SLC transporters have been shown to play a role in cancer pharmacoresistance (Gupta *et al.* 2011), but their potential role in epilepsy pharmacoresistance has not been studied. Twenty-seven different SLC transporters were shown to be dysregulated in the studies included in this analysis. These findings suggest that the search for drug transporters relevant to epilepsy pharmacoresistance needs to be expanded to include SLC transporters.

2.4.3.2 Drug target hypothesis

According to the drug target hypothesis, pharmacoresistance is caused by the modification of one or more drug target molecules.

An important AED drug target is the voltage-gated sodium channel. These channels consist of α and β subunits. To date, nine α-subunits and four β-subunits have been identified. It has been suggested that in pharmacoresistant epilepsy subunit composition of these channels is altered, such that the expression of AED-insensitive subunit combinations is promoted. The SCN1A subunit gene has been the focus of most research so far (Loscher *et al.* 2009). This analysis showed that the expression of SCN2A, SCN3A, SCN3B and SCN8A was also altered in pharmacoresistant epilepsy. SCN2A was downregulated in two different microarray studies included in this analysis. In support of this finding, SCN2A was also downregulated in epileptic foci in a radioactive *in situ* hybridisation study (Whitaker *et al.* 2001). In addition, two different SCN2A polymorphisms have been reported to be associated with epilepsy pharmacoresistance (Kwan *et al.* 2008; Lakhan *et al.* 2009). The role of SCN2A, and the other subunits identified in this analysis, should be confirmed in future studies.

Another important AED target is the $GABA_A$ receptor (Rogawski & Loscher 2004). The pharmacological properties of $GABA_A$ receptors depend on the combination of subunits of which there are more than 20 to choose from (Brooks-Kayal *et al.* 1998). Three $GABA_A$ receptor subunit genes were shown to be downregulated in the studies included in this analysis: *GABRA5, GABRB3 and GABRG2*. Mutations in the *GABRG2* gene are associated with a number of familial epilepsy syndromes: generalized epilepsy with febrile seizures plus, type 3 (Baulac *et al.* 2001; Harkin *et al.* 2002; Carvill *et al.* 2013), childhood absence epilepsy (Wallace *et al.* 2001; Kananura *et al.* 2002), familial febrile seizures (Audenaert *et al.* 2006), and familial idiopathic generalized epilepsy (Lachance-Touchette *et al.* 2011).

GABRA5 was downregulated in four of the included studies. Using immunohistochemical labelling, Bethmann et al., (2008) showed that GABRA5 subunit protein expression in the hippocampi of antiepileptic drug resistant rats was significantly lower than in responsive rats (Bethmann *et al.* 2008). Bonin et al., (2007) have shown that the depolarizing current required to generate an action potential was two-fold greater in neurons from wildtype than from GABRA5 knockout mice (Bonin *et al.* 2007). Phenytoin (Mariotti *et al.* 2010) and carbamazepine (Almgren *et al.* 2008) have been shown to up-regulate GABRA5, and this has been suggested to be involved in their antiepileptic mechanism. GABRA5-selective inverse agonists have been shown to have convulsant or proconvulsant effect in mice in a dose-

dependent manner and proportional to the extent of GABRA5 efficacy (Atack *et al.* 2006). The current analysis reveals strong evidence of downregulation of *GABRA5* in human epileptic tissue. Further studies are needed to look for associations between polymorphisms in the *GABRA5* gene and pharmacoresistance, and for ways to reverse the downregulation of *GABRA5*.

2.4.3.3 Intrinsic severity hypothesis

The observation that a high frequency of seizures prior to onset of treatment is a prognostic signal of increased severity and future drug failure suggests that common neurobiological factors may underlie both disease severity and pharmacoresistance (Schmidt & Loscher 2009). A number of processes are thought to contribute to the development of pharmacoresistant epilepsy through promoting severity: neuroinflammation, enduring increases in excitatory synaptic transmission, changes in GABAergic inhibition, neuronal cell death, and the development of aberrant innervation patterns in part arising from reactive axonal growth (Gall & Lynch 2004). Although this review lends supports to all these processes playing a part, three basic themes were most prominently overrepresented in the gene list assembled:

1. Neuroinflammation
2. Modulation of synaptic transmission
3. Restructuring of neuronal networks

It is worth noting that although the important processes highlighted by this analysis may appear somewhat non-specific, the pattern of genetic changes demonstrated is distinctly different from those seen in other common brain pathologies, as shown by our comparison with an AD dataset.

What follows is a brief summary of the aforementioned three themes, as represented by our analysis. From the themes, we highlight novel and important genes and pathways. This is not a comprehensive précis, and we invite interested researchers to peruse the gene lists and enriched GO terms, pathways and networks to find other genes, pathways and networks of interest.

Neuroinflammation

It is being increasingly recognised that inflammation plays an important role in the pathogenesis of epilepsy (Vezzani & Granata 2005). The role of chemokine- and semaphorin-induced inflammation in the development of refractory epilepsy is little studied (Fabene *et al.* 2010). Our analysis suggests that chemokine and semaphorin signalling plays a significant role in epilepsy pharmacoresistance. We have also identified particular chemokines and semephorins that are potentially important therapeutic targets.

Chemokines

The chemokine signalling canonical pathway was shown to be dysregulated in our analysis, and the chemokine CCL2 and chemokine target Inhibin β-A were prominent in our gene list. Chemokines can induce neuronal hypersynchronization and neuronal epileptiform activity (Seiffert *et al.* 2004; Ivens *et al.* 2007; Marchi *et al.* 2007), leading to seizure generation in animal models of epilepsy (Fabene *et al.* 2008). CCL2 upregulation has been linked to increased susceptibility to seizures in a 'two-hit' seizure model in rats (Somera-Molina *et al.* 2009), and valproic acid has been shown to downregulate CCL2 mRNA in rat brain (Sinn *et al.* 2007). Inhibin β-A is a member of the transforming growth factor-β superfamily (Unsicker & Krieglstein 2002) and may mediate neuroprotective actions of basic fibroblast growth factor (Alzheimer & Werner 2002). Zhang et al., (2009) have identified inhibin β-A as one of a set of neuroprotective genes, termed Activity-regulated Inhibitor of Death (AID) genes, which promote survival of hippocampal neurons after growth factor withdrawal or staurosporine treatment *in vitro* and after kainic acid-induced status epilepticus *in vivo* (Zhang *et al.* 2009).

In addition, the canonical pathway of chemokine receptor CXCR4 was dysregulated. Increased expression of CXCR4 allows for increased CXCL12 binding. CXCL12 induces a slow inward current followed by a spontaneous synaptic activity via ionotropic glutamatergic receptors (Ragozzino *et al.* 2002), and induces microglia to release TNFα, which potentiates prostaglandin-dependent Ca^{++} activation and glutamate release (Lee *et al.* 2007). Other dysregulated chemokines in our analysis were CCL3, CCL4, CCL28, CCL3L1, CX3CL1 and CXCL14.

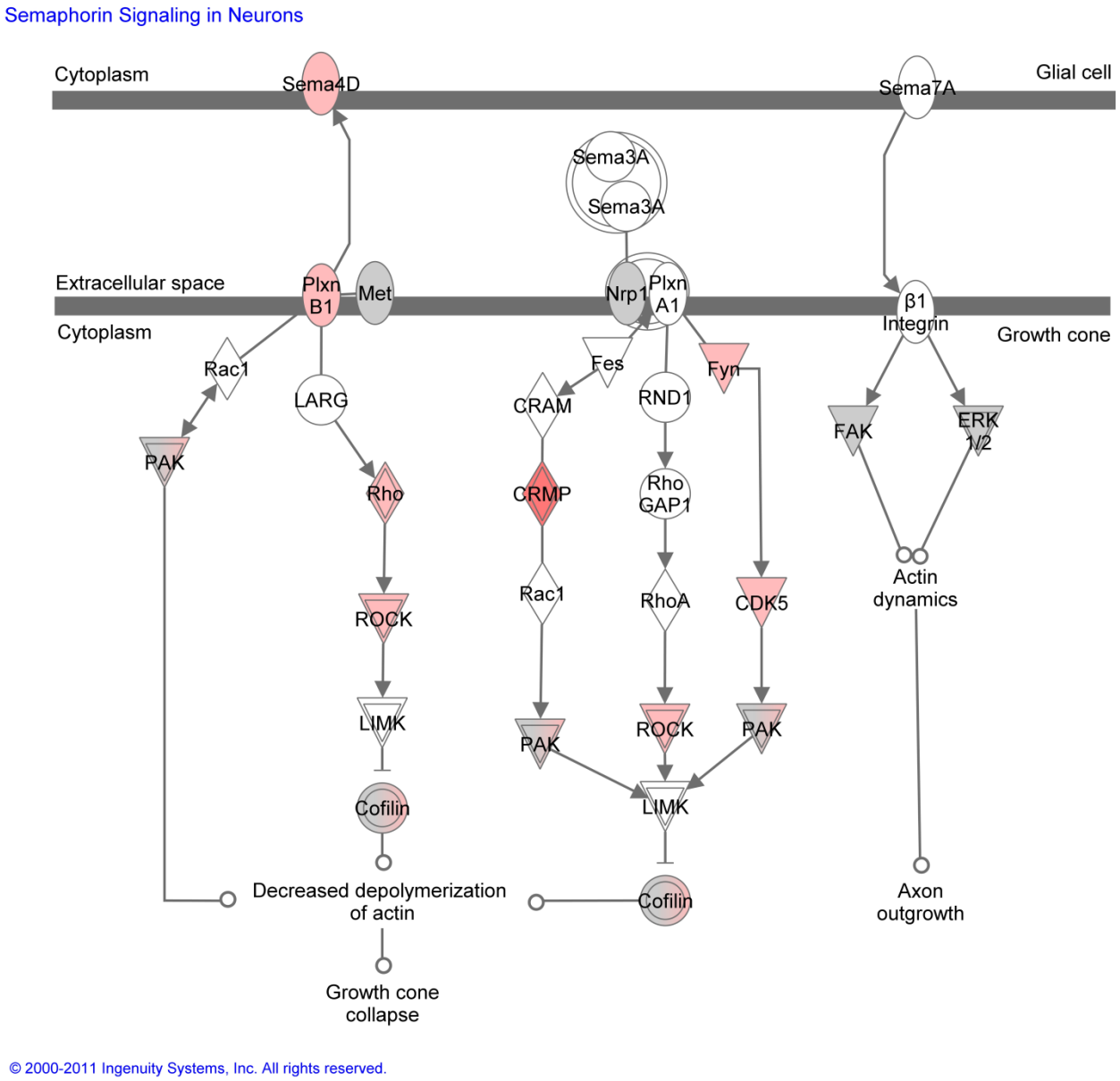


Figure 2.1 Up-regulated genes in the semaphorin pathway. The intensity of the colour indicates the number of studies in which the gene is up-regulated.

Semaphorin Signaling in Neurons

The current review reveals the upregulation of the semaphorin pathway (Figure 2.1), most importantly Sema4D and Plexin B1. Semaphorins are a large family of secreted and transmembrane molecules that function as repulsive axon guidance factors (Kolodkin *et al.* 1993) and modulate dendritic and axonal arborizations of developing neurons. In addition, semaphorins play a significant role in microglia activation and in neuroinflammation. A recent study by (Okuno *et al.* 2010) has shown that Sema4D promoted inducible nitric oxide

synthase expression by primary mouse microglia. This expression was Plexin B1-dependent as it was abolished in Plexin B1-deficient cells. Moreover, during the development of Myelin Oligodendrocyte Glycoprotein-peptide-induced experimental autoimmune encephalomyelitis (EAE), the expression of Sema4D and Plexin B1 was induced in infiltrating mononuclear cells and microglia, respectively (Okuno *et al.* 2010). Wild-type Myelin Oligodendrocyte Glycoprotein-specific T cells adoptively transferred into Plexin B1-deficient mice were not able to induce the disease. Similarly, bone marrow chimeric mice with Plexin B1-deficient central nervous system resident cells were not able to develop EAE. Furthermore, blocking antibodies against Sema4D significantly inhibited neuroinflammation during EAE development. Mutations in members of the semaphorin gene family have been associated with a number of human diseases: *SEMA4A* variants with retinitis pigmentosa, cone-rod dystrophy and congenital blindness (Abid *et al.* 2006), and *SEMA3E* variants with CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) syndrome (Lalani *et al.* 2004). The role semaphorins play in the inflammatory processes associated with epilepsy is not yet known, but deserves further study.

Modulation of synaptic transmission

The cellular component ontology term enrichment shows that some of the most important processes in the development of pharmacoresistance are occurring in membrane-bound vesicles and at the pre- and post-synaptic terminal. The most significant biological processes are cellular cation homeostasis, synaptic transmission and synaptic plasticity. The enriched gene ontology terms suggest that the most important molecular functions in the development of pharmacoresistance are calcium transport and signalling, and transporter activity. Therefore, changes in calcium signalling and in synaptic structure and function were shown to be important in our analysis.

Calcium signalling

Network 3 shows that changes in calcium-mediated cell signalling leading to alterations in neuronal excitability are likely to be important in the development of pharmacoresistance in epilepsy. The network reveals dysregulation of proteins regulating calcium channels (calcium-binding protein 1 and calpastatin), of proteins involved in calcium homeostasis

(inositol 1,4,5-triphosphate receptor, type 1, ATPase Ca^{++} transporting cardiac muscle slow twitch 2, ATPase Ca^{++} transporting plasma membrane 1, solute carrier family 8 sodium/calcium exchanger member 1, calcium channel voltage-dependent alpha 2/delta subunit 1, calcium channel voltage-dependent gamma subunit 3, calcium channel voltage-dependent alpha 2/delta subunit 1, adenosylhomocysteinase-like 1), and of calcium-dependent signalling proteins (calpain 3, and calcium-binding protein 1). Calcium signalling is amongst the top 25 enriched canonical pathways in our gene list. Calcium signalling, therefore, is likely to play a significant part in pharmacoresistance and its manipulation may be an important therapeutic strategy—two potential target genes which overlap in three or more studies are neurogranin and SNAP-25.

Neurogranin has been implicated in the modulation of postsynaptic signal transduction pathways, in synaptic plasticity (Kubota *et al.* 2008), and in the enhancement of long-term potentiation by promoting calcium-mediated signalling (Huang *et al.* 2004), and it is hypothesized that it participates in the action of valproate (Wu *et al.* 2011). SNAP-25 modulates neurotransmitter exocytosis (Schiavo *et al.* 1997; Augustine 2001; Chapman 2002; Zhang *et al.* 2002) and negatively regulates voltage-gated calcium channels (VGCC) in glutamatergic neurons (Condliffe *et al.* 2010). Silencing of endogenous SNAP-25 in glutamatergic neurons leads to an augmentation of VGCC activity, which would increase network excitability. Reductions in SNAP-25 expression have been correlated with neurological conditions characterized by increased network excitability. For example, VGCC currents are up-regulated resulting in absence-like epilepsy and hyperactivity (Risinger & Bennett 1999) in the Coloboma mouse mutant characterized by the heterozygous deletion of the SNAP-25 gene. Also, alterations in SNAP-25 expression have been described in human patients with attention deficit hyperactivity disorder and schizophrenia (Zhang *et al.* 2002).

Alterations in synaptic structure and function

As stated above, GO term enrichment analysis suggests that some of the most important processes in the development of pharmacoresistance are occurring in membrane-bound vesicles and at the pre- and post-synaptic terminal and some of the most significant biological processes are synaptic transmission and synaptic plasticity.

Network 1 reveals that there is dysregulation of proteins involved in maintaining synaptic structure and function. There is dysregulation of pre-synaptic cytomatrix proteins (bassoon, ELKS/RAB6-interacting/CAST family member 2, and piccolo), an active zone protein (PTPRF interacting protein, binding protein 1), a protein which controls presynaptic residual Ca^{++} concentrations (ATPase Ca^{++} transporting plasma membrane 2), proteins in postsynaptic sites which form a multimeric scaffold for the clustering of receptors, ion channels, and associated signaling proteins (calcium/calmodulin-dependent serine protein kinase and DLG2), and proteins which may play a role in clustering of NMDA receptors at excitatory synapses (DLG3). Complexin, a part of the SNARE complex (Neher 2010) which is the core of the synaptic release machinery (Sudhof & Rothman 2009), was downregulated in three studies and is particularly deserving of further study. Perturbations of the complexin proteins causes an increase in spontaneous and asynchronous release of neurotransmitter in some types of synapses, indicating an inhibitory role of the proteins (Neher 2010). Hence, the reduction in complexin 2 expression in the epileptic focus, as demonstrated in this review, could lead to increases in excitatory synaptic transmission, and deserves further study.

Restructuring of neuronal networks

The gene ontology enrichment analysis suggests that some of the most important processes in the development of pharmacoresistance are occurring in the cytoskeleton, the dendrite, the axon, and the growth cone. Some of the most significant biological processes are apoptosis, cytoskeletal organization, neuronal development and differentiation, axonal and dendritic morphogenesis, and one of the most important molecular functions in the development of pharmacoresistance is cytoskeletal function. In support of this, Network 2 reveals dysregulation of proteins involved in apoptotic mechanisms (translocase of inner mitochondrial membrane 23, histone cluster 1 H1c, peptidylprolyl isomerase F, dedicator of cytokinesis 1, and jumonji domain containing 6), in cell growth (protein tyrosine kinase 2, protein tyrosine phosphatase non-receptor type 12, and neural precursor cell expressed developmentally down-regulated 9), and in cell shape maintenance (tensin 1). Network 3 reveals dysregulation of proteins involved in neurogenesis, neurite outgrowth and axon guidance (neurogenic differentiation 2, neurofascin, and dihydropyrimidinase-like 3). Network 1 reveals that there is also dysregulation of proteins involved in axon guidance

(protein tyrosine phosphatase receptor type f and protein tyrosine phosphatase receptor type D). Similarly, the enriched canonical pathways include axonal guidance signalling, RhoA signalling (which plays a significant role in actin stress fibers formation), CDK5 signalling (which regulates neurite outgrowth), and ephrin receptor signalling (which is involved in nervous system development). The cholecystokinin (CCK) signalling pathway was the most significantly enriched canonical pathway in our analysis (Figure 2.2) and has an important, yet understudied, role in the development of aberrant neuronal networks.

Cholecystokinin Signaling

CCK controls the perisomatically targeting inhibitory interneurons of the hippocampus (Lee & Soltesz 2011). In kindling rodent models of epilepsy, there is a reduction of GABA-evoked inhibitory post-synaptic currents in the hippocampus, and this loss corresponds with a reduction in CCK-labelled interneurons (Sayin *et al.* 2003). In humans, the CCK content of cortical tissue from which active epileptic spiking was recorded at the time of surgery was significantly decreased in comparison to tissue samples from patients in whom the lateral temporal cortex was electrographically free of epileptiform spikes (Iadarola & Sherwin 1991). CCK may have anticonvulsant and neuroprotective properties. Pretreatment of hippocampal slices with sulfated CCK blocked the effect of kainic acid on synaptic transmission (Aitken *et al.* 1991). Seizures in a breed of rat with congenital audiogenic seizure were suppressed by cholecystokinin octapeptide injected intraperitoneally (Zhang *et al.* 1993) and by intracerebral injection of a CCK gene vector (Zhang *et al.* 1992). Seizures induced by picrotoxin and electroshock are inhibited by intracerebroventricular administration of cholecystokinin in rats (Kadar *et al.* 1984). There is also a positive influence of CCK on the anticonvulsant efficacy of vigabatrin (Ferraro & Sardo 2009). *CCK* genetic variants have not been studied specifically in epilepsy, but variants in the gene are known to be associated with another common brain disorder: Parkinson's disease (Fujii *et al.* 1999). CCK, therefore, is another potential therapeutic agent that should be further studied.

2.5 Conclusions

Large-scale gene expression profiling studies on brain tissue from epilepsy surgery have been performed largely with the aim of generating hypotheses about the causes of

epileptogenesis and pharmacoresistance. Although there have been at least 12 such studies in the last ten years, they have failed to make a significant impact on our understanding of pharmacoresistance in epilepsy for the reasons which have been outlined in this chapter. Our analysis shows that there is a statistically significant overlap between the gene lists of studies performed on different kind of epileptic foci using different types of microarrays. We have used an inter-study cross-validation technique to simultaneously verify the expression changes of large numbers of genes. We have performed an integrative analysis of the gene lists from different studies to identify the cellular components, biological processes, molecular functions, pathways, networks and individual genes which are likely to be important in the development of refractory epilepsy.

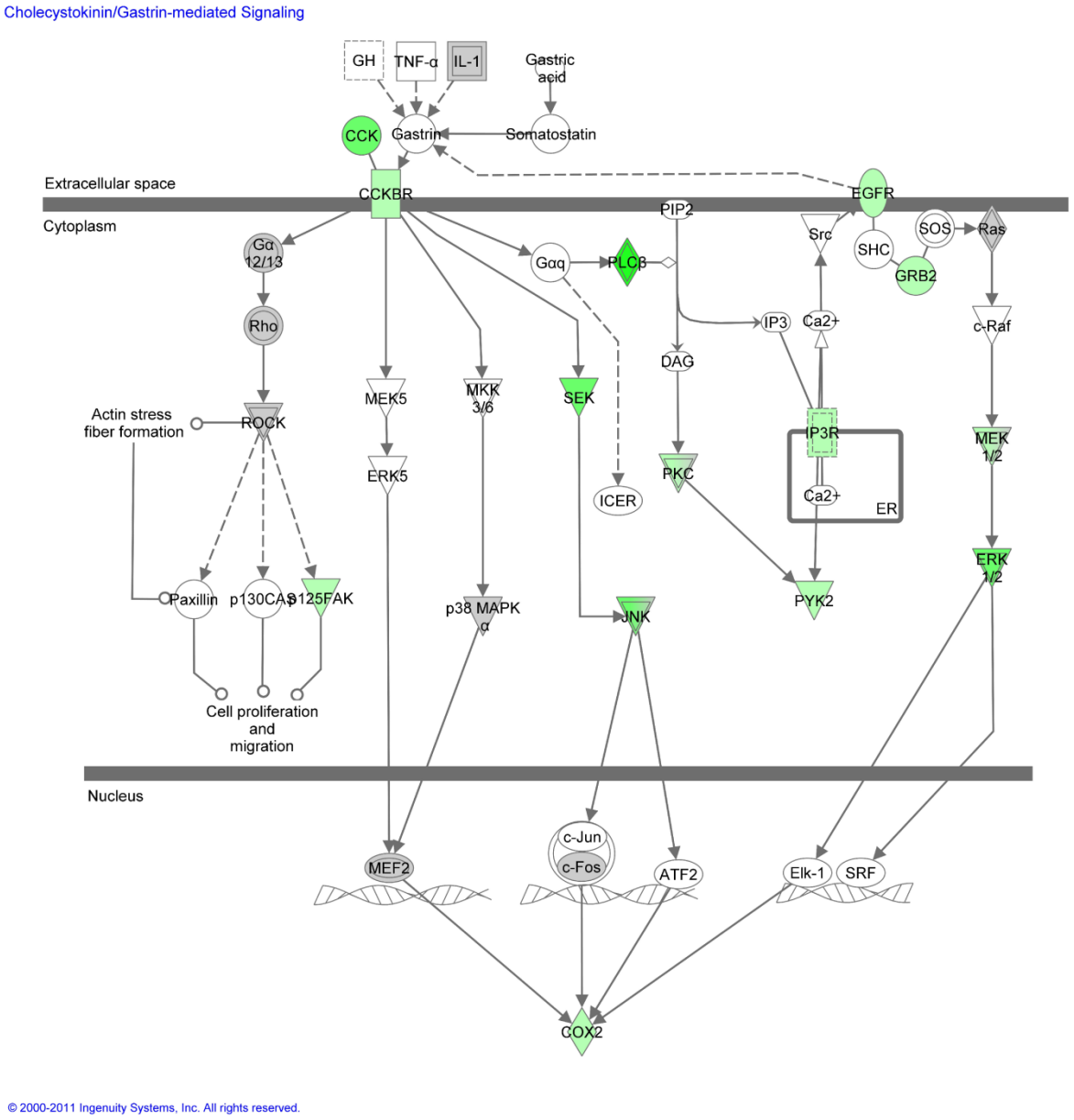


Figure 2.2 Down-regulated genes in the CCK pathway. The intensity of the colour indicates the number of studies in which the gene is down-regulated.

2.6 References

Aitken P.G., Jaffe D.B. & Nadler J.V. (1991) Cholecystokinin blocks some effects of kainic acid in CA3 region of hippocampal slices. *Peptides* **12**, 127-9.

Almgren M., Nyengaard J.R., Persson B. & Lavebratt C. (2008) Carbamazepine protects against neuronal hyperplasia and abnormal gene expression in the megencephaly mouse. *Neurobiol Dis* **32**, 364-76.

Alzheimer C. & Werner S. (2002) Fibroblast growth factors and neuroprotection. *Adv Exp Med Biol* **513**, 335-51.

Arion D., Sabatini M., Unger T., Pastor J., Alonso-Nanclares L., Ballesteros-Yanez I., Garcia Sola R., Munoz A., Mirnics K. & DeFelipe J. (2006) Correlation of transcriptome profile with electrical activity in temporal lobe epilepsy. *Neurobiol Dis* **22**, 374-87.

Atack J.R., Bayley P.J., Fletcher S.R., McKernan R.M., Wafford K.A. & Dawson G.R. (2006) The proconvulsant effects of the GABAA alpha5 subtype-selective compound RY-080 may not be alpha5-mediated. *Eur J Pharmacol* **548**, 77-82.

Augustine G.J. (2001) How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* **11**, 320-6.

Becker A.J., Chen J., Paus S., Normann S., Beck H., Elger C.E., Wiestler O.D. & Blumcke I. (2002) Transcriptional profiling in human epilepsy: expression array and single cell real-time qRT-PCR analysis reveal distinct cellular gene regulation. *Neuroreport* **13**, 1327-33.

Becker A.J., Chen J., Zien A., Sochivko D., Normann S., Schramm J., Elger C.E., Wiestler O.D. & Blumcke I. (2003) Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy. *Eur J Neurosci* **18**, 2792-802.

Benjamini Y. & Hochberg Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* **57**, 289-300.

Bethmann K., Fritschy J.M., Brandt C. & Loscher W. (2008) Antiepileptic drug resistant rats differ from drug responsive rats in GABA A receptor subunit expression in a model of temporal lobe epilepsy. *Neurobiol Dis* **31**, 169-87.

Bonin R.P., Martin L.J., MacDonald J.F. & Orser B.A. (2007) Alpha5GABAA receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. *J Neurophysiol* **98**, 2244-54.

Brooks-Kayal A.R., Shumate M.D., Jin H., Rikhter T.Y. & Coulter D.A. (1998) Selective changes in single cell GABA(A) receptor subunit expression and function in temporal lobe epilepsy. *Nat Med* **4**, 1166-72.

Chapman E.R. (2002) Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? *Nat Rev Mol Cell Biol* **3**, 498-508.

Chayasirisobhon S. (2009) The mechanisms of medically refractory temporal lobe epilepsy. *Acta Neurol Taiwan* **18**, 155-60.

Choi J.K., Yu U., Kim S. & Yoo O.J. (2003) Combining multiple microarray studies and modeling interstudy variation. *Bioinformatics* **19 Suppl 1**, i84-90.

Condliffe S.B., Corradini I., Pozzi D., Verderio C. & Matteoli M. (2010) Endogenous SNAP-25 regulates native voltage-gated calcium channels in glutamatergic neurons. *J Biol Chem* **285**, 24968-76.

Engel J., Jr. (2003) A Greater Role for Surgical Treatment of Epilepsy: Why and When? *Epilepsy Curr* **3**, 37-40.

Fabene P.F., Bramanti P. & Constantin G. (2010) The emerging role for chemokines in epilepsy. *J Neuroimmunol* **224**, 22-7.

Fabene P.F., Navarro Mora G., Martinello M., Rossi B., Merigo F., Ottoboni L., Bach S., Angiari S., Benati D., Chakir A., Zanetti L., Schio F., Osculati A., Marzola P., Nicolato E., Homeister J.W., Xia L., Lowe J.B., McEver R.P., Osculati F., Sbarbati A., Butcher E.C. &

Constantin G. (2008) A role for leukocyte-endothelial adhesion mechanisms in epilepsy. *Nat Med* **14**, 1377-83.

Ferraro G. & Sardo P. (2009) Cholecystokinin-8 sulfate modulates the anticonvulsant efficacy of vigabatrin in an experimental model of partial complex epilepsy in the rat. *Epilepsia* **50**, 721-30.

Gall C.M. & Lynch G. (2004) Integrins, synaptic plasticity and epileptogenesis. *Adv Exp Med Biol* **548**, 12-33.

Gupta S., Burckhardt G. & Hagos Y. (2011) SLC22 transporter family proteins as targets for cytostatic uptake into tumor cells. *Biol Chem* **392**, 117-24.

Hosack D.A., Dennis G., Jr., Sherman B.T., Lane H.C. & Lempicki R.A. (2003) Identifying biological themes within lists of genes with EASE. *Genome Biol* **4**, R70.

Huang K.P., Huang F.L., Jager T., Li J., Reymann K.G. & Balschun D. (2004) Neurogranin/RC3 enhances long-term potentiation and learning by promoting calcium-mediated signaling. *J Neurosci* **24**, 10660-9.

Hughes J.R. (2008) One of the hottest topics in epileptology: ABC proteins. Their inhibition may be the future for patients with intractable seizures. *Neurol Res* **30**, 920-5.

Iadarola M.J. & Sherwin A.L. (1991) Alterations in cholecystokinin peptide and mRNA in actively epileptic human temporal cortical foci. *Epilepsy Res* **8**, 58-63.

Ivens S., Kaufer D., Flores L.P., Bechmann I., Zumsteg D., Tomkins O., Seiffert E., Heinemann U. & Friedman A. (2007) TGF-beta receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. *Brain* **130**, 535-47.

Jamali S., Bartolomei F., Robaglia-Schlupp A., Massacrier A., Peragut J.C., Regis J., Dufour H., Ravid R., Roll P., Pereira S., Royer B., Roeckel-Trevisiol N., Fontaine M., Guye M., Boucraut J., Chauvel P., Cau P. & Szepetowski P. (2006) Large-scale expression study of human mesial temporal lobe epilepsy: evidence for dysregulation of the neurotransmission and complement systems in the entorhinal cortex. *Brain* **129**, 625-41.

Kadar T., Pesti A., Penke B. & Telegdy G. (1984) Inhibition of seizures induced by picrotoxin and electroshock by cholecystokinin octapeptides and their fragments in rats after intracerebroventricular administration. *Neuropharmacology* **23**, 955-61.

King H.C. & Sinha A.A. (2001) Gene expression profile analysis by DNA microarrays: promise and pitfalls. *JAMA* **286**, 2280-8.

Kolodkin A.L., Matthes D.J. & Goodman C.S. (1993) The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75**, 1389-99.

Kubota Y., Putkey J.A., Shouval H.Z. & Waxham M.N. (2008) IQ-motif proteins influence intracellular free Ca2+ in hippocampal neurons through their interactions with calmodulin. *J Neurophysiol* **99**, 264-76.

Kwan P., Poon W.S., Ng H.K., Kang D.E., Wong V., Ng P.W., Lui C.H., Sin N.C., Wong K.S. & Baum L. (2008) Multidrug resistance in epilepsy and polymorphisms in the voltage-gated sodium channel genes SCN1A, SCN2A, and SCN3A: correlation among phenotype, genotype, and mRNA expression. *Pharmacogenet Genomics* **18**, 989-98.

Lakhan R., Kumari R., Misra U.K., Kalita J., Pradhan S. & Mittal B. (2009) Differential role of sodium channels SCN1A and SCN2A gene polymorphisms with epilepsy and multiple drug resistance in the north Indian population. *Br J Clin Pharmacol* **68**, 214-20.

Lazarowski A., Czornyj L., Lubienieki F., Girardi E., Vazquez S. & D'Giano C. (2007) ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. *Epilepsia* **48 Suppl 5**, 140-9.

Lee S.Y. & Soltesz I. (2011) Cholecystokinin: a multi-functional molecular switch of neuronal circuits. *Dev Neurobiol* **71**, 83-91.

Lee T.S., Eid T., Mane S., Kim J.H., Spencer D.D., Ottersen O.P. & de Lanerolle N.C. (2004) Aquaporin-4 is increased in the sclerotic hippocampus in human temporal lobe epilepsy. *Acta Neuropathol* **108**, 493-502.

Lee T.S., Mane S., Eid T., Zhao H., Lin A., Guan Z., Kim J.H., Schweitzer J., King-Stevens D., Weber P., Spencer S.S., Spencer D.D. & de Lanerolle N.C. (2007) Gene expression in temporal lobe epilepsy is consistent with increased release of glutamate by astrocytes. *Mol Med* **13**, 1-13.

Li J.M., Wang X.F., Xi Z.Q., Gong Y., Liu F.Y., Sun J.J., Wu Y., Luan G.M., Wang Y.P., Li Y.L., Zhang J.G., Lu Y. & Li H.W. (2006) Decreased expression of thyroid receptor-associated protein 220 in temporal lobe tissue of patients with refractory epilepsy. *Biochem Biophys Res Commun* **348**, 1389-97.

Liang W.S., Dunckley T., Beach T.G., Grover A., Mastroeni D., Ramsey K., Caselli R.J., Kukull W.A., McKeel D., Morris J.C., Hulette C.M., Schmechel D., Reiman E.M., Rogers J. & Stephan D.A. (2008) Altered neuronal gene expression in brain regions differentially affected by Alzheimer's disease: a reference data set. *Physiol Genomics* **33**, 240-56.

Liu F.Y., Wang X.F., Li M.W., Li J.M., Xi Z.Q., Luan G.M., Zhang J.G., Wang Y.P., Sun J.J. & Li Y.L. (2007) Upregulated expression of postsynaptic density-93 and N-methyl-D-aspartate receptors subunits 2B mRNA in temporal lobe tissue of epilepsy. *Biochem Biophys Res Commun* **358**, 825-30.

Loscher W., Klotz U., Zimprich F. & Schmidt D. (2009) The clinical impact of pharmacogenetics on the treatment of epilepsy. *Epilepsia* **50**, 1-23.

Lukasiuk K. & Pitkanen A. (2004) Large-scale analysis of gene expression in epilepsy research: is synthesis already possible? *Neurochem Res* **29**, 1169-78.

Marchi N., Angelov L., Masaryk T., Fazio V., Granata T., Hernandez N., Hallene K., Diglaw T., Franic L., Najm I. & Janigro D. (2007) Seizure-promoting effect of blood-brain barrier disruption. *Epilepsia* **48**, 732-42.

Mariotti V., Melissari E., Amar S., Conte A., Belmaker R.H., Agam G. & Pellegrini S. (2010) Effect of prolonged phenytoin administration on rat brain gene expression assessed by DNA microarrays. *Exp Biol Med (Maywood)* **235**, 300-10.

Neher E. (2010) Complexin: does it deserve its name? *Neuron* **68**, 803-6.

Okuno T., Nakatsuji Y., Moriya M., Takamatsu H., Nojima S., Takegahara N., Toyofuku T., Nakagawa Y., Kang S., Friedel R.H., Sakoda S., Kikutani H. & Kumanogoh A. (2010) Roles of Sema4D-plexin-B1 interactions in the central nervous system for pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* **184**, 1499-506.

Ozbas-Gerceker F., Redeker S., Boer K., Ozguc M., Saygi S., Dalkara T., Soylemezoglu F., Akalan N., Baayen J.C., Gorter J.A. & Aronica E. (2006) Serial analysis of gene expression in the hippocampus of patients with mesial temporal lobe epilepsy. *Neuroscience* **138**, 457-74.

Ragozzino D., Renzi M., Giovannelli A. & Eusebi F. (2002) Stimulation of chemokine CXC receptor 4 induces synaptic depression of evoked parallel fibers inputs onto Purkinje neurons in mouse cerebellum. *J Neuroimmunol* **127**, 30-6.

Rhodes D.R., Barrette T.R., Rubin M.A., Ghosh D. & Chinnaiyan A.M. (2002) Meta-analysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. *Cancer Res* **62**, 4427-33.

Risinger C. & Bennett M.K. (1999) Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. *J Neurochem* **72**, 614-24.

Rogawski M.A. & Loscher W. (2004) The neurobiology of antiepileptic drugs. *Nat Rev Neurosci* **5**, 553-64.

Sander J.W. (2003) The natural history of epilepsy in the era of new antiepileptic drugs and surgical treatment. *Epilepsia* **44 Suppl 1**, 17-20.

Sayin U., Osting S., Hagen J., Rutecki P. & Sutula T. (2003) Spontaneous seizures and loss of axo-axonic and axo-somatic inhibition induced by repeated brief seizures in kindled rats. *J Neurosci* **23**, 2759-68.

Schiavo G., Stenbeck G., Rothman J.E. & Sollner T.H. (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci U S A* **94**, 997-1001.

Schmidt D. & Loscher W. (2009) New developments in antiepileptic drug resistance: an integrative view. *Epilepsy Curr* **9**, 47-52.

Seiffert E., Dreier J.P., Ivens S., Bechmann I., Tomkins O., Heinemann U. & Friedman A. (2004) Lasting blood-brain barrier disruption induces epileptic focus in the rat somatosensory cortex. *J Neurosci* **24**, 7829-36.

Shorvon S.D. (1996) The epidemiology and treatment of chronic and refractory epilepsy. *Epilepsia* **37 Suppl 2**, S1-S3.

Sinn D.I., Kim S.J., Chu K., Jung K.H., Lee S.T., Song E.C., Kim J.M., Park D.K., Kun Lee S., Kim M. & Roh J.K. (2007) Valproic acid-mediated neuroprotection in intracerebral hemorrhage via histone deacetylase inhibition and transcriptional activation. *Neurobiol Dis* **26**, 464-72.

Somera-Molina K.C., Nair S., Van Eldik L.J., Watterson D.M. & Wainwright M.S. (2009) Enhanced microglial activation and proinflammatory cytokine upregulation are linked to increased susceptibility to seizures and neurologic injury in a 'two-hit' seizure model. *Brain Res* **1282**, 162-72.

Sudhof T.C. & Rothman J.E. (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**, 474-7.

Unsicker K. & Krieglstein K. (2002) TGF-betas and their roles in the regulation of neuron survival. *Adv Exp Med Biol* **513**, 353-74.

van Gassen K.L., de Wit M., Koerkamp M.J., Rensen M.G., van Rijen P.C., Holstege F.C., Lindhout D. & de Graan P.N. (2008) Possible role of the innate immunity in temporal lobe epilepsy. *Epilepsia* **49**, 1055-65.

Vezzani A. & Granata T. (2005) Brain inflammation in epilepsy: experimental and clinical evidence. *Epilepsia* **46**, 1724-43.

Wang Y.Y., Smith P., Murphy M. & Cook M. (2010) Global expression profiling in epileptogenesis: does it add to the confusion? *Brain Pathol* **20**, 1-16.

Whitaker W.R., Faull R.L., Dragunow M., Mee E.W., Emson P.C. & Clare J.J. (2001) Changes in the mRNAs encoding voltage-gated sodium channel types II and III in human epileptic hippocampus. *Neuroscience* **106**, 275-85.

Wu L., Peng J., Wei C., Liu G., Wang G., Li K. & Yin F. (2011) Characterization, using comparative proteomics, of differentially expressed proteins in the hippocampus of the mesial temporal lobe of epileptic rats following treatment with valproate. *Amino Acids* **40**, 221-38.

Xi Z.Q., Xiao F., Yuan J., Wang X.F., Wang L., Quan F.Y. & Liu G.W. (2009) Gene expression analysis on anterior temporal neocortex of patients with intractable epilepsy. *Synapse* **63**, 1017-28.

Xiao F., Wang X.F., Li J.M., Xi Z.Q., Lu Y., Wang L., Zeng Y., Guan L.F. & Yuan J. (2008) Overexpression of N-WASP in the brain of human epilepsy. *Brain Res* **1233**, 168-75.

Zhang L.X., Wu M. & Han J.S. (1992) Suppression of audiogenic epileptic seizures by intracerebral injection of a CCK gene vector. *Neuroreport* **3**, 700-2.

Zhang L.X., Zhou Y., Du Y. & Han J.S. (1993) Effect of CCK-8 on audiogenic epileptic seizure in P77PMC rats. *Neuropeptides* **25**, 73-6.

Zhang S.J., Zou M., Lu L., Lau D., Ditzel D.A., Delucinge-Vivier C., Aso Y., Descombes P. & Bading H. (2009) Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PLoS Genet* **5**, e1000604.

Zhang X., Kim-Miller M.J., Fukuda M., Kowalchyk J.A. & Martin T.F. (2002) Ca2+-dependent synaptotagmin binding to SNAP-25 is essential for Ca2+-triggered exocytosis. *Neuron* **34**, 599-611.

CHAPTER THREE

SLC TRANSPORTERS IN PHARMACORESISTANT EPILEPSY: AN INTEGRATIVE *IN SILICO* & *EX VIVO* ANALYSIS

Contents

Chapter 3: SLC Transporters in Pharmacoresistant Epilepsy: An Integrative *In Silico* & *Ex Vivo* Analysis

3.1 Introduction

Epilepsies are among the most common neurological disorders, affecting up to 1% of the population (Sander 2003). Most patients with epilepsy become seizure-free with antiepileptic drug (AED) therapy. However, approximately 30% of epilepsy patients are pharmacoresistant: they continue to experience seizures even if treated with various AEDs at maximal dosages (Shorvon 1996). A number of hypotheses have been put forward to explain pharmacoresistance (Chayasirisobhon 2009); one of these is the multidrug transporter (MDT) hypothesis. According to this hypothesis, pharmacoresistance results from decreased drug concentrations at the epileptic focus secondary to a localized dysregulation of drug transporters (Chayasirisobhon 2009), which could either increase drug efflux from, or reduce influx into, the epileptic focus.

The largest superfamily of MDTs is the solute carrier (SLC) superfamily which are mainly influx transporters (Huang & Sadee 2006). Downregulation within the epileptic focus of SLC transporters which are normally expressed at significant levels could thus potentially contribute to the development of pharmacoresistance. There are approximately 400 known SLC proteins in total, but very few of these have been studied in epilepsy.

Mesial temporal lobe epilepsy (MTLE), in which seizures originate from the hippocampus, is the most common cause of refractory epilepsy and the most common indication for epilepsy surgery (Engel 2003). In this study, therefore, we focus on SLCs most relevant to this phenotype.

In order to identify the SLCs which are downregulated in the pharmacoresistant epileptic hippocampus, we devised a robust *in silico* approach that exploits relevant published data and builds upon them using cutting-edge computational tools; we then verified the output using a robust *ex vivo* approach. We anticipate that our *in silico* strategy will be adaptable to other pathologies and protein families.

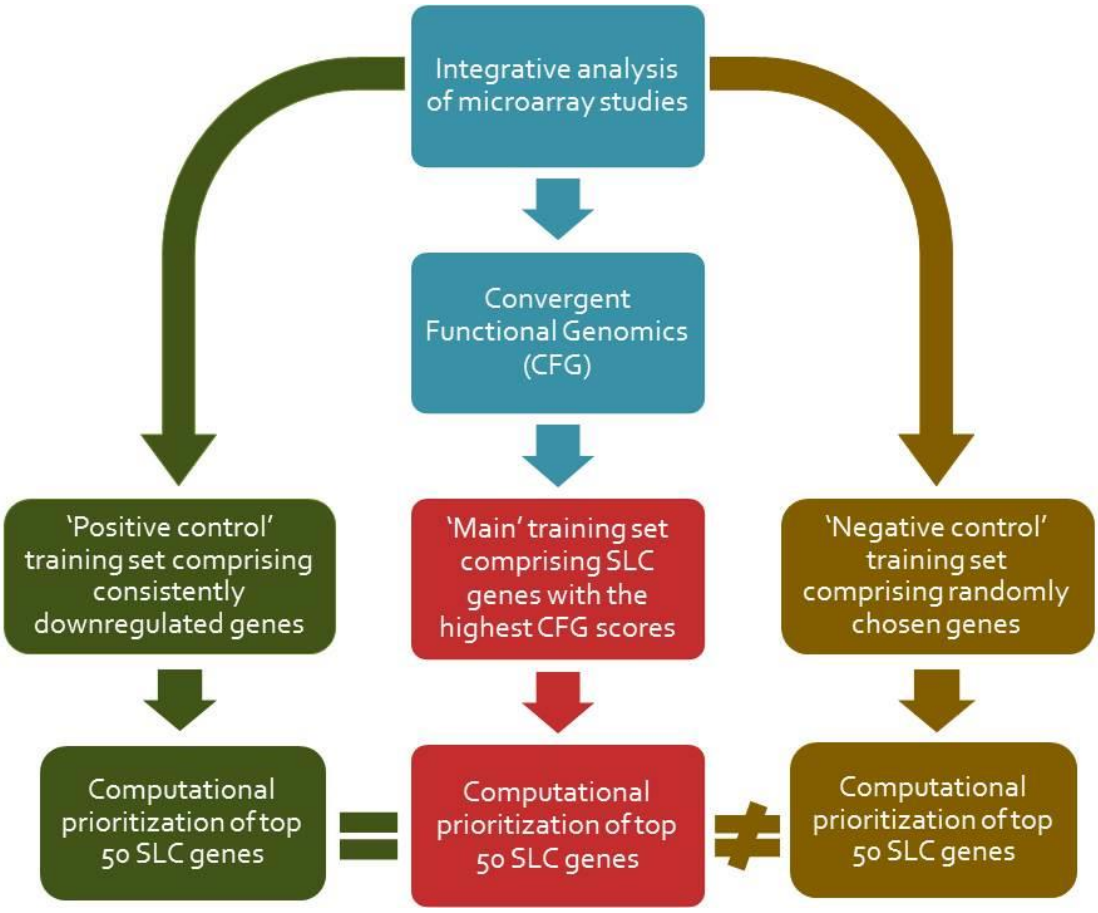


Figure 3.1 Our *in silico* strategy; see text for details. SLC=solute carrier transporter

3.2 Methods:

3.2.1 *In silico* analysis

Our *in silico* strategy was to:

1. rank SLCs based on the strength of the published evidence of their downregulation in pharmacoresistant epileptic foci, if such data is available;
2. for SLCs with no such published data, rank genes based on the computationally-determined likelihood of their downregulation in pharmacoresistant epileptic foci; this step was limited to the SLCs most abundantly expressed in the normal human hippocampus, as these SLCs are most likely to be functionally important in the pharmacoresistant epileptic hippocampus; and
3. based on 1 and 2 above, create a prioritized list of SLCs most likely to be downregulated in pharmacoresistant epileptic foci.

The individual steps and processes are illustrated schematically in Figure 3.1.

Integrative analysis of microarray studies: We started by performing an integrative analysis of large-scale gene expression profiling studies on brain tissue from epilepsy surgery—details of this analysis can be found in Chapter 1. Data relating to SLCs from this analysis was carried forward into the convergent functional genomics step.

Convergent functional genomics (CFG): CFG is an approach for prioritizing candidate genes for complex disorders by integrating and tabulating multiple lines of evidence, such as human and animal-model gene and protein expression data (Le-Niculescu *et al.* 2007). In order to implement the CFG approach, each SLC transporter was scored on the strength of the evidence of down-regulation of the gene or protein in epileptic foci of humans or of animal models of pharmacoresistant epilepsy. The search strategy and search terms used in order to find this data is detailed in Appendix 4. Details of the scoring system are given in Table 3.1.

Table 3.1 Convergent Functional Genomics scoring scheme for SLC transporters

Evidence	Score	Reason
Gene/protein expression data		
Downregulated in ≥2 studies on pharmacoresistant MTLE epileptic foci	1	Maximum weight given to confirmed downregulation in MTLE, which is our phenotype of interest
Downregulated in only 1 study on pharmacoresistant MTLE epileptic foci	0.50	Lower score given to non-replicated evidence
Downregulated in ≥1 study on pharmacoresistant non-MTLE epileptic foci	0.50	Lower score given to evidence in other phenotypes
Evidence of downregulation in brain tissue from animal models of pharmacoresistant epilepsy	0.25	Lowest score for animal models

Six SLC transporters (SLC1A2, SLC1A3, SLC2A3, SLC12A5, SLC17A7 and SLC24A3) were deemed to have strong evidence in support of a role in epilepsy pharmacoresistance (see Discussion below). These six transporters were used as 'training genes' for computational gene prioritization.

Computational gene prioritization (CGP): To perform CGP, we used a bioinformatics software application called Endeavour (Tranchevent *et al.* 2008), whose key feature is that it uses multiple genomic data sources (e.g. sequence, expression, literature and annotation) to estimate how promising a candidate gene is by measuring its similarity with a set of training genes. The training genes are those which are already known to play a role in the biological process under study. The underlying assumption is that the most promising candidate genes are the ones that exhibit most similarities with the training genes. CGP was performed using all available data sources in Endeavour.

Our training gene set comprised the top scoring SLCs from the CFG step: SLC1A2, SLC1A3, SLC2A3, SLC12A5, SLC17A7 and SLC24A3. Our candidate gene set comprised SLCs which are highly likely to be expressed—and abundantly so—in the normal human hippocampus. This candidate gene set was created as described below.

Determining the presence and abundance of SLC transcripts in the normal human hippocampus: In order to determine the presence and abundance of SLC transcripts in the normal human hippocampus, we extracted data from publically-available large-scale microarray studies on the human hippocampus. We searched the ArayExpress (Parkinson *et al.* 2007) and GEO (Edgar *et al.* 2002) databases for studies fulfilling the following criteria:

1. Studies done using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. This chip was chosen as: (i) it is a single-channel array, (ii) it is one the most comprehensive whole human genome expression arrays available, and (iii) it is widely used.
2. Studies which included samples from normal post-mortem human hippocampus in the analysis.
3. Studies for which MIAME-compliant data was publically available.

For the included studies, CEL files relating to normal hippocampal tissue were downloaded for processing. Using this strategy, we obtained 73 CEL files relating to 73 different normal hippocampal tissue samples from four different studies (GEO accession numbers: GSE3526, GSE5281, GSE7307 and GSE11882).

Quality control (QC) analysis and normalization was performed on the downloaded CEL files using the affyAnalysisQC pipeline through the arrayanalysis.org webportal (Eijssen *et al.* 2013). We used the following options: (1) Absent (A), Marginal (M) and Present (P) calls were computed based on the MAS5 function; (2) re-annotation of the probes was performed using a custom Chip Definition File environment from the BrainArray database.

For each gene, the number of P, M and A calls was summed using a value of 1 for P and 0.50 for M, and 0 for A. Genes with a cumulative score of 37 or above were chosen as there was greater evidence of their presence than of their absence. These chosen genes were than ranked by transcript abundance as described below.

We used 'rank product'—a non-parametric statistic that detects items that are consistently highly ranked in a number of lists—to create one combined hierarchical list of transcript abundance. Analysis was performed in R using the RankProd Bioconductor package (Hong *et al.* 2006). The function 'RPadvance', which performs the rank product analysis for multiple-origin data, was used. From this analysis, the 50 most abundant SLCs were chosen as the candidate gene set—all these genes had a false discovery rate (FDR) of less than 5% for being ranked amongst the 50 most abundant SLC genes.

Computational verification of CGP results: To validate our CGP approach and prioritized lists, the following steps were undertaken:

1. The validity of the CGP result is vitally dependent on the quality of the training set. Therefore, to ensure the quality of the training set, we:
    a. performed a comprehensive literature review of our training genes to show that they are highly likely to be involved in epilepsy pharmacoresistance (see details in Discussion section), and
    b. performed leave-one-out analysis (LOOA). LOOA was performed in order to assess the quality (homogeneity) of the training set. LOOA was performed using a specifically designed utility provided by the authors of Endeavour. This analysis comprises multiple automated validation runs. In each validation run, one gene, termed the 'defector' gene, was deleted from a set of training genes and added to 99 randomly selected test genes. The

software then determined the ranking of this defector gene. This procedure makes it possible to assess the quality (homogeneity) of the training set

2. We set up an *in silico* experiment. For this experiment, we created (i) a 'positive control' training set comprising the most consistently down-regulated genes from our previously mentioned integrative analysis of gene-expression profiling studies on brain tissue from epilepsy surgery (Mirza *et al.* 2011), and (ii) a 'negative control' training set consisting of randomly chosen genes from our integrative analysis. Both the control training sets were used to perform CGP of the same 50 SLC genes. The list of genes comprising the control training sets can be found in the Appendix 5. We hypothesized that the 'positive control ranking' would be significantly similar to the original ranking, whereas the 'negative control ranking' would be dissimilar to the original ranking. We compared the original CGP ranking to the positive control and negative control CGP rankings in turn, using the following two methods:
    a. We calculated the size of the overlap between the top 20 genes in the two lists, and calculated the probability of this overlap occurring by chance according to the hypergeometric equation using the function phyper in R (Fury, Batliwalla et al. 2006).
    b. We compared the two gene lists using the Bioconductor package OrderedList (Lottaz, Yang et al. 2006). This package determines if there exist significant order similarities between pairs of gene lists. The package quantifies the similarity between ordered gene lists; the significance of the similarity is estimated from random scores computed on perturbed data.

Creation of the final *in silico* gene list: To create the final *in silico* list of 20 genes, we amalgamated the CFG and CGP results. Giving precedence to genes with direct published evidence of downregulation, CFG genes were placed at the top of the list in rank order, followed by the CGP genes, with a cut-off being applied at a total of 20 genes. As our chosen cut-off of 20 total genes could be deemed arbitrary, we created for testing two further lists of 10 and 30 total genes.

3.2.2 *Ex vivo* analysis

Sample collection: Samples used in the study originated from three UK sites: the Walton Centre for Neurology and Neurosurgery in Liverpool, the Salford Royal Hospital in Salford

and the Southern General Hospital in Glasgow. We aimed to recruit patients of age 5 years and older with pharmacoresistant mesial temporal lobe epilepsy of at least 3 months duration for which a therapeutic temporal lobectomy was being undertaken. As recently proposed by the ILAE, pharmacoresistance was defined as the failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom (Kwan *et al.* 2010). The diagnosis of MTLE was made by the treating clinician based on seizure semiology, MRI brain and EEG characteristics being consistent with a seizure focus in the mesial temporal lobe. Patients suspected of having a neoplastic or malignant temporal lobe lesion were excluded. After surgery, the hippocampus was divided into two portions: (1) one portion was preserved for RNA isolation, (2) the other portion underwent histological analysis by an experienced neuropathologist. Any subjects found to have a neoplastic or malignant lesion on histological analysis were excluded. The portion preserved for RNA isolation was either stored in RNAlater (Liverpool and Glasgow) or frozen directly at -80 ^{O}C (Salford). It should be noted that the quality of the RNA generated from the directly frozen brain samples was not significantly different from RNAlater-preserved samples: mean RIN values were 7.82 and 7.25 respectively, and two-sample t-test p-value for difference between group RINs was 0.07.

Frozen post-mortem histologically-normal hippocampal samples from donors with no known brain diseases were obtained from the MRC Edinburgh Brain Bank (Edinburgh, UK) and the Queen Square Brain Bank (London, UK).

RNA isolation: Brain samples were disrupted and homogenized in an appropriate volume of QIAzol lysis reagent (Qiagen, Crawley, United Kingdom) by using a TissueRuptor handheld rotor-stator homogenizer (Qiagen, Crawley, United Kingdom). Total RNA was extracted from the homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. RNA quality was examined by capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) and Agilent 2100 Expert software was used to calculate the RNA Integrity number (RIN) of each sample. Purity of the RNA sample was assessed using a NanoDrop1000 Spectrophotometer. Capillary electrophoresis traces were also examined. Samples with RNA integrity number scores (RIN) below 6, obvious RNA degradation, significant 18S or 28S ribosomal RNA degradation,

ratio of absorbance at 260nm and 280nm <1.95, or with noticeable DNA or background contaminants did not pass QC, and were withheld from microarray analysis. It should be noted that RNA samples with RIN scores of 6 and lower have been successfully used for 3' microarrays (Hawrylycz *et al.* 2012) and exon microarrays (Trabzuni *et al.* 2011).

Microarrays: The microarrays were processed at the Centre for Genomics Research in the University of Liverpool (http://www.liv.ac.uk/genomic-research/). 50ng of total RNA was amplified and labelled using the Agilent Low Input Quick Amp One-Colour Labeling Kit and labelled RNA was hybridized to Agilent SurePrint G3 Custom Exon 8x60K Microarrays designed to contain probes for each exon of 936 selected genes, including all known SLC genes. Standard Agilent protocols were followed. The quality of the synthesized cRNA was assessed using an Agilent Bioanalyzer 2100—the resulting electropherograms were found to be satisfactory. Each scanned image was viewed for visible artefacts, and if multiple artefacts were present, the array was rejected. Detailed QC reports were generated for each array using the Feature Extraction 11.0.1.1 Software (Agilent, Palo Alto, CA). Based on these reports, we excluded arrays in which more than 1% of features were non-uniform outliers, or the average of the net signals in negative controls was >40, or the average of the background-subtracted signals in negative controls was <-10 or >5, or the standard deviation of the background-subtracted signals in negative controls was >10, or the residual noise after spatial detrending was >15, or the median coefficient of variation for spike-in probes or non-control probes was >12%, or the dose-response curve of the spike-ins had a slope of <0.9 or >1.20, or the spike-in detection limit was <0.01 or >2. One array failed on five of these metrices and, hence, was excluded. Intensity data was extracted from the remaining arrays using the Feature Extraction Software. In line with the manufacturer's recommendations, spatial and multiplicative detrending was applied but background subtraction was not.

Data exported from Feature Extraction Software was imported into GeneSpring GX Software (Agilent, Palo Alto, CA). Features which were population outliers, saturated or non-uniform were flagged as 'Compromised' and filtered out. 75th percentile normalization, the default method recommended by Agilent for their exon arrays, was performed. Gene level values were obtained by summarizing the signal intensity values of probes mapping to various exons in the gene. As GeneSpring does not include functions that allow adjusting for

confounders and covariates, gene-level intensities were exported and further analysed using the limma package in R. This package has been used successfully for exon microarray analysis—see, for example, (Okoniewski *et al.* 2007; Zhang *et al.* 2007; Hall *et al.* 2011; Liu *et al.* 2011; Alagaratnam *et al.* 2013). Covariates included in the model were RIN, age, sex and batch—eight arrays (four cases and four controls) were processed together on each slide, hence, each batch comprised the eight samples processed together on each slide.

From the microarray analysis, we created a list of SLCs significantly downregulated in the pharmacoresistant epileptic hippocampus. To be deemed significantly downregulated, SLC genes had to fulfil the following criteria: (1) strong evidence of expression in normal hippocampal tissue: the gene was flagged 'detected' in more than half of control samples; (2) FDR < 0.05; (3) fold-change ≥ 1.5.

Quantitative Real-Time PCR: For each sample included in the microarray analysis, 1.8 µg of total RNA was reverse transcribed, according to the manufacturer's instructions, using the QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA) with integrated removal of genomic DNA contamination.

Quantitative real-time PCR (qRT-PCR) was performed for three of the most significantly downregulated SLC genes. qRT-PCR was performed using the QuantiFast Multiplex PCR Kit (Qiagen Inc., Valencia, CA). Proprietary primer and FAM-labelled probe sets for target genes SLC24A3 (QF00004536), SLC47A1 (QF0052735) and SLC25A23 (QF00499555) were purchased from Qiagen (Qiagen Inc., Valencia, CA). Proprietary primer and MAX-labelled probe set for endogenous control GAPDH (QF00531132) was bought from Qiagen (Qiagen Inc., Valencia, CA), and for endogenous control CALR (Hs.PT.51.19228618) from IDT (Glasgow, UK). These two genes were chosen as endogenous controls as they are amongst a small group of validated 'high quality' housekeeping genes which have previously been shown to exhibit high expression and low variance (She *et al.* 2009), and they were also found to be invariant between the cohorts in our study after adjusting for covariates (data not shown). Each sample was assayed in triplicate using a duplex format in which a target gene and a control gene are assayed in the same well. The duplex format gives more reliable results as there is no well-to-well variability due to co-amplification of the internal control. To enable detection of contamination, 'no template controls' (containing all the

components of the reaction except for the template), and 'no reverse transcriptase controls' (containing all the components except for the reverse transcriptase) were included in triplicate for each target gene-control gene combination. The qRT-PCR reactions were conducted on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA), using conditions recommended for the QuantiFast Multiplex PCR Kit by its manufacturer. qRT–PCR data were analyzed using the RQ manager version 2.2 (Applied Biosystems, CA, USA). In line with the manufacturer's recommendations, all baseline and threshold values were reviewed and adjusted manually where necessary. Further analysis was performed in DataAssist (Applied Biosystems, CA, USA). Expression levels of each target gene were normalized to the geometric mean of the two endogenous controls. Fold change (relative to the epilepsy group) was calculated, and a p-value was generated based on a two-sample, two-tailed Student's t-test comparing the two groups and then adjusted using the Benjamini-Hochberg False Discovery Rate.

3.2.3 Overlap between computational and microarray results

The statistical significance of the overlap between the computationally-generated gene list and the list of SLCs significantly downregulated in our microarray was calculated using the R function phyper.

3.3 Results

3.3.1 *In silico* analysis

CFG: The results of the CFG analysis are summarized in Table 3.2; references for the data used can be found in Appendix 6. As Table 3.2 shows, there was data in published literature relating only to 17 SLC transporters. We also wished to obtain a prioritized list of SLC transporters that have hence far been overlooked in epilepsy research. To do this, we used the software 'Endeavour' to perform CGP.

Table 3.2 Results of the CFG analysis for SLC transporters. Please see the methods section for a description of the scoring system employed.

Transporter	Total score
SLC1A2	1.5
SLC1A3	1.5
SLC17A7	1.5
SLC24A3	1
SLC2A3	1
SLC12A5	1
SLC30A3	0.5
SLC8A2	0.5
SLC47A1	0.5
SLC4A8	0.5
SLC15A2	0.5
SLC8A1	0.5
SLC17A1	0.5
SLC6A20	0.5
SLC16A1	0.5
SLC1A6	0.5
SLC1A1	0.5

CGP: To create a training gene set, we utilized the six SLC transporters with the highest CFG scores and with replicated evidence in support of a role in epilepsy pharmacoresistance. To assess the quality of our chosen training set, we performed leave-one-out cross-validation. The results of this analysis show that four defector genes were ranked first, while two were ranked second (see Appendix 7). This demonstrates that the training set is homogenous. We also performed a comprehensive literature review of our training genes to show that they

are highly likely to be involved in epilepsy pharmacoresistance (see Discussion section blow).

To compile a candidate gene set for CGP, we created a hierarchical list of the most abundant SLC transporters in the normal human hippocampus. To do this, we performed a 'meta-analysis' of 73 publically-available Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays on the normal human hippocampus, as described in the Methods section. The results of this meta-analysis can be found in the Appendix 8. The 50 most abundant SLC genes were taken forward as the candidate gene set for CGP. All these genes had an FDR of less than 5% for being ranked amongst the 50 most abundant SLC genes.

To verify the results of the above CGP, we performed a second CGP of the same candidate SLC genes using a different and independently selected 'positive control' training set: the 10 genes most consistently downregulated in the integrative analysis of large-scale microarray studies on brain tissue from epilepsy surgery presented in Chapter 1. We compared the results of the two CGPs (original and positive control): of the top 20 genes in each list, 13 were common to both lists. The probability of this overlap occurring by chance alone is 0.004, according to the hypergeometric equation. We also compared the two prioritized gene lists using the Bioconductor package OrderedList (Lottaz *et al.* 2006). The order of genes in the two lists was significantly similar (p-value=0.01). In contrast, when the original CGP ranking was compared with the 'negative control' CGP ranking, generated using a 'negative control' training set of randomly chosen genes from our integrative analysis, there was no significant overlap in the top 20 genes of the two lists (p=0.614) and no significant similarity between the order of genes in the two lists (p=0.10).

Creation of the final *in silico* gene list: To create the final *in silico* gene list, we amalgamated the CFG and CGP results. Giving precedence to genes with direct published evidence of downregulation, CFG genes were placed at the top of the list in rank order, followed by the CGP genes. In order to test the accuracy of *in silico* gene lists of varying sizes, we created lists of 10, 20 and 30 genes (Table 3.3).

3.3.2 *Ex vivo* analysis

For *ex vixo* validation, we collected 24 hippocampal samples from surgery for pharmacoresistant mesial temporal lobe epilepsy and obtained 24 histologically-normal post-mortem hippocampal samples from donors with no known brain disease. Important patient and donor individual and sample characteristics are summarized in Table 3.4.

There was no statistically significant difference in the sex distribution of the two groups (the number of males in cases was 12, and in controls was 18, χ^2= 2.2222, df = 1, p-value = 0.136). There was a statistically significant difference in the mean RIN values for the two groups (7.487500 in cases and 6.383333 in controls, 2-sample t-test p-value < 0.05). RIN was included as a covariate in the linear model used to analyse the microarray data. There was also a statistically significant difference in the mean age of the two groups (36.33333 years in cases and 53.95833 years in controls, 2-sample t-test p-value <0.05). Again, age was included as a covariate in the linear model used to analyse the microarray data.

One of the control samples failed microarray QC evaluation (see Methods section) and, hence, was excluded, leaving 24 disease samples and 23 normal control samples for subsequent analysis. We filtered out SLC genes lacking strong evidence of expression in the control samples (see Methods section) and utilized linear models implemented in the R Bioconductor package limma for data analysis, including as covariates batch, RIN, age and sex. 18 SLC genes (Table 3.5) were significantly downregulated (FDR < 0.05, fold change ≥ 1.5). qRT-PCR results for the three tested SLC genes were highly concordant with the microarray results (Table 3.5)

3.3.3 Overlap between computational and microarray results

We calculated the size of the overlap between the 18 SLC genes shown to be down-regulated by our microarray and our *in silico* gene lists of 10, 20 and 30 SLC genes respectively. All three overlaps were highly significant, with p-values of 8.4×10^{-7}, 7.9×10^{-6} and 9.9×10^{-7} respectively.

Table 3.3 *In silico* gene list of 30 genes.

***In silico* list of 30 genes**
SLC1A2
SLC1A3
SLC17A7
SLC24A3
SLC2A3
SLC12A5
SLC30A3
SLC8A2
SLC47A1
SLC4A8
SLC15A2
SLC8A1
SLC17A1
SLC6A20
SLC16A1
SLC1A6
SLC1A1
SLC1A4
SLC6A1
SLC24A2
SLC12A7
SLC25A22
SLC6A8
SLCO1C1
SLC7A5
SLCO3A1
SLC7A11
SLC25A11
SLC4A3
SLC20A1

Table 3.4 Sample characteristics. RIN=RNA Integrity Number

Sample	Phenotype	Age	Sex	RIN
D1	Case	41	F	7.4
D2	Case	23	F	6.8
D3	Case	51	M	6
D4	Case	49	F	6.9
D5	Case	50	F	7.8
D6	Case	45	F	6.6
D7	Case	12	M	7
D8	Case	29	F	7.8
D9	Case	33	M	6.8
D10	Case	25	F	7.1
D11	Case	34	M	7
D12	Case	33	M	8.8
D13	Case	33	M	8.6
D14	Case	22	F	7.3
D15	Case	48	M	7.6
D16	Case	39	F	7.4
D17	Case	29	F	7.1
D18	Case	44	M	7.9
D19	Case	40	F	6.6
D20	Case	48	M	8.5
D21	Case	23	M	8.4
D22	Case	63	M	7.9
D23	Case	31	M	8.2
D24	Case	27	F	8.2

Sample	Phenotype	Age	Sex	RIN
N1	control	81	M	6.5
N2	control	78	F	6.2
N3	control	84	F	6.6
N4	control	91	F	6.7
N5	control	88	M	6.3
N6	control	38	M	6.1
N7	control	50	M	6.2
N8	control	45	M	6.3
N9	control	39	M	6.1
N10	control	40	M	6
N11	control	61	M	6.2
N12	control	63	F	6.2
N13	control	66	M	6.2
N14	control	22	F	6.3
N15	control	27	M	6.3
N16	control	45	M	6.9
N17	control	44	F	6.7
N18	control	50	M	6.5
N19	control	43	M	6.6
N20	control	46	M	6.8
N21	control	51	M	6.2
N22	control	48	M	6
N23	control	43	M	6.6

Table 3.5 Microarray and qRT-PCR results. FDR=false discovery rate

Gene Symbol	Microarray results		qRT-PCR results	
	FDR	Fold-change	FDR	Fold-change
SLC24A3	1.65×10^{-4}	3.7	0	3.7
SLC47A1	1.49×10^{-3}	2.6	1.60×10^{-3}	2.8
SLC25A23	3.38×10^{-4}	2.3	1.00×10^{-4}	1.9
SLC8A2	1.64×10^{-5}	2.0		
SLC17A7	3.89×10^{-3}	1.8		
SLC25A41	1.20×10^{-5}	1.6		
SLC26A10	5.28×10^{-5}	1.6		
SLC4A3	8.02×10^{-5}	1.6		
SLC4A7	2.26×10^{-2}	1.6		
SLC12A5	1.24×10^{-2}	1.6		
SLC7A1	6.81×10^{-5}	1.6		
SLC16A2	3.38×10^{-4}	1.6		
SLC25A22	1.88×10^{-4}	1.6		
SLC29A4	1.22×10^{-3}	1.5		
SLC8A1	1.24×10^{-2}	1.5		
SLC35E2	6.80×10^{-4}	1.5		
SLC4A8	1.83×10^{-4}	1.5		
SLC18A2	2.89×10^{-2}	1.5		

3.4 Discussion:

Our contributions, in the present work, are twofold. The first contribution is methodological—we have developed a novel *in silico* strategy that can aid epilepsy research by prioritizing specific genes for study from within large gene sets. The second contribution is biological—we have identified the most significantly downregulated SLCs in the pharmacoresistant epileptic human hippocampus.

3.4.*1 In silico* strategy

There are more than 400 known SLC proteins. From amongst these, we were able to make a prioritized list of 20 SLCs, using our *in silico* approach, which was highly predictive of our robust exon microarray results. We wish to highlight the following novel and noteworthy elements of our *in silico* strategy that are particularly responsible for its success:

1. We validated the output from each step of our strategy, before carrying that output forward into the next step:

a. The 'training gene-set' created through CFG was validated using LOOA and a detailed literature review.
b. The prioritized gene list from the CGP step was validated using a completely novel approach, by performing an *in silico* experiment. We prioritized the candidate genes using a robust independent 'positive control' training set (containing the most consistently downregulated genes from nine microarray studies) and showed that there was significant overlap and rank order similarity between the two prioritized lists. To compare, we prioritized the same genes using a 'negative control' training set consisting of a random selection of differentially regulated genes from the aforementioned nine microarray studies; the latter prioritized gene list had neither a significant overlap nor significant rank order similarity with our first list.

2. When creating the candidate gene list for CGP, we adopted a robust filtering approach, which is discussed in detail below.

Filtering the candidate genes for CGP: SLC proteins are, in the main, influx transporters. We were interested, therefore, in SLC transporters that are most highly expressed in the normal human hippocampus, but downregulated in the pharmacoresistant epileptic focus. To create a hierarchical list of the most abundant SLC transporters in the normal hippocampus, we extracted data from four different single-channel large-scale microarray studies. In this way, we were able to combine the gene expression data for 73 different donor samples and, hence, obtain a robust estimate of the relative abundance of genes in the human hippocampus.

Before creating the hierarchical list of transcript abundance, we filtered out non-expressed genes—not all genes are expressed at levels that are biologically significant in any particular tissue—based on 'fraction present' according to Affymetrix Microarray Suite 5.0 (MAS5) detection call. The MAS5 data processing algorithm provides a qualitative detection call (Absent, Present or Marginal) for each probe set on each array (Shi *et al.* 2006); this detection call is based on a non-parametric statistical test (Wilcoxon signed rank test) (Shi *et al.* 2006) of whether significantly more perfect matches show hybridization signal than their corresponding mismatches. We retained genes that were called Present in more than 50% of the samples, i.e. there was greater evidence of their presence than of their absence.

Filtering on detection call has been demonstrated to reduce the number of false positive findings (McClintick *et al.* 2003; McClintick & Edenberg 2006; Pepper *et al.* 2007; Hackstadt & Hess 2009). Cross-platform correlation also increases as the percent present call filter is increased (Shippy *et al.* 2004).

To create one hierarchical list of transcript abundance for the retained genes, we used 'rank product'—a non-parametric statistic that detects items that are consistently highly ranked in a number of lists to create one combined list (Hong *et al.* 2006). The premise of the rank product approach is that highly expressed genes should be highly ranked in each independent biological replicate set. The rank product method entails ranking genes by expression level within each replicate, then computing the product of the ranks across the replicates; the rank product value for each gene increases if the gene is consistently present at the top of the lists. The rank product can be translated into a measure of statistical significance by comparing the observed rank product statistic to a rank product statistic obtained from a large number of simulated data sets. On this basis, a 'percent false positive' value is calculated for each gene as an estimate of the FDR. Converting expression values into ranks increases robustness against noise and heterogeneity across studies. The rank product method has high sensitivity and specificity and desirable operating characteristics, as demonstrated in extensive numerical studies (Breitling & Herzyk 2005; Hong *et al.* 2006; Jeffery *et al.* 2006; Hong & Breitling 2008). Although developed originally for microarrays, the rank product method has found widespread acceptance in diverse settings, for example RNAi analysis (Birmingham *et al.* 2009), proteomics (Wiederhold *et al.* 2009) and machine learning model selection (Hoefsloot *et al.* 2008). The 50 most abundant SLC genes were taken forward for CGP. All these genes had an FDR of less than 5% for being ranked amongst the 50 most abundant genes.

Our robust approach to candidate gene set selection improved the results of the CGP—by filtering out SLCs from the candidate set that were less likely to be relevant, more relevant SLCs were ranked more favourably. If no filters were applied to the candidate gene set, the overlap between the top CGP and microarray results was not statistically significant ($p=0.3861$). But by filtering into the candidate set genes which were present in more than 50% of meta-analysis samples and were amongst the 50 most abundant SLCs, the overlap of the top CGP and microarray results became statistically significant ($p=0.0087$).

Limitations of our *in silico* approach: One possible criticism of our CFG-cum-CP approach is that as we have prioritized genes based on a training set derived from data mining, any functionally important SLC proteins that have been neglected in epilepsy research to date will also be overlooked in this study. However, we feel that our data collection and collation methodology minimizes the effect of this limitation as we have gathered data not only from studies focusing on one or more selected genes but also from genome-wide microarray studies. Furthermore, our CFG-cum-CP gene list compares favourably with the prioritized gene list created using an independent training set derived from nine microarray studies; the latter training set does not contain any SLC genes.

It should be noted that for collating published MDT gene and protein expression data, we included studies not only on microvascular endothelial (blood-brain barrier) cells isolated from epileptic foci, but also studies on brain parenchymal tissue obtained from epilepsy surgery. This is because in epileptic foci, MDTs have been shown to be dysregulated not only in the capillary endothelial cells (Tishler *et al.* 1995; Dombrowski *et al.* 2001; Calatozzolo *et al.* 2005; van Vliet *et al.* 2005; Volk & Loscher 2005; Sisodiya *et al.* 2006), but also in the perivascular glia (Tishler *et al.* 1995; Aronica *et al.* 2003b; Lu *et al.* 2004; Calatozzolo *et al.* 2005), and even in the neurons (Seegers *et al.* 2002; Aronica *et al.* 2003a; Aronica *et al.* 2004; Lazarowski *et al.* 2004a; Lazarowski *et al.* 2004b; Lazarowski *et al.* 2004c; Marchi *et al.* 2004; Marchi *et al.* 2005; Hoffmann *et al.* 2006). MDTs expressed in the lesional perivascular glia may facilitate the transport of AEDs between the extraneuronal space and the blood. Furthermore, altered expression of MDTs in neurons also contributes to AED resistance as demonstrated in a recent study on the Drosophila epilepsy model (Bao *et al.* 2011).

Effectiveness of our *in silico* approach: Our *in silico* predictive gene lists of various sizes—10, 20 or 30 genes—were very significantly enriched for truly downregulated SLCs, with p-values as significant as 8.4×10^{-7}. This demonstrates that our *in silico* strategy shows much promise for the task of prioritizing specific genes for study from within very large datasets. Its performance with different phenotypes and gene groups will vary depending on the quality and quantity of base data available. However, the approach is intuitive, pragmatic and practical, and includes suggested methods for computational verification of outputs making it more attractive to future researchers.

3.4.2 *Ex vivo* strategy

To ensure that our *ex vivo* analysis was robust, we chose to make use of an exon array. The whole-transcript amplification protocol of exon arrays allows more accurate measurement of gene expression than standard microarrays (Kapur *et al.* 2007; Xing *et al.* 2007; Lockstone 2011). Stringent QC filters were applied to arrays and individual features, and a 'percent present' filter was applied to the genes to reduce false positives. Finally, important technical (batch and RIN) and clinical (age and sex) covariates were included in the linear model; this reduced the false positive rate significantly. For example, without adjusting for these important covariates, 540 out of 936 genes (56%) covered by the exon array would be differentially expressed at an FDR of 0.05 (detailed data not shown); such a high rate of differential expression clearly indicates the presence of false positive results. After adjusting for these covariates, only 56 out of 936 genes (6%) are differentially expressed at an FDR of 0.05.

It is interesting to note that three training genes (SLC1A2, SLC1A3 and SLC2A3), created from CFG and used for CGP, were not significantly dysregulated in our exon array. SLC1A2 and SLC1A3 were not significantly downregulated in any previous epilepsy microarray study, although they were downregulated in previous PCR studies; this may be a result of the wider dynamic range of PCR assays. SLC2A3 was significantly downregulated in two previous microarray studies and was also borderline significant in our study, but failed to fulfil our stringent cut-off criteria—p-value was 0.02, and fold-change was 1.3 in our analysis.

3.4.3 SLC proteins downregulated in the pharmacoresistant epileptic human hippocampus

Table 3.6 summarizes the functions of the 18 SLC proteins downregulated in our exon array. As examples, three of the proteins are discussed in greater detail below, in order to illustrate their potential role in epilepsy pharmacoresistance.

SLC12A5 is a neuron-specific potassium-chloride symporter which is expressed throughout the central nervous system (Payne *et al.* 1996; Lu *et al.* 1999; Williams *et al.* 1999). As demonstrated by our analysis, it is one of the most abundant SLC transporter transcripts in the hippocampus and its downregulation in pharmacoresistant epilepsy has been confirmed in independent studies (Palma *et al.* 2006; Lee *et al.* 2007). Studies in animal models reveal that a reduction in SLC12A5 expression results in an increased susceptibility to the

development of seizures: complete deletion of SLC12A5 is incompatible with life (Hubner *et al.* 2001), a 95% reduction in SLC12A5 expression results in handling-induced seizure behaviour (Woo *et al.* 2002), and heterozygous animals have a lower threshold for epileptic seizures—electrophysiological measurements in the hippocampus show hyperexcitability and animals demonstrate a twofold increase in pentylenetetrazole-induced seizures (Woo *et al.* 2002).

SLC17A7 encodes vesicular glutamate transporter isoform 1 (vGLUT1). There are two other known isoforms of vGLUT: vGLUT2 and and vGLUT3. vGLUTs mediate the uptake of glutamate into synaptic vesicles. The three vesicular glutamate transporters are expressed in distinct populations of neurons, and SLC17A7 is the predominant vGLUT in the hippocampus (Rasmussen *et al.* 2007). SLC17A7 was downregulated in pharmacoresistant MTLE in a large-scale microarray study (Lee *et al.* 2007). In agreement with this, both SLC17A7 transcript and protein levels were downregulated in another study that used immunohistochemistry, immunofluorescence, in situ hybridization, Western blotting, and quantitative polymerase chain reaction to quantitate SLC17A7 in pharmacoresistant MTLE (van der Hel *et al.* 2009). Reduced vGLUT1 immunoreactivity was also found in epileptic peritumoural neocortex (Alonso-Nanclares & De Felipe 2005). In a recent study, SLC17A7-heterozygous mice displayed increased anxiety (Tordera *et al.* 2007), but the authors did not determine if there was neuronal hyperexcitability or if there was a reduced seizure threshold in these animals. Interestingly, mice heterozygous for SLC17A6 (which encodes vGLUT2) show increased susceptibility to clonic seizures induced by pentylenetetrazol (Schallier *et al.* 2009), while mice lacking SLC17A8 (which encodes vGLUT3) exhibit primary generalized epilepsy (Seal *et al.* 2008). Further studies are needed to determine if downregulation of SLC17A7 predisposes to epilepsy.

SLC24A3 encodes the plasma membrane sodium/calcium exchanger NCKX3 (Blaustein & Lederer 1999). SLC24A3 transcripts are most abundant in the brain, with highest levels found in hippocampal CA1 neurons (Kraev *et al.* 2001). SLC24A3 was downregulated in two independent microarray studies on the pharmacoresistant epileptic hippocampus (Lee *et al.* 2007; van Gassen *et al.* 2008). Although SLC24A3 has not been studied specifically in epilepsy research, supportive evidence for its possible role in epilepsy comes from the observation that the downregulation of the functionally-similar sodium/calcium exchanger

NCX3 leads to the hyperexcitability of hippocampal neurones in gerbils—NCX3 immunoreactivity in the hippocampus of seizure-sensitive gerbils is lower than that of seizure-resistant gerbils (Park *et al.* 2011).

As can be seen from Table 3.6, the vast majority of the identified SLC proteins are either small metal ion exchangers or transporters of neurotransmitters, particularly glutamate. Given the key roles played by ionic transport and gluatmatergic transmission in neuronal function, it stands to reason that these should be the most important SLC proteins in the epileptic hippocampus. However, for SLC transporters of metal ions, there is as yet no evidence of non-endogenous substrate transport—their small endogenous ionic substrates are markedly dissimilar to most xenobiotics, so it might be expected that they are not readily involved in xenobiotic transport (Dobson & Kell 2008). Similarly, there is no evidence as yet that the transporters of glutamate or of other neurotransmitters are able to transport therapeutic drugs. Therefore, while these SLCs potentially mediate pharmacoresistance in epilepsy, it is unlikely that they do this through altered transport of AEDs, but rather by enhancing the intrinsic severity of epilepsy (Schmidt & Loscher 2009).

3.5 Conclusions

We have developed a novel *in silico* strategy that can aid epilepsy research by prioritizing specific genes for study from within large gene sets. We have identified the most significantly downregulated SLCs in the pharmacoresistant epileptic human hippocampus. The role of these SLCs in the epileptic hippocampus will need to be defined through future functional studies.

Table 3.6 Functions of the 18 SLC proteins downregulated in the exon microarray. Data from Gene (http://www.ncbi.nlm.nih.gov/gene) and UniPort (http://www.uniprot.org/help/uniprotkb).

Gene Symbol	Function
SLC24A3	Calcium, potassium:sodium antiporter activity
SLC47A1	Monovalent cation:hydrogen antiporter activity
SLC25A23	Calcium-dependent mitochondrial solute carrier activity
SLC8A2	Sodium:calcium exchange activity
SLC17A7	L-glutamate transmembrane transporter activity
SLC25A41	Mitochondrial ATP-magnesium:phosphate carrier activity
SLC26A10	Anion exchange activity
SLC4A3	Anion exchange activity
SLC4A7	Sodium bicarbonate cotransporter activity
SLC12A5	Potassium:chloride transporter activity
SLC7A1	Transport of the cationic amino acids (arginine, lysine and ornithine)
SLC16A2	Thyroid hormone transporter
SLC25A22	Transport of glutamate across the inner mitochondrial membrane
SLC29A4	Reuptake of monoamines into presynaptic neurons
SLC8A1	Sodium:calcium exchanger activity
SLC35E2	Transport of nucleotide sugars into endoplasmic reticulum and Golgi bodies
SLC4A8	Sodium- and carbonate-dependent chloride:bicarbonate exchange activity
SLC18A2	Vesicular transport of biogenic amine neurotransmitters

3.6 References

Alagaratnam S., Harrison N., Bakken A.C., Hoff A.M., Jones M., Sveen A., Moore H.D., Andrews P.W., Lothe R.A. & Skotheim R.I. (2013) Transforming Pluripotency: An Exon-Level Study of Malignancy-Specific Transcripts in Human Embryonal Carcinoma and Embryonic Stem Cells. *Stem Cells Dev*.

Alonso-Nanclares L. & De Felipe J. (2005) Vesicular glutamate transporter 1 immunostaining in the normal and epileptic human cerebral cortex. *Neuroscience* **134**, 59-68.

Aronica E., Gorter J.A., Jansen G.H., van Veelen C.W., van Rijen P.C., Leenstra S., Ramkema M., Scheffer G.L., Scheper R.J. & Troost D. (2003a) Expression and cellular distribution of multidrug transporter proteins in two major causes of medically intractable epilepsy: focal cortical dysplasia and glioneuronal tumors. *Neuroscience* **118**, 417-29.

Aronica E., Gorter J.A., Ramkema M., Redeker S., Ozbas-Gerceker F., van Vliet E.A., Scheffer G.L., Scheper R.J., van der Valk P., Baayen J.C. & Troost D. (2004) Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia* **45**, 441-51.

Aronica E., Gorter J.A., van Vliet E.A., Spliet W.G., van Veelen C.W., van Rijen P.C., Leenstra S., Ramkema M.D., Scheffer G.L., Scheper R.J., Sisodiya S.M. & Troost D. (2003b) Overexpression of the human major vault protein in gangliogliomas. *Epilepsia* **44**, 1166-75.

Bao G.S., Wang W.A., Wang T.Z., Huang J.K., He H., Liu Z. & Huang F.D. (2011) Overexpression of Human MRP1 in Neurons causes resistance to Antiepileptic Drugs in Drosophila Seizure Mutants. *J Neurogenet*.

Birmingham A., Selfors L.M., Forster T., Wrobel D., Kennedy C.J., Shanks E., Santoyo-Lopez J., Dunican D.J., Long A., Kelleher D., Smith Q., Beijersbergen R.L., Ghazal P. & Shamu C.E. (2009) Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* **6**, 569-75.

Blaustein M.P. & Lederer W.J. (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* **79**, 763-854.

Breitling R. & Herzyk P. (2005) Rank-based methods as a non-parametric alternative of the T-statistic for the analysis of biological microarray data. *J Bioinform Comput Biol* **3**, 1171-89.

Calatozzolo C., Gelati M., Ciusani E., Sciacca F.L., Pollo B., Cajola L., Marras C., Silvani A., Vitellaro-Zuccarello L., Croci D., Boiardi A. & Salmaggi A. (2005) Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 and GST-pi in human glioma. *J Neurooncol* **74**, 113-21.

Chayasirisobhon S. (2009) The mechanisms of medically refractory temporal lobe epilepsy. *Acta Neurol Taiwan* **18**, 155-60.

Dobson P.D. & Kell D.B. (2008) Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat Rev Drug Discov* **7**, 205-20.

Dombrowski S.M., Desai S.Y., Marroni M., Cucullo L., Goodrich K., Bingaman W., Mayberg M.R., Bengez L. & Janigro D. (2001) Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42**, 1501-6.

Edgar R., Domrachev M. & Lash A.E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**, 207-10.

Eijssen L.M., Jaillard M., Adriaens M.E., Gaj S., de Groot P.J., Muller M. & Evelo C.T. (2013) User-friendly solutions for microarray quality control and pre-processing on ArrayAnalysis.org. *Nucleic Acids Res* **41**, W71-6.

Engel J., Jr. (2003) A Greater Role for Surgical Treatment of Epilepsy: Why and When? *Epilepsy Curr* **3**, 37-40.

Hackstadt A.J. & Hess A.M. (2009) Filtering for increased power for microarray data analysis. *BMC Bioinformatics* **10**, 11.

Hall J.S., Leong H.S., Armenoult L.S., Newton G.E., Valentine H.R., Irlam J.J., Moller-Levet C., Sikand K.A., Pepper S.D., Miller C.J. & West C.M. (2011) Exon-array profiling unlocks

clinically and biologically relevant gene signatures from formalin-fixed paraffin-embedded tumour samples. *Br J Cancer* **104**, 971-81.

Hawrylycz M.J., Lein E.S., Guillozet-Bongaarts A.L., Shen E.H., Ng L., Miller J.A., van de Lagemaat L.N., Smith K.A., Ebbert A., Riley Z.L., Abajian C., Beckmann C.F., Bernard A., Bertagnolli D., Boe A.F., Cartagena P.M., Chakravarty M.M., Chapin M., Chong J., Dalley R.A., Daly B.D., Dang C., Datta S., Dee N., Dolbeare T.A., Faber V., Feng D., Fowler D.R., Goldy J., Gregor B.W., Haradon Z., Haynor D.R., Hohmann J.G., Horvath S., Howard R.E., Jeromin A., Jochim J.M., Kinnunen M., Lau C., Lazarz E.T., Lee C., Lemon T.A., Li L., Li Y., Morris J.A., Overly C.C., Parker P.D., Parry S.E., Reding M., Royall J.J., Schulkin J., Sequeira P.A., Slaughterbeck C.R., Smith S.C., Sodt A.J., Sunkin S.M., Swanson B.E., Vawter M.P., Williams D., Wohnoutka P., Zielke H.R., Geschwind D.H., Hof P.R., Smith S.M., Koch C., Grant S.G. & Jones A.R. (2012) An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* **489**, 391-9.

Hoefsloot H.C., Smit S. & Smilde A.K. (2008) A classification model for the Leiden proteomics competition. *Stat Appl Genet Mol Biol* **7**, Article8.

Hoffmann K., Gastens A.M., Volk H.A. & Loscher W. (2006) Expression of the multidrug transporter MRP2 in the blood-brain barrier after pilocarpine-induced seizures in rats. *Epilepsy Res* **69**, 1-14.

Hong F. & Breitling R. (2008) A comparison of meta-analysis methods for detecting differentially expressed genes in microarray experiments. *Bioinformatics* **24**, 374-82.

Hong F., Breitling R., McEntee C.W., Wittner B.S., Nemhauser J.L. & Chory J. (2006) RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* **22**, 2825-7.

Huang Y. & Sadee W. (2006) Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. *Cancer Lett* **239**, 168-82.

Hubner C.A., Stein V., Hermans-Borgmeyer I., Meyer T., Ballanyi K. & Jentsch T.J. (2001) Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron* **30**, 515-24.

Jeffery I.B., Higgins D.G. & Culhane A.C. (2006) Comparison and evaluation of methods for generating differentially expressed gene lists from microarray data. *BMC Bioinformatics* **7**, 359.

Kapur K., Xing Y., Ouyang Z. & Wong W.H. (2007) Exon arrays provide accurate assessments of gene expression. *Genome Biol* **8**, R82.

Kraev A., Quednau B.D., Leach S., Li X.F., Dong H., Winkfein R., Perizzolo M., Cai X., Yang R., Philipson K.D. & Lytton J. (2001) Molecular cloning of a third member of the potassium-dependent sodium-calcium exchanger gene family, NCKX3. *J Biol Chem* **276**, 23161-72.

Kwan P., Arzimanoglou A., Berg A.T., Brodie M.J., Allen Hauser W., Mathern G., Moshe S.L., Perucca E., Wiebe S. & French J. (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. *Epilepsia* **51**, 1069-77.

Lazarowski A., Lubieniecki F., Camarero S., Pomata H., Bartuluchi M., Sevlever G. & Taratuto A.L. (2004a) Multidrug resistance proteins in tuberous sclerosis and refractory epilepsy. *Pediatr Neurol* **30**, 102-6.

Lazarowski A., Massaro M., Schteinschnaider A., Intruvini S., Sevlever G. & Rabinowicz A. (2004b) Neuronal MDR-1 gene expression and persistent low levels of anticonvulsants in a child with refractory epilepsy. *Ther Drug Monit* **26**, 44-6.

Lazarowski A., Ramos A.J., Garcia-Rivello H., Brusco A. & Girardi E. (2004c) Neuronal and glial expression of the multidrug resistance gene product in an experimental epilepsy model. *Cell Mol Neurobiol* **24**, 77-85.

Le-Niculescu H., McFarland M.J., Mamidipalli S., Ogden C.A., Kuczenski R., Kurian S.M., Salomon D.R., Tsuang M.T., Nurnberger J.I., Jr. & Niculescu A.B. (2007) Convergent Functional Genomics of bipolar disorder: from animal model pharmacogenomics to human genetics and biomarkers. *Neurosci Biobehav Rev* **31**, 897-903.

Lee T.S., Mane S., Eid T., Zhao H., Lin A., Guan Z., Kim J.H., Schweitzer J., King-Stevens D., Weber P., Spencer S.S., Spencer D.D. & de Lanerolle N.C. (2007) Gene expression in temporal lobe epilepsy is consistent with increased release of glutamate by astrocytes. *Mol Med* **13**, 1-13.

Liu S., Lin L., Jiang P., Wang D. & Xing Y. (2011) A comparison of RNA-Seq and high-density exon array for detecting differential gene expression between closely related species. *Nucleic Acids Res* **39**, 578-88.

Lockstone H.E. (2011) Exon array data analysis using Affymetrix power tools and R statistical software. *Brief Bioinform* **12**, 634-44.

Lottaz C., Yang X., Scheid S. & Spang R. (2006) OrderedList--a bioconductor package for detecting similarity in ordered gene lists. *Bioinformatics* **22**, 2315-6.

Lu J., Karadsheh M. & Delpire E. (1999) Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J Neurobiol* **39**, 558-68.

Lu Y., Yan Y. & Wang X.F. (2004) Antiepileptic drug-induced multidrug resistance P-glycoprotein overexpression in astrocytes cultured from rat brains. *Chin Med J (Engl)* **117**, 1682-6.

Marchi N., Guiso G., Rizzi M., Pirker S., Novak K., Czech T., Baumgartner C., Janigro D., Caccia S. & Vezzani A. (2005) A pilot study on brain-to-plasma partition of 10,11-dyhydro-10-hydroxy-5H-dibenzo(b,f)azepine-5-carboxamide and MDR1 brain expression in epilepsy patients not responding to oxcarbazepine. *Epilepsia* **46**, 1613-9.

Marchi N., Hallene K.L., Kight K.M., Cucullo L., Moddel G., Bingaman W., Dini G., Vezzani A. & Janigro D. (2004) Significance of MDR1 and multiple drug resistance in refractory human epileptic brain. *BMC Med* **2**, 37.

McClintick J.N. & Edenberg H.J. (2006) Effects of filtering by Present call on analysis of microarray experiments. *BMC Bioinformatics* **7**, 49.

McClintick J.N., Jerome R.E., Nicholson C.R., Crabb D.W. & Edenberg H.J. (2003) Reproducibility of oligonucleotide arrays using small samples. *BMC Genomics* **4**, 4.

Mirza N., Vasieva O., Marson A.G. & Pirmohamed M. (2011) Exploring the genomic basis of pharmacoresistance in epilepsy: an integrative analysis of large-scale gene expression profiling studies on brain tissue from epilepsy surgery. *Hum Mol Genet* **20**, 4381-94.

Okoniewski M.J., Hey Y., Pepper S.D. & Miller C.J. (2007) High correspondence between Affymetrix exon and standard expression arrays. *Biotechniques* **42**, 181-5.

Palma E., Amici M., Sobrero F., Spinelli G., Di Angelantonio S., Ragozzino D., Mascia A., Scoppetta C., Esposito V., Miledi R. & Eusebi F. (2006) Anomalous levels of Cl- transporters in the hippocampal subiculum from temporal lobe epilepsy patients make GABA excitatory. *Proc Natl Acad Sci U S A* **103**, 8465-8.

Park D.K., Park K.H., Ko J.S. & Kim D.S. (2011) Alteration in NCX-3 immunoreactivity within the gerbil hippocampus following spontaneous seizures. *BMB Rep* **44**, 306-11.

Parkinson H., Kapushesky M., Shojatalab M., Abeygunawardena N., Coulson R., Farne A., Holloway E., Kolesnykov N., Lilja P., Lukk M., Mani R., Rayner T., Sharma A., William E., Sarkans U. & Brazma A. (2007) ArrayExpress--a public database of microarray experiments and gene expression profiles. *Nucleic Acids Res* **35**, D747-50.

Payne J.A., Stevenson T.J. & Donaldson L.F. (1996) Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem* **271**, 16245-52.

Pepper S.D., Saunders E.K., Edwards L.E., Wilson C.L. & Miller C.J. (2007) The utility of MAS5 expression summary and detection call algorithms. *BMC Bioinformatics* **8**, 273.

Rasmussen M., Kong L., Zhang G.R., Liu M., Wang X., Szabo G., Curthoys N.P. & Geller A.I. (2007) Glutamatergic or GABAergic neuron-specific, long-term expression in neocortical neurons from helper virus-free HSV-1 vectors containing the phosphate-activated glutaminase, vesicular glutamate transporter-1, or glutamic acid decarboxylase promoter. *Brain Res* **1144**, 19-32.

Sander J.W. (2003) The natural history of epilepsy in the era of new antiepileptic drugs and surgical treatment. *Epilepsia* **44 Suppl 1**, 17-20.

Schallier A., Massie A., Loyens E., Moechars D., Drinkenburg W., Michotte Y. & Smolders I. (2009) vGLUT2 heterozygous mice show more susceptibility to clonic seizures induced by pentylenetetrazol. *Neurochem Int* **55**, 41-4.

Schmidt D. & Loscher W. (2009) New developments in antiepileptic drug resistance: an integrative view. *Epilepsy Curr* **9**, 47-52.

Seal R.P., Akil O., Yi E., Weber C.M., Grant L., Yoo J., Clause A., Kandler K., Noebels J.L., Glowatzki E., Lustig L.R. & Edwards R.H. (2008) Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. *Neuron* **57**, 263-75.

Seegers U., Potschka H. & Loscher W. (2002) Expression of the multidrug transporter P-glycoprotein in brain capillary endothelial cells and brain parenchyma of amygdala-kindled rats. *Epilepsia* **43**, 675-84.

She X., Rohl C.A., Castle J.C., Kulkarni A.V., Johnson J.M. & Chen R. (2009) Definition, conservation and epigenetics of housekeeping and tissue-enriched genes. *BMC Genomics* **10**, 269.

Shi L., Reid L.H., Jones W.D., Shippy R., Warrington J.A., Baker S.C., Collins P.J., de Longueville F., Kawasaki E.S., Lee K.Y., Luo Y., Sun Y.A., Willey J.C., Setterquist R.A., Fischer G.M., Tong W., Dragan Y.P., Dix D.J., Frueh F.W., Goodsaid F.M., Herman D., Jensen R.V., Johnson C.D., Lobenhofer E.K., Puri R.K., Schrf U., Thierry-Mieg J., Wang C., Wilson M., Wolber P.K., Zhang L., Amur S., Bao W., Barbacioru C.C., Lucas A.B., Bertholet V., Boysen C., Bromley B., Brown D., Brunner A., Canales R., Cao X.M., Cebula T.A., Chen J.J., Cheng J., Chu T.M., Chudin E., Corson J., Corton J.C., Croner L.J., Davies C., Davison T.S., Delenstarr G., Deng X., Dorris D., Eklund A.C., Fan X.H., Fang H., Fulmer-Smentek S., Fuscoe J.C., Gallagher K., Ge W., Guo L., Guo X., Hager J., Haje P.K., Han J., Han T., Harbottle H.C., Harris S.C., Hatchwell E., Hauser C.A., Hester S., Hong H., Hurban P., Jackson S.A., Ji H., Knight C.R., Kuo W.P., LeClerc J.E., Levy S., Li Q.Z., Liu C., Liu Y., Lombardi M.J., Ma Y., Magnuson S.R., Maqsodi B., McDaniel T., Mei N., Myklebost O., Ning B., Novoradovskaya N., Orr M.S., Osborn T.W., Papallo A., Patterson T.A., Perkins R.G., Peters E.H., Peterson R., Philips K.L., Pine P.S., Pusztai L., Qian F., Ren H., Rosen M., Rosenzweig B.A., Samaha R.R., Schena M., Schroth G.P., Shchegrova S., Smith D.D., Staedtler F., Su Z., Sun H., Szallasi Z., Tezak Z., Thierry-Mieg D., Thompson K.L., Tikhonova I., Turpaz Y., Vallanat B., Van C., Walker S.J.,

Wang S.J., Wang Y., Wolfinger R., Wong A., Wu J., Xiao C., Xie Q., Xu J., Yang W., Zhong S., Zong Y. & Slikker W., Jr. (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* **24**, 1151-61.

Shippy R., Sendera T.J., Lockner R., Palaniappan C., Kaysser-Kranich T., Watts G. & Alsobrook J. (2004) Performance evaluation of commercial short-oligonucleotide microarrays and the impact of noise in making cross-platform correlations. *BMC Genomics* **5**, 61.

Shorvon S.D. (1996) The epidemiology and treatment of chronic and refractory epilepsy. *Epilepsia* **37 Suppl 2**, S1-S3.

Sisodiya S.M., Martinian L., Scheffer G.L., van der Valk P., Scheper R.J., Harding B.N. & Thom M. (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathol Appl Neurobiol* **32**, 51-63.

Tishler D.M., Weinberg K.I., Hinton D.R., Barbaro N., Annett G.M. & Raffel C. (1995) MDR1 gene expression in brain of patients with medically intractable epilepsy. *Epilepsia* **36**, 1-6.

Tordera R.M., Totterdell S., Wojcik S.M., Brose N., Elizalde N., Lasheras B. & Del Rio J. (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). *Eur J Neurosci* **25**, 281-90.

Trabzuni D., Ryten M., Walker R., Smith C., Imran S., Ramasamy A., Weale M.E. & Hardy J. (2011) Quality control parameters on a large dataset of regionally dissected human control brains for whole genome expression studies. *J Neurochem* **119**, 275-82.

Tranchevent L.C., Barriot R., Yu S., Van Vooren S., Van Loo P., Coessens B., De Moor B., Aerts S. & Moreau Y. (2008) ENDEAVOUR update: a web resource for gene prioritization in multiple species. *Nucleic Acids Res* **36**, W377-84.

van der Hel W.S., Verlinde S.A., Meijer D.H., de Wit M., Rensen M.G., van Gassen K.L., van Rijen P.C., van Veelen C.W. & de Graan P.N. (2009) Hippocampal distribution of vesicular glutamate transporter 1 in patients with temporal lobe epilepsy. *Epilepsia* **50**, 1717-28.

van Gassen K.L., de Wit M., Koerkamp M.J., Rensen M.G., van Rijen P.C., Holstege F.C., Lindhout D. & de Graan P.N. (2008) Possible role of the innate immunity in temporal lobe epilepsy. *Epilepsia* **49**, 1055-65.

van Vliet E.A., Redeker S., Aronica E., Edelbroek P.M. & Gorter J.A. (2005) Expression of multidrug transporters MRP1, MRP2, and BCRP shortly after status epilepticus, during the latent period, and in chronic epileptic rats. *Epilepsia* **46**, 1569-80.

Volk H.A. & Loscher W. (2005) Multidrug resistance in epilepsy: rats with drug-resistant seizures exhibit enhanced brain expression of P-glycoprotein compared with rats with drug-responsive seizures. *Brain* **128**, 1358-68.

Wiederhold E., Gandhi T., Permentier H.P., Breitling R., Poolman B. & Slotboom D.J. (2009) The yeast vacuolar membrane proteome. *Mol Cell Proteomics* **8**, 380-92.

Williams J.R., Sharp J.W., Kumari V.G., Wilson M. & Payne J.A. (1999) The neuron-specific K-Cl cotransporter, KCC2. Antibody development and initial characterization of the protein. *J Biol Chem* **274**, 12656-64.

Woo N.S., Lu J., England R., McClellan R., Dufour S., Mount D.B., Deutch A.Y., Lovinger D.M. & Delpire E. (2002) Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. *Hippocampus* **12**, 258-68.

Xing Y., Ouyang Z., Kapur K., Scott M.P. & Wong W.H. (2007) Assessing the conservation of mammalian gene expression using high-density exon arrays. *Mol Biol Evol* **24**, 1283-5.

Zhang X., Liu G., Lenburg M.E. & Spira A. (2007) Comparison of smoking-induced gene expression on Affymetrix Exon and 3'-based expression arrays. *Genome Inform* **18**, 247-57.

CHAPTER FOUR

A NEW MICROARRAY ANALYSIS OF BRAIN TISSUE FROM EPILEPSY SURGERY

Contents

Chapter 4: A New Microarray Analysis of Brain Tissue from Epilepsy Surgery

4.1 Introduction

Genome-wide transcriptomic studies continue to be a cornerstone in the investigation of mechanisms underlying complex diseases. Because of their whole genome approach, microarray studies enable researchers to identify not only differentially expressed genes, but also dysregulated pathways and networks. Such a holistic approach is essential for uncovering the many interlinked biological processes that are likely to be underlying a multifactorial condition like pharmacoresistant epilepsy.

There are a number of published microarray studies on brain tissue from surgery for pharmacoresistant epilepsy; these studies have been extensively examined in Chapter 2. Although the previously published microarray studies have provided important insights into this condition, they suffer from a number of important methodological weaknesses, of which the following are particularly noteworthy:

1. The sample sizes have been small: the largest published study on hippocampal tissue from pharmacoresistant mesial temporal lobe epilepsy to date included 8 disease samples only.
2. In recent years, it has become widely recognized that adjusting for batch effect, hidden sources of heterogeneity and polymorphisms-in-probes is essential in microarray analysis; previously published microarray studies on human epilepsy have not accounted for these confounders.
3. The previous microarray studies have not attempted to objectively demonstrate the therapeutic and causal relevance of the identified differentially expressed genes.
4. The previous microarray studies have been limited to an analysis of differential expression; differential connectivity has not been studied and, hence, much of the information contained in the gene expression datasets has been ignored.
5. A few previous microarray studies have included pathway-based analyses. However, as individual pathways do not function in isolation, it is useful to construct a network

of pathways and to identify the most central pathways in this network; this has not been attempted before.

In order to resolve these deficiencies, we have performed the largest and most robust microarray analysis of brain tissue from surgery for pharmacoresistant mesial temporal lobe epilepsy.

4.2 Aims

1. To perform a robust microarray analysis of hippocampal tissue from surgery for pharmacoresistant mesial temporal lobe epilepsy.
2. To objectively demonstrate the therapeutic and causal relevance of the differentially expressed genes from the microarray study.
3. To identify differentially expressed pathways.
4. To identify differentially connected pathways.
5. To construct a network of differentially expressed and differentially connected pathways.
6. To identify the 'hub' pathways.

4.3 Methods

4.3.1 Sample collection

Samples used in the study originated from three UK sites: the Walton Centre for Neurology and Neurosurgery in Liverpool, the Salford Royal Hospital in Salford and the Southern General Hospital in Glasgow. We aimed to recruit patients of age 5 years and older with pharmacoresistant mesial temporal lobe epilepsy of at least 3 months durations for which a therapeutic temporal lobectomy was being undertaken. As recently proposed by the ILAE, pharmacoresistance was defined as the failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom. The diagnosis of MTLE was made by the treating clinician based on seizure semiology, MRI brain and EEG characteristics being consistent with a seizure focus in the mesial temporal lobe. Patients suspected of having a neoplastic or malignant temporal lobe lesion were excluded. After surgery, the hippocampus was divided into two portions: (1) one portion was preserved for RNA

isolation, (2) the other portion underwent histological analysis by an experienced neuropathologist. Any subjects found to have a neoplastic or malignant lesion on histological analysis were excluded. The portion preserved for RNA isolation was either stored in RNAlater (Liverpool and Glasgow) or frozen directly at -80 ^{O}C (Salford). It should be noted that the quality of the RNA generated from the directly frozen brain samples was not significantly different from RNAlater-preserved samples: mean RIN values were 7.82 and 7.25 respectively, and two-sample t-test p-value for difference between group RINs was 0.06554.

Frozen post-mortem histologically-normal hippocampal samples from donors with no known brain diseases were obtained from the MRC Edinburgh Brain Bank (Edinburgh, UK) and the Queen Square Brain Bank (London, UK).

4.3.2 RNA isolation

Brain samples were disrupted and homogenized in an appropriate volume of QIAzol lysis reagent (Qiagen, Crawley, United Kingdom) by using a TissueRuptor handheld rotor-stator homogenizer (Qiagen, Crawley, United Kingdom). Total RNA was extracted from the homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. RNA quality was examined by capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) and Agilent 2100 Expert software was used to calculate the RNA Integrity number (RIN) of each sample. Purity of the RNA sample was assessed using a NanoDrop1000 Spectrophotometer. Capillary electrophoresis traces were also examined. Samples with RNA integrity number scores (RIN) below 6, obvious RNA degradation, significant 18S or 28S ribosomal RNA degradation, ratio of absorbance at 260nm and 280nm <1.95, or with noticeable DNA or background contaminants did not pass QC, and were withheld from microarray analysis. It should be noted that RNA samples with RIN scores of 6 and lower have been successfully used for 3' microarrays (Hawrylycz *et al.* 2012) and exon microarrays (Trabzuni *et al.* 2011).

4.3.3 Microarray processing and quality control

The microarrays were processed at the Centre for Genomics Research in the University of Liverpool. 50ng of total RNA was amplified and labelled using the Agilent Low Input Quick Amp One-Colour Labeling Kit and labelled RNA was hybridized to Agilent SurePrint G3

Custom Exon 8x60K Microarrays. Standard Agilent protocols were followed. The quality of the synthesized cRNA was assessed using an Agilent Bioanalyzer 2100—the resulting electropherograms were found to be satisfactory. Each scanned image was viewed for visible artefacts, and if multiple artefacts were present, the array was rejected. Detailed QC reports were generated for each array using the Feature Extraction 11.0.1.1 Software (Agilent, Palo Alto, CA). Based on these reports, we excluded arrays in which more than 1% of features were non-uniform outliers, or the average of the net signals in negative controls was >40, or the average of the background-subtracted signals in negative controls was <-10 or >5, or the standard deviation of the background-subtracted signals in negative controls was >10, or the residual noise after spatial detrending was >15, or the median coefficient of variation for spike-in probes or non-control probes was >12%, or the dose-response curve of the spike-ins had a slope of <0.9 or >1.20, or the spike-in detection limit was <0.01 or >2. One array failed on five of these metrices and, hence, was excluded. Intensity data was extracted from the remaining arrays using the Feature Extraction Software. Spatial and multiplicative detetrending and local background substraction was applied.

We employed an unbiased approach for the detection of outlier samples: samples with average inter-array correlation (IAC) ≤ 2 standard deviations below the mean were removed. This approach has been used successfully in published studies (Yang *et al.* 2006a; Flight & Wentzell 2010; Yang *et al.* 2012).

4.3.4 Data normalization, adjustment and filtering

Data exported from Feature Extraction Software was imported into GeneSpring GX Software (Agilent, Palo Alto, CA). Features which were population outliers, saturated or non-uniform were flagged as 'Compromised' and filtered out. Non-expressed probes were filtered out: a probe was deemed expressed only if it was flagged 'Detected' in at least 25% of control or case samples. Quantile normalization was applied. As GeneSpring does not include functions that allow adjusting for confounders and covariates, probe-level intensities were exported for further processing.

Batch effect was corrected using the ComBat (Johnson *et al.* 2007) algorithm implemented in the SVA Bioconductor package. Eight arrays (four cases and four controls) were processed per slide; hence, each batch comprised the eight samples processed together on a slide. To

adjust for known and unknown confounders, Independent Surrogate Variable Analysis (ISVA) (Teschendorff *et al.* 2011) was applied; RIN, age and sex were explicitly included in the ISVA adjustment. Where multiple probes mapped to the same gene and mRNA variant, the probe with the highest variance was retained, as the most variant probe is likely to be most informative.

4.3.5 Assessing the influence of confounders

To determine if there was residual confounding after data adjustment, we used the phenoTest Bioconductor package to test for association between probes and confounders (batch, RIN, age and sex).

4.3.6 Assessing the influence of polymorphisms-in-probes (PIP)

Polymorphisms present in the probe-target sequences have been shown to alter probe hybridization affinities, leading to reduced signal intensity measurements and resulting in false-positive results, at least for Affymetrix microarrays (Benovoy *et al.* 2008). Similar studies have not been performed for Agilent microarrays so far. To establish if PIP could be leading to false-positive results in our analysis, we used a recently devised tool (Ramasamy *et al.* 2013) to determine which probes in our microarray had polymorphisms located within the probe sequences; we then determined if these probes with polymorphisms were overrepresented within the list of differentially expressed probes.

4.3.7 Differential expression analysis

Differential expression analysis of the adjusted dataset was performed using the limma package (Smyth 2004). A probe was considered differentially expressed if FDR was < 0.05 and FC was ≥ 1.5.

4.3.8 External validation of differential expression

As robust validation of our differential expression results, we determined if there was significant overlap and rank order similarity between our results and the results from three previous microarray studies on hippocampal tissue from epilepsy surgery (Lee *et al.* 2007; van Gassen *et al.* 2008; Venugopal *et al.* 2012). For comparison, we determined if our results show a significant overlap or a significant rank order similarity with a previously published microarray study on hippocampal tissue from Alzheimer's disease sufferers (Liang *et al.* 2008).

Statistical significance of overlap was calculated using the hypergeometric equation, and statistical significance of rank order similarity was calculated using the OrderedList Bioconductor package (Yang *et al.* 2006b).

4.3.9 Assessing the potential therapeutic relevance of differentially expressed genes

We created a comprehensive list of the targets of all current AEDs by searching three publically-available databases of drug targets: Stitch, SuperTarget and DrugBank. We then determined if our microarray results were enriched with AED targets by calculating the statistical significance of the overlap between AED targets and our DEG-list. For comparison, we did the same analysis for all previously published microarray studies on brain tissue from epilepsy surgery.

4.3.10 Assessing the potential causative relevance of differentially expressed genes

We performed a genome-wide association study (GWAS) of pharmacoresistant epilepsy (described in detail in Chapter 5 of the current thesis). We determined if our DEG-set was significantly enriched in the GWAS. For comparison, we did the same analysis for all previously published microarray studies on brain tissue from epilepsy surgery.

4.3.11 Differential connectivity

For performing differential connectivity analysis, three algorithms were compared: Differential Correlation in Expression for meta-module Recovery (DICER) (Amar *et al.* 2013), coXpress (Watson 2006) and DiffCoEx (Tesson *et al.* 2010).

Including all microarray probes in the analysis was not computationally feasible: the time and memory requirements were both prohibitive. Hence, the probes were sequentially filtered as follows: (1) only probes mapping to genes with valid current Entrez numbers and HUGO Gene Nomenclature Committee gene symbols were retained, (2) where multiple probes mapped to the same gene, the most variant probe only was retained, (3) the 10,000 probes with the highest variance were retained. Hence, after filtering, 10,000 probes with high variance remained, each mapping to a unique gene.

DICER was the best differential connectivity algorithm (see below). The genes in differentially connected clusters from DICER were used to perform pathway enrichment

analysis. For the enriched pathways, differential connectivity was further confirmed using the Gene Set Co-Expression Analysis (Choi & Kendziorski 2009) protocol.

4.3.12 Pathway-enrichment analysis

Pathway-enrichment analysis was performed using the Reactome database on the Gene Set Enrichment Analysis website.

4.3.13 Network of pathways and network analysis

Enrichment Map (Merico *et al.* 2010) tool was used to determine the connections between enriched pathways. Enrichment Map was used with default settings. Specifically, we employed an overlap coefficient cut-off of 0.5—two pathways were deemed connected only if the ratio of the size of the intersection over the size of the smallest pathway was 0.5 or more. The Network Analysis tool was used to calculate network parameters, including 'betweenness centrality' of nodes.

4.4 Results

4.4.1 Detecting Outlier Arrays Using Inter-Array Correlation (IAC)

Mean IAC for control samples was 0.98 (range 0.96 – 0.98), while mean IAC for disease samples was 0.95 (range 0.95 – 0.98). As there were no arrays with IAC ≤ 2 standard deviations below the mean, no arrays were deemed to be outliers and no arrays were excluded.

Important patient and donor individual and sample characteristics are summarized in Table 4.1.

4.4.2 Effective Adjustment for Confounders

We confirmed that, after ComBat and ISVA treatment, confounders had been adjusted for appropriately: we found that no probes were associated with RIN, age, batch or sex at an FDR<0.10 in the adjusted data.

Table 4.1 Sample characteristics. RIN=RNA Integrity Number

Sample	Phenotype	Age	Sex	RIN		Sample	Phenotype	Age	Sex	RIN
D1	Case	41	F	7.4		**N1**	control	81	M	6.5
D2	Case	23	F	6.8		**N2**	control	78	F	6.2
D3	Case	51	M	6		**N3**	control	84	F	6.6
D4	Case	49	F	6.9		**N4**	control	91	F	6.7
D5	Case	50	F	7.8		**N5**	control	88	M	6.3
D6	Case	45	F	6.6		**N6**	control	38	M	6.1
D7	Case	12	M	7		**N7**	control	50	M	6.2
D8	Case	29	F	7.8		**N8**	control	45	M	6.3
D9	Case	33	M	6.8		**N9**	control	39	M	6.1
D10	case	25	F	7.1		**N10**	control	40	M	6
D11	case	34	M	7		**N11**	control	61	M	6.2
D12	case	33	M	8.8		**N12**	control	63	F	6.2
D13	case	33	M	8.6		**N13**	control	66	M	6.2
D14	case	22	F	7.3		**N14**	control	22	F	6.3
D15	case	48	M	7.6		**N15**	control	27	M	6.3
D16	case	39	F	7.4		**N16**	control	45	M	6.9
D17	case	29	F	7.1		**N17**	control	44	F	6.7
D18	case	44	M	7.9		**N18**	control	50	M	6.5
D19	case	40	F	6.6		**N19**	control	43	M	6.6
D20	case	48	M	8.5		**N20**	control	46	M	6.8
D21	case	23	M	8.4		**N21**	control	51	M	6.2
D22	case	63	M	7.9		**N22**	control	48	M	6
D23	case	31	M	8.2		**N23**	control	43	M	6.6
D24	case	27	F	8.2						

4.4.3 Effect of PIP

We found that, as opposed to Affymetrix microarrays (Merico *et al.* 2010), PIP are not a source of false-positive results in our Agilent microarray. Of all the probes included in our microarray, 18% had polymorphisms located within the probe sequences. We found that probes with polymorphisms were not overrepresented within the list of differentially expressed probes, for varying definitions of differential expression (Table 4.2).

4.4.4 Differential Expression: External Replication

There was a highly statistically significant overlap between the DEG-list from our study and from three previously published microarray studies on pharmacoresistant epileptic hippocampal tissue (Table 4.3). Furthermore, the rank order of the DEGs from our study and

the previously published studies was statistically significant (Table 4.3). For comparison, we demonstrated that our results show neither a significant overlap nor a significant rank order similarity with a previously published microarray study on hippocampal tissue from Alzheimer's disease sufferers.

Table 4.2 Proportion of probes with polymorphisms

	Total probes	Probes with polymorphisms	Proportion
All probes on microarray	61341	11027	18.0
Differentially expressed probes: FDR<0.05	22281	3872	17.4
Differentially expressed probes: FDR<0.05 & FC≥1.5	1729	304	17.6
Differentially expressed probes: FDR<0.05 & FC≥2	310	52	16.8
Differentially expressed probes: FDR<0.03	19144	3310	17.3
Differentially expressed probes: FDR<0.03 & FC≥2	308	53	17.2
Differentially expressed probes: FDR<0.01 & FC≥1.5	1541	258	16.7
Differentially expressed probes: FDR<0.01 & FC≥2	304	50	16.4

Table 4.3 Statistical significance (FDR) of overlap and rank order similarity between the DEGs from the current microarray study and previously published microarrays on hippocampal tissue. AD= Alzheimer's disease

Phenotype	Study	Statistical significance of overlap	Statistical significance of rank order similarity
Epilepsy	van Gassen *et al*., 2008	6.191×10^{-7}	0
Epilepsy	Venugopal *et al*., 2012	2.245×10^{-8}	0.027
Epilepsy	Lee *et al*., 2007	3.272×10^{-14}	0.010
AD	Liang *et al*., 2008	1	0.707

4.4.5 Differential Expression: Evidence of Potential Therapeutic Relevance

We found that the DEG-lists from a number of microarray studies on brain tissue from epilepsy surgery have statistically significant overlaps with targets of current AEDs, but the most significant overlap by far was for our microarray study (Table 4.4 and Figure 4. 1).

Table 4.4 Enrichment within microarray DEGs for targets of current AEDs. FDR=false discovery rate

Study	FDR
Current study	$2.75x10^{-6}$
Jamali et al., 2006	0.0008
Lee et al., 2007	0.0013
Arion et al., 2006	0.0024
van Gassen et al., 2008	0.0306
Ozbas-Gerceker et al., 2006	0.0956
Becker et al., 2003	0.1445
Xiao et al., 2008	0.6933
Becker et al., 2002	1
Venugopal et al., 2012	1

Table 4.5 GWAS association of microarray DEG-sets. FDR=false discovery rate

Study	FDR
Current study	$1.92x10^{-18}$
Lee et al., 2007	$3.14x10^{-7}$
van Gassen et al., 2008	$1.43x10^{-2}$
Arion et al., 2006	$2.62x10^{-2}$
Jamali et al., 2006	$2.44x10^{-1}$
Venugopal et al., 2012	$2.84Ex10^{-1}$
Xiao et al., 2008	$4.20Ex10^{-1}$
Becker et al., 2003	$4.96Ex10^{-1}$
Becker et al., 2002	$2.75Ex10^{-1}$
Ozbas-Gerceker et al., 2006	$2.75Ex10^{-1}$

4.4.6 Differential Expression: Evidence of Potential Causative Significance

We found that the DEG-sets from a number of microarray studies on brain tissue from epilepsy surgery were significantly enriched in the focal epilepsy GWAS study, but the most significant enrichment by far was for our microarray study (Table 4.5 and Figure 4.2).

Figure 4.1 Statistical significance of overlaps between targets of current AEDs and DEG-lists from a number of microarray studies on brain tissue from epilepsy surgery. Red line demarcates the level of statistical significance at FDR=0.05.

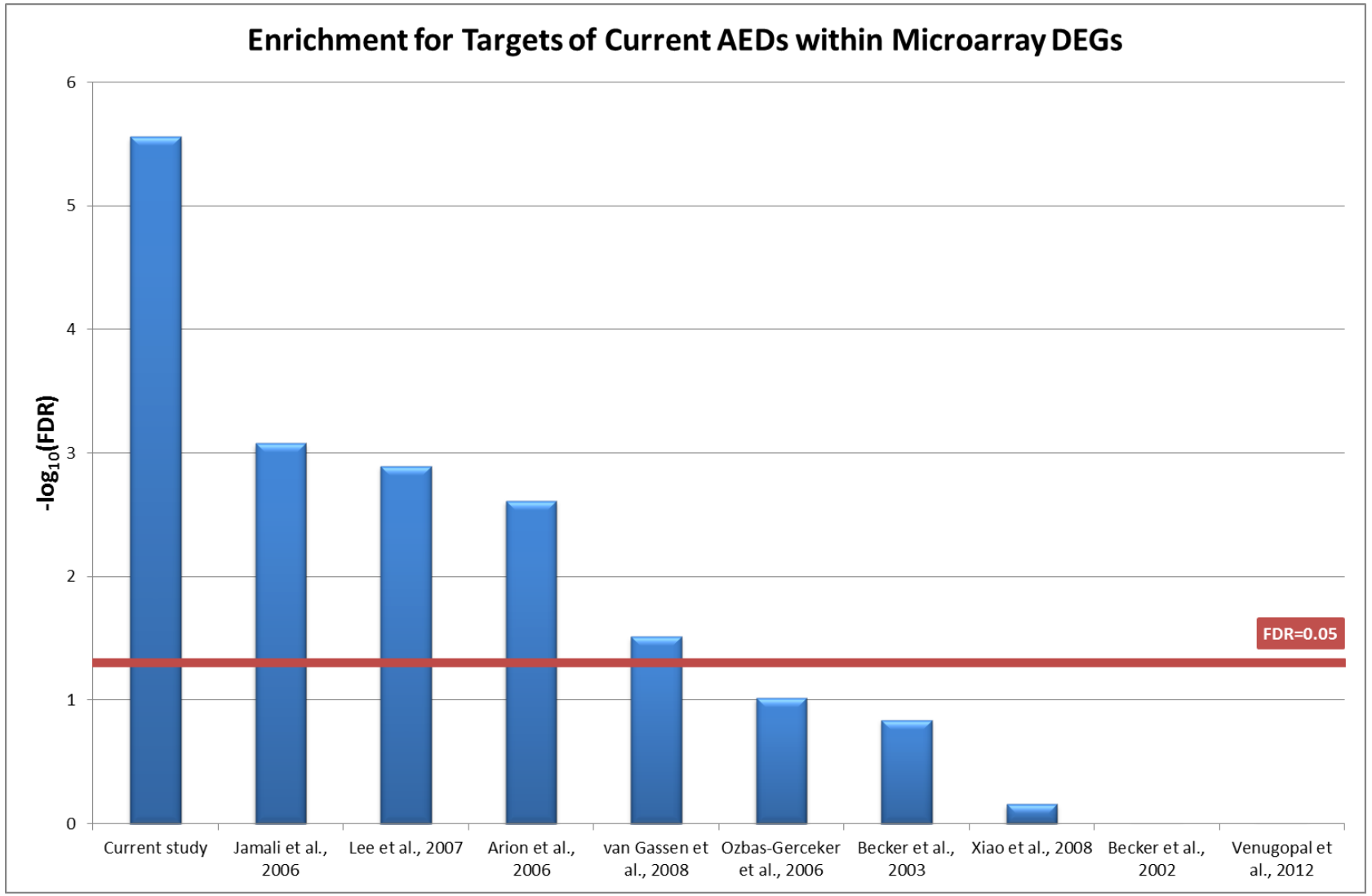
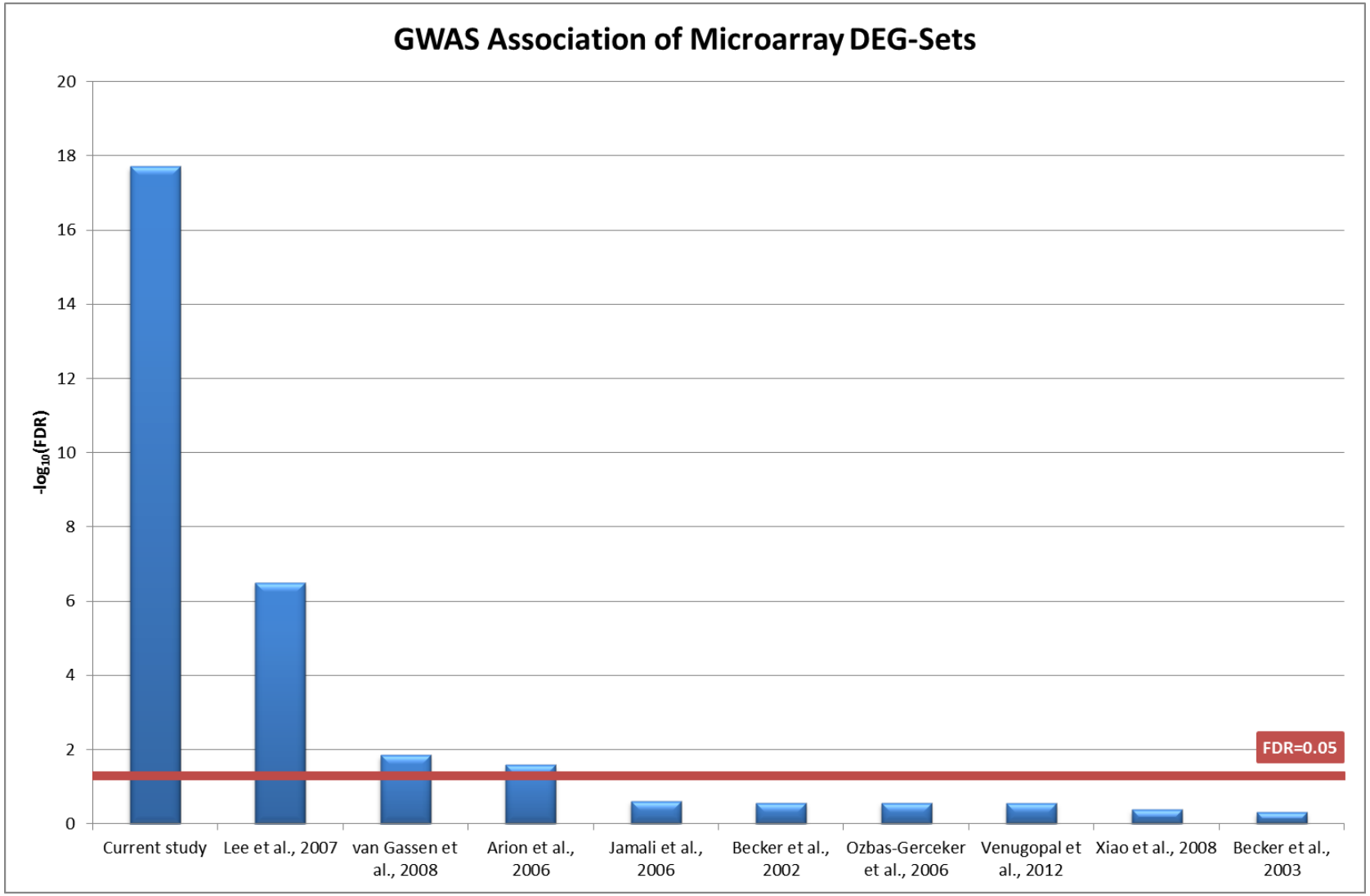


Figure 4.2 GWAS enrichment of microarray DEG-sets. Red line demarcates the level of statistical significance at FDR=0.05.



4.4.7 Differential Expression: Pathway-Enrichment Analysis

Pathway-enrichment analysis of the 1010 DEGs with valid gene symbols revealed 91 significantly enriched pathways (FDR<0.05). The top 10 significantly-enriched pathways are listed in Table 4.6, while the remaining can be found in Appendix 9.

Table 4.6 The 10 most significantly enriched Reactome pathways amongst DEGs.

Gene set name	FDR q-value
Signalling by GPCR	0
GPCR downstream signalling	0
Transmembrane transport of small molecules	0
GPCR ligand binding	0
Class A1 rhodopsin-like receptors	0
Neuronal system	0
Transmission across chemical synapses	1.07×10^{-14}
Peptide ligand binding receptors	8.89×10^{-13}
G alpha I signalling events	1.85×10^{-12}
SLC-mediated transmembrane transport	4.40×10^{-12}

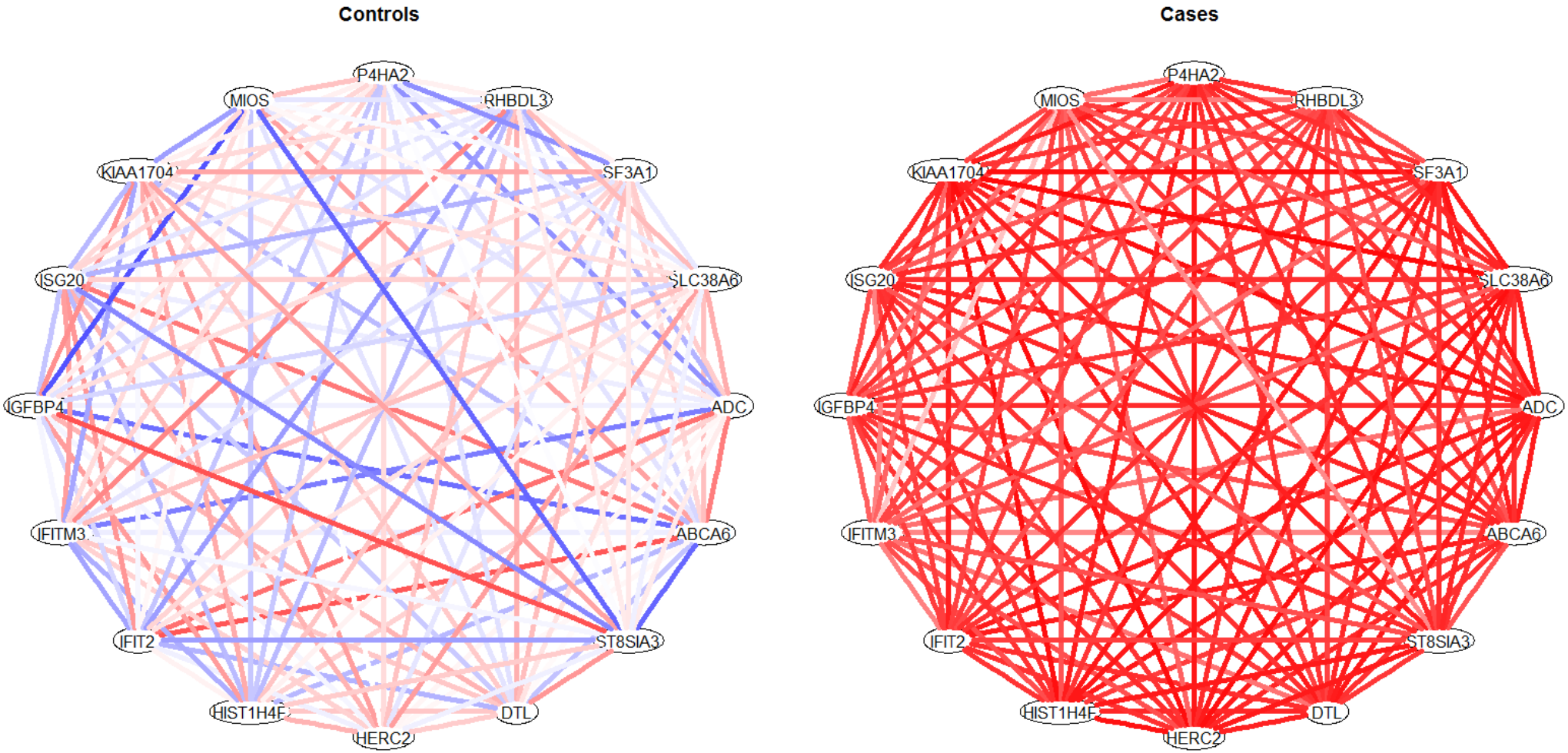
4.4.8 Differential Connectivity: Clusters and Genes

We compared differentially connected clusters produced by DICER, coXpress and DiffCoEx. Pathway and GWAS enrichment analysis revealed that the results produced by DICER were the most functionally coherent and potentially causative (coXpress and DiffCoEx results not shown).

DICER produced 36 differentially connected clusters, ranging in size from 15 genes to 102 genes. 17 clusters exhibited increased connectivity in disease tissue, while 19 clusters exhibited decreased connectivity. The contents of each cluster are tabulated in Appendix 10. The plot in Figure 4.3 illustrates, as an example, the clearly contrasting connectivity between normal and disease tissue for one of the clusters. Similar plots for all identified clusters are shown in Appendix 11.

In total, 890 of the 10,000 genes (9%) included in the DICER analysis were differentially connected. While 211 of these genes were also found in our list of differentially expressed genes, the majority (76%) were differentially connected without being significantly differentially expressed.

Figure 4.3 Plot illustrating differential connectivity in one of the identified clusters. Nodes represent genes and edges represent connectivity between genes. Edges are coloured from blue to red indicating correlation value of -1 to 1.



4.4.9 Differential Connectivity: Pathway-Enrichment Analysis

Pathway-enrichment analysis of the 890 differentially connected genes revealed 75 significantly enriched pathways (FDR<0.05). The top 10 significantly-enriched pathways are listed in Table 4.7, while the remaining can be found in the Appendix 9. We further confirmed that these pathways are differentially connected (FDR<0.05) using the GSCA package (see Appendix 9).

Table 4.7 Differential connectivity: The top 10 significantly enriched Reactome pathways.

Pathway	FDR
Neuronal system	2.73×10^{-08}
Transmembrane transport of small molecules	8.85×10^{-08}
Developmental biology	2.23×10^{-06}
Axon guidance	5.79×10^{-06}
Transmission across chemical synapses	9.58×10^{-06}
Generic transcription pathway	1.01×10^{-05}
SLC-mediated transmembrane transport	4.68×10^{-05}
Semaphorin interactions	1.25×10^{-04}
Sema3a PAK dependent axon repulsion	2.12×10^{-04}
Amino acid and oligopeptide SLC transporters	7.78×10^{-04}

4.4.10 Differential Connectivity: Evidence of Genetic Basis and Potential Causality

The differentially connected gene-set was significantly enriched in the focal epilepsy GWAS ($p= 4.50 \times 10^{-10}$). We created a further gene-set consisting of genes which were differentially connected but not differentially expressed—this latter gene set was also significantly enriched in the GWAS ($p=6.52 \times 10^{-06}$).

4.4.11 Identifying a Network of Pathways

Enrichment Map analysis revealed a highly interconnected central network of pathways (Figure 4.4). In this central network, each pathway is directly connected to, on average, 10.5 other pathways. In order to identify the most central 'hub' pathways, 'betweeness centrality' network analysis was performed; the results are illustrated in Figure 4.5 and the top 15 pathways are listed in Table 4.8. The complete ranked list can be found in the Appendix 9.

Figure 4.4 Network of pathways. Nodes with a red centre represent differentially expressed pathways, nodes with a blue border represent differentially connected pathways, and nodes with both a red centre and blue border represent pathways which are both differentially expressed and connected. Major functional groups containing at least three pathways are labelled.

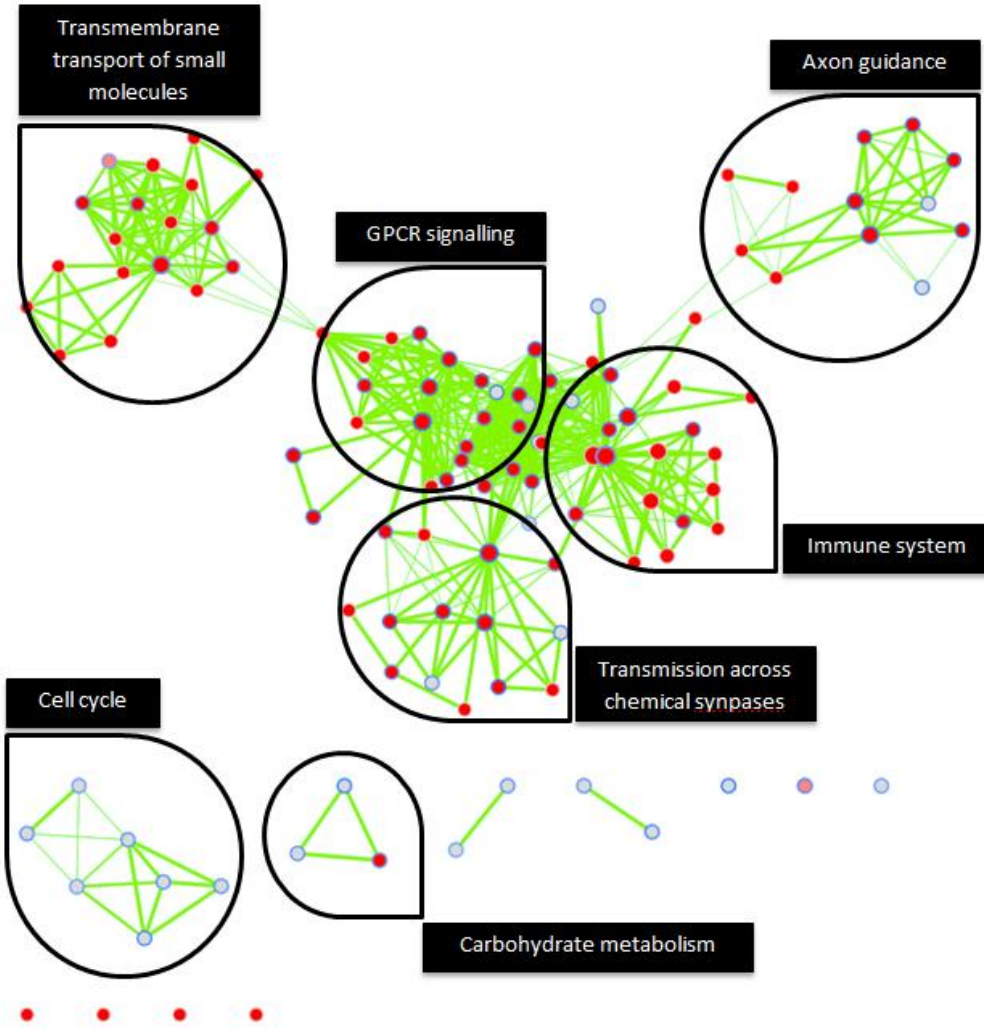


Figure 4.5 The central network of interconnected pathways. Node sizes are proportional to their betweenness centrality. Major functional groups of pathways are labelled.

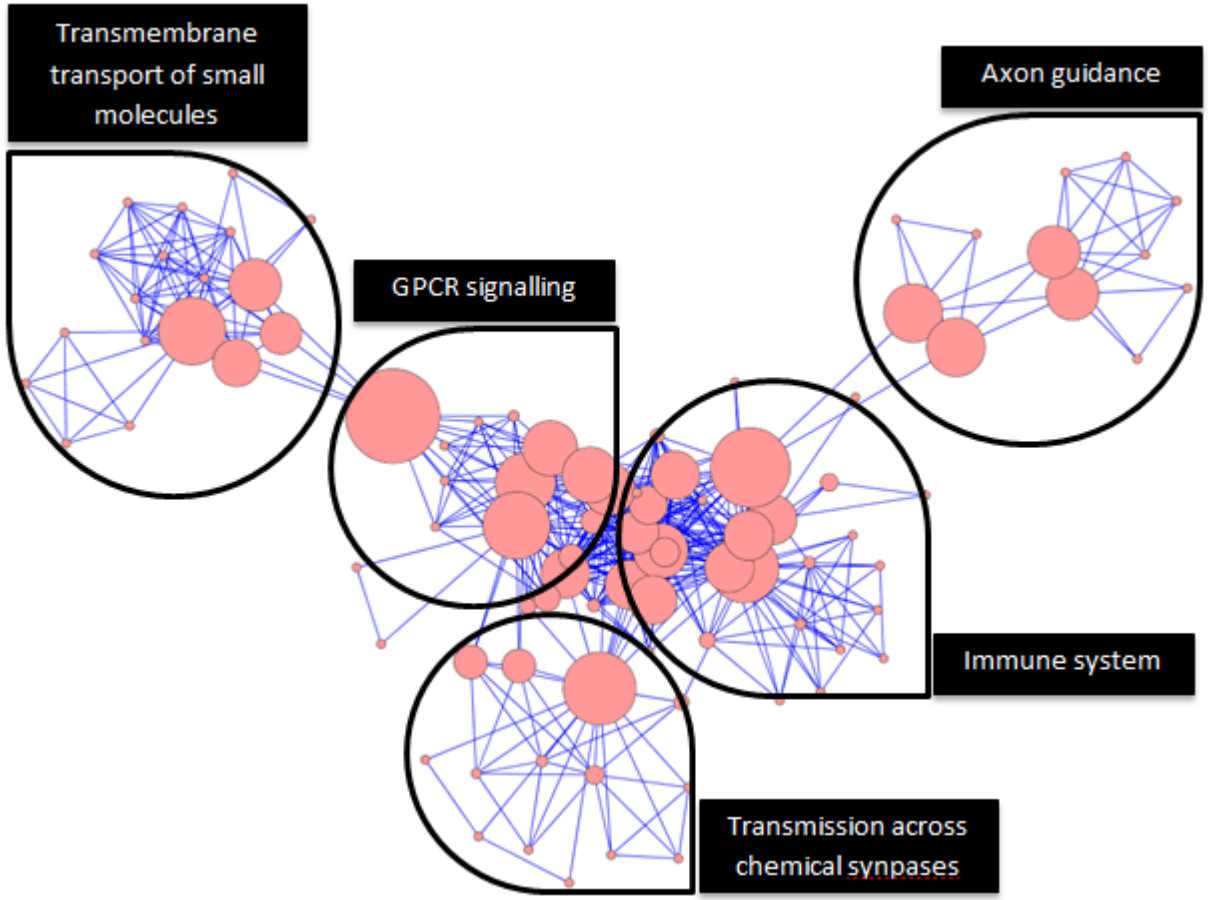


Table 4.8 Betweenness centrality

Pathway	Betweenness Centrality
Signalling by NOTCH	0.6
CREB phosphorylation through the activation of CAMKII	0.31572334
Signalling by PDGF	0.22722128
Nephrin interactions	0.225894
Transmembrane transport of small molecules	0.18478239
Signalling by GPCR	0.16146105
Immune system	0.15259226
Neuronal system	0.15097778
Signalling by NOTCH3	0.15
Receptor ligand binding initiates the second proteolytic cleavage of notch receptor	0.15
Transmission across chemical synapses	0.10843453
NCAM signalling for neurite out growth	0.09770921
NCAM1 interactions	0.09770921
Post NMDA receptor activation events	0.08083733
Neurotransmitter receptor binding and downstream transmission in the postsynaptic cell	0.07588839

4.5 Discussion

Although there have been a number of previous microarray studies on brain tissue from surgery for pharmacoresistant epilepsy, the current analysis advances this field of study in a number of significant and novel ways, of which the following aspects are especially noteworthy:

1. This is the largest transcriptomic study of hippocampal tissue from pharmacoresistant mesial temporal lobe epilepsy to date, having thrice the number of disease samples of the previous largest study.
2. We have externally validated our results by showing statistically significant similarities with previously published studies (see results).
3. Cutting edge analytical techniques have been applied which improve the sensitivity and reliability of our results:
    a. Batch effects have now been recognized as an important but overlooked source of confounding in transcriptomic studies; we have accounted and

corrected for batch effects using careful study design and appropriate data processing.

   b. We effectively adjusted for known confounders. We confirmed that, after ComBat and ISVA treatment, no probes were associated with RIN, age, batch or sex at an FDR<0.10 in the adjusted data. It should also be noted that Agilent microarrays are robust to differences in RIN values between compared samples (Opitz *et al.* 2010).
   c. We have adjusted for unknown confounders using the cutting edge Independent Surrogate Variable Analysis technique.
   d. Polymorphisms-in-probes (PIP) have recently been established as a potential source of false-positive results in many microarray studies; we have carefully assessed the influence of PIP in our study.

4. We have shown the therapeutic and causal relevance of our results:
   a. We have demonstrated the functional and therapeutic relevance of our results by showing that they are enriched with antiepileptic drug targets and more so than any previous microarray study.
   b. We have demonstrated the causal relevance of our results by showing their enrichment in a genome-wide genetic association study of pharmacoresistant partial epilepsy.
5. In addition to a differential expression analysis, we have performed a 'differentially connectivity' analysis; the first such analysis in epilepsy.
6. We have revealed the extensively interconnected network of pathways underlying pharmacoresistant epilepsy and identified the 'hub' pathways.

Some of these aspects are discussed in greater detail below.

4.5.1 Adjustment for batch effect

Batch effect refers to the systematic error introduced when samples are processed in multiple batches (Chen *et al.* 2011). Although batch effects can be reduced by careful experimental design, they cannot be eliminated unless the whole study is done in a single batch. Hence, batch effects are almost inevitable as practical considerations limit the number of samples that can be amplified and hybridized at one time. Combining data from different batches without carefully removing batch effects can give rise to misleading

results, since the bias introduced by the non-biological nature of the batch effects can be strong enough to mask, or confound true biological differences. However, of the thousands of DNA microarray papers published every year, few address the problem (Chen *et al.* 2011). Certainly, none of the human epilepsy transcriptomic studies published hence far have addressed this issue.

A number of approaches have been developed for identifying and removing batch effects from microarray data. In the current study, we have utilized an empirical Bayes method called ComBat (Johnson *et al.* 2007), which has been shown to outperform other programs (Chen *et al.* 2011), and is currently the most widely used method for batch effect correction.

After completing the data adjustment, we have demonstrated that our data has been effectively corrected by showing that the probes are not associated with batch or with other known confounding factors (age, sex and RNA quality).

4.5.2 Adjustment for hidden confounders

It is relatively straightforward to account for those confounders that are known and measured. When measured, inclusion of these known factors in the analytical model allows for a more sensitive analysis. For example, it is standard procedure to include covariates such as age and gender in the analysis. In practise it is not possible to measure or even be aware of all potential sources of variation. Unobserved hidden genetic, environmental, demographic, and technical factors may have substantial effects on gene expression levels, and may create spurious false associations or mask real genetic association signals (Leek & Storey 2007). These factors cannot be directly included in modelling if they are not measured, and techniques that have been used to explicitly adjust for known factors (for example, ComBat for batch effect) cannot be used. A number of statistical approaches have been developed to account for such hidden determinants of expression variation (Leek & Storey 2007; Kang *et al.* 2008; Teschendorff *et al.* 2011; Fusi *et al.* 2012; Stegle *et al.* 2012). Of these approaches, the most popular and widely is surrogate variable analysis (SVA) (Leek & Storey 2007), which has been shown to lead to improved estimates of statistical significance, biological accuracy and reproducibility. SVA uses the expression data itself to identify groups of genes affected by each unobserved factor and estimates the factor based on the expression of those genes; the confounding factors can then be regressed out of the

dataset. A recent improvement upon the SVA protocol is Independent Surrogate Variable Analysis (ISVA), which has been shown to outperform SVA (Teschendorff *et al.* 2011). ISVA is the protocol employed in the current study.

4.5.3 Checking for the presence of false-positive results caused by polymorphisms-in-probes

Polymorphisms present in the probe-target sequences have been shown to alter probe hybridization affinities, leading to reduced signal intensity measurements and resulting in false-positive results in Affymetrix (Benovoy *et al.* 2008) and, to a lesser extent, in Illumina (Ramasamy *et al.* 2013) microarrays. Similar studies have not been performed for Agilent microarrays so far. To establish if PIP could be leading to false-positive results in our analysis, we used a recently devised tool (Ramasamy *et al.* 2013) to determine which probes in our microarray had polymorphisms located within the probe sequences; we then determined if these probes with polymorphisms were overrepresented within the list of differentially expressed probes.

We have shown for the first time that Agilent gene expression microarrays are not deleteriously affected by PIP. This is an important methodological contribution to the analysis of Agilent microarrays. The differing susceptibility of the various microarray platforms to the PIP problem can be explained by the different lengths of the probes used. The presence of a polymorphism in a longer sequence has a less pronounced effect on the binding affinity than in a shorter sequence (Rennie *et al.* 2008). It has previously been shown that results generated using the Affymetrix array (25-mer probe design) are much more affected by the PIP problem than those results generated using the Illumina array (50-mer probe design). We have now demonstrated that results generated using the Agilent array (60-mer probe design) are not significantly affected by PIP. However, previous pharmacoresistant human epilepsy microarray studies which were performed using the Affymetrix platform (Becker *et al.* 2003; Arion *et al.* 2006; Lee *et al.* 2007), and perhaps studies performed on other non-Agilent platforms, are likely to have been affected by the PIP problem.

4.5.4 Demonstrating the potential therapeutic and causal significance of our results

The discovery of new therapeutic targets is one of the main motivations behind conducting microarray studies in pharmacoresistant epilepsy. From this point of view, we wished to determine if the DEGs we have identified are potentially enriched with novel AED targets. We reasoned that if our DEGs are enriched with targets of current AEDs, then the DEGs are also likely to be harbouring future AED targets. We created a comprehensive list of the known targets of current AEDs using an objective unbiased unsupervised methodology. We found that the DEG-lists from a number of microarray studies on brain tissue from epilepsy surgery have statistically significant overlaps with targets of current AEDs, but the most significant overlap by far was for our microarray study (Table 4.3 and Figure 4.1). This shows that our list of DEGs is likely to be a highly valuable source for future drug discovery.

A limitation inherent in transcriptomic studies is that the gene expression changes identified can be a consequence, rather than a cause, of the disease. In order to demonstrate that the DEGs identified are causally relevant, we have devised a novel strategy: we have determined if the DEG-set is enriched in a GWAS study of pharmacoresistant partial epilepsy (described in Chapter 5). We found that the DEG-sets from a number of microarray studies on brain tissue from epilepsy surgery were significantly enriched in the focal epilepsy GWAS, but the most significant enrichment by far was for our microarray study (Table 4.4 and Figure 4.2).

The above observations illustrate that our results are clearly more therapeutically and causally relevant than any previous microarray study on brain tissue from epilepsy surgery. We feel that our superior results can be attributed to our stringent QC and cutting edge data processing strategies (for example, adjustment for batch effect and hidden confounders, described above), which ensure that more false positive are filtered out from our results and our results are more enriched with true positives.

4.5.5 Differential Connectivity

All published transcriptomic studies on human epileptic brain tissue, and the vast majority of transcriptomic studies in general, have focused on identifying genes or gene sets showing average expression levels that vary across biological conditions. Although such differential expression approaches have been very successful, much of the information contained in

gene expression datasets is ignored. Disease genes are often not differentially expressed in diseases because genetic variations in the coding region can affect the function of the gene without affecting its expression level. Furthermore, a variety of post-translational modifications can affect regulatory activities of a gene product independently of its expression level. Hence, manifestation of disease can result from a de- or reregulation of genes that does not significantly affect each gene's average expression level. For these reasons, it is being increasingly appreciated that focusing solely on differential expression and overlooking other types of differential regulation, such as differential connectivity (also known as differential co-expression or differential correlation) can be critically limiting (Mentzen *et al.* 2009).

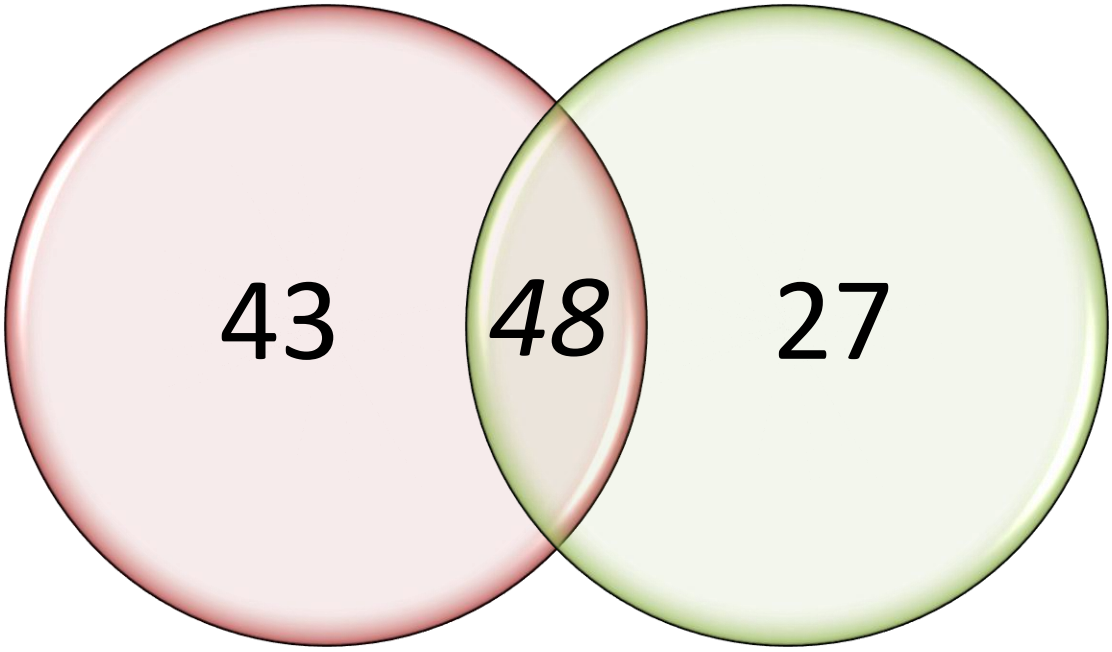
Differential connectivity is defined as a change in the correlation relationships between genes. Transcriptomic correlation analysis identifies sets of genes that are expressed in a coordinated fashion. Such correlation is considered evidence for possible coregulation under the principle of guilt-by-association (Chu *et al.* 1998). Hence, major changes in correlation patterns between classes may indicate changes in regulation.

Differential connectivity analysis algorithms can be divided into two distinct types: (1) targeted approaches study gene modules that are defined *a priori*, while (2) untargeted approaches aim at grouping genes into modules on the basis of their differential correlation status. In order to ensure that our results are reliable, we utilized both an untargeted and a targeted approach. Starting with an untargeted approach, we used the program 'Differential Correlation in Expression for meta-module Recovery' (DICER), to identify differentially connected clusters. The plot in Figure 4.3 illustrates, as an example, the clearly contrasting connectivity between normal and disease tissue for one of the clusters. We confirmed the causal relevance of the differentially connected genes identified using DICER by showing that they are enriched in a GWAS study of pharmacoresistant partial epilepsy. The differentially connected gene-set remains enriched in the GWAS study even when we exclude those genes which are also differentially expressed. Pathway-enrichment analysis of the differentially connected genes revealed 75 significantly enriched pathways. We further confirmed that these pathways are differentially connected using a targeted algorithm, implemented in the package Gene Set Co-Expression Analysis (GSCA) (Choi & Kendziorski 2009).

We have revealed for the first time that there is widespread differential connectivity in the pharmacoresistant epileptic hippocampus. In fact, we have shown that differential connectivity is more widespread than differential expression. Of the 10,000 probes subjected to DICER analysis, 890 (9%) were differentially connected. Of the 29,003 probes included in the differential expression analysis, 1176 probes (4%) were differentially expressed.

4.5.6 Identifying a network of pathways and 'hub' pathways

Figure 4.6 Differential expression (red) and connectivity (green) pathway-analysis confirm and complement each other.



As illustrated in Figure 4.6, differential expression and differential connectivity pathway-analyses confirm and complement each other. We identified 118 unique differentially regulated (differentially expressed or differentially connected) Reactome pathways. Out of these, 48 pathways (41%) were both differentially expressed and differentially connected, 41 pathways (36%) were only differentially expressed, and 27 pathways (23%) were solely differentially connected (Figure 4.6). This point is further highlighted by looking at the ten most differentially expressed and the ten most differentially connected pathways (Tables 4.6 and 4.7). Common to both Tables are pathways related to the neuronal system, transmission across chemical synapses and SLC-mediated transmembrane transport of small

molecules. However, prominent amongst the most differentially expressed pathways, but not amongst the most differentially connected pathways, are pathways related to ligand binding to G protein-coupled receptors (GPCR), especially class A1 rhodopsin-like receptors such as chemokine receptor 1 (CCR1), and the resultant downstream signalling events. By contrast, prominent amongst the most differentially connected pathways, but not amongst the most differentially expressed pathways, are pathways related to axon guidance, especially via semaphorins. These observations demonstrate that considering only one form of differential regulation (that is, only differential expression or only differential connectivity) may lead to important biological processes being overlooked.

The differentially expressed and differentially connected Reactome pathways identified in our microarray analysis (Tables 4.6 and 4.7, and Appendix 9) could be deemed to represent disparate and disconnected processes, for example 'axon guidance', 'transmembrane transport of small molecules' and 'class A1 rhodopsin-like receptors'. Using the Enrichment Map utility, we have shown that these differentially expressed and differentially connected pathways in fact form a highly interconnected central network (Figure 4.4). In this central network, each pathway is directly connected, on average, to 10.5 other pathways. The seemingly unrelated pathways, therefore, form a coherent whole and it can be expected that changes in one pathway in this network will have a cascading effect on the rest of the network.

We acknowledge that alternative methods of network construction are available (see Chapter 1). We have adopted a strategy that begins with the identification of enriched biological pathways within the transcriptomic data and then constructs a network of the enriched pathways. This methodology is well suited to addressing a key unanswered question in epilepsy research: which pathways and processes are most central in the dysregulated transcriptomic network underlying pharmacoresistant epilepsy? The current analysis and previously published studies have shown that numerous functionally diverse genes are dysregulated in the epileptic human hippocampus. Starting with a pathway-enrichment analysis approach allows immediate functional characterisation of these complex transcriptomic results, as each biological pathway serves a clearly defined purpose. Subsequent construction of a network of dysregulated pathways and analysis of network properties allows the identification of the most important hub pathways within the

network. In order to construct a network of dysregulated pathways, we have opted for a simple and intuitive approach not reliant on strong assumptions. The underlying assumption is that the dysfunction of a pathway will spread to overlapping pathways with which it shares constituent genes.

Of particular importance in this network are likely to be the 'hub pathways' identified using betweenness centrality network analysis. Betweenness is a measure of the centrality of a node in a network, and is calculated as the fraction of shortest paths between node pairs that pass through the node of interest. Hence, betweenness is a measure of the influence a node has over the spread of information through the network. The most central pathway by far was 'signalling by Notch'. We have conducted a literature review in order to highlight any published evidence of the potential role of Notch signalling in epilepsy.

4.5.7 Notch signalling pathway in epilepsy: a literature review

It is important to note that the differential expression of the Notch signalling pathway reported here can be independently and robustly confirmed: in a study published recently, it has been shown using Western Blots that Notch signalling is significantly upregulated in the pharmacoresistant epileptic human hippocampus (Sha *et al.* 2013).

Notch is a cell surface receptor that engages at least two signal transduction pathways: one that controls nuclear gene expression and another that directly targets the cytoskeleton (Giniger 2012). Notch signalling plays critical roles in both the developing and the adult brain. In the developing brain, Notch regulates specification of neuronal identity and neuronal division, survival and migration, as well as axon guidance, morphogenesis of dendritic arbors and weighting of synapse strength (Giniger 2012). Notch pathway components are expressed throughout the adult brain (Ables *et al.* 2011). In the adult hippocampus and other brain regions, Notch is localized at synapses and is an essential contributor to synaptic plasticity (Wang *et al.* 2004; Dahlhaus *et al.* 2008; Alberi *et al.* 2011), and is involved in neuronal excitability and discharges (Alberi *et al.* 2011; Lieber *et al.* 2011). Using the kainate murine model of temporal lobe epilepsy, Sha and colleagues (Sha *et al.* 2013) have recently shown that Notch is activated in response to seizure activity, and that the pharmacological induction of Notch expression has proconvulsant effects, while the inhibition of Notch suppresses seizures. The authors show that Notch enhances

transmission at glutamatergic synapses of CA1 pyramidal neurons. In summary, this data demonstrates that Notch is activated in response to seizure activity and that its activation in turn promotes seizures.

Notch is known to modulate a number of transcription factors and this list is constantly growing (Borggrefe & Oswald 2009). Mutations in the *NOTCH3* gene have been associated with human brain disorders: cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (Rutten *et al.* 2014), and migraine (Menon *et al.* 2011). Based on the preceding observations, it can be postulated that the Notch signalling pathway occupies a central position in the transcriptional network that underlies the development of pharmacoresistant epilepsy.

4.6 Conclusions

We have performed the largest and most robust transcriptomic analysis of hippocampal tissue from pharmacoresistant mesial temporal lobe epilepsy to date. We have externally validated our results using previously published studies. We have shown the causal relevance of our results using novel objective methodology, and we have demonstrated that our list of DEGs is likely to be a highly valuable source for future drug discovery. For the first time, we have identified the differentially connected pathways in the pharmacoresistant epileptic hippocampus, and revealed the network of differentially expressed and differentially connected pathways underlying this disease, and identified the hub pathways.

4.7 References

Ables J.L., Breunig J.J., Eisch A.J. & Rakic P. (2011) Not(ch) just development: Notch signalling in the adult brain. *Nat Rev Neurosci* **12**, 269-83.

Alberi L., Liu S., Wang Y., Badie R., Smith-Hicks C., Wu J., Pierfelice T.J., Abazyan B., Mattson M.P., Kuhl D., Pletnikov M., Worley P.F. & Gaiano N. (2011) Activity-induced Notch signaling in neurons requires Arc/Arg3.1 and is essential for synaptic plasticity in hippocampal networks. *Neuron* **69**, 437-44.

Amar D., Safer H. & Shamir R. (2013) Dissection of regulatory networks that are altered in disease via differential co-expression. *PLoS Comput Biol* **9**, e1002955.

Arion D., Sabatini M., Unger T., Pastor J., Alonso-Nanclares L., Ballesteros-Yanez I., Garcia Sola R., Munoz A., Mirnics K. & DeFelipe J. (2006) Correlation of transcriptome profile with electrical activity in temporal lobe epilepsy. *Neurobiol Dis* **22**, 374-87.

Becker A.J., Chen J., Zien A., Sochivko D., Normann S., Schramm J., Elger C.E., Wiestler O.D. & Blumcke I. (2003) Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy. *Eur J Neurosci* **18**, 2792-802.

Benovoy D., Kwan T. & Majewski J. (2008) Effect of polymorphisms within probe-target sequences on olignonucleotide microarray experiments. *Nucleic Acids Res* **36**, 4417-23.

Borggrefe T. & Oswald F. (2009) The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol Life Sci* **66**, 1631-46.

Chen C., Grennan K., Badner J., Zhang D., Gershon E., Jin L. & Liu C. (2011) Removing batch effects in analysis of expression microarray data: an evaluation of six batch adjustment methods. *PLoS One* **6**, e17238.

Choi Y. & Kendziorski C. (2009) Statistical methods for gene set co-expression analysis. *Bioinformatics* **25**, 2780-6.

Chu S., DeRisi J., Eisen M., Mulholland J., Botstein D., Brown P.O. & Herskowitz I. (1998) The transcriptional program of sporulation in budding yeast. *Science* **282**, 699-705.

Dahlhaus M., Hermans J.M., Van Woerden L.H., Saiepour M.H., Nakazawa K., Mansvelder H.D., Heimel J.A. & Levelt C.N. (2008) Notch1 signaling in pyramidal neurons regulates synaptic connectivity and experience-dependent modifications of acuity in the visual cortex. *J Neurosci* **28**, 10794-802.

Flight R.M. & Wentzell P.D. (2010) Preliminary exploration of time course DNA microarray data with correlation maps. *OMICS* **14**, 99-107.

Fusi N., Stegle O. & Lawrence N.D. (2012) Joint modelling of confounding factors and prominent genetic regulators provides increased accuracy in genetical genomics studies. *PLoS Comput Biol* **8**, e1002330.

Giniger E. (2012) Notch signaling and neural connectivity. *Curr Opin Genet Dev* **22**, 339-46.

Hawrylycz M.J., Lein E.S., Guillozet-Bongaarts A.L., Shen E.H., Ng L., Miller J.A., van de Lagemaat L.N., Smith K.A., Ebbert A., Riley Z.L., Abajian C., Beckmann C.F., Bernard A., Bertagnolli D., Boe A.F., Cartagena P.M., Chakravarty M.M., Chapin M., Chong J., Dalley R.A., Daly B.D., Dang C., Datta S., Dee N., Dolbeare T.A., Faber V., Feng D., Fowler D.R., Goldy J., Gregor B.W., Haradon Z., Haynor D.R., Hohmann J.G., Horvath S., Howard R.E., Jeromin A., Jochim J.M., Kinnunen M., Lau C., Lazarz E.T., Lee C., Lemon T.A., Li L., Li Y., Morris J.A., Overly C.C., Parker P.D., Parry S.E., Reding M., Royall J.J., Schulkin J., Sequeira P.A., Slaughterbeck C.R., Smith S.C., Sodt A.J., Sunkin S.M., Swanson B.E., Vawter M.P., Williams D., Wohnoutka P., Zielke H.R., Geschwind D.H., Hof P.R., Smith S.M., Koch C., Grant S.G. & Jones A.R. (2012) An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* **489**, 391-9.

Johnson W.E., Li C. & Rabinovic A. (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8**, 118-27.

Kang H.M., Ye C. & Eskin E. (2008) Accurate discovery of expression quantitative trait loci under confounding from spurious and genuine regulatory hotspots. *Genetics* **180**, 1909-25.

Lee T.S., Mane S., Eid T., Zhao H., Lin A., Guan Z., Kim J.H., Schweitzer J., King-Stevens D., Weber P., Spencer S.S., Spencer D.D. & de Lanerolle N.C. (2007) Gene expression in temporal lobe epilepsy is consistent with increased release of glutamate by astrocytes. *Mol Med* **13**, 1-13.

Leek J.T. & Storey J.D. (2007) Capturing heterogeneity in gene expression studies by surrogate variable analysis. *PLoS Genet* **3**, 1724-35.

Liang W.S., Dunckley T., Beach T.G., Grover A., Mastroeni D., Ramsey K., Caselli R.J., Kukull W.A., McKeel D., Morris J.C., Hulette C.M., Schmechel D., Reiman E.M., Rogers J. & Stephan D.A. (2008) Altered neuronal gene expression in brain regions differentially affected by Alzheimer's disease: a reference data set. *Physiol Genomics* **33**, 240-56.

Lieber T., Kidd S. & Struhl G. (2011) DSL-Notch signaling in the Drosophila brain in response to olfactory stimulation. *Neuron* **69**, 468-81.

Mentzen W.I., Floris M. & de la Fuente A. (2009) Dissecting the dynamics of dysregulation of cellular processes in mouse mammary gland tumor. *BMC Genomics* **10**, 601.

Merico D., Isserlin R., Stueker O., Emili A. & Bader G.D. (2010) Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One* **5**, e13984.

Opitz L., Salinas-Riester G., Grade M., Jung K., Jo P., Emons G., Ghadimi B.M., Beissbarth T. & Gaedcke J. (2010) Impact of RNA degradation on gene expression profiling. *BMC Med Genomics* **3**, 36.

Ramasamy A., Trabzuni D., Gibbs J.R., Dillman A., Hernandez D.G., Arepalli S., Walker R., Smith C., Ilori G.P., Shabalin A.A., Li Y., Singleton A.B., Cookson M.R., Hardy J., Ryten M. & Weale M.E. (2013) Resolving the polymorphism-in-probe problem is critical for correct interpretation of expression QTL studies. *Nucleic Acids Res* **41**, e88.

Rennie C., Noyes H.A., Kemp S.J., Hulme H., Brass A. & Hoyle D.C. (2008) Strong position-dependent effects of sequence mismatches on signal ratios measured using long oligonucleotide microarrays. *BMC Genomics* **9**, 317.

Sha L., Wu X., Yao Y., Wen B., Feng J., Sha Z., Wang X., Xing X., Dou W., Jin L., Li W., Wang N., Shen Y., Wang J., Wu L. & Xu Q. (2013) Notch Signaling Activation Promotes Seizure Activity in Temporal Lobe Epilepsy. *Mol Neurobiol*.

Smyth G.K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3.

Stegle O., Parts L., Piipari M., Winn J. & Durbin R. (2012) Using probabilistic estimation of expression residuals (PEER) to obtain increased power and interpretability of gene expression analyses. *Nat Protoc* **7**, 500-7.

Teschendorff A.E., Zhuang J. & Widschwendter M. (2011) Independent surrogate variable analysis to deconvolve confounding factors in large-scale microarray profiling studies. *Bioinformatics* **27**, 1496-505.

Tesson B.M., Breitling R. & Jansen R.C. (2010) DiffCoEx: a simple and sensitive method to find differentially coexpressed gene modules. *BMC Bioinformatics* **11**, 497.

Trabzuni D., Ryten M., Walker R., Smith C., Imran S., Ramasamy A., Weale M.E. & Hardy J. (2011) Quality control parameters on a large dataset of regionally dissected human control brains for whole genome expression studies. *J Neurochem* **119**, 275-82.

van Gassen K.L., de Wit M., Koerkamp M.J., Rensen M.G., van Rijen P.C., Holstege F.C., Lindhout D. & de Graan P.N. (2008) Possible role of the innate immunity in temporal lobe epilepsy. *Epilepsia* **49**, 1055-65.

Venugopal A.K., Sameer Kumar G.S., Mahadevan A., Selvan L.D., Marimuthu A., Dikshit J.B., Tata P., Ramachandra Y., Chaerkady R., Sinha S., Chandramouli B., Arivazhagan A., Satishchandra P., Shankar S. & Pandey A. (2012) Transcriptomic Profiling of Medial Temporal Lobe Epilepsy. *J Proteomics Bioinform* **5**.

Wang Y., Chan S.L., Miele L., Yao P.J., Mackes J., Ingram D.K., Mattson M.P. & Furukawa K. (2004) Involvement of Notch signaling in hippocampal synaptic plasticity. *Proc Natl Acad Sci U S A* **101**, 9458-62.

Watson M. (2006) CoXpress: differential co-expression in gene expression data. *BMC Bioinformatics* **7**, 509.

Yang Q., Orman M.A., Berthiaume F., Ierapetritou M.G. & Androulakis I.P. (2012) Dynamics of short-term gene expression profiling in liver following thermal injury. *J Surg Res* **176**, 549-58.

Yang S., Guo X., Yang Y.C., Papcunik D., Heckman C., Hooke J., Shriver C.D., Liebman M.N. & Hu H. (2006a) Detecting outlier microarray arrays by correlation and percentage of outliers spots. *Cancer Inform* **2**, 351-60.

Yang X., Bentink S., Scheid S. & Spang R. (2006b) Similarities of ordered gene lists. *J Bioinform Comput Biol* **4**, 693-708.

CHAPTER FIVE

A GENOME-WIDE ASSOCIATION STUDY OF PHARMACORESISTANT PARTIAL EPILEPSY: PATHWAY AND NETWORK ANALYSIS

Chapter 5: A Genome-Wide Association Study of Pharmacoresistant Partial Epilepsy: *Pathway and Network Analysis*

5.1 Introduction

It has been shown that genetic factors have a major influence on epilepsy prognosis (Johnson *et al.* 2003). However, for many complex traits, including pharmacoresistant epilepsy, the underlying pathobiology is not well understood, making it difficult to select candidate genes for analysis. For such traits, performing genome-wide association studies (GWAS) is an important initial strategy for identifying susceptibility genes. This approach can be quite successful—several highly significant SNP-disease associations have been found through GWAS that have been replicated across studies (Manolio *et al.* 2008), for example the association between interleukin-23 receptor polymorphisms and inflammatory bowel disease (Duerr *et al.* 2006; Raelson *et al.* 2007). Despite the success of single-marker association tests, this strategy has limited power to identify disease genes, given the hundreds of thousands of SNP markers used in most GWA studies. Some genes may be genuinely associated with disease status but may not reach a stringent genome-wide significance threshold in any GWAS. Most genetic associations have a small effect size and require very large sample sizes across several cohorts to be robustly identified. Furthermore, the small effect sizes for these associations do not explain the observed heritability of most traits (Maher 2008). In addition, it is can be challenging to translate a genetic association into a functional connection with the trait based only on the annotation of a single gene.

In pathway-based association tests for GWAS, researchers examine a collection of predefined gene sets; each set represents a pathway (based on prior biological knowledge); and the significance of each pathway can be summarized based on the disease association of markers in or near genes that are components of that pathway (Wang *et al.* 2010). Intuitively, it seems likely that susceptibility markers for any given disorder are not randomly distributed among genes but instead, are distributed among one (or more) set(s) of genes whose functions are to some extent related (Holmans 2010). Hence, GWAS pathway

analysis can assess whether a group of genes with related functions are jointly associated with a trait of interest.

The pathway-based GWAS approach offers several advantages over single-marker analysis. Firstly, pathway-based methods boost the power to identify genetic associations (Wang *et al.* 2007). Genetic heterogeneity may cause any one causal variant to exhibit only modest disease risk in the sample as a whole, since different individuals may possess different disease risk polymorphisms at different loci in the same gene, or in different genes. This will reduce power to detect any one variant by traditional association methods. However, if the genes in question are members of the same biological pathway, then considering the pathway as the unit of analysis may increase power to detect association between the genes and disease. For similar reasons, association of disease with biological pathways may be easier to replicate across different studies than association with individual SNPs. This was clearly shown in an analysis of Crohn's disease (Wang *et al.* 2009), where the IL12/IL23 pathway showed evidence of enrichment in four independent GWAS, despite the genes and SNPs involved differing between the studies. In addition, by identifying additional susceptibility genes, pathway-based analysis can be used to fill-in part of the 'missing heritability' described above (Fridley & Biernacka 2011). Furthermore, compared with SNP-based analysis, pathway-based analysis may yield more secure insights into disease biology since an associated pathway is likely to implicate function better than a hit in a single gene that may have many functional possibilities. Finally, as the most associated gene in a pathway might not be the best candidate for therapeutic intervention, targeting susceptibility pathways might also have clinical implications for finding additional drug targets.

To date, there has been only one GWAS of response to antiepileptic drugs. No single variant achieved genome-wide significance in this study, although a number of significant pathways were identified (Speed *et al.* 2013). The current study is distinct from the previously published work in three important ways: (1) we have included only patients with partial epilepsy, (2) we have applied stringent definitions of pharmacoresistance (see below), and (3) we have used normal subjects as controls. These characteristics mean that the design of our GWAS closely mirrors the design of our transcriptomic study (Chapter 4), and the two types of studies can be effectively integrated (in Chapter 7).

5.2 Aims

1. To perform a GWAS of pharmacoresistant partial epilepsy.
2. To perform objective validation of a gene-set based analysis approach of our GWAS.
3. To perform pathway-level enrichment analysis of GWAS results.
4. To demonstrate pathway-level replication of GWAS results.
5. To create a network of enriched pathways and identify 'hub' pathways.

5.3 Methods

5.3.1 Patients

The control dataset consisted of 3000 subjects from the Wellcome Trust Case Control Consortium (WTCCC) 1958 British Birth Cohort (dataset EGAD00000000022). This dataset was downloaded from the European Genome-Phenome Archive after obtaining the requisite approval from the Wellcome Trust Case Control Consortium Data Access Committee.

The epilepsy cases used in the discovery cohort were from amongst the patients recruited into the Standard and New Antiepileptic Drugs (SANAD) trial (Marson *et al.* 2007a, b). SANAD was an unblinded randomised controlled trial in hospital-based outpatient clinics in the UK which examined the longer-term outcomes of standard versus new antiepileptic drugs. Patients were included in SANAD if they had a history of two or more clinically definite unprovoked epileptic seizures in the previous year. This allocation allowed inclusion of patients with newly diagnosed epilepsy, patients who had failed treatment with previous monotherapy, and patients who had entered a period of remission from seizures but had relapsed after withdrawal of treatment. Patients were excluded if the clinician or patient felt that treatment was contraindicated, if all their seizures had been acute symptomatic seizures (including febrile seizures), they were aged 4 years or younger, or if there was a history of progressive neurological disease. Clinicians were asked to classify seizures and epilepsy syndromes by International League Against Epilepsy (ILAE) classifications(1981; 1989) as far as was possible, and at least to differentiate between partial onset (focal) or generalised onset seizures. Patients were seen for follow-up at 3 months, 6 months, 1 year, and at successive yearly intervals from the date of randomisation. At every visit, details of

the occurrence of seizures, adverse events, hospital admissions, and antiepileptic drug treatment were documented.

For the purposes of our GWAS, we extracted subjects from the SANAD trial with a history of pharmacoresistant partial epilepsy. Pharmacoresistant epilepsy was defined as failure to achieve seizure freedom with two appropriately chosen antiepileptic drugs (used as monotherapies or in combination) at their minimum therapeutic doses or above for at least three months, in keeping with ILAE guidelines (Kwan *et al.* 2010). Seizure freedom was defined as freedom from all seizures for a minimum period of 12 months. Total daily minimum therapeutic doses of antiepileptic drugs used in our classification protocol were taken from the British National Formulary 60 (September 2010), and are listed in Table 5.1.

Table 5.1 Total daily minimum therapeutic doses of antiepileptic drugs used in our classification of pharmacoresistance.

Antiepileptic drug	Minimum therapeutic dose (mg)
Carbamazepine	600
Clobazam	20
Clonazepam	4
Eslicarbazepine	800
Ethosuximide	1000
Gabapentin	1200
Lacosamide	200*
Lamotrigine	150
Levetiracetam	1000*
Oxcarbazepine	900
Phenobarbital	60
Phenytoin	200
Pregabalin	300
Primidone	750
Tiagabine	15§
Topiramate	150
Valproate	1000
Vigabatrin	1000
Zonisamide	150

*Minimum therapeutic dose not stated in the British National Formulary and taken from product literature. §30mg in patients receiving enzyme inducing drugs.

The epilepsy cases used in the replication cohort were from amongst the patients recruited into the 'Pharmacogenetics of GABAergic Mechanisms of Benefit and Harm in Epilepsy'

study. The aims of the study were: (i) to determine a genomic profile that predicts therapeutic response to clobazam (CLB), and (ii) to develop a gene-test for predicting vigabatrin (VGB) related visual field defects. Patients were recruited from hospital-based outpatient clinics in the UK. Patients were invited to join the CLB arm of the study if they had continued seizures despite current treatment with between one and three conventional antiepileptic drugs and the addition of CLB to the existing antiepileptic regime was clinically indicated. Patients were asked to join the VGB arm of the study if they were over the age of 12 years and had taken VGB for at least 6 months. For the CLB and VGB studies, the dose and duration of use details for previously prescribed antiepileptic drugs was not recorded. Hence, it was not possible to make use of the ILAE definition stated above for the identification of pharmacoresistant patients. However, both CLB and VGB are used in the UK as adjunctive treatments in cases where first- and second-line antiepileptic drugs have proven ineffective. The chief investigator for the study, who is familiar with clinical practice at the participating hospitals, confirmed that both CLB and VGB are prescribed in pharmacoresistant patients in the concerned centres. To further ensure that we included only a pharmacoresistant phenotype in our GWAS, we selected only those patients who had active epilepsy for at least 10 years and were still uncontrolled at the time of initiating CLB or VGB. The epilepsy syndrome of the recruited patients was recorded as part of the CLB and VGB studies, and we included in our GWAS only those with documented partial epilepsy.

5.3.2 Genotyping, imputation and quality control

DNA was extracted from blood samples using standard procedures. All samples from patients with epilepsy were genotyped at the Wellcome Trust Sanger Institute on an Illumina 670K chip. Our standard data quality control (QC) procedures were as follows. Individuals were excluded on the basis of discordance between reported and observed sex, SNP call rate <95%, outlying autosomal heterozygosity rates (>3 standard deviations from the mean), inadvertent subject duplication or cryptic relatedness based on pairwise identity-by-descent estimations (the individual with greater missing data was removed from each pair with π-hat >0.1875), being outliers from the European cluster in a plot of the first two principal components of genotypes for samples from the study and HapMap Phase III. SNPs were excluded on the basis of genotype missing rate ≥5% if minor allele frequency (MAF)

was ≥0.05 and missing rate ≥1% if MAF was <0.05, observed minor allele frequency <0.01, significant deviation (P $<10^{-4}$) from Hardy-Weinberg equilibrium, significant difference (P $<10^{-4}$) in the genotype missing rate in cases compared to controls, and all non-autosomal SNPs. As an additional filter against false-positive associations resulting from genotyping errors, genotype imputation was used as a strategy for detecting genotyping errors (Marchini & Howie 2010). Samples were pre-phased using ShapeIt version 2 (Delaneau *et al.* 2013) and then imputed using Impute2 (Howie *et al.* 2009), with 1000 Genomes Project haplotypes (released in March 2012) as the reference panel. Impute2 produces a concordance metric: each genotyped SNP in the study dataset is masked one at a time, and then imputed using information from the reference data and nearby study variants; concordance between imputed and original genotypes is then calculated. SNPs imputed with high certainty (Impute2 'info' score >0.9) but with low concordance (<0.9) were excluded.

For controls, we obtained the WTCCC 1958 British Birth Cohort dataset genotyped on the Illumina 1.2M chip. This dataset was chosen as the SNPs on our Illumina 670K chip are a complete subset of those on the Illumina 1.2M chip. This ensures maximal overlap between the genotyped SNPs for the control and epilepsy samples. Hence, we reduced the SNPs in the control dataset to those also found on the Illumina 670K chip. For QC, we started by excluding all 231 individuals listed as 'individual exclusions' in the data release documentation. We then applied our standard per-individual QC filters stated above—this led to the exclusion of only two additional individuals, based on outlying ancestry. We excluded all SNPs listed as 'SNP exclusions' in the WTCCC data release documentation. We then applied our standard per-SNP QC filters stated above. In total 68114 SNPs were excluded, 53326 of which were in the suggested SNP exclusions from the Wellcome Trust data release documentation, and 1209 were identified using the imputation-based filter.

In the SANAD cohort, there were 84 genotyped samples with pharmacoresistant partial epilepsy. The final dataset, after applying the QC filters described above, included 76 samples.

In the combined clobazam and vigabatrin cohort, there were 385 genotyped samples with pharmacoresistant partial epilepsy. The final dataset, after applying the QC filters described above, included 345 samples.

For case-control analysis, our 'discovery' dataset comprised the SANAD cohort and half of the WTCCC 1958 British Birth Cohort chosen at random. Our 'replication' dataset comprised the combined clobazam and vigabatrin cohort and the other half of the WTCCC 1958 British Birth Cohort. Our standard QC filters were applied again to the discovery and replication cohorts. In the end, our discovery cohort consisted of 76 cases and 1312 controls genotyped over 509319 SNPs, and our replication cohort consisted of 345 cases and 1312 controls genotyped over 509269 SNPs.

We also created a 'mega-analysis' dataset by merging the SANAD, clobazam, vigabatrin and WTCCC 1958 British Birth cohorts. After QC, this dataset comprised 421 cases and 2624 controls genotyped over 509534 SNPs.

Genotype imputation was performed in order to boost power and to aid fine mapping of any association signals (Marchini & Howie 2010). The 'mega-analysis' dataset were pre-phased using ShapeIt version 2 (Delaneau *et al.* 2013) and then imputed using Impute2 (Howie *et al.* 2009), with 1000 Genomes Project haplotypes (released in March 2012) as the reference panel. The QCTOOL software was used to exclude SNPs with an 'info' score of <0.9. GTOOL software was then used to transform the imputed data to PED format, with a posterior probability of 0.9 used as a threshold to call genotypes. In the end, our standard per-SNP QC filters, described above, were applied. The final dataset included 5,519,310 genotyped and imputed SNPs.

5.3.3 Association, pathway and network analysis

SNP-level association analysis was performed using the chi-squared (χ^2) test in Plink version 1.07 (Purcell *et al.* 2007). Based on the results of the χ^2 test, genomic inflation factor (λ) was calculated and quantile-quantile (QQ) plots were generated. Where results were inflated due to population stratification, the association analysis was performed using logistic regression with significant principal components (PCs) as covariates. Specifically, PC analysis was performed using the package SNPRelate (Zheng *et al.* 2012). Tracy-Widom (TW) statistic (Patterson *et al.* 2006) and significance for the eigenvalue of each PC was calculated using

the package EigenCorr (Lee *et al.* 2011); PCs with statistically significant eigenvalues (FDR<0.05) were included as covariates in the logistic regression analysis. Sex was not included as a covariate as this can substantially reduce power for the identification of associated variants when the disease prevalence is lower than a few percent (Pirinen *et al.* 2012; Bezzina *et al.* 2013).

We wished to demonstrate that GWAS gene-set analysis was able to discriminate functionally relevant from irrelevant gene-sets. For this purpose, we used a robust objective methodology for compiling a highly functionally relevant gene-set: we created a comprehensive list of the targets of all current AEDs by searching three publically-available databases of drug targets: Stitch, SuperTarget and DrugBank. For comparison, we divided the rest of the genome randomly into 122 gene-sets of the same size as our functional gene-set. We determined the degree of enrichment of these gene-sets with our discovery and cohort association results.

Gene-set and pathway-level enrichment analysis was performed using the widely used program GSA-SNP (Nam *et al.* 2010). For SNP-to-gene mapping, we used a mapping distance of 20kb, and compared results obtained with using the first and second most significant SNP. In order to maintain consistency with our transcriptomic pathway analysis, the Reactome pathways database downloaded from the Gene Set Enrichment Analysis website was used in the enrichment analysis.

The statistical significance of the overlap between the list of enriched pathways in the discovery and replication cohorts was calculated using the hypergeometric equation.

As described for our transcriptomic analysis, Enrichment Map (Merico *et al.* 2010) tool was used to determine the connections between enriched pathways. Enrichment Map was used with default settings. Specifically, we employed an overlap coefficient cut-off of 0.5—two pathways were deemed connected only if the ratio of the size of the intersection over the size of the smallest pathway was 0.5 or more. The Network Analysis tool was used to calculate network parameters, including ‘betweenness centrality’ of nodes.

5.4 Results

5.4.1 SNP-level analysis

For the discovery cohort χ^2 association analysis, the λ was 1.01 and the QQ plot of the observed p-values revealed a good fit with the null distribution (Figure 5.1). For the replication cohort χ^2 association analysis, the λ was 1.04. The eigenvalues for the first two PCs were statistically significant; these PCs were included as covariates in a logistic regression analysis. After PCA-adjustment, both the λ (1.03) and the QQ plot showed appropriate improvement (Figure 5.2), suggesting that there was little evidence of residual inflation due to population stratification. For the combined cohort χ^2 association analysis, the λ was 1.05. The eigenvalues for the first eight PCs were significant; these PCs were included as covariates in a logistic regression analysis. After PCA-adjustment, both the λ (1.03) and the QQ plot (Figure 5.3) showed appropriate improvement, suggesting that there was little evidence of residual inflation due to population stratification. The association results of the combined imputed dataset were adjusted by including the same eight PCs as covariates in the logistic regression analysis. Again, the the λ (1.03) and the QQ plot (Figure 5.4) showed little evidence of residual inflation due to population stratification.

None of the p-values in our discovery or replication studies reached the now widely-accepted 5×10^{-8} threshold for genome-wide significance in association studies (McCarthy *et al.* 2008), nor the 9.8×10^{-8} threshold required to achieve significance after applying Bonferroni correction for the 509316 tests in our discovery analysis or the 509266 tests in our replication analysis. 54 SNPs reached a suggestive ($<5x10^{-5}$) level of significance in the discovery cohort and 39 in the replication cohort. There was no overlap between the suggestive SNPs in the two cohorts.

In the combined analysis, one SNP (rs35452866) reached genome-wide significance ($p=1.6x10^{-9}$), while 29 SNPs reached a suggestive level of significance (Table 5.2 and Figure 5.5). In the combined imputed analysis, one SNP (rs35452866) reached genome-wide significance ($p=3.4x10^{-9}$), while 402 reached a suggestive level of significance (Figure 5.6).

5.4.2 Gene-set enrichment validation

We demonstrated that a gene-set comprised of all known targets of current AEDs is significantly enriched in both the discovery (FDR<0.005) and the replication (FDR<0.0004) GWAS (Figure 5.7). By contrast, out of 122 random gene-sets of the same size as our AED-targets gene-set, none was significantly enriched in either GWAS (smallest FDR=0.15).

Table 5.2 SNPs reaching genome-wide or suggestive level of significance in the combined analysis. BP: base-pair position. OR: odds ratio.

Chromosome	SNP	BP	OR	P
4	rs35452866	97950633	2.717	1.59×10^{-9}
2	rs2176529	194613887	1.701	1.33×10^{-7}
5	rs10069413	84055052	1.649	1.32×10^{-6}
8	rs6470428	87256166	1.451	5.77×10^{-6}
8	rs7815102	87270243	1.447	6.83×10^{-6}
15	rs12323994	44770917	1.423	9.89×10^{-6}
1	rs10493734	83221230	1.389	1.43×10^{-5}
1	rs1144256	83160157	1.385	1.73×10^{-5}
17	rs4789963	74741972	1.39	1.78×10^{-5}
5	rs1979006	156666964	1.845	1.88×10^{-5}
22	rs763280	23923452	1.381	2.39×10^{-5}
11	rs11606985	2800252	1.428	2.44×10^{-5}
1	rs11163625	83210060	1.374	2.65×10^{-5}
1	rs1144269	83169134	1.373	2.79×10^{-5}
20	rs6110905	16035405	0.6973	2.80×10^{-5}
4	rs2242226	189709167	1.525	2.88×10^{-5}
7	rs722037	7282916	1.367	3.39×10^{-5}
16	rs2531995	3953468	1.371	3.42×10^{-5}
1	rs11163602	83137141	1.366	3.56×10^{-5}
8	rs6996246	87281129	1.375	3.60×10^{-5}
16	rs2230742	3956677	1.507	3.60×10^{-5}
7	rs11974777	80050992	1.673	3.82×10^{-5}
7	rs1638210	7284485	1.364	3.95×10^{-5}
22	rs6000762	36248418	1.41	3.95×10^{-5}
13	rs7990091	25645138	1.455	4.27×10^{-5}
3	rs11710045	57212261	0.7202	4.45×10^{-5}
14	rs12586261	44062009	2.628	4.51×10^{-5}
3	rs269392	19824546	1.419	4.53×10^{-5}
3	rs4060726	57310916	0.7227	4.61×10^{-5}
20	rs1418925	52259138	1.357	4.70×10^{-5}

Figure 5.1 QQ plot for GWAS discovery cohort

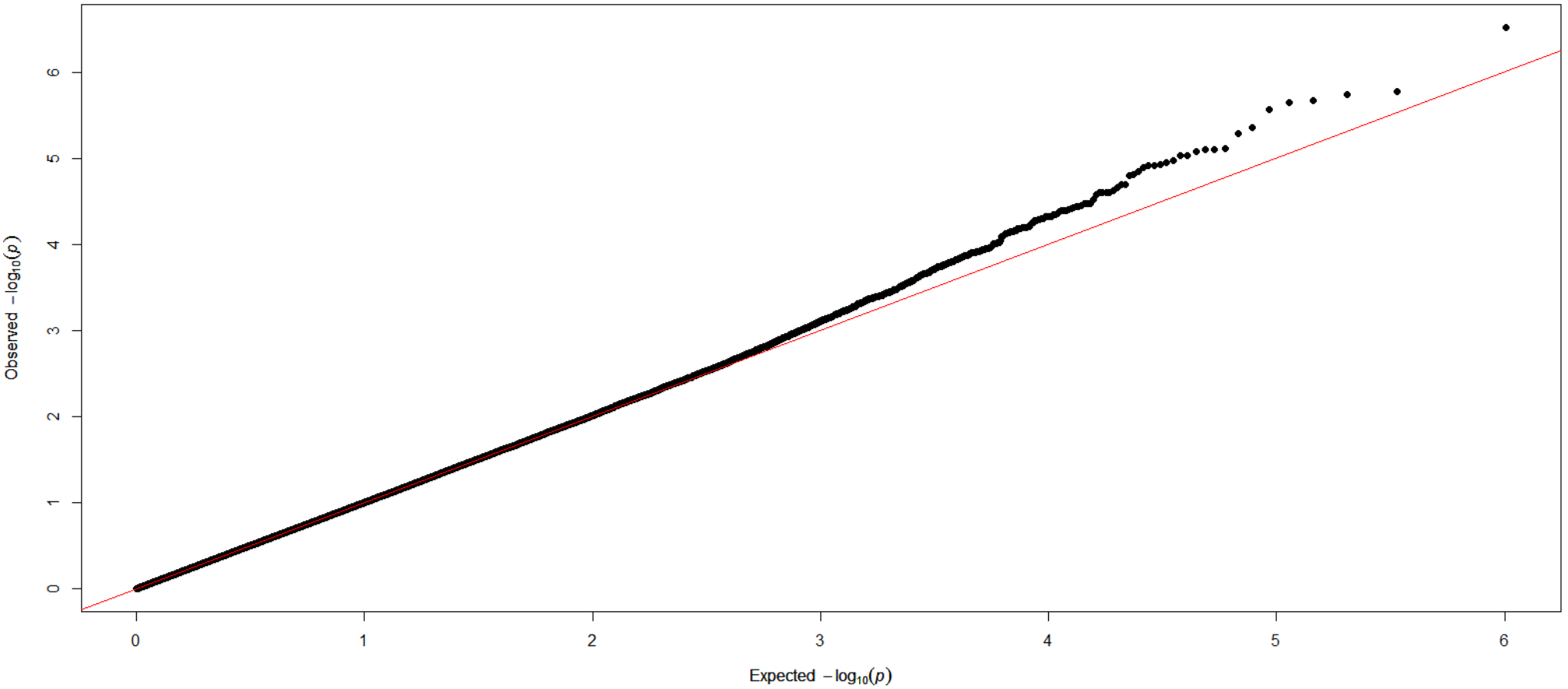


Figure 5.2 QQ plot of GWAS replication cohort

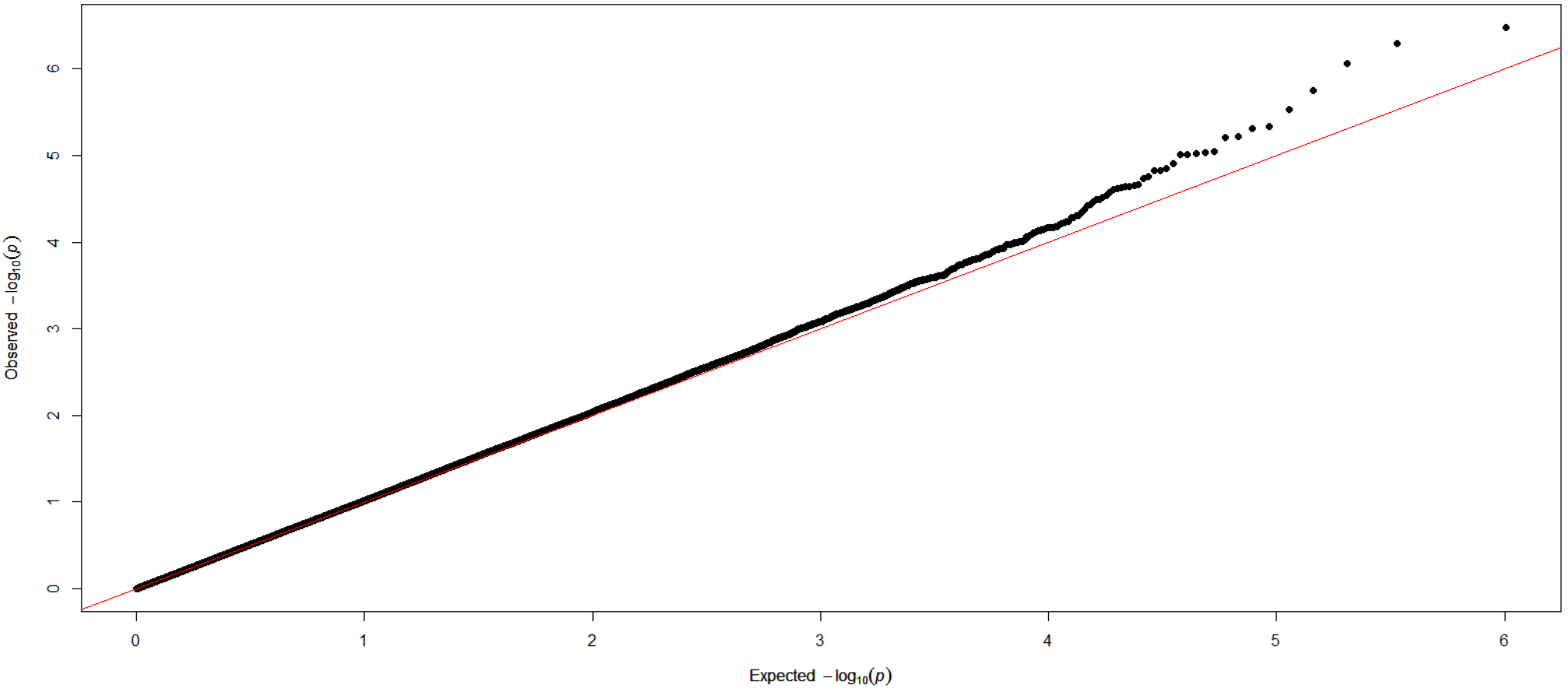


Figure 5.3 QQ plot for combined GWAS cohort

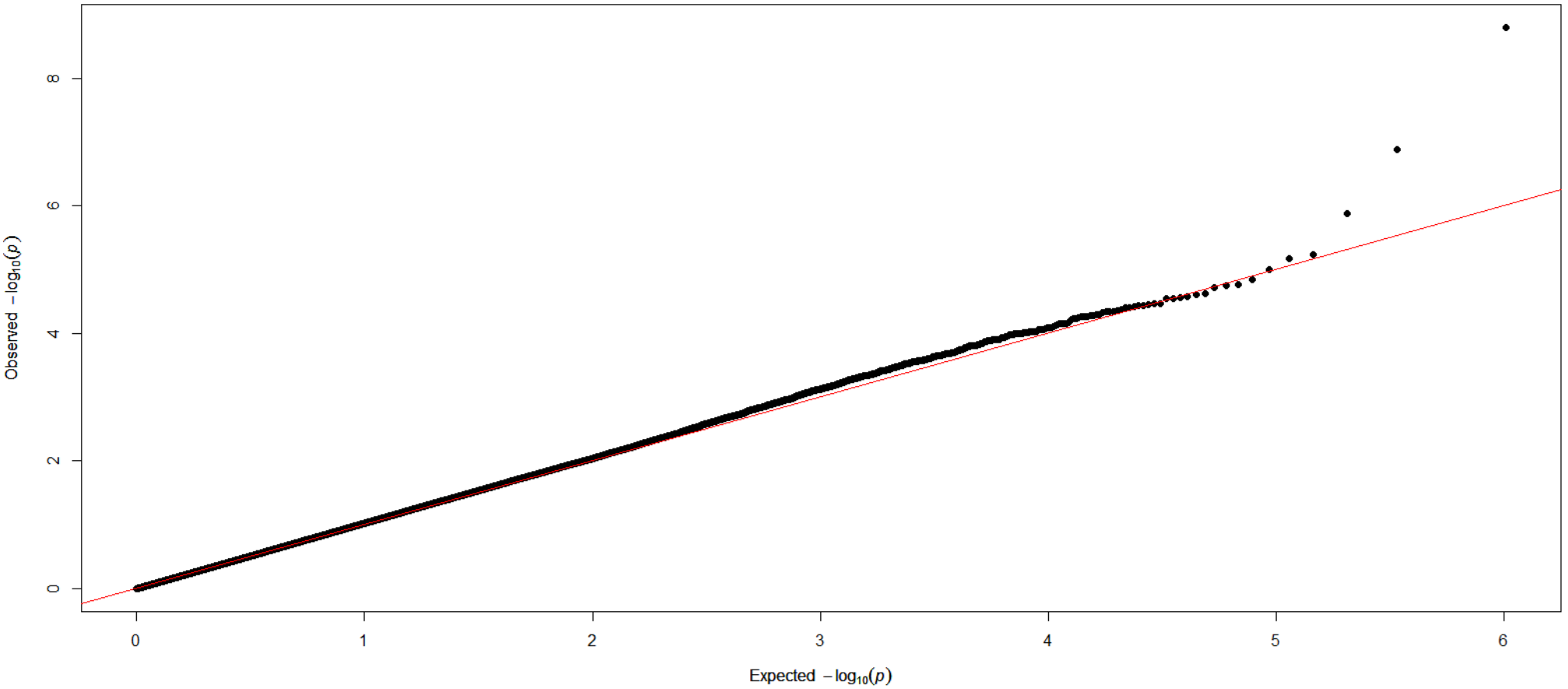


Figure 5.4 QQ plot for combined imputed GWAS dataset

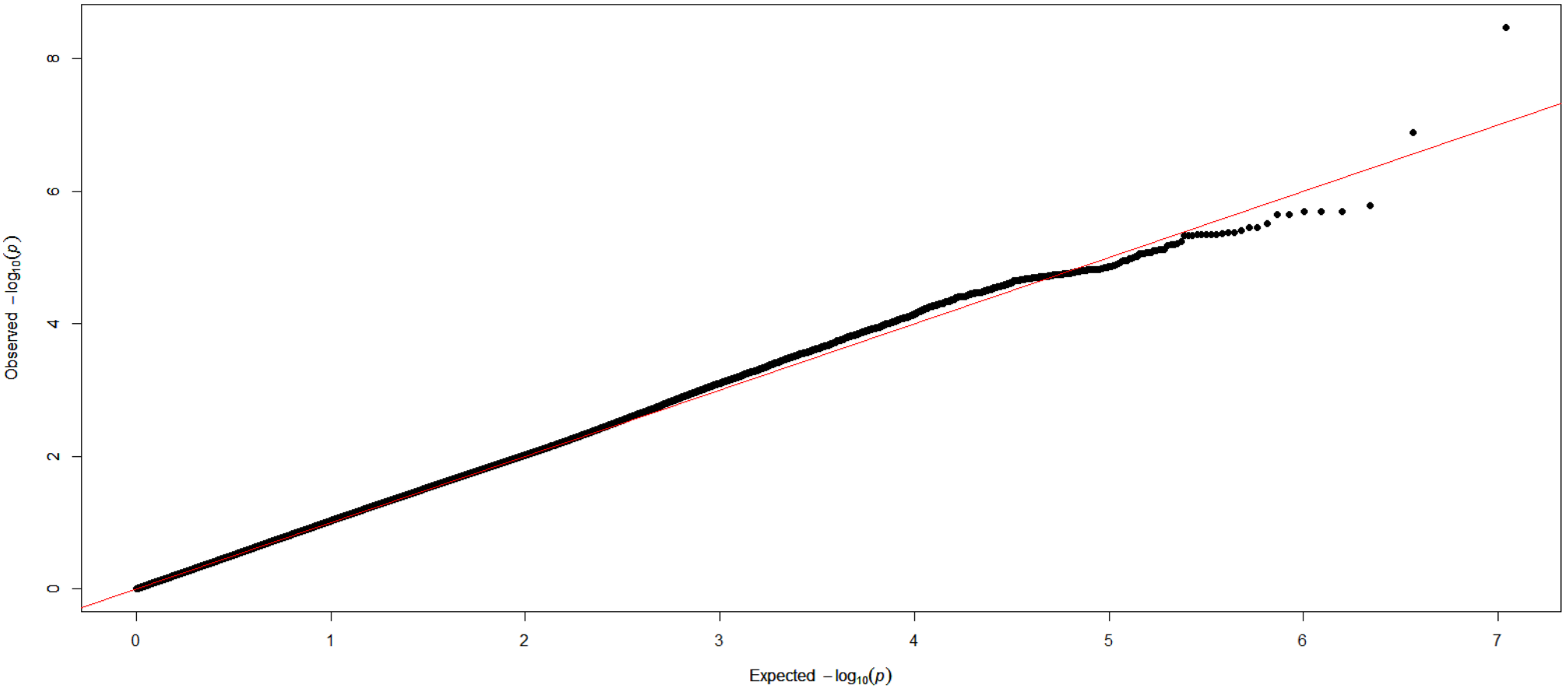


Figure 5.5 Manhattan plot of association results for the combined cohort

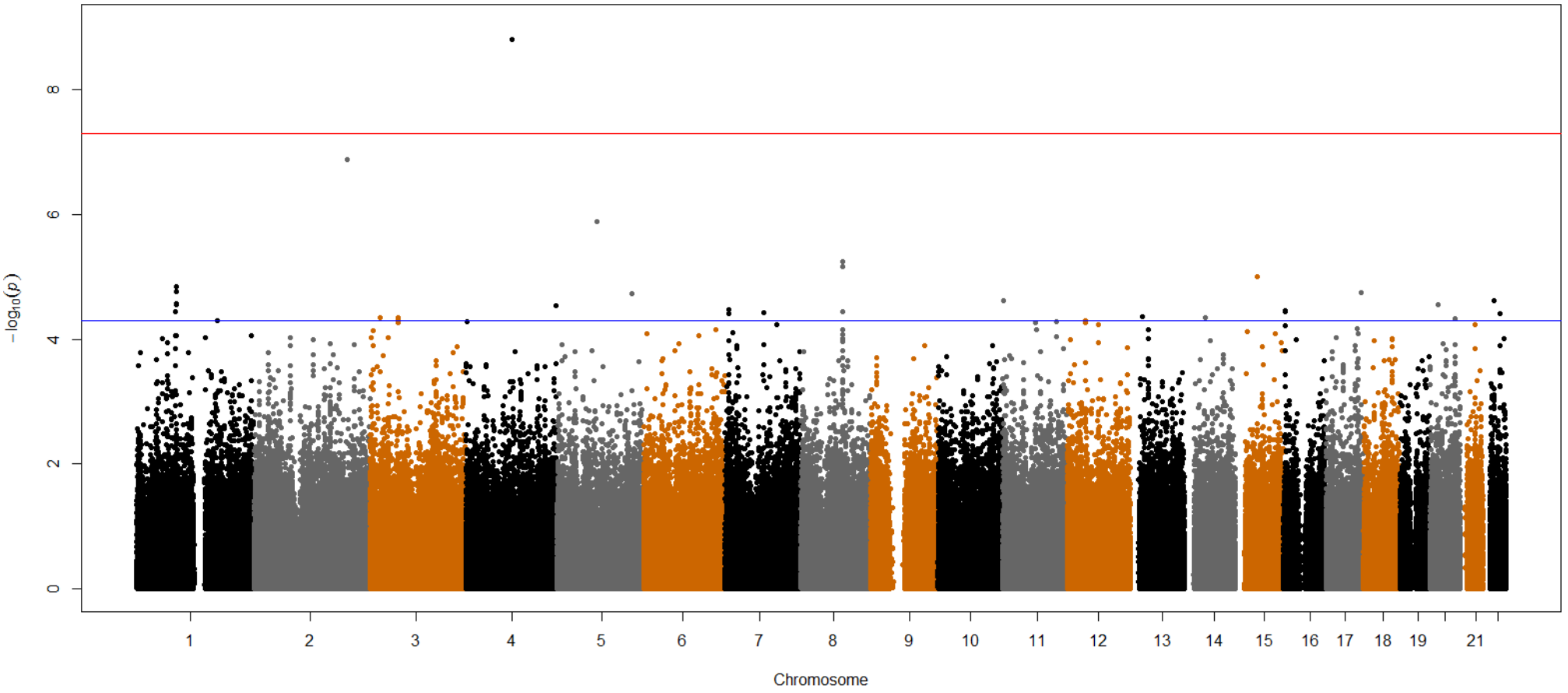


Figure 5.6 Manhattan plot of association results for the combined imputed cohort

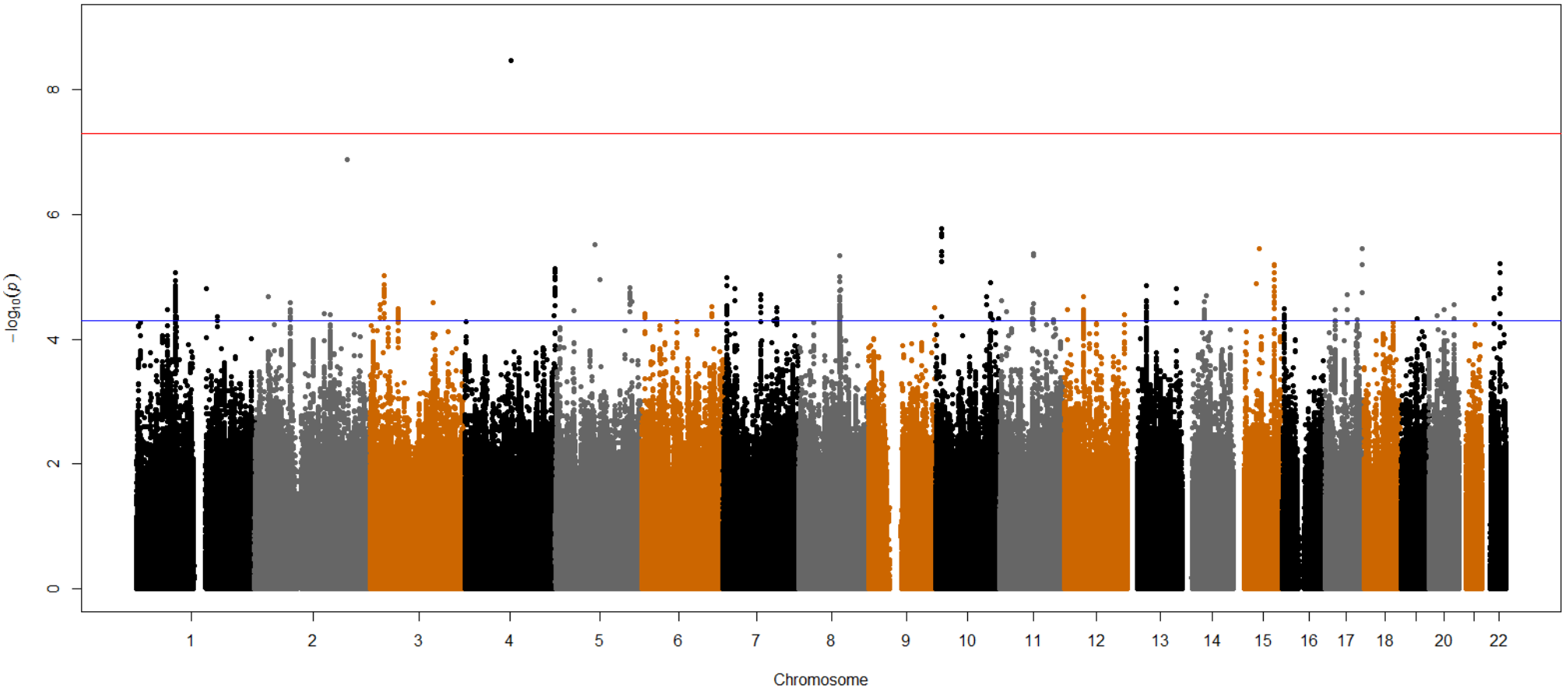
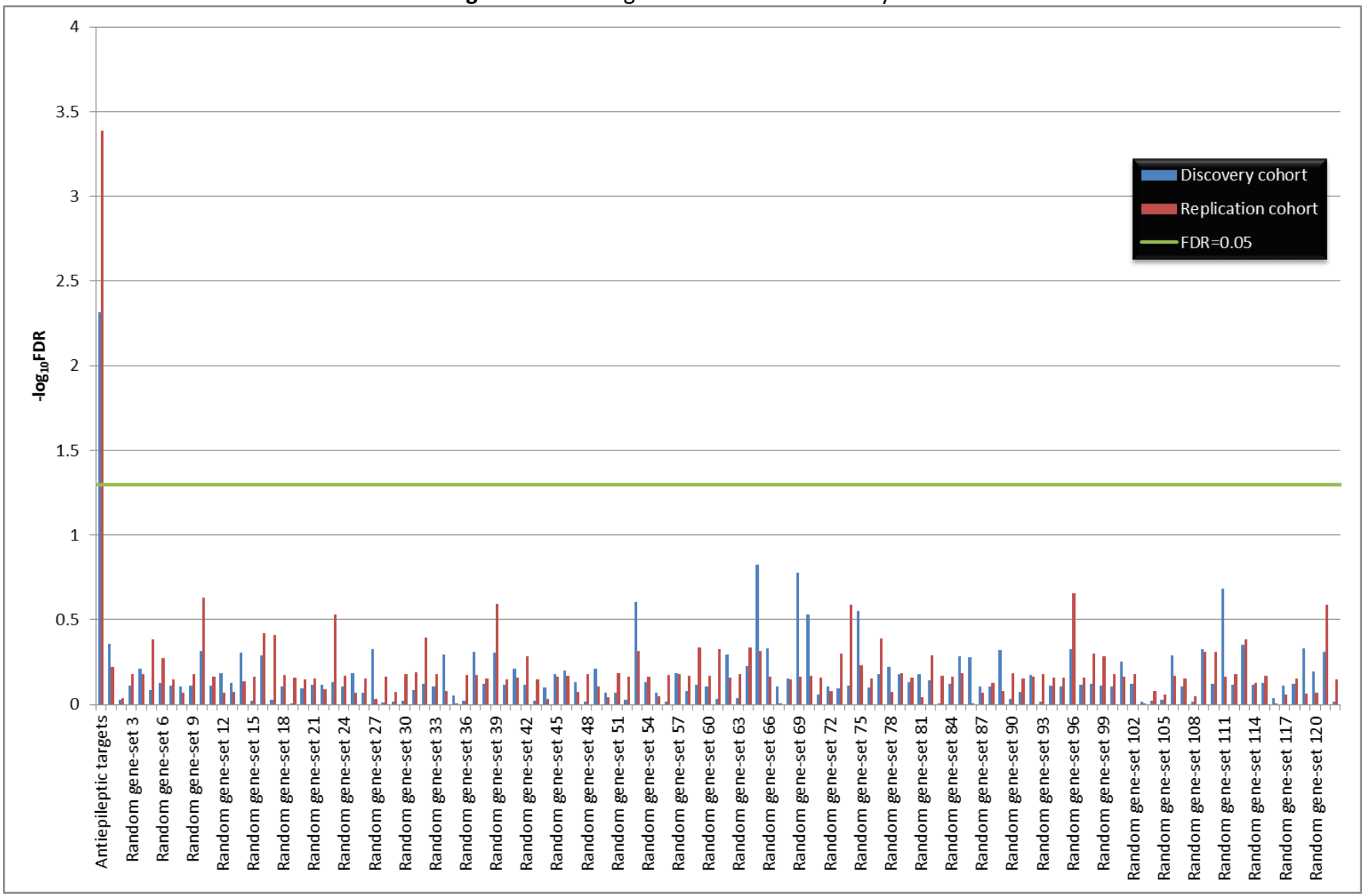


Figure 5.7 GWAS gene-set enrichment analysis



5.4.3 Gene-set and pathway level replication of results

The gene-set analysis p-values for the AED-targets gene-set and 122 random gene-sets were highly significantly correlated between the discovery and replication cohorts (Pearson's correlation coefficient=0.8, one-sided p-value<2.2×10^{-16}).

For the Reactome pathway enrichment analysis, 49 pathways were enriched in the discovery cohort and 73 pathways were enriched in the replication cohort. Out of the top 10 discovery cohort enriched pathways, all were replicated in the replication cohort; out of the top 20 discovery cohort enriched pathways, 17 were replicated in the replication cohort. In total 28 of the discovery cohort enriched pathways were replicated in the replication cohort (overlap hypergeometric p-value <3.7×10^{-17}).

The combined GWAS was also used to perform pathway enrichment analysis. The top 10 pathways are listed in Table 5.3, while the rest can be found in Appendix 12.

Table 5.3 Top 10 enriched Reactome pathways for the combined GWAS analysis. FDR=False Discovery Rate

Pathway	FDR
Axon guidance	8.62×10^{-10}
Developmental biology	3.33×10^{-8}
Neuronal system	2.79×10^{-8}
Signalling by Rho GTPases	2.29×10^{-8}
Transmembrane transport of small molecules	3.67×10^{-7}
NRAGE signals death through JNK	4.10×10^{-7}
Netrin1 signalling	1.86×10^{-6}
Transmission across chemical synapses	1.24×10^{-5}
Cell death signalling via NRAGE, NRIF and NADE	3.27×10^{-5}
G_{12}/G_{13} α subunits signalling events	3.26×10^{-5}

5.4.4 Identifying a Network of Pathways

Enrichment Map analysis revealed a highly interconnected central network of pathways (Figure 5.8). In this central network, each pathway is directly connected to, on average, 8.4 other pathways. In order to identify the most central 'hub' pathways, 'betweeness centrality' network analysis was performed; the results are illustrated in Figure 5.9 and the top 10 pathways are listed in Table 5.4. The complete ranked list can be found in Appendix 12.

Table 5.4 The top 10 pathways according to betweenness centrality

Pathway	Betweenness Centrality
Signalling by NGF	0.343469
DSCAM interactions	0.273255
Transmembrane transport of small molecules	0.238506
Developmental biology	0.185783
Signalling by GPCR	0.183818
Haemostasis	0.085772
Glucagon signalling in metabolic regulation	0.070287
PKA mediated phosphorylation of CREB	0.0569
G-α-z signalling events	0.056336
Neuronal system	0.05371

Figure 5.8 Central network of pathways enriched in combined GWA analysis results

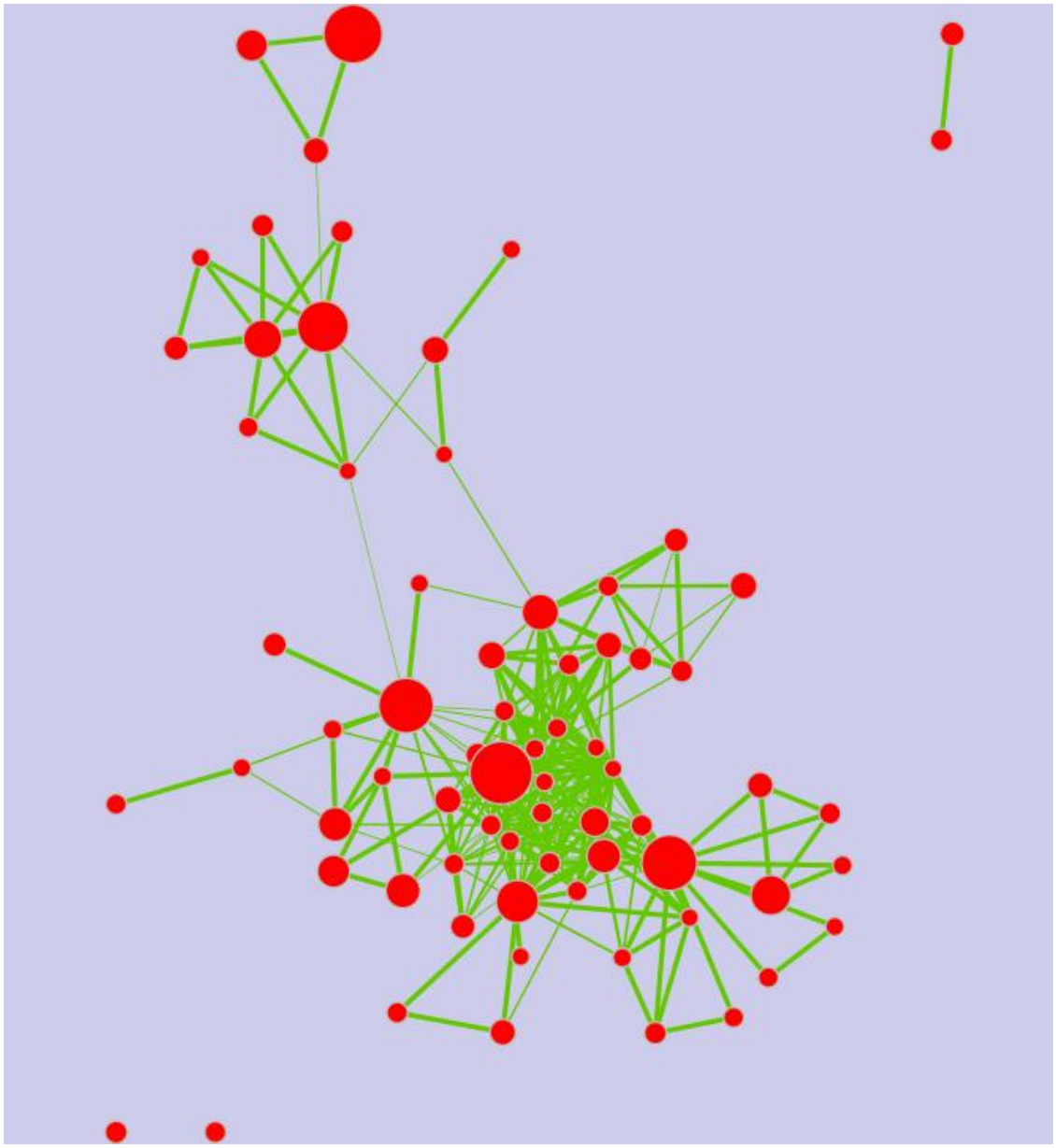
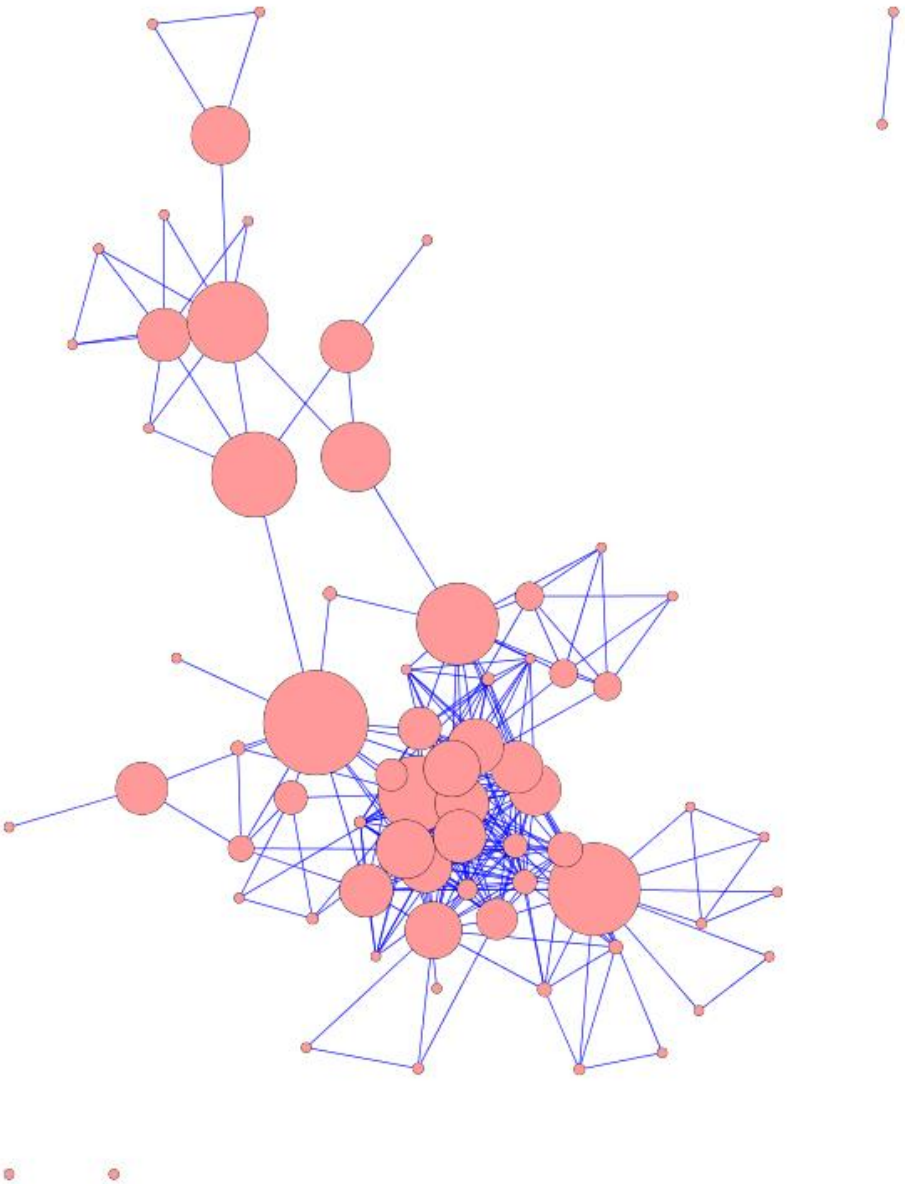


Figure 5.9 The central network of interconnected pathways. Node sizes are proportional to their betweenness centrality.



5.5 Discussion

We have performed the first GWAS of pharmacoresistant partial epilepsy.

One SNP (rs35452866) reached genome-wide significance in the combined 'mega-analysis'. rs35452866 is an intergenic SNP. Its flanking genes (PDHA2 and COX7AP2) have no known role in epilepsy, and its neighbouring SNPs did not reach a genome-wide or suggestive level of significance (Figures 5.5 and 5.6). It would be advisable, therefore, to exercise caution in interpreting the importance to this SNPs, until it is replicated in future independent studies. We went on to perform a gene-set or pathway-level analysis.

5.5.1 Validation of GWAS Gene-Set Analysis

Although GWAS gene-set enrichment analysis is an increasingly utilized tool, we are not aware of any published study demonstrating objectively that this technique can successfully discriminate functionally relevant from irrelevant gene-sets. In previously published studies, the assertion that significantly enriched gene-sets are truly linked to the disease phenotype has been based on conjecture, sometimes supported by evidence picked from a literature-review. However, the use of an inappropriate clinical cohort, genotyping errors, or employing a flawed GWAS gene-set analysis protocol could all render the GWAS gene-set enrichment analysis results unreliable.

We, therefore, devised a novel strategy to show that both our discovery and replication GWAS analyses results are enriched with a gene-set of obvious objective relevance to drug-resistant epilepsy: the targets of antiepileptic drugs. This gene-set was created in an unsupervised and unbiased manner using three different publically-available drug-target databases. This gene-set was significantly enriched in both our discovery and replication cohorts. We divided the rest of the genome into 122 random gene-sets of the same size as the antiepileptic-targets gene-set: none of these random gene-sets was enriched in either the discovery or the replication dataset. We consider this to be an objective validation of our clinical cohorts and of the GWAS gene-set analysis technique.

5.5.2 Gene-set and pathway level replication of results

Although there was no overlap between the suggestive SNPs in the discovery and replication cohorts, there was significant replication of results at the gene-set and pathway level. There was a striking correlation (Figure 5.7) between the discovery and replication

cohorts in the gene-set analysis results for the AED-targets gene-set and 122 random gene-sets (Pearson's correlation coefficient=0.8, one-sided p-value<$2.2x10^{-16}$). Furthermore, out of the top 10 discovery cohort enriched pathways, all were replicated in the replication cohort; and out of the top 20 discovery cohort enriched pathways, 17 were replicated in the replication cohort. Overall, there was a highly significant overlap between the enriched pathways for the discovery and replication cohorts (hypergeometric distribution p-value <$3.7x10^{-17}$).

5.5.3 Identifying a Network of Pathways and Hub Pathways

The Reactome pathways enriched in the GWAS (Table 5.3 and Appendix 12) could be deemed to represent disparate and disconnected processes, for example 'axon guidance', 'transmembrane transport of small molecules' and 'cell death signalling via NRAGE, NRIF and NADE'. Using the Enrichment Map utility, we have shown that these enriched pathways in fact form a highly interconnected central network (Figure 5.8). In this central network, each pathway is directly connected, on average, to 8.4 other pathways. The seemingly unrelated pathways, therefore, form a coherent whole and it can be expected that changes in one pathway in this network will have a cascading effect on the rest of the network.

Of particular importance in this network are likely to be the 'hub pathways' identified using betweenness centrality network analysis. Betweenness is a measure of the centrality of a node in a network, and is calculated as the fraction of shortest paths between node pairs that pass through the node of interest. Hence, betweenness is a measure of the influence a node has over the spread of information through the network.

5.5.4 The most important pathways: a literature review

The ten most enriched pathways are shown in Table 5.3 and the ten most important pathways according to 'betweenness centrality' are listed in Table 5.4. It is remarkable that the enriched and central pathways identified are directly related to the formation and function of neuronal and supporting tissues. The relevance of pathways such as 'axon guidance', 'neuronal system' and 'transmission across chemical synapses' is self-evident. We will examine below the evidence from literature in support of a role in epilepsy pharmacoresistance for a number of the other pathways.

One of the most enriched pathways is 'signalling by Rho GTPases'. The Rho family of GTPases and related molecules play an important role in various aspects of neuronal development, including neurite outgrowth and differentiation, axon path finding, and dendritic spine formation and maintenance (Govek *et al.* 2005). A recent GWAS found a SNP within the Rho GTPase ARHGAP11B to be associated with epilepsy prognosis at a suggestive level of significance (Speed *et al.* 2013). Genetic variants in *ARHGEF9* and *ARHGEF6*, both members of the Rho GTPase signalling pathway, have been associated with early infantile epileptic encephalopathy (Harvey *et al.* 2004) and mental retardation with refractory seizures (Shimojima *et al.* 2011), respectively.

One of the enriched pathways is 'PPAR-α activates gene expression'. Peroxisome proliferator-activated receptor-α (PPAR-α) is one of three subtypes of the nuclear receptor PPAR family. PPAR-α is expressed at functionally significant levels in neuronal tissue (Cullingford 2008). PPAR-α may have antiepileptic properties via modulation of gene expression. PPAR-α can regulate genes encoding enzymes of neurotransmitter metabolism in the brain (Cullingford 2004). It can also regulate inflammatory pathways (Delerive *et al.* 2001; Moraes *et al.* 2006). In addition, PPAR-α directly interacts with proteins involved in acute voltage-dependent neuronal responses (LoVerme *et al.* 2006). Hence, PPAR-α may favourably perturb neurotransmitter concentrations (Cullingford 2004); PPAR-α-induced anti-inflammatory actions may protect against convulsion-induced cell damage (Chen *et al.* 2007); and PPAR-α-induced action on membrane channels may favourably perturb the nerve-cell membrane potential. In keeping with these observations, a single dose of PPAR-α agonist WY14643 or chronic administration of PPAR-α-agonist fenofibrate significantly reduces behavioural and EEG expression of nicotine-induced seizures (Puligheddu *et al.* 2013). PPAR-α exhibits a broad ligand tolerance, including valproate, phenytoin, and polyunsaturated fatty acids (as present in the ketogenic diet) (Cullingford 2008). Thus PPAR-α has potential relevance to the mechanism of several antiepileptic classes. Finally, it is worth mentioning that common human PPAR-α polymorphisms, such as L162V, lead to different basal and ligand-induced PPAR-α responses (Sapone *et al.* 2000) and, hence, are of

potential relevance in patients exhibiting diverse responses to the ketogenic diet and antiepileptics.

Two other significantly enriched pathways are 'cell death signalling via NRAGE, NRIF and NADE' and 'NRAGE signals death through JNK'. One of the most central pathways was 'Signalling by NGF'. The p75 neurotrophin receptor ($p75^{NTR}$) is a key regulator of neuronal apoptosis, both during development and after injury. Apoptosis is triggered by binding of either mature neurotrophin, for example nerve growth factor (NGF), or proneurotrophin, for example proNGF, and requires activation of c-JUN N-terminal Kinase (JNK). The $p75^{NTR}$ signals by recruiting intracellular binding proteins. Many proteins have been identified that can interact with the $p75^{NTR}$ intracellular domain, and several of these proteins have been implicated in apoptotic signalling, such as neurotrophin receptor-interacting factor (NRIF), $p75^{NTR}$-associated Cell Death Executor (NADE) and neurotrophin receptor–interacting MAGE homolog (NRAGE). NRIF is required for $p75^{NTR}$-mediated apoptosis following seizures, as pilocarpine treatment of $NRIF^{-/-}$ mice yields significantly fewer dying neurons compared with wild-type mice (Volosin *et al.* 2008). NADE can be co-induced with $p75^{NTR}$ following kainate-induced seizures (Yi *et al.* 2003), and may also play a role in cell death signalling. In addition, NRAGE has been detected in a complex with NRIF following proNGF stimulation (Volosin *et al.* 2008). Therefore, these $p75^{NTR}$ adapter proteins may act together in a complex to stimulate downstream apoptotic signalling (Friedman 2010). Genetic variants in *NGF* genes have been associated with a neurological disorder hereditary sensory and autonomic neuropathy type V (Einarsdottir *et al.* 2004; Carvalho *et al.* 2011).

One of the most significantly enriched pathways was 'Netrin1 signalling' and two central pathways were 'DCC mediated attractive signalling' and 'DSCAM interactions'. All of these pathways play essential roles in appropriate axon guidance within the hippocampus. Proper axonal targeting is fundamental to the establishment of functional neural circuits. In temporal lobe epilepsy, there is aberrant axonal targeting, known as mossy fibre sprouting, which results in the formation of hyperexcitable recurrent networks (Muramatsu *et al.* 2010). Netrins are secreted proteins that play an important role in neuronal migration and in axon guidance. Netrin1 is the most studied member of the family and has been shown to

play a crucial role in neuronal navigation through its interaction with its receptors. In the human hippocampus, netrin1 attracts mossy fibres to CA3, which is their normal target under physiological conditions (Muramatsu *et al.* 2010). The deleted in colorectal cancer receptor (DCC) is required for netrin-induced axon attraction (Muramatsu *et al.* 2010). Another netrin1 receptor is the Down syndrome cell adhesion molecule (DSCAM), which induces dendritic arborization. DSCAM expression in the epileptic foci of intractable epilepsy patients is significantly higher compared to controls (Shen *et al.* 2011). In addition, DSCAM is also highly expressed in the rat brain during the different phases of the epileptogenic and epileptic process; this suggests that DSCAM may be involved in the generation and the development of pharmacoresistant epilepsy (Shen *et al.* 2011). Furthermore, DSCAM was associated with susceptibility to epilepsy at a suggestive level of significance in a recent GWAS (Guo *et al.* 2012).

5.6 Conclusions

We have performed the first ever GWAS of pharmacoresistant partial epilepsy, and the first ever objective validation of the GWAS gene-set analysis approach. We have identified the most significant polymorphisms and pathways underlying pharmacoresistant partial epilepsy, and we have demonstrated that there is significant replication of the most significant pathways. Furthermore, we have shown that the enriched pathways form a highly interconnected central network and, finally, we have identified the hub pathways in this network.

Potential future avenues of research, for further prioritizing from amongst the pathways identified in this work, would involve identifying 'causal' pathways (which we aim to do in Chapter 7) and performing functional studies in animal models of epilepsy to determine if genetic manipulation of key pathways leads to changes in drug responsiveness.

5.7 References

(1981) Proposal for revised clinical and electroencephalographic classification of epileptic seizures. From the Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* **22**, 489-501.

(1989) Proposal for revised classification of epilepsies and epileptic syndromes. Commission on Classification and Terminology of the International League Against Epilepsy. *Epilepsia* **30**, 389-99.

Bezzina C.R., Barc J., Mizusawa Y., Remme C.A., Gourraud J.B., Simonet F., Verkerk A.O., Schwartz P.J., Crotti L., Dagradi F., Guicheney P., Fressart V., Leenhardt A., Antzelevitch C., Bartkowiak S., Schulze-Bahr E., Zumhagen S., Behr E.R., Bastiaenen R., Tfelt-Hansen J., Olesen M.S., Kaab S., Beckmann B.M., Weeke P., Watanabe H., Endo N., Minamino T., Horie M., Ohno S., Hasegawa K., Makita N., Nogami A., Shimizu W., Aiba T., Froguel P., Balkau B., Lantieri O., Torchio M., Wiese C., Weber D., Wolswinkel R., Coronel R., Boukens B.J., Bezieau S., Charpentier E., Chatel S., Despres A., Gros F., Kyndt F., Lecointe S., Lindenbaum P., Portero V., Violleau J., Gessler M., Tan H.L., Roden D.M., Christoffels V.M., Le Marec H., Wilde A.A., Probst V., Schott J.J., Dina C. & Redon R. (2013) Common variants at SCN5A-SCN10A and HEY2 are associated with Brugada syndrome, a rare disease with high risk of sudden cardiac death. *Nat Genet*.

Chen X.R., Besson V.C., Palmier B., Garcia Y., Plotkine M. & Marchand-Leroux C. (2007) Neurological recovery-promoting, anti-inflammatory, and anti-oxidative effects afforded by fenofibrate, a PPAR alpha agonist, in traumatic brain injury. *J Neurotrauma* **24**, 1119-31.

Cullingford T. (2008) Peroxisome proliferator-activated receptor alpha and the ketogenic diet. *Epilepsia* **49 Suppl 8**, 70-2.

Cullingford T.E. (2004) The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. *Prostaglandins Leukot Essent Fatty Acids* **70**, 253-64.

Delaneau O., Zagury J.F. & Marchini J. (2013) Improved whole-chromosome phasing for disease and population genetic studies. *Nat Methods* **10**, 5-6.

Delerive P., Fruchart J.C. & Staels B. (2001) Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* **169**, 453-9.

Duerr R.H., Taylor K.D., Brant S.R., Rioux J.D., Silverberg M.S., Daly M.J., Steinhart A.H., Abraham C., Regueiro M., Griffiths A., Dassopoulos T., Bitton A., Yang H., Targan S., Datta L.W., Kistner E.O., Schumm L.P., Lee A.T., Gregersen P.K., Barmada M.M., Rotter J.I., Nicolae D.L. & Cho J.H. (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* **314**, 1461-3.

Fridley B.L. & Biernacka J.M. (2011) Gene set analysis of SNP data: benefits, challenges, and future directions. *Eur J Hum Genet* **19**, 837-43.

Friedman W.J. (2010) Proneurotrophins, seizures, and neuronal apoptosis. *Neuroscientist* **16**, 244-52.

Govek E.E., Newey S.E. & Van Aelst L. (2005) The role of the Rho GTPases in neuronal development. *Genes Dev* **19**, 1-49.

Guo Y., Baum L.W., Sham P.C., Wong V., Ng P.W., Lui C.H., Sin N.C., Tsoi T.H., Tang C.S., Kwan J.S., Yip B.H., Xiao S.M., Thomas G.N., Lau Y.L., Yang W., Cherny S.S. & Kwan P. (2012) Two-stage genome-wide association study identifies variants in CAMSAP1L1 as susceptibility loci for epilepsy in Chinese. *Hum Mol Genet* **21**, 1184-9.

Holmans P. (2010) Statistical methods for pathway analysis of genome-wide data for association with complex genetic traits. *Adv Genet* **72**, 141-79.

Howie B.N., Donnelly P. & Marchini J. (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet* **5**, e1000529.

Johnson M.R., Milne R.L., Torn-Broers Y., Hopper J.L., Scheffer I.E. & Berkovic S.F. (2003) A twin study of genetic influences on epilepsy outcome. *Twin Res* **6**, 140-6.

Kwan P., Arzimanoglou A., Berg A.T., Brodie M.J., Allen Hauser W., Mathern G., Moshe S.L., Perucca E., Wiebe S. & French J. (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. *Epilepsia* **51**, 1069-77.

Lee S., Wright F.A. & Zou F. (2011) Control of population stratification by correlation-selected principal components. *Biometrics* **67**, 967-74.

LoVerme J., Russo R., La Rana G., Fu J., Farthing J., Mattace-Raso G., Meli R., Hohmann A., Calignano A. & Piomelli D. (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor-alpha. *J Pharmacol Exp Ther* **319**, 1051-61.

Maher B. (2008) Personal genomes: The case of the missing heritability. *Nature* **456**, 18-21.

Manolio T.A., Brooks L.D. & Collins F.S. (2008) A HapMap harvest of insights into the genetics of common disease. *J Clin Invest* **118**, 1590-605.

Marchini J. & Howie B. (2010) Genotype imputation for genome-wide association studies. *Nat Rev Genet* **11**, 499-511.

Marson A.G., Al-Kharusi A.M., Alwaidh M., Appleton R., Baker G.A., Chadwick D.W., Cramp C., Cockerell O.C., Cooper P.N., Doughty J., Eaton B., Gamble C., Goulding P.J., Howell S.J., Hughes A., Jackson M., Jacoby A., Kellett M., Lawson G.R., Leach J.P., Nicolaides P., Roberts R., Shackley P., Shen J., Smith D.F., Smith P.E., Smith C.T., Vanoli A. & Williamson P.R. (2007a) The SANAD study of effectiveness of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, or topiramate for treatment of partial epilepsy: an unblinded randomised controlled trial. *Lancet* **369**, 1000-15.

Marson A.G., Al-Kharusi A.M., Alwaidh M., Appleton R., Baker G.A., Chadwick D.W., Cramp C., Cockerell O.C., Cooper P.N., Doughty J., Eaton B., Gamble C., Goulding P.J., Howell S.J., Hughes A., Jackson M., Jacoby A., Kellett M., Lawson G.R., Leach J.P., Nicolaides P., Roberts R., Shackley P., Shen J., Smith D.F., Smith P.E., Smith C.T., Vanoli A. & Williamson P.R. (2007b) The SANAD study of effectiveness of valproate, lamotrigine, or topiramate for generalised and unclassifiable epilepsy: an unblinded randomised controlled trial. *Lancet* **369**, 1016-26.

McCarthy M.I., Abecasis G.R., Cardon L.R., Goldstein D.B., Little J., Ioannidis J.P. & Hirschhorn J.N. (2008) Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat Rev Genet* **9**, 356-69.

Merico D., Isserlin R., Stueker O., Emili A. & Bader G.D. (2010) Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One* **5**, e13984.

Moraes L.A., Piqueras L. & Bishop-Bailey D. (2006) Peroxisome proliferator-activated receptors and inflammation. *Pharmacol Ther* **110**, 371-85.

Muramatsu R., Nakahara S., Ichikawa J., Watanabe K., Matsuki N. & Koyama R. (2010) The ratio of 'deleted in colorectal cancer' to 'uncoordinated-5A' netrin-1 receptors on the growth cone regulates mossy fibre directionality. *Brain* **133**, 60-75.

Nam D., Kim J., Kim S.Y. & Kim S. (2010) GSA-SNP: a general approach for gene set analysis of polymorphisms. *Nucleic Acids Res* **38**, W749-54.

Patterson N., Price A.L. & Reich D. (2006) Population structure and eigenanalysis. *PLoS Genet* **2**, e190.

Pirinen M., Donnelly P. & Spencer C.C. (2012) Including known covariates can reduce power to detect genetic effects in case-control studies. *Nat Genet* **44**, 848-51.

Puligheddu M., Pillolla G., Melis M., Lecca S., Marrosu F., De Montis M.G., Scheggi S., Carta G., Murru E., Aroni S., Muntoni A.L. & Pistis M. (2013) PPAR-alpha agonists as novel antiepileptic drugs: preclinical findings. *PLoS One* **8**, e64541.

Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira M.A., Bender D., Maller J., Sklar P., de Bakker P.I., Daly M.J. & Sham P.C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**, 559-75.

Raelson J.V., Little R.D., Ruether A., Fournier H., Paquin B., Van Eerdewegh P., Bradley W.E., Croteau P., Nguyen-Huu Q., Segal J., Debrus S., Allard R., Rosenstiel P., Franke A., Jacobs G., Nikolaus S., Vidal J.M., Szego P., Laplante N., Clark H.F., Paulussen R.J., Hooper J.W., Keith T.P., Belouchi A. & Schreiber S. (2007) Genome-wide association study for Crohn's disease in the Quebec Founder Population identifies multiple validated disease loci. *Proc Natl Acad Sci U S A* **104**, 14747-52.

Sapone A., Peters J.M., Sakai S., Tomita S., Papiha S.S., Dai R., Friedman F.K. & Gonzalez F.J. (2000) The human peroxisome proliferator-activated receptor alpha gene: identification and functional characterization of two natural allelic variants. *Pharmacogenetics* **10**, 321-33.

Shen L., Xiao Z., Pan Y., Fang M., Li C., Chen D., Wang L., Xi Z., Xiao F. & Wang X. (2011) Altered expression of Dscam in temporal lobe tissue from human and experimental animals. *Synapse* **65**, 975-82.

Speed D., Hoggart C., Petrovski S., Tachmazidou I., Coffey A., Jorgensen A., Eleftherohorinou H., De Iorio M., Todaro M., De T., Smith D., Smith P.E., Jackson M., Cooper P., Kellett M., Howell S., Newton M., Yerra R., Tan M., French C., Reuber M., Sills G.E., Chadwick D., Pirmohamed M., Bentley D., Scheffer I., Berkovic S., Balding D., Palotie A., Marson A., O'Brien T.J. & Johnson M.R. (2013) A genome-wide association study and biological pathway analysis of epilepsy prognosis in a prospective cohort of newly treated epilepsy. *Hum Mol Genet*.

Volosin M., Trotter C., Cragnolini A., Kenchappa R.S., Light M., Hempstead B.L., Carter B.D. & Friedman W.J. (2008) Induction of proneurotrophins and activation of p75NTR-mediated apoptosis via neurotrophin receptor-interacting factor in hippocampal neurons after seizures. *J Neurosci* **28**, 9870-9.

Wang K., Li M. & Bucan M. (2007) Pathway-based approaches for analysis of genomewide association studies. *Am J Hum Genet* **81**, 1278-83.

Wang K., Li M. & Hakonarson H. (2010) Analysing biological pathways in genome-wide association studies. *Nat Rev Genet* **11**, 843-54.

Wang K., Zhang H., Kugathasan S., Annese V., Bradfield J.P., Russell R.K., Sleiman P.M., Imielinski M., Glessner J., Hou C., Wilson D.C., Walters T., Kim C., Frackelton E.C., Lionetti P., Barabino A., Van Limbergen J., Guthery S., Denson L., Piccoli D., Li M., Dubinsky M., Silverberg M., Griffiths A., Grant S.F., Satsangi J., Baldassano R. & Hakonarson H. (2009) Diverse genome-wide association studies associate the IL12/IL23 pathway with Crohn Disease. *Am J Hum Genet* **84**, 399-405.

Yi J.S., Lee S.K., Sato T.A. & Koh J.Y. (2003) Co-induction of p75(NTR) and the associated death executor NADE in degenerating hippocampal neurons after kainate-induced seizures in the rat. *Neurosci Lett* **347**, 126-30.

Zheng X., Levine D., Shen J., Gogarten S.M., Laurie C. & Weir B.S. (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics* **28**, 3326-8.

CHAPTER SIX

GENETIC REGULATION OF GENE EXPRESSION IN THE PHARMACORESISTANT EPILEPTIC HUMAN HIPPOCAMPUS

Contents

Chapter 6: Genetic Regulation of Gene Expression in the Pharmacoresistant Epileptic Human Hippocampus

6.1 Introduction

Genetic contribution to phenotypic diversity can result from changes in amounts of proteins as from functional modifications in them (Li & Burmeister 2005). Studies in animal and human tissues have revealed that the expression of a high percentage of genes is substantially influenced by DNA variants (Romanoski *et al.* 2010; van Nas *et al.* 2010; Orozco *et al.* 2012). Most significant SNPs in genome-wide association studies (GWAS) map outside protein-coding regions, and >75% of significant GWAS SNPs map to functional regulatory elements that have been identified in the Encyclopedia of DNA Elements (ENCODE) project (Schaub *et al.* 2012). Also, in contrast to polymorphisms that change the amino acid sequence of genes, genetic variants that are responsible for changes in gene expression might have more subtle phenotypic effects that better explain complex genetic diseases. These observations suggest that genetic variants that alter gene expression, rather than variants that alter protein sequences, form the primary basis of natural variation in complex traits. Hence, genetic variation driving gene expression is likely to be integral to the pathogenic processes active within the pharmacoresistant epileptic human hippocampus.

We have performed whole-genome genotyping and transcriptome-expression analysis on a series of control and pharmacoresistant epileptic human hippocampal samples to reveal expression quantitative trait loci (eQTLs) that influence gene expression within the pharmacoresistant epileptic human hippocampus. eQTLs are genomic sequence variants (as determined by genome-wide polymorphism analysis) that correlate with gene-expression levels (as determined by genome-wide microarray analysis). eQTL studies are similar to traditional genetic-association studies, but instead of associating genetic variants with discrete traits such as disease status, eQTL studies correlate genetic variants with quantitative gene-expression levels. The outputs from this type of analysis are SNP-transcript pairs in which gene expression is correlated with genotype in a dose-dependent fashion. 'Cis' associations are between a gene's expression level and a nearby SNP (one located within an arbitrarily defined distance, such as 500kb). 'Trans' associations include all

non-cis pairs, and can be between the expression of a gene on one chromosome and a SNP located on another chromosome.

In complex diseases, truly trait-associated SNPs are more likely to be eQTLs (Nicolae *et al.* 2010), and it has been suggested that eQTL analysis is a powerful approach for the detection of novel disease risk loci (Schadt 2005). Furthermore, determining the relationship between disease-associated SNPs and gene expression levels offers potential insights into the pathological mechanisms associated with these SNPs.

It has been shown that 69% to 80% of eQTLs operate in a cell-type specific manner (Dimas *et al.* 2009). We are aware of only one previously published eQTL study on human hippocampal tissue (Kim *et al.* 2012). This study was limited to cis-eQTL analysis. There are currently no published trans-eQTL analyses of hippocampal tissue, and no eQTL analyses of the epileptic human hippocampus. Our study not only examines eQTLs in hippocampal tissue, but specifically in samples with the disease, a strategy which may be necessary for the identification of the disease-related eQTLs (Ertekin-Taner 2011).

6.2 Aims

- To identify eQTLs within the pharmacoresistant epileptic human hippocampus.
- To validate the identified eQTLs using previously published data.
- To determine if the eQTLs are relevant to disease by establishing if
    - eQTLs are enriched within disease-associated SNPs.
    - more eQTLs converge on differentially-expressed than non-differentially-expressed genes
- To identify the most important eQTLs and determine the biological processes enriched within these eQTLs.
- To illustrate the practical utility of our eQTL dataset by using it to prioritize GWAS SNPs and reveal their functional implications.

6.3 Methods

The normalized microarray gene expression data generated previously (Chapter 3) was utilized for the eQTL analysis. The methods used for generating this data are repeated below for the reader's convenience.

6.3.1 Sample collection

Samples used in the study originated from three UK sites: the Walton Centre for Neurology and Neurosurgery in Liverpool, the Salford Royal Hospital in Salford and the Southern General Hospital in Glasgow. We aimed to recruit patients of age 5 years and older with pharmacoresistant mesial temporal lobe epilepsy of at least 3 months durations for which a therapeutic temporal lobectomy was being undertaken. As recently proposed by the ILAE, pharmacoresistance was defined as the failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom. The diagnosis of MTLE was made by the treating clinician based on seizure semiology, MRI brain and EEG characteristics being consistent with a seizure focus in the mesial temporal lobe. Patients suspected of having a neoplastic or malignant temporal lobe lesion were excluded. After surgery, the hippocampus was divided into two portions: (1) one portion was preserved for RNA isolation, (2) the other portion underwent histological analysis by an experienced neuropathologist. Any subjects found to have a neoplastic or malignant lesion on histological analysis were excluded. The portion preserved for RNA isolation was either stored in RNAlater (Liverpool and Glasgow) or frozen directly at -80^{o}C (Salford). It should be noted that the quality of the RNA generated from the directly frozen brain samples was not significantly different from RNAlater-preserved samples: mean RIN values were 7.82 and 7.25 respectively, and two-sample t-test p-value for difference between group RINs was 0.06554.

Frozen post-mortem histologically-normal hippocampal samples from donors with no known brain diseases were obtained from the MRC Edinburgh Brain Bank (Edinburgh, UK) and the Queen Square Brain Bank (London, UK).

6.3.2 RNA isolation

Brain samples were disrupted and homogenized in an appropriate volume of QIAzol lysis reagent (Qiagen, Crawley, United Kingdom) by using a TissueRuptor handheld rotor-stator homogenizer (Qiagen, Crawley, United Kingdom). Total RNA was extracted from the homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. RNA quality was examined by capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) and Agilent 2100

Expert software was used to calculate the RNA Integrity number (RIN) of each sample. Purity of the RNA sample was assessed using a NanoDrop1000 Spectrophotometer. Capillary electrophoresis traces were also examined. Samples with RNA integrity number scores (RIN) below 6, obvious RNA degradation, significant 18S or 28S ribosomal RNA degradation, ratio of absorbance at 260nm and 280nm <1.95, or with noticeable DNA or background contaminants did not pass QC, and were withheld from microarray analysis. It should be noted that RNA samples with RIN scores of 6 and lower have been successfully used for 3' microarrays (Hawrylycz *et al.* 2012) and exon microarrays (Trabzuni *et al.* 2011).

6.3.3 Microarray processing and quality control

The microarrays were processed at the Centre for Genomics Research in the University of Liverpool. 50ng of total RNA was amplified and labelled using the Agilent Low Input Quick Amp One-Colour Labeling Kit and labelled RNA was hybridized to Agilent SurePrint G3 Custom Exon 8x60K Microarrays designed to contain probes for each exon of 936 selected genes, including all known SLC genes. Standard Agilent protocols were followed. The quality of the synthesized cRNA was assessed using an Agilent Bioanalyzer 2100—the resulting electropherograms were satisfactory. Each scanned image was viewed for visible artefacts, and if multiple artefacts were present, the array was rejected. Detailed QC reports were generated for each array using the Feature Extraction 11.0.1.1 Software (Agilent, Palo Alto, CA). Based on these reports, we excluded arrays in which more than 1% of features were non-uniform outliers, or the average of the net signals in negative controls was >40, or the average of the background-subtracted signals in negative controls was <-10 or >5, or the standard deviation of the background-subtracted signals in negative controls was >10, or the residual noise after spatial detrending was >15, or the median coefficient of variation for spike-in probes or non-control probes was >12%, or the dose-response curve of the spike-ins had a slope of <0.9 or >1.20, or the spike-in detection limit was <0.01 or >2. One array failed on five of these metrices and, hence, was excluded. Intensity data was extracted from the remaining arrays using the Feature Extraction Software. Spatial and multiplicative detetrending and local background substraction was applied.

We employed an unbiased approach for the detection of outlier samples: samples with average inter-array correlation (IAC) ≤ 2 standard deviations below the mean were

removed. This approach has been used successfully in published studies (Yang *et al.* 2006; Flight & Wentzell 2010; Yang *et al.* 2012).

6.3.4 Data normalization, adjustment and filtering

Data exported from Feature Extraction Software was imported into GeneSpring GX Software (Agilent, Palo Alto, CA). Features which were population outliers, saturated or non-uniform were flagged as 'Compromised' and filtered out. Non-expressed probes were filtered out: a probe was deemed expressed only if it was flagged 'Detected' in at least 25% of control or case samples. Quantile normalization was applied. As GeneSpring does not include functions that allow adjusting for confounders and covariates, probe-level intensities were exported for further processing.

Batch effect was corrected using the ComBat (Johnson *et al.* 2007) algorithm implemented in the SVA Bioconductor package. Eight arrays (four cases and four controls) were processed per slide; hence, each batch comprised the eight samples processed together on a slide. To adjust for known and unknown confounders, Independent Surrogate Variable Analysis (ISVA) (Teschendorff *et al.* 2011) was applied; RIN, age and sex were explicitly included in the ISVA adjustment. Where multiple probes mapped to the same gene, the probe with the highest variance was retained, as the most variant probe is likely to be most informative.

6.3.5 Excluding probes-with-polymorphisms (PwP)

Polymorphisms present in the probe-target sequences have been shown to alter probe hybridization affinities, leading to reduced signal intensity measurements and resulting in false-positive results (Ramasamy *et al.* 2013). We used the 'PIP Finder' tool (Ramasamy *et al.* 2013) to determine which probes in our microarray had polymorphisms with a minor allele frequency >1% in Europeans located within the probe sequences; all such probes were excluded from the eQTL analysis in keeping with current practice.

6.3.6 Assessing the influence of confounders

To determine if there was residual confounding after data adjustment, we used the phenoTest Bioconductor package to test for association between probes and confounders (batch, RIN, age and sex).

6.3.7 DNA extraction

Cortical brain samples were available for some subjects; DNA was extracted from these cortical samples using the DNeasy Blood & Tissue Kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's recommended protocol. For other subjects, for whom cortical brain samples were not available, DNA was extracted from hippocampal homogenate in QIAzol that was prepared during RNA isolation, according to the protocol provide by Qiagen: In 1ml of homogenate, 200µl of chloroform was added, shaken vigorously and incubated at room temperature for 3 minutes, followed by centrifugation at 12,000x*g* for 15 min at 4°C. The upper, aqueous phase was transferred to a new tube and stored at -80^{o}C for future use. To precipitate the DNA pellet, 0.3ml of 100% ethanol was added to the interphase and phenol phase, the sample was incubated at room temperature 3 min, and then centrifuged at 2,000x*g* for 2 min at 4°C. The DNA pellet was washed twice in sodium citrate: for each wash 1 ml sodium citrate solution was added to the pellet, followed by incubation at room temperature for 30 min, with mixing by inversion every 5 min, and then centrifugation at 2000x*g* for 5 min at 4°C. The DNA pellet was then washed in 75% ethanol: 2 ml of 75% ethanol was added to the DNA pellet, followed by incubation at room temperature for 20 min, with mixing by inversion every 5 min, and then centrifugation at 2,000x*g* for 5 min at 4°C. The DNA pellet was air-dried and then re-dissolved in 500µl of 8mM NaOH. The solution was centrifuged at 14,000x*g* for 10 min at room temperature. The supernatant, containing the DNA, was removed. 60µl of 0.1M HEPES and 5.5µl of 100mM EDTA was added to the DNA solution. Extracted DNA was quantitated using the PicoGreen® dsDNA Quantitation Kit (Invitrogen, UK).

6.3.8 Genotyping and Quality Control

All samples were genotyped on the Illumina Infinium HumanOmniExpressExome BeadChip in the ARK Genomics facility at the Roslin Institute, UK

Our quality control (QC) procedures were as follows. Individuals were excluded on the basis of discordance between reported and observed sex, SNP call rate <95%, outlying autosomal heterozygosity rates (>3 standard deviations from the mean), inadvertent subject duplication or cryptic relatedness based on pairwise identity-by-descent estimations (the individual with greater missing data was removed from each pair with π-hat >0.1875), being outliers from the European cluster in a plot of the first two principal components of

genotypes for samples from the study and HapMap Phase III. Three individuals were excluded from the eQTL analysis on the basis of these QC filters, and one further individual was excluded from the eQTL analysis having failed the microarray QC criteria. SNPs were excluded on the basis of genotype missing rate ≥5% if minor allele frequency (MAF) was ≥0.05 and missing rate ≥1% if MAF was <0.05, observed minor allele frequency <0.01, significant deviation ($P < 10^{-4}$) from Hardy-Weinberg equilibrium, significant difference ($P < 10^{-4}$) in the genotype missing rate in cases compared to controls, and all non-autosomal SNPs. 250,985 SNPs were excluded on the basis of these QC criteria. The final data-set, after QC filtering, included 22 controls and 22 cases genotyped over 550,043 SNPs.

6.3.9 Imputation

Genotype imputation was performed using Impute2(Howie *et al.* 2009), with 1000 Genomes Project haplotypes (March 2012) as the reference panel. The QCTOOL software was used to exclude SNPs with an 'info' score of <0.9. GTOOL software was then used to transform the imputed data to PED format, with a posterior probability of 0.9 used as a threshold to call genotypes. Our standard per-SNP QC filters, described above, were then applied. The final dataset included 4,663,226 genotyped and imputed SNPs.

6.3.10 eQTL analysis

eQTL analysis was performed using the linear model in the program Matrix eQTL (Shabalin 2012). To investigate potential confounding effects from population stratification, the principal components (PC) of variance of the genotyping dataset were calculated using the package SNPRelate (Zheng *et al.* 2012), and then the Tracy-Widom (TW) statistic (Patterson *et al.* 2006) and significance for the eigenvalue of each PC was calculated using the package EigenCorr (Lee *et al.* 2011). Furthermore, the top ten PCs were separately correlated with the probe expression profiles in R.

6.3.11 External validation of eQTL results

We are aware of only one previously published eQTL study on human hippocampal tissue (Kim *et al.* 2012). In this study, 61 hippocampal samples were analysed in total, obtained from post-mortem donors with schizophrenia and normal controls. This study was limited to cis-eQTL analysis. In order to perform a comparison with this study, we also performed a cis-eQTL analysis using the linear model in the program Matrix eQTL (Shabalin 2012). cis-eQTLs

were defined as eQTLs that map within 20kb upstream or downstream of the physical location of the probe on the genomic sequence. To find 'conditional' eQTLs—eQTLs which are active in either the normal or the pharmacoresistant partial epilepsy phenotype only—a further analysis was carried out which included phenotype as an interaction term in the model (Ackermann *et al.* 2013); this analysis was limited to significant cis-eQTLs only as the computational requirements for analysing all SNPs and probes were prohibitive.

We calculated the statistical significance of the overlap between the cis-eQTL genes discovered in our study and the study by Kim and colleagues.

6.3.12 Enrichment of eQTLs within significant GWAS results

We wished to determine if significant GWAS SNPs are enriched with significant eQTLs. We calculated the number of significant eQTLs within 'suggestive' GWAS SNPs (those with a p-value $<5x10^{-5}$). We calculated the statistical significance of the size of this enrichment by using permutation-based methods. There were 403 suggestive SNPs within our GWAS. We employed two permutation-based methods: (1) we generated 10,000 random sets of 403 GWAS SNPs and then determined how many sets were as (or more) enriched with eQTLs as the suggestive SNPs, and (2) the GWAS SNPs were sorted in descending order of statistical significance and divided into 10,000 sets of 403 SNPs each, and we then determined how many sets were as (or more) enriched with eQTLs as the suggestive SNPs.

6.3.13 eQTL genes and disease-associated genes under eQTL influence

We created a list of eQTL genes: eQTL SNPs within 20kb of the flanking sequence of a gene were mapped to the gene. We calculated the number of probes whose expression is effected by each eQTL gene. We also determined how many disease-associated genes (genes differentially expressed at FDR<0.05 and FC≥1.5) are under significant eQTL influence, and how many eQTL genes influence each disease-associated gene. Gene ontology analysis of the most prominent eQTL genes and targets was done using Broad Institute's Gene-Set Enrichment Analysis web-tool.

6.3.14 Utilizing eQTL data to prioritize GWAS SNPs and reveal their functional implications: practical examples

There were no SNPs associated at a genome-wide level of significance ($p<5x10^{-8}$) in our GWAS of pharmacoresistant partial epilepsy. We demonstrated how the eQTL data we have

generated can be utilised in order to select genetic variants for further study from amongst the large number of SNPs with a 'suggestive' level of significance ($p<5x10^{-5}$). We determined which of these suggestive SNPs are significant eQTLs and which of these eQTLs influence the expression of genes which are differentially-expressed in the pharmacoresistant epileptic hippocampus. Finally, based on a literature-review, we examined the potential functional importance of this latter set of genes.

Similarly, we extracted from the online 'Catalog of Published Genome-Wide Association Studies' a list of SNPs associated with schizophrenia at a suggestive level of significance. Again, we determined if any of these suggestive SNPs are significant eQTLs according to our data. Finally, we extracted from the same online database a list of variants associated with schizophrenia at a genome-wide level of significance, determined which of these SNPs are significant eQTLs, and examined the potential functional importance of the genes under eQTL influence based on a literature-review.

6.4 Results

6.4.1 Final datasets

The final genetic and genomic datasets included 22 cases and 22 controls. The participant characteristics are shown in Table 6.1. The final genetic dataset included 4,663,226 genotyped and imputed SNPs. The final genomic dataset, after applying the variance-based filter described above and removing probes-with-polymorphhisms, included 18,916 unique probes.

Table 6.1 Sample characteristics. RIN=RNA Integrity Number. The sample labels used in Chapter 3 have been maintained.

Sample	Phenotype	Age	Sex	RIN
D1	case	41	F	7.4
D2	case	23	F	6.8
D3	case	51	M	6
D4	case	49	F	6.9
D5	case	50	F	7.8
D6	Case	45	F	6.6
D7	Case	12	M	7
D8	Case	29	F	7.8
D9	Case	33	M	6.8
D11	Case	34	M	7
D12	Case	33	M	8.8
D14	Case	22	F	7.3
D15	Case	48	M	7.6
D16	Case	39	F	7.4
D17	Case	29	F	7.1
D18	Case	44	M	7.9
D19	Case	40	F	6.6
D20	Case	48	M	8.5
D21	Case	23	M	8.4
D22	Case	63	M	7.9
D23	Case	31	M	8.2
D24	Case	27	F	8.2

Sample	Phenotype	Age	Sex	RIN
N1	control	81	M	6.5
N2	control	78	F	6.2
N3	control	84	F	6.6
N4	control	91	F	6.7
N5	control	88	M	6.3
N6	control	38	M	6.1
N7	control	50	M	6.2
N8	control	45	M	6.3
N9	control	39	M	6.1
N10	control	40	M	6
N11	control	61	M	6.2
N12	control	63	F	6.2
N13	control	66	M	6.2
N14	control	22	F	6.3
N15	control	27	M	6.3
N16	control	45	M	6.9
N18	control	50	M	6.5
N19	control	43	M	6.6
N20	control	46	M	6.8
N21	control	51	M	6.2
N22	control	48	M	6
N23	control	43	M	6.6

6.4.2 Adjustment for confounders and population stratification

After ComBat and ISVA treatment of the genomic dataset, confounders had been adjusted for appropriately: we found that no probes were associated with RIN, age, batch or sex at an FDR<0.10 in the adjusted dataset.

Similarly, we found no evidence of confounding effects from population stratification. The eigenvalues of none of the PCs reached statistical significance. Furthermore, none of the probe expression profiles were significantly correlated with any of the top ten PCs, demonstrating that the gene expression profile was not significantly influenced by population stratification in this cohort (Fairfax *et al.* 2012). Hence, the PCs were not included as covariates in the analysis.

6.4.3 External validation of eQTL results

We are aware of only one previously published eQTL study on human hippocampal tissue (Kim *et al.* 2012). In this study, 61 hippocampal samples were analysed in total, obtained from post-mortem donors with schizophrenia and normal controls. This study was limited to cis-eQTL analysis. In order to perform a comparison with this study, we also performed a cis-eQTL analysis. Kim and colleagues found 281 significant cis-eQTL genes. 55 of these genes were also significant cis-eQTLs in our study; this overlap is highly statistically significant (hypergeometric distribution p-value 1.9×10^{-34}).

We also determined which of our significant cis-eQTLs are 'conditional'—eQTLs which are active in either the normal or the pharmacoresistant partial epilepsy phenotype only—by performing a further analysis which included phenotype as an interaction term in the model. We found that only 4% of significant cis-eQTLs in our study are conditional.

6.4.4 cis- and trans-eQTLs

We found 298,703 SNPs (6.4% of all genotyped or imputed SNPs included in the eQTL analysis) to be significant (FDR<0.05) cis- or trans-eQTLs. 7516 probes were under significant eQTL influence; 5680 of these probes correspond to protein-coding genes, and the remaining probes correspond to long non-coding RNAs. The full list of eQTL SNPs and associated probes is provided in the Appendix 13.

6.4.5 Enrichment of eQTLs within significant GWAS results

There were 403 'suggestive' SNPs (p-value $<5 \times 10^{-5}$) in our GWAS; 53 (13%) of these were significant eQTLs. In comparison, from the 5,519,309 SNPs in our GWAS as a whole, 200,835 (3.6%) were eQTLs. This difference is statistically significant (p-value <0.001 for one-tailed test for significance of the difference between two proportions).

Similarly, the 'suggestive' GWAS SNPs were significantly enriched with eQTLs based on our permutation-based tests: p-value <0.0001 based on 10,000 sets of 403 random SNPs, and p-value <0.009 based on 10,000 sets of 403 SNPs ordered according to GWAS statistical significance.

6.4.6 eQTL 'hub' genes

We created a list of eQTL genes: eQTL SNPs within 20kb of the flanking sequence of a gene were mapped to the gene. The median number of probes influenced by each eQTL gene was 7, with a range of 1 to 284. We consider eQTL genes influencing 100 or more probes to be 'hub' genes. Out of all the eQTL genes, 0.7% (158 genes, see list in Appendix 14) were hub genes. Out of eQTL genes which were also associated with the phenotype at an empirical p-value<0.05 in our GWAS, 0.9% were hub genes; this proportion is significantly different compared to the hub genes amongst all eQTL genes (p=0.02, test for equality of proportions). Out of eQTL genes which were also associated with the phenotype at an empirical p-value<0.005 in our GWAS, 1.6% were hub genes; this proportion is significantly different compared to the hub genes amongst all eQTL genes ($p=4.2x10^{-7}$, test for equality of proportions). In order to gain an understanding of the biological processes eQTL hub genes are involved in, we performed gene ontology enrichment analysis using all 158 eQTL hub genes; the results are shown in Table 6.2.

6.4.7 Disease-associated genes under eQTL influence

Of the 1010 significantly (FDR<0.05 and FC≥1.5) differentially-expressed genes from our microarray (see Chapter 4), 369 genes are under significant eQTL influence (FDR<0.05); we term these 369 genes 'differentially-expressed plus genetically-regulated (DE+GR)'. We found that 3569 eQTL genes significantly (FDR<0.05) effect the expression of the 369 DE+GR genes (9.7 eQTL genes per target DE+GR gene). 16429 genes significantly (FDR<0.05) affect the expression of the 7147 genes which are not differentially-expressed (2.3 eQTL genes per target gene). Hence, there are 4.2 fold greater eQTL genes converging on each differentially-expressed gene; this difference is statistically significant (one-tail two-sample test for equality of proportions p-value < $2.2x10^{-16}$). The difference becomes even more prominent if we restrict the eQTL genes to those that are also associated with pharmacoresistant focal epilepsy in our GWAS: eQTL genes which are also associated with the pharmacoresistant focal epilepsy phenotype at an empirical p-value of <0.05 converge 4.5 fold more on genes which are differentially-expressed than on those not differentially-expressed, and eQTL genes which are associated with the pharmacoresistant focal epilepsy phenotype at an empirical p-value of <0.005 converge 5 fold more on genes which are differentially-expressed than on those not differentially-expressed

Table 6.2 Biological processes enriched with eQTL hub genes. GO=gene ontology

GO Biological Process	GO Accession	FDR
Extracellular structure organization and biogenesis	GO:0043062	$1.19x10^{-9}$
Synaptogenesis	GO:0007416	$1.44x10^{-9}$
Synapse organization and biogenesis	GO:0050808	$5.18x10^{-9}$
Nervous system development	GO:0007399	0.000106
Anatomical structure development	GO:0048856	0.000209
System development	GO:0048731	0.000209
Synaptic transmission	GO:0007268	0.000575
Cell-cell signalling	GO:0007267	0.000718
Transmission of nerve impulse	GO:0019226	0.000718
Multicellular organismal development	GO:0007275	0.000865
Anti-apoptosis	GO:0006916	0.000865
Regulation of developmental process	GO:0050793	0.00097
Negative regulation of cellular process	GO:0048523	0.00208
Negative regulation of apoptosis	GO:0043066	0.00208
Negative regulation of programmed cell death	GO:0043069	0.00208
Negative regulation of biological process	GO:0048519	0.00245
Cell development	GO:0048468	0.00461
Negative regulation of developmental process	GO:0051093	0.00608
Signal transduction	GO:0007165	0.00827
Regulation of apoptosis	GO:0042981	0.00827
Regulation of programmed cell death	GO:0043067	0.00827
Generation of precursor metabolites and energy	GO:0006091	0.00942
Neurological system process	GO:0050877	0.0131
Defence response	GO:0006952	0.0194
Negative regulation of cell proliferation	GO:0008285	0.0204
Apoptosis go	GO:0006915	0.0221
Programmed cell death	GO:0012501	0.0221
Biopolymer metabolic process	GO:0043283	0.0271
Lipid metabolic process	GO:0006629	0.0367
Regulation of gene expression	GO:0010468	0.039
Positive regulation of transcription factor activity	GO:0051091	0.0425
Positive regulation of DNA binding	GO:0043388	0.0483

6.4.8 Utilizing eQTL data to prioritize GWAS SNPs and reveal their functional implications: practical examples

There were 403 SNPs within our GWAS with a 'suggestive' level of significance ($p<5x10^{-5}$). 53 of these SNPs were also significant (FDR<0.05) eQTLs in our genetical genomics study (see Appendix 14 for full list). From these 53 SNPs, one (rs11163824) was chosen as an example to demonstrate how eQTL data can be used to prioritize GWAS SNPs and reveal their functional implications.

We extracted from the online 'Catalog of Published Genome-Wide Association Studies' a list of 125 SNPs associated with schizophrenia at a suggestive or genome-wide level of significance (see Appendix 14). Six of these SNPs are significant eQTLs according to our data (see Appendix 14). One SNP (rs11038167) associated with schizophrenia (Yue *et al.* 2011) at a genome-wide level of significance (p-value $1x10^{-11}$), is an eQTL according to our data. This SNP has a significant influence on the expression levels of genes SYNDIG1L and RFX4 according to our study. We examined the potential functional importance of these genes through a literature-review.

6.5 Discussion

We present the first ever study to analyse trans-eQTLs in human hippocampal tissue and the first ever eQTL study to include epileptic human brain tissue.

6.5.1 External validation and comparison

Before proceeding to draw conclusions from our study, we have carried out external validation of our results. We are aware of only one previously published eQTL study on human hippocampal tissue (Kim *et al.* 2012). In this study, 61 hippocampal samples were analysed in total, obtained from post-mortem donors with schizophrenia and normal controls. This study was limited to cis-eQTL analysis. In order to perform a comparison with this study, we also performed a cis-eQTL analysis. Kim and colleagues found 281 significant cis-eQTL genes. Our study uncovered 577 cis-eQTL genes (not including lincRNAs, which were not assayed in the study by Kim and colleagues).

Firstly, it is worth considering why we have discovered a much higher number of cis-eQTLs than Kim *et al*. One potential reason is than Kim and colleagues' study included only 309,531 SNPs, while our analysis included 4,663,227 SNPs. There are 15-fold fewer SNPs in Kim and

colleagues' study and, hence, in their analysis there are likely to be more genes without SNPs within or near them. The 15-fold greater SNP coverage in our study also means that we are likely to capture associated genetic variants which are not covered in Kim and colleagues' SNP panel. It should be noted that although we include 15-fold higher SNPs in our analysis, we only discover 2.3-fold more cis-eQTL genes. This suggests that our multiple-testing correction methodology adequately controls the false-discovery rate from the significantly higher number of tests in our analysis.

It may be argued that the overlap between the significant cis-eQTL genes from our study and Kim and colleagues' study is small. However, this overlap is highly statistically significant (hypergeometric distribution p-value 1.9×10^{-34}) and, hence, unlikely to be a chance occurrence.

On the other hand, it may be argued that such a significant overlap between two eQTL studies on tissue from two different pathologies is unexpected. By including phenotype as an interaction term in our eQTL analysis model, we were able to show that only 4% of significant cis-eQTLs are 'conditional'. Conditional eQTLs influence gene expression in only one of the two conditions in our study. The remaining 96% of significant cis-eQTLs are 'static'—they influence gene expression under any condition. In addition to this, it is being increasingly recognised that schizophrenia and epilepsy have common underlying causal mechanisms, including genetic mechanisms (Chang *et al.* 2011). Hence, it is possible that some of the conditional eQTLs relevant to epilepsy are also relevant to schizophrenia.

6.5.2 eQTLs and their targets: characteristics and patterns

We found that eQTLs are enriched within the most significantly trait-associated SNPs from our GWAS of pharmacoresistant partial epilepsy. This is consistent with the previously published observation that, in complex diseases, truly trait-associated SNPs are more likely to be eQTLs (Nicolae *et al.* 2010). This feature can be exploited in order to select genetic variants for further study from amongst a large number of SNPs with a 'suggestive' level of significance, as demonstrated in an example below.

It is worth noting that not all eQTL genes and the genes under their influence ('target genes') have an exclusive one-to-one relationship; one-to-many and many-to-one relationships are a prominent feature of the genetic regulation of gene expression.

We found 158 eQTL-hub genes which effect the expression of 100 or more target genes each. These eQTL-hubs are likely to be of particular importance in the genetic regulation of gene expression and clinical traits. As shown in Table 6.2, the eQTL-hubs are enriched in many regulatory processes, such as 'regulation of gene expression' and 'regulation of apoptosis'. The proportion of eQTL-hubs increased significantly amongst genes which were more strongly associated with the phenotype in our GWAS analysis.

On the other hand, multiple eQTL genes often converge on the same target gene. We found that the number of convergent eQTL genes is significantly greater if the target gene is disease-linked (differentially-expressed in diseased tissue). We found that there were, on average, 9.7 eQTL genes targeting each DE+GR gene, which was over four fold greater than the number of eQTL genes targeting non-differentially expressed genes. This fold difference became even more prominent if we limited our analysis to those eQTL genes which are more strongly associated with the disease in our GWAS analysis. Similar patterns have previously been noted within eQTLs in the peripheral blood (Fehrmann *et al.* 2011). It has previously been suggested (Fehrmann *et al.* 2011) that for a particular phenotype the different associated genetic variants eventually converge on the same downstream target genes; our results are consistent with this observation.

Taken together, the above observations suggest that in the pharmacoresistant epileptic human hippocampus: (1) genetic variants most significantly associated with the clinical phenotype are more likely to be eQTLs, (2) genes more significantly associated with the clinical phenotype are more likely to be eQTL-hubs, and (3) genes which are differentially expressed in this phenotype are under the influence of significantly higher numbers of disease-associated eQTLs. This demonstrates that the eQTLs identified in our study are relevant to the clinical phenotype.

6.5.3 Applying the eQTL data

In complex diseases, truly trait-associated SNPs are more likely to be eQTLs (Nicolae *et al.* 2010). This feature can be exploited in order to select genetic variants for further study from amongst a large number of SNPs with a 'suggestive' level of significance. From the SNPs with a suggestive level of significance ($p<5x10^{-5}$) in our pharmacoresistant epilepsy GWAS 'mega-analysis', 53 SNPs were also significant (FDR<0.05) eQTLs in our genetical genomics study.

One of these 53 SNPs, rs11163824, was associated with the expression of gene ALDH5A1, which was significantly down regulated (FDR<0.05) in our microarray study. It is interesting to note that rs11163824 lies within a long non-coding RNA (lincRNA:chr1:84106887-84316837). It can be postulated that altered activity of this lincRNA leads to differential expression of ALDH5A1. ALDH5A1 (also known as succinic semialdehyde dehydrogenase) is involved in the degradation of the inhibitory neurotransmitter GABA and patients with ALDH5A1 deficiency (an autosomal recessive disorder) suffer from seizures (Lorenz *et al.* 2006). Furthermore, genetic variations within ALDH5A1 were among a set of SNPs found to contribute to disease predisposition in sporadic epilepsy (Cavalleri *et al.* 2007).

As shown above, epilepsy and schizophrenia share many eQTLs. Hence, our dataset will prove useful for the study of schizophrenia and other diseases in which the hippocampus is affected. As an example, one SNP (rs11038167) associated with schizophrenia at a genome-wide level of significance (p-value $1x10^{-11}$) in a previous study (Yue *et al.* 2011) is an eQTL according to our data. This SNP lies within the TSPAN18 gene and has a significant influence on the expression levels of genes SYNDIG1L and RFX4 according to our dataset. We examined the potential functional importance of these genes through a literature-review. TSPAN18 encodes one member of a large family of transmembrane proteins (tetraspanins) found in all multicellular eukaryotes. Expressed widely and in diverse cell types, the tetraspanins appear to affect cellular penetration, adhesion, motility, and signal conduction, and are thought to be influential in diverse physiologic processes and diseases (Charrin *et al.* 2009; Rubinstein 2011). TSPAN18 genetic variants have been associated with both bipolar disorder and schizophrenia (Scholz *et al.* 2010; Yue *et al.* 2011). RFX4 is a transcription factor and is differentially expressed in patients with schizophrenia (Glatt *et al.* 2011). Interestingly, RFX4 is located in the linkage region of bipolar affective disorder (Ewald *et al.* 1998) and schizophrenia (Holmans *et al.* 2009), and a RFX4 haplotype confers bipolar disorder disease risk (Glaser *et al.* 2005). SYNDIG1L is the Synaptic Differentiation Induced Gene I-Like gene. SYNDIG1 encodes a novel postsynaptic transmembrane protein that plays a critical role in excitatory synapse development (Diaz 2010).

6.6 Conclusions

We have presented the first ever study to analyse trans-eQTLs in human hippocampal tissue and the first ever eQTL study to include epileptic human brain tissue. We have shown that

these eQTLs are relevant to disease: genetic variants most significantly associated with the clinical phenotype are more likely to be eQTLs, genes more significantly associated with the clinical phenotype are more likely to be eQTL-hubs, and genes which are differentially expressed in this phenotype are under the influence of significantly higher numbers of disease-associated eQTLs. We have also identified the most enriched biological processes for eQTL-hubs. Finally, we have demonstrated how this eQTL dataset can be utilized in order to interpret and prioritize GWAS results from epilepsy and other disease.

6.7 References

Ackermann M., Sikora-Wohlfeld W. & Beyer A. (2013) Impact of natural genetic variation on gene expression dynamics. *PLoS Genet* **9**, e1003514.

Cavalleri G.L., Weale M.E., Shianna K.V., Singh R., Lynch J.M., Grinton B., Szoeke C., Murphy K., Kinirons P., O'Rourke D., Ge D., Depondt C., Claeys K.G., Pandolfo M., Gumbs C., Walley N., McNamara J., Mulley J.C., Linney K.N., Sheffield L.J., Radtke R.A., Tate S.K., Chissoe S.L., Gibson R.A., Hosford D., Stanton A., Graves T.D., Hanna M.G., Eriksson K., Kantanen A.M., Kalviainen R., O'Brien T.J., Sander J.W., Duncan J.S., Scheffer I.E., Berkovic S.F., Wood N.W., Doherty C.P., Delanty N., Sisodiya S.M. & Goldstein D.B. (2007) Multicentre search for genetic susceptibility loci in sporadic epilepsy syndrome and seizure types: a case-control study. *Lancet Neurol* **6**, 970-80.

Chang Y.T., Chen P.C., Tsai I.J., Sung F.C., Chin Z.N., Kuo H.T., Tsai C.H. & Chou I.C. (2011) Bidirectional relation between schizophrenia and epilepsy: a population-based retrospective cohort study. *Epilepsia* **52**, 2036-42.

Charrin S., le Naour F., Silvie O., Milhiet P.E., Boucheix C. & Rubinstein E. (2009) Lateral organization of membrane proteins: tetraspanins spin their web. *Biochem J* **420**, 133-54.

Diaz E. (2010) SynDIG1 regulation of synaptic AMPA receptor targeting. *Commun Integr Biol* **3**, 347-9.

Dimas A.S., Deutsch S., Stranger B.E., Montgomery S.B., Borel C., Attar-Cohen H., Ingle C., Beazley C., Gutierrez Arcelus M., Sekowska M., Gagnebin M., Nisbett J., Deloukas P., Dermitzakis E.T. & Antonarakis S.E. (2009) Common regulatory variation impacts gene expression in a cell type-dependent manner. *Science* **325**, 1246-50.

Ertekin-Taner N. (2011) Gene expression endophenotypes: a novel approach for gene discovery in Alzheimer's disease. *Mol Neurodegener* **6**, 31.

Ewald H., Degn B., Mors O. & Kruse T.A. (1998) Significant linkage between bipolar affective disorder and chromosome 12q24. *Psychiatr Genet* **8**, 131-40.

Fairfax B.P., Makino S., Radhakrishnan J., Plant K., Leslie S., Dilthey A., Ellis P., Langford C., Vannberg F.O. & Knight J.C. (2012) Genetics of gene expression in primary immune cells identifies cell type-specific master regulators and roles of HLA alleles. *Nat Genet* **44**, 502-10.

Fehrmann R.S., Jansen R.C., Veldink J.H., Westra H.J., Arends D., Bonder M.J., Fu J., Deelen P., Groen H.J., Smolonska A., Weersma R.K., Hofstra R.M., Buurman W.A., Rensen S., Wolfs M.G., Platteel M., Zhernakova A., Elbers C.C., Festen E.M., Trynka G., Hofker M.H., Saris C.G., Ophoff R.A., van den Berg L.H., van Heel D.A., Wijmenga C., Te

Meerman G.J. & Franke L. (2011) Trans-eQTLs reveal that independent genetic variants associated with a complex phenotype converge on intermediate genes, with a major role for the HLA. *PLoS Genet* **7**, e1002197.

Flight R.M. & Wentzell P.D. (2010) Preliminary exploration of time course DNA microarray data with correlation maps. *OMICS* **14**, 99-107.

Glaser B., Kirov G., Bray N.J., Green E., O'Donovan M.C., Craddock N. & Owen M.J. (2005) Identification of a potential bipolar risk haplotype in the gene encoding the winged-helix transcription factor RFX4. *Mol Psychiatry* **10**, 920-7.

Glatt S.J., Stone W.S., Nossova N., Liew C.C., Seidman L.J. & Tsuang M.T. (2011) Similarities and differences in peripheral blood gene-expression signatures of individuals with schizophrenia and their first-degree biological relatives. *Am J Med Genet B Neuropsychiatr Genet* **156B**, 869-87.

Hawrylycz M.J., Lein E.S., Guillozet-Bongaarts A.L., Shen E.H., Ng L., Miller J.A., van de Lagemaat L.N., Smith K.A., Ebbert A., Riley Z.L., Abajian C., Beckmann C.F., Bernard A., Bertagnolli D., Boe A.F., Cartagena P.M., Chakravarty M.M., Chapin M., Chong J., Dalley R.A., Daly B.D., Dang C., Datta S., Dee N., Dolbeare T.A., Faber V., Feng D., Fowler D.R., Goldy J., Gregor B.W., Haradon Z., Haynor D.R., Hohmann J.G., Horvath S., Howard R.E., Jeromin A., Jochim J.M., Kinnunen M., Lau C., Lazarz E.T., Lee C., Lemon T.A., Li L., Li Y., Morris J.A., Overly C.C., Parker P.D., Parry S.E., Reding M., Royall J.J., Schulkin J., Sequeira P.A., Slaughterbeck C.R., Smith S.C., Sodt A.J., Sunkin S.M., Swanson B.E., Vawter M.P., Williams D., Wohnoutka P., Zielke H.R., Geschwind D.H., Hof P.R., Smith S.M., Koch C., Grant S.G. & Jones A.R. (2012) An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* **489**, 391-9.

Holmans P.A., Riley B., Pulver A.E., Owen M.J., Wildenauer D.B., Gejman P.V., Mowry B.J., Laurent C., Kendler K.S., Nestadt G., Williams N.M., Schwab S.G., Sanders A.R., Nertney D., Mallet J., Wormley B., Lasseter V.K., O'Donovan M.C., Duan J., Albus M., Alexander M., Godard S., Ribble R., Liang K.Y., Norton N., Maier W., Papadimitriou G., Walsh D., Jay M., O'Neill A., Lerer F.B., Dikeos D., Crowe R.R., Silverman J.M. & Levinson D.F. (2009) Genomewide linkage scan of schizophrenia in a large multicenter pedigree sample using single nucleotide polymorphisms. *Mol Psychiatry* **14**, 786-95.

Howie B.N., Donnelly P. & Marchini J. (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet* **5**, e1000529.

Johnson W.E., Li C. & Rabinovic A. (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8**, 118-27.

Kim S., Cho H., Lee D. & Webster M.J. (2012) Association between SNPs and gene expression in multiple regions of the human brain. *Transl Psychiatry* **2**, e113.

Lee S., Wright F.A. & Zou F. (2011) Control of population stratification by correlation-selected principal components. *Biometrics* **67**, 967-74.

Li J. & Burmeister M. (2005) Genetical genomics: combining genetics with gene expression analysis. *Hum Mol Genet* **14 Spec No. 2**, R163-9.

Lorenz S., Heils A., Taylor K.P., Gehrmann A., Muhle H., Gresch M., Becker T., Tauer U., Stephani U. & Sander T. (2006) Candidate gene analysis of the succinic semialdehyde dehydrogenase gene (ALDH5A1) in patients with idiopathic generalized epilepsy and photosensitivity. *Neurosci Lett* **397**, 234-9.

Nicolae D.L., Gamazon E., Zhang W., Duan S., Dolan M.E. & Cox N.J. (2010) Trait-associated SNPs are more likely to be eQTLs: annotation to enhance discovery from GWAS. *PLoS Genet* **6**, e1000888.

Orozco L.D., Bennett B.J., Farber C.R., Ghazalpour A., Pan C., Che N., Wen P., Qi H.X., Mutukulu A., Siemers N., Neuhaus I., Yordanova R., Gargalovic P., Pellegrini M., Kirchgessner T. & Lusis A.J. (2012) Unraveling inflammatory responses using systems genetics and gene-environment interactions in macrophages. *Cell* **151**, 658-70.

Patterson N., Price A.L. & Reich D. (2006) Population structure and eigenanalysis. *PLoS Genet* **2**, e190.

Ramasamy A., Trabzuni D., Gibbs J.R., Dillman A., Hernandez D.G., Arepalli S., Walker R., Smith C., Ilori G.P., Shabalin A.A., Li Y., Singleton A.B., Cookson M.R., Hardy J., Ryten M. & Weale M.E. (2013) Resolving the polymorphism-in-probe problem is critical for correct interpretation of expression QTL studies. *Nucleic Acids Res* **41**, e88.

Romanoski C.E., Lee S., Kim M.J., Ingram-Drake L., Plaisier C.L., Yordanova R., Tilford C., Guan B., He A., Gargalovic P.S., Kirchgessner T.G., Berliner J.A. & Lusis A.J. (2010) Systems genetics analysis of gene-by-environment interactions in human cells. *Am J Hum Genet* **86**, 399-410.

Rubinstein E. (2011) The complexity of tetraspanins. *Biochem Soc Trans* **39**, 501-5.

Schadt E.E. (2005) Exploiting naturally occurring DNA variation and molecular profiling data to dissect disease and drug response traits. *Curr Opin Biotechnol* **16**, 647-54.

Schaub M.A., Boyle A.P., Kundaje A., Batzoglou S. & Snyder M. (2012) Linking disease associations with regulatory information in the human genome. *Genome Res* **22**, 1748-59.

Scholz C.J., Jacob C.P., Buttenschon H.N., Kittel-Schneider S., Boreatti-Hummer A., Zimmer M., Walter U., Lesch K.P., Mors O., Kneitz S., Deckert J. & Reif A. (2010) Functional variants of TSPAN8 are associated with bipolar disorder and schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* **153B**, 967-72.

Shabalin A.A. (2012) Matrix eQTL: ultra fast eQTL analysis via large matrix operations. *Bioinformatics* **28**, 1353-8.

Teschendorff A.E., Zhuang J. & Widschwendter M. (2011) Independent surrogate variable analysis to deconvolve confounding factors in large-scale microarray profiling studies. *Bioinformatics* **27**, 1496-505.

Trabzuni D., Ryten M., Walker R., Smith C., Imran S., Ramasamy A., Weale M.E. & Hardy J. (2011) Quality control parameters on a large dataset of regionally dissected human control brains for whole genome expression studies. *J Neurochem* **119**, 275-82.

van Nas A., Ingram-Drake L., Sinsheimer J.S., Wang S.S., Schadt E.E., Drake T. & Lusis A.J. (2010) Expression quantitative trait loci: replication, tissue- and sex-specificity in mice. *Genetics* **185**, 1059-68.

Yang Q., Orman M.A., Berthiaume F., Ierapetritou M.G. & Androulakis I.P. (2012) Dynamics of short-term gene expression profiling in liver following thermal injury. *J Surg Res* **176**, 549-58.

Yang S., Guo X., Yang Y.C., Papcunik D., Heckman C., Hooke J., Shriver C.D., Liebman M.N. & Hu H. (2006) Detecting outlier microarray arrays by correlation and percentage of outliers spots. *Cancer Inform* **2**, 351-60.

Yue W.H., Wang H.F., Sun L.D., Tang F.L., Liu Z.H., Zhang H.X., Li W.Q., Zhang Y.L., Zhang Y., Ma C.C., Du B., Wang L.F., Ren Y.Q., Yang Y.F., Hu X.F., Wang Y., Deng W., Tan L.W., Tan Y.L., Chen Q., Xu G.M., Yang G.G., Zuo X.B., Yan H., Ruan Y.Y., Lu T.L., Han X., Ma

X.H., Cai L.W., Jin C., Zhang H.Y., Yan J., Mi W.F., Yin X.Y., Ma W.B., Liu Q., Kang L., Sun W., Pan C.Y., Shuang M., Yang F.D., Wang C.Y., Yang J.L., Li K.Q., Ma X., Li L.J., Yu X., Li Q.Z., Huang X., Lv L.X., Li T., Zhao G.P., Huang W., Zhang X.J. & Zhang D. (2011) Genome-wide association study identifies a susceptibility locus for schizophrenia in Han Chinese at 11p11.2. *Nat Genet* **43**, 1228-31.

Zheng X., Levine D., Shen J., Gogarten S.M., Laurie C. & Weir B.S. (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics* **28**, 3326-8.

CHAPTER SEVEN

TESTING THE INTRINSIC SEVERITY HYPOTHESIS AND IDENTIFYING THE CAUSAL PATHWAYS UNDERLYING INTRINSIC SEVERITY

Contents

Chapter 7: Testing the Intrinsic Severity Hypothesis and Identifying the Causal Pathways Underlying Intrinsic Severity

7.1 Introduction

In the current chapter, I will integrate the data and results from the previous chapters in order to present a combined view of the genetic and genomic changes which underlie pharmacoresistance in epilepsy.

The analyses presented in the preceding chapters demonstrate that underlying epilepsy pharmacoresistance, at both genetic and transcriptomic levels, are diverse pathways and processes pertaining to disparate domains, such as axon guidance, neuroinflammation, transmembrane transport of small molecules and synaptic neurotransmitter release machinery. This observation lends credence to the intrinsic severity hypothesis of pharmacoresistance in epilepsy, which states that pharmacoresistance is the results of increased dysfunction of the neurobiological processes underlying epilepsy (please see Chapter 1 for a detailed discussion of this hypothesis). It has previously been suggested that genetic variations are likely to be key components of the biological factors that mediate increased severity and intractability (Rogawski 2013). The results presented in Chapters 5 and 6 tend to support this suggestion, but this idea has never been objectively examined. In this chapter, I will objectively test the hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy. In order to achieve this aim, I will first create a gene-set that represents the main pathways and processes underlying epilepsy, and then determine if this gene-set is more enriched in a GWAS of pharmacoresistant epilepsy than in a GWAS of pharmacoresponsive epilepsy.

Following on from the above analysis, I will identify the 'causal' pathways underlying pharmacoresistant epilepsy, using the data presented in the previous chapters.

7.2 Aims

I. To test the hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy.

II. To identify the causal pathways underlying pharmacoresistance in epilepsy.

7.3 Methods

7.3.1 Methods overview

We wished to demonstrate that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy. In order to show this, we first identified the single most functionally-relevant gene-set in the epileptic human hippocampus and then determined if this was affected by genetic variation more in drug resistant than in drug responsive epilepsy. Putative functionally-relevant gene-sets were identified by performing Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder & Horvath 2008) on our microarray data from Chapter 4, and the most important gene-set was chosen (see below for details). In order to identify the genetic variations associated with drug responsive epilepsy, a genome-wide association study (GWAS) was performed of drug responsive epilepsy. Similarly, in order to identify the genetic variations associated with drug resistant epilepsy, a GWAS was performed of drug resistant epilepsy. We then compared the degree of enrichment of the functionally-relevant gene-set in the two GWAS. Finally, in order to perform a head-to-head comparison of drug responsive and resistant partial epilepsy, a GWAS of drug responsive versus resistant partial epilepsy was performed.

In order to identify 'causal' pathways, we determined the Reactome pathways enriched in genes which are significant in the pharmacoresistant focal epilepsy GWAS and are also significant eQTLs for differentially-expressed genes. We also identified 'intermediate' pathways: the Reactome pathways enriched in genes which are genetically regulated by eQTLs and are also differentially expressed in the pharmacoresistant epileptic hippocampus.

7.3.2 Weighted Gene Co-expression Network Analysis (WGCNA)

The microarray data was adjusted for batch effects and for known and unknown confounders using ComBat and ISVA, as described in Chapter 4. Including all microarray probes in the WGCNA was not computationally feasible: the time and memory requirements were both prohibitive. Hence, the probes were sequentially filtered as follows: (1) only probes mapping to genes with valid current Entrez numbers and HUGO Gene Nomenclature Committee gene symbols were retained, (2) where multiple probes mapped to the same gene, the most variant probe only was retained, (3) 75% of the probes with the highest variance were retained. Hence, after filtering, 13,367 probes with high variance remained, each mapping to a unique gene.

WGCNA was performed using the WGCNA R package according to the authors' instructions. WCGNA network construction and module detection was carried out using the one-step automatic network construction and module detection approach, with a soft threshold of $\beta=9$ and a minimum module size of 30. The most important module, from amongst those detected, was identified using techniques devised and validated by the authors of WGCNA (Langfelder & Horvath 2008) and used successfully by others. Briefly, the most important module was identified based on (a) the significance of its association with the phenotype of interest and lack of association with confounders, and (b) the strength of the correlation between module membership and gene significance. Gene significance of a gene is defined as $\{GeneSignificance(i)=|cor(x_i, T)|\}$ where x_i is the gene expression profile of Gene *i* and T is the trait of interest. The module membership of a gene is the correlation between its gene expression profile and module eigengene of a given module; the module eigengene corresponds to the first principal component of a given module.

Pathway-enrichment analysis for the most important module was performed using the Reactome database on the Gene Set Enrichment Analysis website.

7.3.3 External validation of the chosen WGCNA module

Before using the chosen WGCNA module in our own GWAS gene-set enrichment analysis, we validated the module using an independent previously published partial epilepsy GWAS dataset (Kasperaviciute *et al.* 2010). Complete genome-wide results for this study were downloaded from the authors' website. The size of our chosen WGCNA module was 1594

genes. The rest of the genome was divided into 12 random gene-sets of 1594 genes. The chosen WGCNA module and the 12 random gene-sets were then tested for enrichment in the previously published independent GWAS using the GSA-SNP software.

7.3.4 GWAS study cohorts

Three GWAS analyses were carried out:

i. Drug responsive partial epilepsy versus normal population controls (NPC)
ii. Drug resistant partial epilepsy versus NPC
iii. An extreme discordant phenotype (EDP) analysis: Drug resistant versus drug responsive partial epilepsy

The epilepsy cases used in the drug responsive cohort were from amongst the patients recruited into the Standard and New Antiepileptic Drugs (SANAD) trial (Marson *et al.* 2007); this study has been described in detail in Chapter 5. For the purposes of this analysis, subjects were said to have drug responsive epilepsy if they entered 12-month remission immediately upon starting treatment. On this basis, 155 subjects from the SANAD study were classified as having drug responsive partial epilepsy. The epilepsy cases used in the drug resistant cohort were from amongst the patients recruited into the SANAD and 'Pharmacogenetics of GABAergic Mechanisms of Benefit and Harm in Epilepsy' studies; these studies have been described in detail in Chapter 5. We utilized subjects included in our 'mega-analysis' (please see Chapter 5 for details). The definitions of drug resistance used in our analysis have been stated in Chapter 5. The control dataset consisted of subjects from the Wellcome Trust Case Control Consortium (WTCCC) 1958 British Birth Cohort (dataset EGAD00000000022). We used the 2624 subjects passing our quality control (QC) filters, as described in Chapter 5.

7.3.5 GWAS genotyping and QC

The genotyping and QC procedures employed have been detailed in Chapter 5.

After per-individual QC, 146 cases remained in the drug responsive cohort. In order to allow a fair comparison, for the drug resistant partial epilepsy versus NPC analysis, 146 cases were chosen at random from the 421 total subjects in the drug resistant cohort. Hence, the final drug responsive partial epilepsy versus NPC dataset included 146 cases and 2624 normal

controls, and the final drug resistant partial epilepsy versus NPC dataset similarly included 146 cases and 2624 normal controls. The EDP analysis included 146 drug responsive and 421 drug resistant subjects.

After per-SNP QC, there were 510501 SNPs in the drug responsive cohort and 509534 SNPs in the drug resistant cohort. In order to allow a fair comparison, both datasets were reduced to the 509534 SNPs which were common to both cohorts.

7.3.6 Association and gene-set analysis

Association analysis was performed using the chi-squared (χ^2) test in Plink. In order to ensure consistency in correcting for inflation due to population stratification, the genomic control method, which has proven effectiveness (Bouaziz *et al.* 2011), was applied to both the drug responsive partial epilepsy versus NPC analysis and the drug resistant partial epilepsy versus NPC analysis.

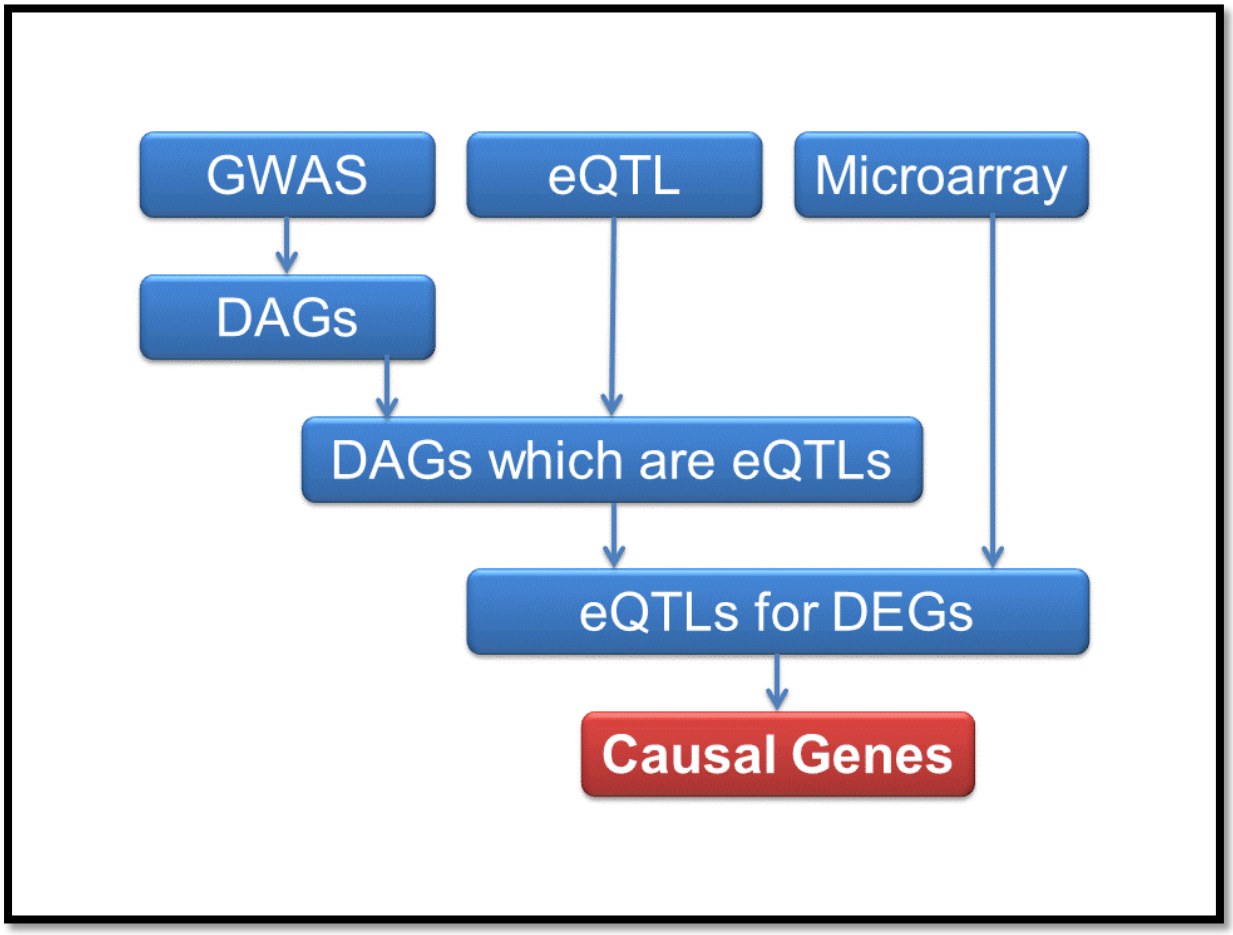
We tested the chosen WGCNA module for enrichment in three GWAS analyses using the program GSA-SNP.

7.3.7 Causal genes and pathways

The step-wise scheme for identifying causal genes is illustrated in Figure 7.1. In this approach, potentially causal genes are those that are (1) disease-associated, (2) eQTLs, and (3) influence the transcript levels of genes differentially-expressed in pharmacoresistant epilepsy.

SNPs within 20kb of the flanking sequence of a gene were mapped to the gene. The results of the GWAS mega-analysis (Chapter 5) were used to identify disease-associated genes. In order to select a GWAS p-value cut-off for defining disease associated genes, we created sets of genes at various GWAS p-values cut-offs (0.05, 0.005, 0.0005), and then determined how enriched each set was with disease-specific eQTLs (eQTLs which regulate differentially-expressed genes). After this process, we used an empirical p-value <0.005 cut-off to define disease-associated genes. Then, we retained the subset of disease-associated genes which are significant eQTLs (Chapter 6). Then, we retained the subset of eQTLs which regulate differentially-expressed genes (DEGs) identified through microarray analysis (Chapter 4); this final subset (721 genes) represents causal genes.

Figure 7.1 Step-wise scheme for the identification of causal genes. First, we used Genome-Wide Association Study (GWAS) to identify diseased-associated genes (DAGs)—those bearing SNPs associated with the clinical phenotype. Then, we retained the subset of DAGs which are significant expression quantitative trait loci (eQTL) genes. Then, we retained the subset of eQTLs which regulate differentially-expressed genes (DEGs) identified through microarray analysis; this subset represents causal genes.



As our initial GWAS empirical p-value<0.005 cut-off could be considered somewhat arbitrary, we determined if the chosen 721 genes were significantly associated with the pharmacoresistant focal epilepsy phenotype at the gene-set level, by analysing the 721 genes as one set in the program GSA-SNP. For comparison, we divided the rest of the genome into 25 gene-sets of 721 genes and analysed them similarly in GSA-SNP.

We performed Reactome pathway enrichment analysis on this set of 721 genes using the Gene Set Enrichment Analysis website. These pathways were designated 'causal' pathways, as they are likely to represent the genetic changes which influence the transcriptomic

intermediate phenotype which, in turn, contributes to the development of the clinical phenotype. Enrichment Map (Merico *et al.* 2010) tool was used to determine the connections between enriched pathways. Enrichment Map was used with default settings. Betweenness centrality network analysis was used to identify the most central pathways in this network.

7.3.8 Intermediate genes and pathways

We collated the list of genes which are under significant (FDR<0.05) eQTL control of causal genes and significantly differentially expressed (FDR<0.05 and FC≥1.5) in pharmacoresistant epileptic tissue. We performed Reactome pathway enrichment analysis on this set of genes using the Gene Set Enrichment Analysis website. These pathways were designated 'intermediate' pathways, as they are likely to represent the true transcriptomic intermediate phenotype which contributes to the development of the clinical phenotype of interest. Enrichment Map (Merico *et al.* 2010) tool was used to determine the connections between enriched pathways. Enrichment Map was used with default settings.

7.4 Results

7.4.1 WGCNA

WGCNA revealed 18 distinct modules. Out of the 18 modules detected, the blue module was deemed most relevant and important because (1) it was the module most significantly (p-value=7.1×10^{-7}) associated with the phenotype, and it was not associated with any confounders (Appendix 15), and (2) module membership in the blue module was highly correlated with gene-significance (correlation=0.94, p<1×10^{-200}, Figure 7.2). The genes included in the blue module, and all other detected modules, are tabulated in the Appendix 15. The Reactome pathways enriched in the blue module are also listed in the Appendix 15.

7.4.2 External validation of the chosen WGCNA module

We tested the blue module and 11 other random gene-sets of equal size for enrichment within the results of a previously published GWAS study of partial epilepsy. We found that while the blue module was highly significantly enriched in this GWAS analysis (FDR<3×10^{-17}), none of the other gene-sets showed significant enrichment (minimum FDR 0.15); the gene-set enrichment analysis results can be found in the Appendix 15.

7.4.3 GWAS analyses of drug responsive and drug resistant epilepsy

The quantile-quantile (QQ) plots of the GWAS analysis of drug responsive partial epilepsy versus NPC and drug resistant partial epilepsy versus NPC are shown in the Appendix 15. Gene-set analysis of the blue module showed that there was greater enrichment for these genes in the drug resistant ($p=2.6x10^{-14}$) than in the drug responsive ($p=1.1x10^{-9}$) GWAS study.

The genomic inflation factor (1.01) and the QQ plot (Figure 7.3) for the EDP GWAS study showed that there no significant inflation in the results and, hence, the no further adjustments were applied to the analysis. Gene-set analysis showed significant enrichment ($p<8.5x10^{-10}$) of the blue module in the EDP GWAS analysis.

7.4.4 Causal genes and pathways

We found 721 genes that associated with the pharmacoresistant focal epilepsy phenotype in our genome-wide genetic-association (GWAS) analysis at an empirical p-value <0.005 and were also significant (FDR<0.05) eQTLs for differentially-expressed genes. This particular GWAS p-value cut-off was chosen for the following reason: as increasingly stringent GWAS p-values are used, the proportion of selected genes which are also eQTLs for DEGs increases; GWAS p-value <0.005 is the final cut-off at which this proportion is significantly increased compared to more lenient cut-offs (Bonferroni corrected p-value $6.7x10^{-16}$ for 2-sample test for equality of proportions). We also determined if these 721 genes were significantly associated in the pharmacoresistant epilepsy GWAS at the gene-set level. These 721 genes were significantly associated with the pharmacoresistant focal epilepsy phenotype at the gene-set level ($FDR<5x10^{-20}$), while 25 random sets of 721 genes were not (minimum FDR=0.7). We performed Reactome pathway enrichment analysis on this set of 721 genes. We term these enriched pathways 'causal' pathways—the 10 most enriched pathways are shown in Table 7.1 and the complete list in the Appendix 15. Enrichment Map analysis revealed a highly interconnected network of pathways (Figure 7.4). In this network, each pathway is directly connected to, on average, 9 other pathways. In order to identify the most central 'hub' pathways, 'betweeness centrality' network analysis was performed; the top 10 most central pathways are listed in Table 7.2, and the complete ranked list can be found in the Appendix 15.

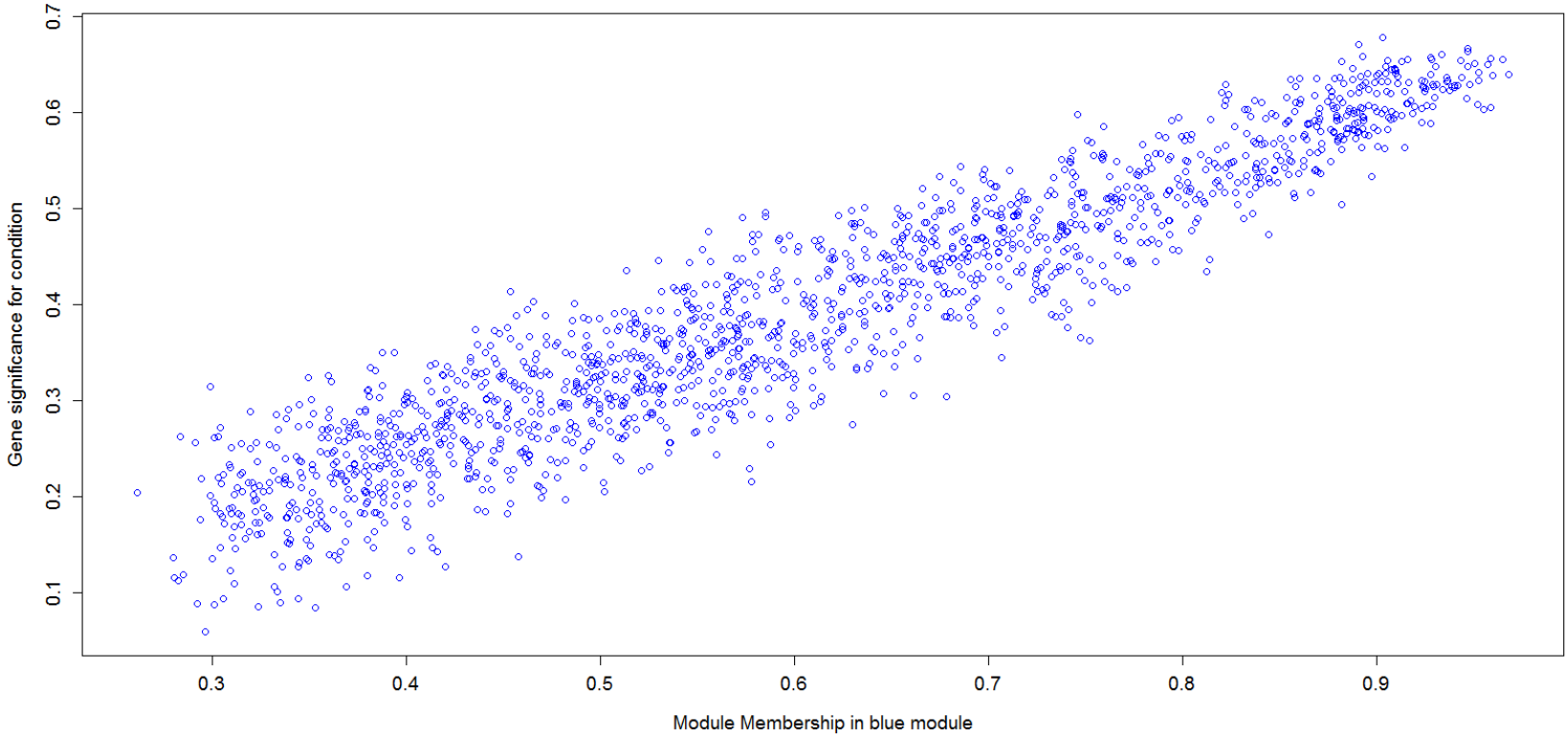


Figure 7.2 Module membership versus gene significance for the blue module

Table 7.1 The 10 most enriched 'causal' pathways: enriched Reactome pathways for 721 genes that are significant (FDR<0.05) eQTLs for differentially-expressed genes and also significantly associated with the pharmacoresistant focal epilepsy phenotype at the gene-level (empirical p-value <0.005) and gene-set level (FDR<$5x10^{-20}$).

Pathway	P-Value	FDR
Signalling by NGF	$5.66x10^{-9}$	$3.81x10^{-6}$
Axon guidance	$4.40x10^{-8}$	$1.48x10^{-5}$
Developmental biology	$2.21x10^{-7}$	$4.95x10^{-5}$
GPVI-mediated activation cascade	$1.28x10^{-6}$	$1.99x10^{-4}$
Regulation of signalling by CBL	$1.47x10^{-6}$	$1.99x10^{-4}$
Nephrin interactions	$2.62x10^{-6}$	$2.94x10^{-4}$
Metabolism of lipids and lipoproteins	$3.59x10^{-6}$	$3.46x10^{-4}$
p75-neurotrophin receptor mediated signalling	$4.38x10^{-6}$	$3.69x10^{-4}$
Netrin1 signalling	$7.07x10^{-6}$	$4.97x10^{-4}$
Transmembrane transport of small molecules	$7.37x10^{-6}$	$4.97x10^{-4}$

Table 7.2 Top 10 central causal pathways

Pathway
Transmembrane transport of small molecules
DCC mediated attractive signalling
Axon guidance
Cell-cell communication
Developmental biology
Signalling by NGF
Immune system
TIE2 signalling
Signalling by constitutively active EGFR
CREB phosphorylation through the activation of RAS

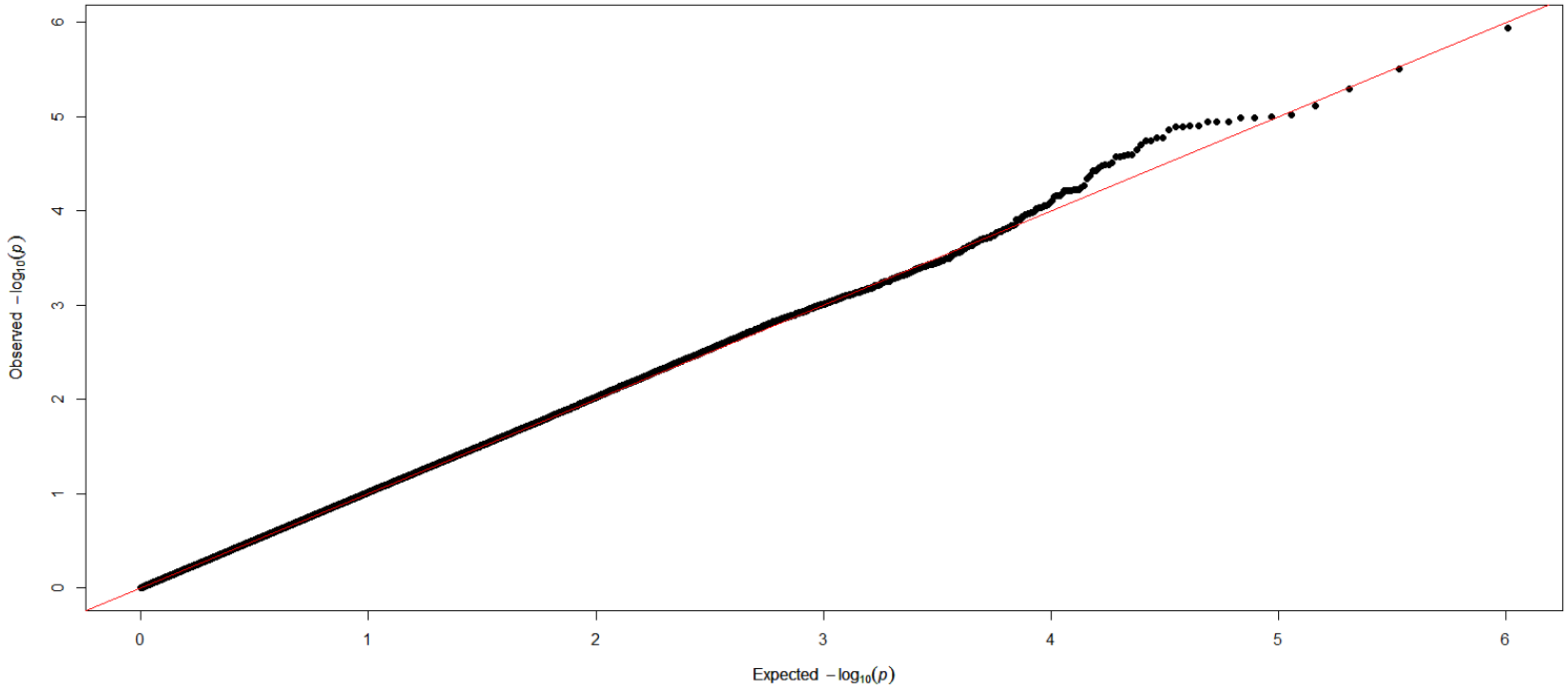


Figure 7.3 Quantile-quantile plot for extreme discordant phenotype genome-wide genetic association analysis

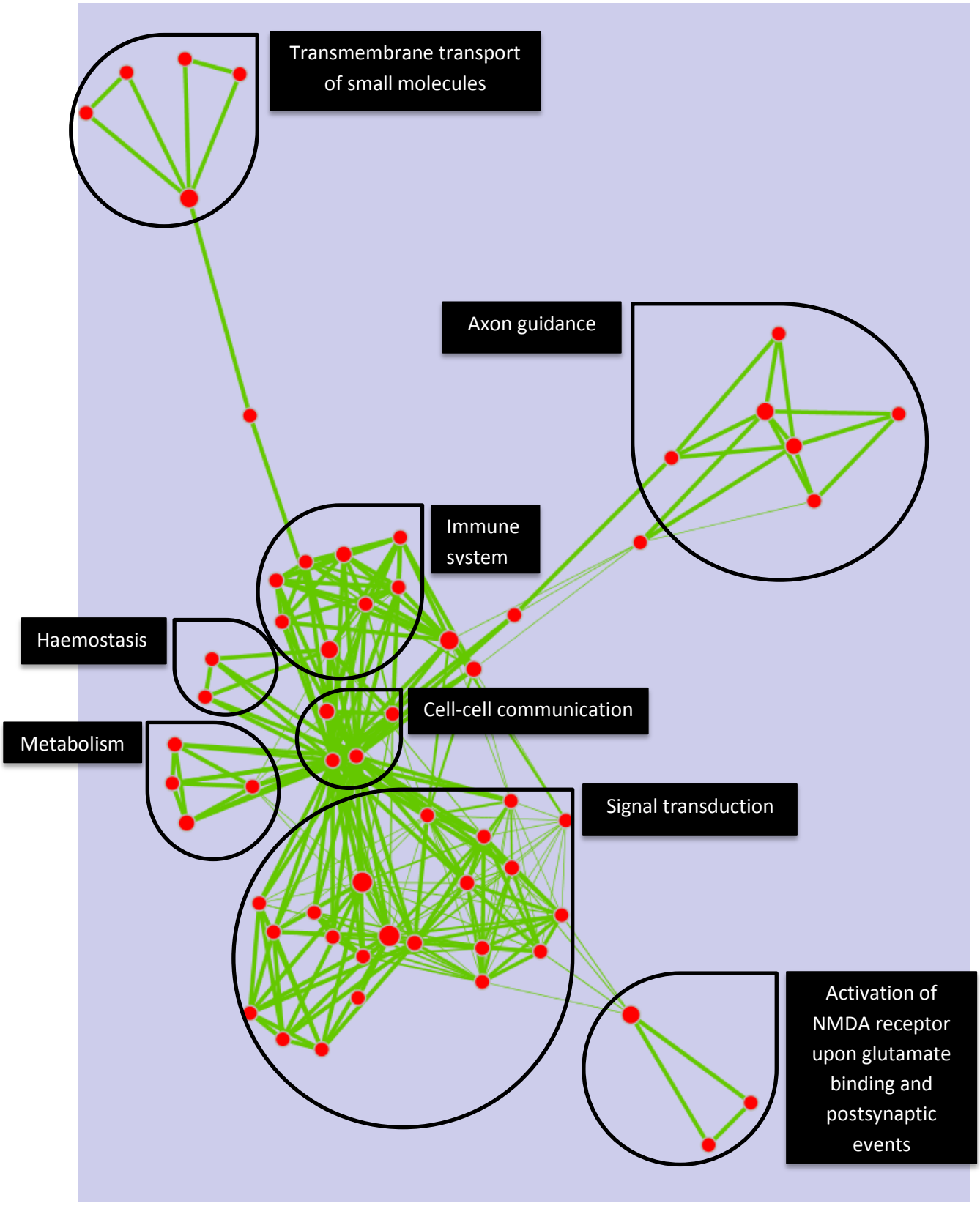


Figure 7.4 Network of causal pathways. Major functional groups of pathways are labelled.

7.4.5 Intermediate genes and pathways

We collated the list of genes which are both differentially expressed (FDR<0.05 and FC≥1.5) and significantly (FDR<0.05) regulated by causal genes. Of the 1010 genes which are differentially expressed (FDR<0.05 and FC≥1.5), the expression of 369 (37%) is significantly (FDR<0.05) influenced by causal genes (see list in Appendix 15). This set of 369 genes is highly functionally coherent, being enriched (FDR<0.05) with 58 Reactome pathways. We term these enriched pathways 'intermediate' pathways—the 10 most enriched pathways are shown in Table 7.3, and the full list of 58 enriched pathways is tabulated in the Appendix 15. Enrichment Map analysis revealed a highly interconnected network of pathways (Figure 7.5). In this network, each pathway is directly connected to, on average, 10 other pathways.

Table 7.3 Top 10 'intermediate' pathways: enriched Reactome pathways for 369 differentially-expressed genetically-regulated genes

Pathway	p-value	FDR
Transmembrane transport of small molecules	2.22×10^{-16}	1.50×10^{-13}
Neuronal system	6.66×10^{-16}	2.24×10^{-13}
Class A1 rhodopsin-like receptors	4.00×10^{-15}	8.98×10^{-13}
Signalling by GPCR	1.13×10^{-14}	1.91×10^{-12}
GPCR downstream signalling	7.33×10^{-14}	9.88×10^{-12}
SLC-mediated transmembrane transport	9.53×10^{-14}	1.07×10^{-11}
GPCR ligand binding	1.16×10^{-12}	1.12×10^{-10}
Transport of inorganic cations, anions and amino acids oligopeptides	2.34×10^{-12}	1.97×10^{-10}
Transmission across chemical synapses	3.74×10^{-12}	2.80×10^{-10}
Axon guidance	2.63×10^{-10}	1.77×10^{-8}

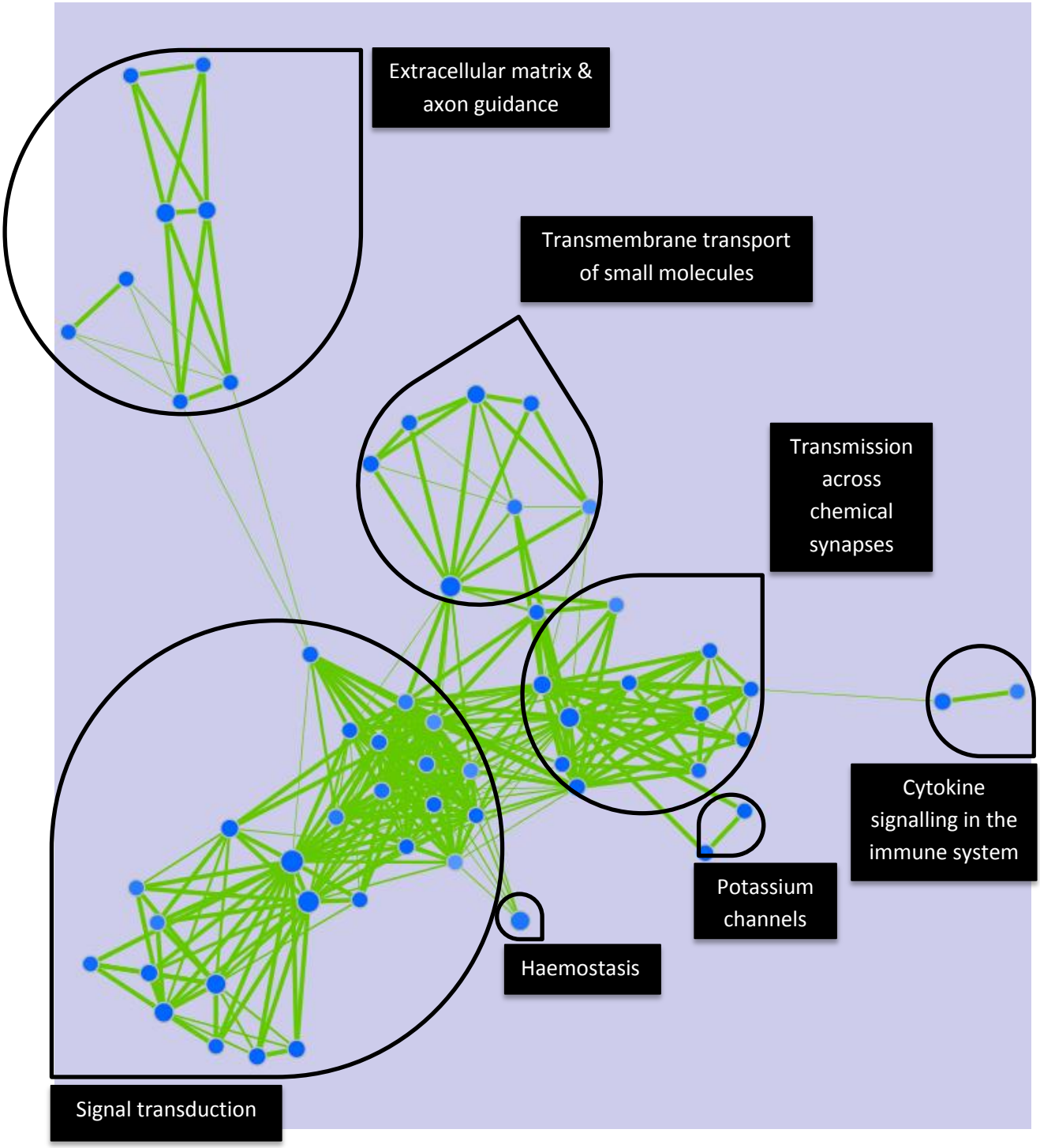


Figure 7.5 Network of intermediate pathways. Major functional groups of pathways are labelled.

7.5 Discussion

Our first task was to objectively test the hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within

the genes that constitute the core pathways and processes underlying epilepsy. In order to achieve this aim, we first created a gene-set that represents the main pathways and processes underlying epilepsy, and then determined:

1. If this gene-set is more enriched in a GWAS of pharmacoresistant epilepsy than in a GWAS of pharmacoresponsive epilepsy
2. If this gene-set is enriched in a GWAS of pharmacoresponsive vs pharmacoresistant epilepsy.

7.5.1 Creating a gene-set that represents the main pathways and processes underlying epilepsy

It has previously been shown in a number of studies that WGCNA can identify functionally and causally relevant gene modules. For example, it has been demonstrated that a significant WGCNA module from a microarray study of mouse weight is enriched with eQTLs for mouse weight (Ghazalpour *et al.* 2006). Similar to our findings in epilepsy, Roussos and colleagues found that WGCNA modules from transcriptomic profiling of post-mortem brain samples in schizophrenia are enriched in an independent schizophrenia GWAS (Roussos *et al.* 2012).

Before using the selected WGCNA module for testing our hypothesis, we took two steps to 'validate' it—we confirmed that it was a functionally relevant and causally significant gene-set:

1. We analysed the Reactome pathways enriched in this module (please see Appendix 15), and found that they were appropriate and consistent with our previous pathways analyses in Chapters 1, 4, 5 and 6.
2. We demonstrated that this module was highly enriched in an independent previously published GWAS of partial epilepsy, while 12 random gene-sets of equal size were not.

7.5.2 Testing our hypothesis using GWAS enrichment analysis

We showed that the selected WGCNA module was significantly enriched in the pharmacoresponsive epilepsy GWAS ($p=1.1x10^{-9}$), but considerably more enriched in the pharmacoresistant epilepsy GWAS ($p=2.6x10^{-14}$). To ensure that the comparison was fair,

both GWAS datasets had genotype data for the same SNPs and the same normal population control samples and the same number of disease samples. Additionally, in order to ensure consistency in correcting for inflation due to population stratification, the stringent genomic control method, which has proven effectiveness (Bouaziz *et al.* 2011), was applied to both the GWAS analyses. In the genomic control method, one calculates the λGC (the ratio of the observed-to-expected median $\chi 2$ test statistic) and divides all of the test statistics by the λGC (Devlin & Roeder 1999).

The above findings suggest that the same gene-set potentially plays a causal role in both pharmacoresponsive and pharmacoresistant epilepsy, but is considerably more enriched with genetic variations in the latter condition. This lends support to our hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy.

To confirm that there is a significant difference in the burden of genetic variations within this gene-set between the two conditions, we performed a further 'extreme discordant phenotype' GWAS analysis with pharmacoresistant cases and pharmacoresponsive controls; the gene-set was highly enriched in this analysis.

7.5.3 Causal pathways

It has previously been suggested that an eQTL that maps to a disease locus can be considered a likely causal gene underlying the disease (Thessen Hedreul *et al.* 2013). We have advanced this concept in two novel respects:

1. We have made this concept more stringent by only including eQTLs that target significantly differentially expressed genes (FDR<0.05, fold change ≥ 1.5) in the disease tissue.
2. We have extended this concept to the gene-set and pathway level, by identifying the pathways enriched within the causal genes.

721 genes fulfilled the criteria of being significantly associated with the pharmacoresistant focal epilepsy phenotype at the gene-level (empirical p-value <0.005) and gene-set level

(FDR<$5x10^{-20}$), and also being significant (FDR<0.05) eQTLs for differentially-expressed genes. 61 Reactome pathways were enriched within these 721 genes.

The 61 enriched Reactome pathways form a highly interconnected network, which is shown in Figure 7.4 with the major groups labelled. This figure illustrates the major causal processes in pharmacoresistant epilepsy, for example immune system and activation of NMDA receptors. The 10 most enriched individual pathways are shown in Table 7.1, and the 10 most central pathways are shown in Table 7.2

7.5.4 Intermediate genes and pathways

Table 7.4 Pathways which are both causal and intermediate.

Pathway
Axon guidance
CREB phosphorylation through the activation of RAS
Cytokine signalling in immune system
Developmental biology
Downstream signal transduction
GPCR downstream signalling
Haemostasis
Interaction between L1 and ankyrins
L1CAM interactions
Neuronal system
RAS activation upon calcium influx through NMDA receptor
Signalling by FGFR
Signalling by FGFR in disease
Signalling by GPCR
Signalling by PDGF
Transmembrane transport of small molecules

Gene expression is an informative intermediate phenotype that links variation in genetic information to human disease (Williams *et al.* 2007; Fehrmann *et al.* 2011). Of the genes that were significantly differentially expressed (FDR<0.05 and FC≥1.5), 369 were under significant cis- or trans-eQTL (FDR<0.05) control of causal genes; we term these 'intermediate' genes. This gene-set is highly functionally coherent, being enriched (FDR<0.05) with 58 Reactome pathways. We term these enriched pathways 'intermediate' pathways. These 58 enriched Reactome pathways form a highly interconnected network, which is shown in Figure 7.5 with the major groups labelled. This Figure illustrates the major

intermediate processes in pharmacoresistant epilepsy, for example cytokine signalling in the immune system. The most enriched individual pathways are shown in Table 7.3.

16 Reactome pathways (Table 7.4) were both causal and intermediate pathways and, hence, can be considered of special interest, as therapeutic targets, because they bear not only causal genetic variations but also the transcriptomic changes that link causal genetic variations to the disease.

As an example, we demonstrate below, using a literature review, the potential relevance of a pathway which is both a causal and an intermediate pathway.

7.5.5 Fibroblast growth factors in epilepsy

Fibroblast growth factors (FGFs) are a family of growth factors. Many members of the FGF family are dysregulated in brain tissue from epilepsy surgery (Paradiso *et al.* 2013), for example FGF2 (Sugiura *et al.* 2008; Ueda *et al.* 2011). FGFs have been implicated in a number of morphological and functional alterations associated with epileptogenesis in the hippocampus, including apoptosis, astrocytosis, blood-brain barrier disruption, changes in synaptogenesis, axonal sprouting, and aberrant neurogenesis (Paradiso *et al.* 2013). The therapeutic potential of FGFs has also been demonstrated in an animal model of epilepsy: injecting a viral vector expressing FGF2, as well as brain-derived neurotrophic factor, into the hippocampus of pilocarpine-treated rats significantly reduces neuronal loss and prevents the emergence of spontaneous recurrent seizures (Paradiso *et al.* 2011). Genetic variants in a fibroblast growth factor *FGF14* are associated with a human neurological disorder: spinocerebellar ataxia 27 (van Swieten *et al.* 2003; Dalski *et al.* 2005).

7.6 Conclusions

Using novel bioinformatics strategies, we have presented evidence in support of the hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy. We have identified the causal and intermediate pathways that are likely to be of particular importance in the development of pharmacoresistant epilepsy.

7.7 References

Abo R., Jenkins G.D., Wang L. & Fridley B.L. (2012) Identifying the genetic variation of gene expression using gene sets: application of novel gene Set eQTL approach to PharmGKB and KEGG. *PLoS One* **7**, e43301.

Bouaziz M., Ambroise C. & Guedj M. (2011) Accounting for population stratification in practice: a comparison of the main strategies dedicated to genome-wide association studies. *PLoS One* **6**, e28845.

Devlin B. & Roeder K. (1999) Genomic control for association studies. *Biometrics* **55**, 997-1004.

Fehrmann R.S., Jansen R.C., Veldink J.H., Westra H.J., Arends D., Bonder M.J., Fu J., Deelen P., Groen H.J., Smolonska A., Weersma R.K., Hofstra R.M., Buurman W.A., Rensen S., Wolfs M.G., Platteel M., Zhernakova A., Elbers C.C., Festen E.M., Trynka G., Hofker M.H., Saris C.G., Ophoff R.A., van den Berg L.H., van Heel D.A., Wijmenga C., Te Meerman G.J. & Franke L. (2011) Trans-eQTLs reveal that independent genetic variants associated with a complex phenotype converge on intermediate genes, with a major role for the HLA. *PLoS Genet* **7**, e1002197.

Ghazalpour A., Doss S., Zhang B., Wang S., Plaisier C., Castellanos R., Brozell A., Schadt E.E., Drake T.A., Lusis A.J. & Horvath S. (2006) Integrating genetic and network analysis to characterize genes related to mouse weight. *PLoS Genet* **2**, e130.

Kasperaviciute D., Catarino C.B., Heinzen E.L., Depondt C., Cavalleri G.L., Caboclo L.O., Tate S.K., Jamnadas-Khoda J., Chinthapalli K., Clayton L.M., Shianna K.V., Radtke R.A., Mikati M.A., Gallentine W.B., Husain A.M., Alhusaini S., Leppert D., Middleton L.T., Gibson R.A., Johnson M.R., Matthews P.M., Hosford D., Heuser K., Amos L., Ortega M., Zumsteg D., Wieser H.G., Steinhoff B.J., Kramer G., Hansen J., Dorn T., Kantanen A.M., Gjerstad L., Peuralinna T., Hernandez D.G., Eriksson K.J., Kalviainen R.K., Doherty C.P., Wood N.W., Pandolfo M., Duncan J.S., Sander J.W., Delanty N., Goldstein D.B. & Sisodiya S.M. (2010) Common genetic variation and susceptibility to partial epilepsies: a genome-wide association study. *Brain* **133**, 2136-47.

Langfelder P. & Horvath S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559.

Marson A.G., Al-Kharusi A.M., Alwaidh M., Appleton R., Baker G.A., Chadwick D.W., Cramp C., Cockerell O.C., Cooper P.N., Doughty J., Eaton B., Gamble C., Goulding P.J., Howell S.J., Hughes A., Jackson M., Jacoby A., Kellett M., Lawson G.R., Leach J.P., Nicolaides P., Roberts R., Shackley P., Shen J., Smith D.F., Smith P.E., Smith C.T., Vanoli A. & Williamson P.R. (2007) The SANAD study of effectiveness of carbamazepine, gabapentin, lamotrigine, oxcarbazepine, or topiramate for treatment of partial epilepsy: an unblinded randomised controlled trial. *Lancet* **369**, 1000-15.

Paradiso B., Zucchini S. & Simonato M. (2013) Implication of fibroblast growth factors in epileptogenesis-associated circuit rearrangements. *Front Cell Neurosci* **7**, 152.

Paradiso B., Zucchini S., Su T., Bovolenta R., Berto E., Marconi P., Marzola A., Navarro Mora G., Fabene P.F. & Simonato M. (2011) Localized overexpression of FGF-2 and BDNF in hippocampus reduces mossy fiber sprouting and spontaneous seizures up to 4 weeks after pilocarpine-induced status epilepticus. *Epilepsia* **52**, 572-8.

Rogawski M.A. (2013) The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. *Epilepsia* **54 Suppl 2**, 33-40.

Roussos P., Katsel P., Davis K.L., Siever L.J. & Haroutunian V. (2012) A system-level transcriptomic analysis of schizophrenia using postmortem brain tissue samples. *Arch Gen Psychiatry* **69**, 1205-13.

Sugiura C., Miyata H., Ueda M., Ohama E., Vinters H.V. & Ohno K. (2008) Immunohistochemical expression of fibroblast growth factor (FGF)-2 in epilepsy-associated malformations of cortical development (MCDs). *Neuropathology* **28**, 372-81.

Thessen Hedreul M., Moller S., Stridh P., Gupta Y., Gillett A., Daniel Beyeen A., Ockinger J., Flytzani S., Diez M., Olsson T. & Jagodic M. (2013) Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity. *Hum Mol Genet*.

Ueda M., Sugiura C., Ohno K., Kakita A., Hori A., Ohama E., Vinters H.V. & Miyata H. (2011) Immunohistochemical expression of fibroblast growth factor-2 in developing human cerebrum and epilepsy-associated malformations of cortical development. *Neuropathology* **31**, 589-98.

Williams R.B., Chan E.K., Cowley M.J. & Little P.F. (2007) The influence of genetic variation on gene expression. *Genome Res* **17**, 1707-16.

CHAPTER EIGHT

DISCUSSION

Contents

Chapter 8: Discussion

Epilepsy effects up to 1% of the population, and up to a third of people with epilepsy are pharmacoresistant—they continue to experience seizures despite treatment with maximal doses of multiple antiepileptic drugs with differing molecular targets and mechanisms of action. As detailed in Chapter 1, two different theories have gained the widest acceptance as possible explanations of pharmacoresistance: the multidrug transporter hypothesis and the intrinsic severity hypothesis. In the present work, we started by studying previously unexplored aspects of the multidrug transporter hypothesis, and then focused our attention on the intrinsic severity hypothesis.

8.1 An *in silico* and *ex vivo* analysis of SLC transporters in the pharmacoresistant epileptic hippocampus

According to the MDT hypothesis, pharmacoresistance results from decreased drug concentrations at the epileptic focus secondary to a localized dysregulation of drug transporters (Chayasirisobhon 2009), which could either increase drug efflux from, or reduce influx into, the epileptic focus. There are two main superfamilies of drug transporters: ABC proteins and SLC proteins. Research on multidrug transporters in epilepsy pharmacoresistance has been focused almost exclusively on ABC transporters. In spite of twenty years of research on the role of ABC proteins in intractable epilepsy, there remains a lack of convincing evidence that these transporters mediate pharmacoresistance. Meanwhile, SLC proteins, which constitute a much larger superfamily of transporters—there are over 400 known SLC proteins and less than 50 known ABC proteins—have hence far been neglected within epilepsy research. In order to identify the SLCs which are dysregulated in the pharmacoresistant epileptic hippocampus, we devised a robust *in silico* approach that exploits relevant published data and builds upon them using cutting-edge computational tools; we then verified the output using a robust *ex vivo* approach.

It has been recognized in the study of cancers that SLCs typically mediate uptake of drugs (Huang & Sadee 2006; Nakanishi 2007), and lower activity of these transporters may result in resistance to chemotherapy (Rochat 2009; Rosenbaum 2011). As SLCs are primarily influx transporters, we focused on SLCs that are significantly downregulated in the

pharmacoresistant epileptic hippocampus. We extracted data relating to downregulated SLCs from (1) our integrative analysis of microarray studies on brain tissue from epilepsy surgery (Chapter 2), and (2) a comprehensive review of published literature on epilepsy pharmacoresistance, and integrated all the data using 'convergent functional genomics (CFG)'—a validated technique (Bertsch *et al.* 2005) for prioritizing genes involved in complex diseases by collating evidence using a pre-defined scoring system. To identify SLCs which have not been studied in epilepsy pharmacoresistance but could potentially be involved, we employed a computational gene prioritization tool called Endeavour (Tranchevent *et al.* 2008), using SLCs with the highest CFG-scores as the training genes. We validated this computationally prioritized gene list by (1) prioritizing the same candidate genes using a robust independent 'positive control' training set comprising the 10 most consistently downregulated genes from the aforementioned integrative analysis and, then, demonstrating significant rank order similarity with the original list, and (2) prioritizing the same candidate genes using a 'negative control' training set comprising 10 randomly chosen genes from the aforementioned integrative analysis and demonstrating no significant rank order similarity with the original list.

As our *in* silico strategy was novel, we validated our *in silico* strategy by *ex vivo* analysis of human brain tissue. We used a custom one-colour Agilent oligonucletoide microarray containing exon probes for all known SLCs to analyse 24 hippocampal samples obtained from surgery for pharmacoresistant mesial temporal lobe epilepsy and 24 hippocampal samples from normal post-mortem controls. The whole-transcript amplification protocol of exon arrays allows more accurate measurement of gene expression than standard microarrays (Kapur *et al.* 2007; Xing *et al.* 2007; Lockstone 2011). Stringent QC filters were applied to arrays and individual features, and a 'percent present' filter was applied to the genes to reduce false positives. Finally, important technical (batch and RIN) and clinical (age and sex) covariates were included in the linear model; this reduced the false positive rate significantly. Our exon array identified 18 SLCs significantly (FDR <0.05) downregulated in the epileptic hippocampus by 1.5 fold or more. There was a highly significant overlap between the genes identified by our *in silico* and *ex vivo* strategies: $p < 9.0 \times 10^{-7}$ (hypergeometric distribution) for the overlap between the top bioinformatically-identified SLCs and the 18 experimentally-identified SLCs. Our successful *in silico* strategy can be

adapted in order to prioritize genes relevant to epilepsy from other gene families. qRT-PCR results for the three tested SLC genes were highly concordant with the microarray results.

The vast majority of the identified SLC proteins are either small metal ion exchangers or transporters of neurotransmitters, particularly glutamate (see Table 3.6 in Chapter 3). Given the key roles played by ionic transport and gluatmatergic transmission in neuronal function, it stands to reason that these should be the most important SLC proteins in the epileptic hippocampus. However, for SLC transporters of metal ions, there is as yet no evidence of non-endogenous substrate transport—their small endogenous ionic substrates are markedly dissimilar to most xenobiotics, so it might be expected that they are not readily involved in xenobiotic transport (Dobson & Kell 2008). Similarly, there is no evidence as yet that the transporters of glutamate or of other neurotransmitters are able to transport therapeutic drugs. Therefore, while these SLCs potentially mediate pharmacoresistance in epilepsy, it is unlikely that they do this through altered transport of AEDs, but rather by enhancing the intrinsic severity of epilepsy (Schmidt & Loscher 2009).

8.2 Objective evidence in support of the intrinsic severity hypothesis

According to the intrinsic severity hypothesis, pharmacoresistance in epilepsy is the manifestation of increased dysfunction of the processes and pathways which underlie epilepsy. It has previously been suggested that genetic variations are likely to play a key role in producing increased dysfunction of these pathways and processes (Rogawski 2013). We are not aware of any previous attempts to test the hypothesis that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy. In order to test this hypothesis, we first identified the single most functionally-relevant gene-set that represents the main pathways and processes underlying epilepsy, through the widely-used and validated WGCNA, and then determined if this gene-set was affected by genetic variation more in drug resistant than in drug responsive epilepsy. In order to identify the genetic variations associated with drug responsive epilepsy, a GWAS was performed of drug responsive epilepsy. Similarly, in order to identify the genetic variations associated with drug resistant epilepsy, a GWAS was performed of drug resistant epilepsy. We then compared the degree of enrichment of the functionally-relevant gene-set in the two GWAS. We showed that the gene-set was significantly enriched in the

pharmacoresponsive epilepsy GWAS ($p=1.1x10^{-9}$), but considerably more enriched in the pharmacoresistant epilepsy GWAS ($p=2.6x10^{-14}$). To ensure that the comparison was fair, both GWAS datasets had genotype data for the same SNPs and the same normal population control samples and the same number of disease samples. To confirm that there is a significant difference in the burden of genetic variations within this gene-set between the two conditions, we performed a further 'extreme discordant phenotype' GWAS analysis with pharmacoresistant cases and pharmacoresponsive controls; the gene-set was highly enriched in this analysis. Using this strategy, we have demonstrated that pharmacoresistance is the result of accumulation of deleterious genetic variations of increasing severity and/or numbers within the genes that constitute the core pathways and processes underlying epilepsy.

What are the different pathways underlying pharmacoresistant epilepsy and which of these are most important? In order to answer these questions, we have taken a step-wise analytical approach:

1. Complexity: Identifying the different and diverse dysfunctional pathways underlying pharmacoresistant epilepsy.
2. Coherence: Mapping a coherent interconnected network formed by the above dysfunctional pathways.
3. Centrality: Identifying the most central 'hub' pathways in the above network.

8.3 Complexity: The pathways underlying epilepsy pharmacoresistance are diverse

The pathways underlying epilepsy belong to diverse functional domains—this was suggested by our integrative analysis of previously published large-scale gene expression profiling studies on brain tissue from epilepsy surgery (Chapter 2), and confirmed by our microarray analysis (Chapter 4), and further supported by our GWAS analysis (Chapter 5).

In the integrative analysis of previously published large-scale gene expression profiling studies on brain tissue from epilepsy surgery (Chapter 2), we integrated the lists of differentially expressed genes from nine previously published microarray studies. The validity of our integrative approach was demonstrated by using an inter-study cross-

validation technique and by demonstrating a statistically significant overlap with CarpeDB, a dynamic continuously updated epilepsy genetics database. The integrated gene list was then subjected to pathway and gene ontology analysis. The enriched pathways belonged to a number of different functional domains, but three domains were most prominently overrepresented in the integrated gene list: neuro-inflammation, modulation of synaptic transmission, and restructuring of neuronal networks.

Although, as stated above, there have been a number of previous microarray studies on brain tissue from surgery for pharmacoresistant epilepsy, we went onto perform our own microarray analysis that is superior to the previous studies in a number of significant and novel ways. This is the largest transcriptomic study of hippocampal tissue from pharmacoresistant mesial temporal lobe epilepsy to date, having thrice the number of disease samples of the previous largest study. Furthermore, cutting edge analytical techniques have been applied which improve the sensitivity and reliability of our results: we have accounted and corrected for batch effects using careful study design and appropriate data processing; we have adjusted for unknown confounders using the cutting edge Independent Surrogate Variable Analysis technique; we have demonstrated the functional and therapeutic relevance of our results by showing that they are enriched with antiepileptic drug targets and more so than any previous microarray study; we have demonstrated the causal relevance of our results by showing their enrichment in a GWAS of pharmacoresistant partial epilepsy; and we have performed a 'differentially connectivity' analysis, which is the first such analysis in epilepsy. We identified 118 unique dysregulated (differentially expressed or differentially connected) pathways. As found in the integrative analysis of previously published microarray studies, the enriched pathways identified in our microarray analysis belong to diverse functional domains, for example 'axon guidance', 'transmembrane transport of small molecules' and 'class A1 rhodopsin-like receptors'.

We performed the first ever genome-wide association study (GWAS) of pharmacoresistant partial epilepsy, with discovery and replication cohorts including, in total, 421 cases and 2624 normal population controls, and 5,519,310 genotyped and imputed SNPs. At the single SNP level, there was no overlap in the top results of the two cohorts. We went on to perform gene-set and pathway-level analyses. We carried out the first ever objective validation of the GWAS gene-set analysis approach by showing that a gene-set comprising

antiepileptic drug targets is very significantly enriched in the GWAS, while random gene-sets of equal size are not. We then demonstrated that, at the gene-set and pathway level, there is clear replication of results between the two cohorts: there was a striking correlation between the discovery and replication cohorts in the gene-set analysis results (Pearson's correlation coefficient=0.8, one-sided p-value<2.2×10^{-16}). Furthermore, out of the top 10 enriched pathways in the discovery cohort, all were replicated in the replication cohort. Overall, there was a highly significant overlap between the enriched pathways for the discovery and replication cohorts (hypergeometric distribution p-value <3.7×10^{-17}). The combined GWAS 'mega-analysis' was used to perform pathway enrichment analysis. Again, the pathways enriched in the GWAS represent disparate processes, for example 'axon guidance', 'transmembrane transport of small molecules' and 'cell death signalling via NRAGE, NRIF and NADE'.

8.4 Coherence: the dysfunctional pathways underlying epilepsy form a coherent interconnected network

Enrichment Map (Merico *et al.* 2010) tool was used to determine the connections between enriched pathways. Enrichment Map was used with default settings. Specifically, we employed an overlap coefficient cut-off of 0.5. Given sets A and B, and the cardinality operator | | where |X| equals to the number of elements within set X, the overlap coefficient (OC) is defined as:

$$OC = \frac{|A \cap B|}{Min(|A|, |B|)}$$

In other words, two pathways were deemed connected only if the ratio of the size of the intersection over the size of the smallest pathway was 0.5 or more

Enrichment Map analysis of our transcriptomic study revealed a highly interconnected central network of pathways in which each pathway is directly connected to, on average, 10.5 other pathways. Similarly, Enrichment Map analysis of our GWAS study revealed a highly interconnected central network of pathways in which each pathway is directly connected to, on average, 8.4 other pathways.

The above analysis shows that the dysregulated pathways underlying epilepsy pharmacoresistance, though seemingly unrelated, in fact form a coherent whole, and it can be expected that changes in one pathway in this network will have a cascading effect on the rest of the network.

8.5 Centrality: Identifying the most central 'hub' pathways

The Network Analysis tool was used to calculate the 'betweenness centrality' of nodes (individual pathways) in the networks. Betweenness is a measure of the centrality of a node in a network, and is calculated as the fraction of shortest paths between node pairs that pass through the node of interest. Hence, betweenness is a measure of the influence a node has over the spread of information through the network.

In our transcriptomic analysis, the most central pathway by far was 'signalling by Notch'. In our genetic analysis, the most central pathway by far was 'signalling by NGF'. We have conducted literature reviews in order to highlight previously published evidence of the potential role of Notch signalling (see Chapter 4) and signalling by NGF (see Chapter 5) in epilepsy.

It must be noted, however, that the central pathways identified are not necessarily causal. For example, it is possible that some of the gene expression changes identified in our transcriptomic analysis are the consequence, rather than the cause, of refractory seizures. Similarly, some of the genetic changes identified in our GWAS might be 'bystander' variations that do not have a causal link with the phenotype. In order to identify, with greater certainty, the pathways which are likely to be causal, we performed an expression quantitative trait loci (eQTL) analysis, and integrated the results with our genetic and transcriptomic analyses.

8.6 Causality

Our eQTL analysis included hippocampal samples from 22 patients with mesial temporal lobe epilepsy and from 22 normal controls. It has been shown that 69% to 80% of eQTLs operate in a cell-type specific manner (Dimas *et al.* 2009). There is only one previously published eQTL study on human hippocampal tissue (Kim *et al.* 2012); this study was limited to cis-eQTL analysis. There are currently no published trans-eQTL analyses of hippocampal

tissue, and no eQTL analyses of the epileptic human hippocampus. Our study not only examines eQTLs in hippocampal tissue, but specifically in samples with the disease, a strategy which may be necessary for the identification of the disease-related eQTLs (Ertekin-Taner 2011).

In complex diseases, truly trait-associated SNPs are more likely to be eQTLs (Nicolae *et al.* 2010), and eQTL analysis is a powerful approach for the detection of novel disease risk loci (Schadt 2005). It has been suggested that an eQTL that maps to a disease locus can be considered a likely causal gene underlying the disease (Thessen Hedreul *et al.* 2013). We found 721 genes that were associated with the pharmacoresistant focal epilepsy phenotype in our GWAS analysis at an empirical p-value <0.005 and were also significant (FDR<0.05) eQTLs for differentially-expressed genes from our microarray study. Because our chosen GWAS p-value cut-off of 0.005 could be criticised for being uncorrected, we determined if these genes were significantly associated with pharmacoresistant epilepsy at the gene-set level. These 721 genes were significantly associated with the pharmacoresistant focal epilepsy phenotype at the gene-set level (FDR<$5x10^{-20}$), while 25 random sets of 721 genes were not (minimum FDR=0.7). 61 Reactome pathways were enriched within these 721 genes. These 61 pathways were designated 'causal' pathways, as they are likely to represent the genetic changes which influence the transcriptomic intermediate phenotype which, in turn, contributes to the development of the clinical phenotype. As shown previously for our genetic and transcriptomic pathway analysis, we demonstrated again that these 61 causal pathways, although apparently diverse and disparate, form a highly interconnected coherent network. The top 10 most central pathways in this causal network are shown in Table 8.1

We also collated the list of genes which are under significant (FDR<0.05) eQTL control of causal genes and significantly differentially expressed (FDR<0.05 and FC≥1.5) in pharmacoresistant epileptic tissue. We performed Reactome pathway enrichment analysis on this set of genes. These pathways were designated 'intermediate' pathways, as they are likely to represent the true transcriptomic intermediate phenotype which contributes to the development of the clinical phenotype of interest. 16 Reactome pathways (see Table 7.3 in Chapter 7) were both causal and intermediate pathways and, hence, can be considered of

special interest, as therapeutic targets, because they bear not only causal genetic variations but also the transcriptomic changes that link causal genetic variations to the disease.

Table 8.1 Top 10 central causal pathways

Pathway
Transmembrane transport of small molecules
DCC mediated attractive signalling
Axon guidance
Cell-cell communication
Developmental biology
Signalling by NGF
Immune system
TIE2 signalling
Signalling by constitutively active EGFR
CREB phosphorylation through the activation of RAS

8.7 Limitations

The potential limitations of this work should be considered.

The first point to consider is the type of control tissue used in the transcriptomic analysis. Table 8.2 lists the desired features of the 'ideal' control tissue for a transcriptomic study on the causes of pharmacoresistant MTLE.

Table 8.2 Desired features of the 'ideal' control tissue for a transcriptomic study on the causes of pharmacoresistant MTLE

Desired features of the 'ideal' control tissue
1. Tissue is from donors: a. with drug responsive epilepsy b. exposed to the same range of AEDs as the disease group 2. Epileptic hippocampus; free from other diseases 3. Surgically resected and stored under conditions similar to disease tissue 4. Histologically similar to the disease/pharmacoresistant group 5. Comparable subsections of the hippocampus are used for both disease and control groups 6. Tissue is available for research studies!

Considering the features listed in table 8.2, it is evident that the ideal control tissue for a transcriptomic study on the causes of pharmacoresistant MTLE is non-existent and

unobtainable. For example, patients with drug responsive epilepsy will not be exposed to the same range of AEDs as those with drug resistant epilepsy, and patients with drug responsive epilepsy will not undergo epilepsy surgery. Hence, various types of alternative brain tissue have been tried by epilepsy researchers (see Table 2.1 in Chapter 2). For example, Lee and colleagues (Lee *et al.* 2007) utilized hippocampi from surgery for drug resistant MTLE that were not affected by hippocampal sclerosis, even though the hippocampi were thought to be the epileptic foci. However, comparing drug resistant epileptic hippocampi in such a way runs the risk of failing to discover important transcriptional changes contributing to pharmacoresistant epilepsy. Becker and colleagues (Becker *et al.* 2002) used hippocampal tissue adjacent to and resected along with brain tumours. This hippocampal tissue was thought to be normal. This approach also poses certain difficulties. It is impossible to be certain that there is no cancerous contamination within the hippocampal tissue which lies just adjacent to the tumour. Even if there is no overt histological evidence of contamination with cancer cells within the portions of hippocampal tissue being used for transcriptomic analysis, some of these hippocampal cells may be in a 'pre-cancerous' state at the transcriptomic level. Becker and colleagues (Becker *et al.* 2003) also tried using different subsections of the same hippocampus for disease and control tissue: CA1 formation was treated as disease tissue, while the dentate gyrus was treated as control tissue. This methodology is also flawed. Firstly, the denate gyrus also plays a role in MTLE (Kralic *et al.* 2005; Sloviter *et al.* 2012). Secondly, the transcriptomic profiles of two histologically distinct tissues will be significantly dissimilar.

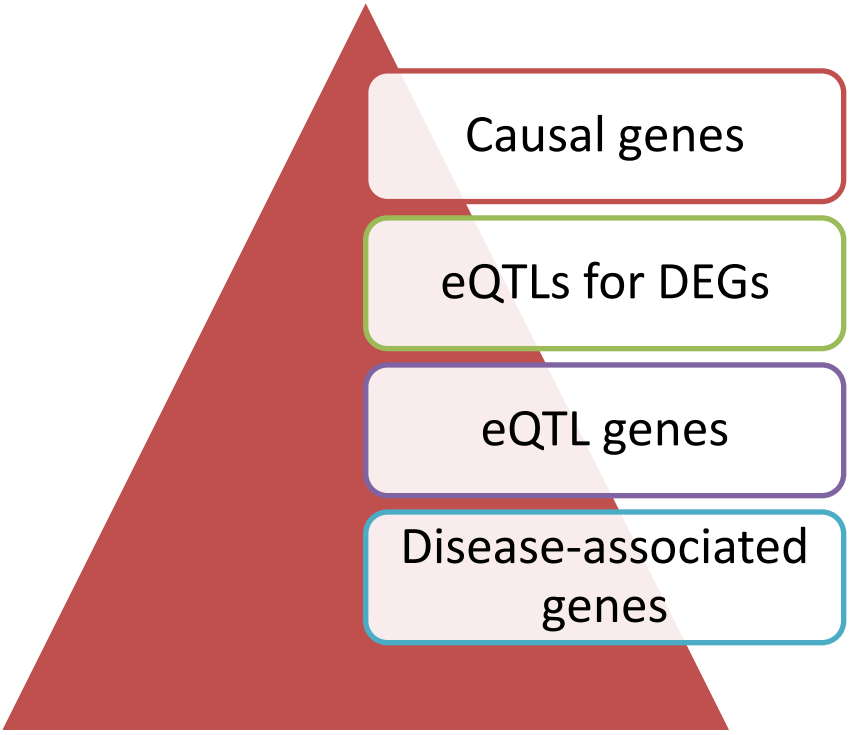
Normal post-mortem hippocampal tissue has been used as the control in a number of studies (Ozbas-Gerceker *et al.* 2006; van Gassen *et al.* 2008; Venugopal *et al.* 2012). The use of such control tissue offers a number of benefits. Various parts of the donor brain have been histologically studied to ensure that they are free from pathology. Also, the whole hippocampus is available and, hence, equivalent anatomical subsections of the hippocampus can be used for control and disease groups. Furthermore, utilizing normal hippocampus, rather than drug resistant epileptic hippocampus, does not run the risk of obscuring important transcriptional changes contributing to epilepsy pharmacoresistance. On the other hand, some will argue that comparing pharmacoresistant epileptic hippocampus with normal hippocampus will reveal changes underlying epilepsy in general

and not epilepsy pharmacoresistance in particular. However, as we have shown in the present work, epilepsy pharmacoresistance results from the increased dysfunction of the same pathways and processes that underlie epilepsy. This being the case, using normal post-mortem hippocampal control tissue is likely to be the most effective and efficient strategy for uncovering the causes of epilepsy and epilepsy pharmacoresistance.

The final point presented in the above discussion is also applicable to GWAs analyses. We used normal controls in the GWAS analysis presented in Chapter 5. Again, a possible criticism is that comparing pharmacoresistant epilepsy with normal controls will reveal changes underlying epilepsy in general and not epilepsy pharmacoresistance in particular. However, as stated above, epilepsy pharmacoresistance results from the increased dysfunction of the same pathways and processes that underlie epilepsy and, hence, studying epilepsy and epilepsy pharmacoresistance in unison, rather than in artificial isolation, is likely to be most fruitful and efficient. This can be achieved by comparing pharmacoresistant epilepsy with normal controls

Finally, in previous studies by other researchers (Thessen Hedreul *et al.* 2013), an eQTL that maps to a disease locus has been considered potentially causal. We have adopted an even more robust and selective approach to identifying potentially causal genes. In this approach, (Figure 8.1) potentially causal genes are those that are (1) disease-associated, (2) eQTLs, and (3) influence the transcript levels of genes differentially-expressed in pharmacoresistant epilepsy. However, even such a stringent approach is, on its own, insufficient for confirming the causal influence of the identified genes. Causality can only be confirmed using functional studies; one such functional strategy is proposed in the next section.

Figure 8.1 Overall scheme for the identification of causal genes. eQTL: expression quantitative trait locus; DEGs: differentially expressed genes.



8.8 Coming full circle and future work

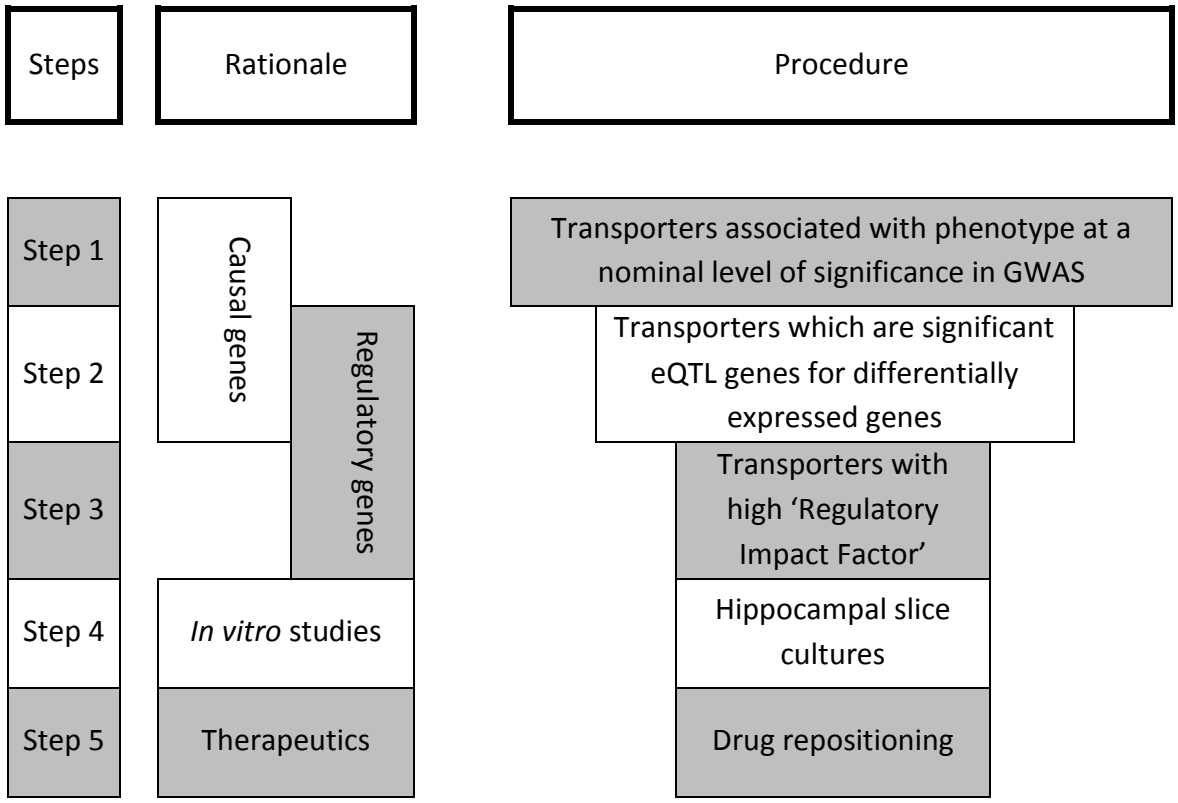
We started this work by focusing on the multidrug transporter hypothesis. Specifically, we determined which SLC proteins are dysregulated in the pharmacoresistant epileptic hippocampus and, based on current knowledge about substrates of these proteins, we judged whether they are likely to transport antiepileptic drugs. We found a number of SLC proteins to be dysregulated in the pharmacoresistant epileptic hippocampus. However, these particular SLC proteins are highly unlikely to be transporters of xenobiotics (although we cannot completely exclude this as we did not undertake transport studies). Instead, as we suggested in Chapter 2, these SLC proteins are more likely to contribute to the development of pharmacoresistance through altered transport of small metal ions or neurotransmitters.

We then turned our attention to the intrinsic severity hypothesis, and used genome-wide genetic, transcriptomic and integrative systems level approaches to find the most important pathways underlying epilepsy pharmacoresistance. At the genetic level, the

'transmembrane transport of small molecules' pathway was one of the most significant in our GWAS of pharmacoresistant epilepsy and one of the most central. Similarly, at the transcriptomic level, this pathway was one of the most significantly differentially expressed, differentially connected and central pathways. Most importantly, in our integrative systems level analysis for discovering 'causal' pathways, we found the 'transmembrane transport of small molecules' pathway to be most central. As expected, this pathway was also one of the 'intermediate' pathways, which represent the true transcriptomic intermediate phenotype which contributes to the development of the clinical phenotype of interest.

This brings us back full circle to the conclusion drawn from our initial exon array analysis: altered transport of small molecules plays an important role in the development of pharmacoresistant epilepsy. The central position of transporters of small molecules within a network comprising diverse and distinct biological pathways suggests a potential role for these transporters in the regulation of many processes. How can we identify the key transporters which are likely to be most influential? A proposed integrative schema is illustrated in Figure 8.2. Steps 1 and 2 of this schema represent the strategy employed for finding causal genes in Chapter 7; this strategy is based on the observation that an eQTL that maps to a disease locus can be considered a likely causal gene underlying the disease (Thessen Hedreul *et al.* 2013). Steps 2 and 3 represent two complimentary and proven methods for identifying genes with the most prominent regulatory roles. Step 2 identifies important eQTLs: transporters whose genotype is significantly correlated with differentially expressed genes. Step 3 utilises a recently developed innovative method for identifying genes with an important regulatory role: the 'Regulatory Impact Factor (RIF)' (Reverter *et al.* 2010). RIF identifies the regulator genes that are most consistently differentially co-expressed with differentially expressed genes. RIF analysis has been shown to recover well-recognised experimentally validated regulator genes for the processes studied, for example PPAR signalling in adipose development and the importance of transduction of oestrogen signals in breast cancer survival and sexual differentiation (Reverter *et al.* 2010).

Figure 8.2 Suggested schema for the identification of transporters of causal and regulatory importance in pharmacoresistant epilepsy.



After the identification of transporters of putative causal and regulatory importance, *in vitro* studies can be performed to confirm the functional significance of these proteins in epilepsy. As the transporters potentially exert an influence upon diverse functional domains, including electrophysiological and histological, they must be studied in an *in vitro* model that allows detailed investigation of cellular and molecular features of epilepsy, yet is amenable and rapidly responsive to genetic and pharmacological manipulation. The organotypic hippocampal slice culture (OHSC) is such a model.

OHSCs are becoming an increasingly popular tool in the study of epileptogenesis, chronic epilepsy and antiepileptics. OHSCs are a robust and rapid model of epileptogenesis, and can be maintained for weeks or longer (Dyhrfjeld-Johnsen *et al.* 2010). The key features of human epileptogenesis are reproduced in this model (Berdichevsky *et al.* 2012): latency (the cultures become spontaneously epileptic after a latent period *in vitro*), electrographic spiking prior to the onset of spontaneous seizures, clustering of seizure activity, suppression of seizures but not interictal spikes by anticonvulsants and the gradual development of anticonvulsant resistance. Advantages of this preparation include dramatically improved

experimental accessibility (Wahab *et al.* 2010): organotypic cultures easily lend themselves to continuous recordings of electrical activity, imaging of cellular plasticity, gene transfer and transduction, and chronic application of drugs. Given the convenience of *in vitro* preparations and the relative compressed time frame of epileptogenesis in OHSCs, this model could serve for high-throughput screening of candidate antiepileptic drugs. The best candidates from the initial *in vitro* screen could then be tested further in traditional *in vivo* models of epilepsy.

A number of different transporters are targeted by widely-used medicinal agents, for example cardiac glycosides and proton pump inhibitors (Alexander 2011). Hence, after the identification of transporters of putative clinical significance in epilepsy, the first strategy should be to employ computational drug repositioning (Hurle *et al.* 2013) in order to repurpose therapeutic agents that can be used to tackle the problem of pharmacoresistant epilepsy.

8.9 References

Alexander S. (2011) Transporters are an under-developed therapeutic target. Discuss. *Br J Pharmacol* **164**, 1751-2.

Becker A.J., Chen J., Paus S., Normann S., Beck H., Elger C.E., Wiestler O.D. & Blumcke I. (2002) Transcriptional profiling in human epilepsy: expression array and single cell real-time qRT-PCR analysis reveal distinct cellular gene regulation. *Neuroreport* **13**, 1327-33.

Becker A.J., Chen J., Zien A., Sochivko D., Normann S., Schramm J., Elger C.E., Wiestler O.D. & Blumcke I. (2003) Correlated stage- and subfield-associated hippocampal gene expression patterns in experimental and human temporal lobe epilepsy. *Eur J Neurosci* **18**, 2792-802.

Berdichevsky Y., Dzhala V., Mail M. & Staley K.J. (2012) Interictal spikes, seizures and ictal cell death are not necessary for post-traumatic epileptogenesis in vitro. *Neurobiol Dis* **45**, 774-85.

Bertsch B., Ogden C.A., Sidhu K., Le-Niculescu H., Kuczenski R. & Niculescu A.B. (2005) Convergent functional genomics: a Bayesian candidate gene identification approach for complex disorders. *Methods* **37**, 274-9.

Chayasirisobhon S. (2009) The mechanisms of medically refractory temporal lobe epilepsy. *Acta Neurol Taiwan* **18**, 155-60.

Dimas A.S., Deutsch S., Stranger B.E., Montgomery S.B., Borel C., Attar-Cohen H., Ingle C., Beazley C., Gutierrez Arcelus M., Sekowska M., Gagnebin M., Nisbett J., Deloukas P., Dermitzakis E.T. & Antonarakis S.E. (2009) Common regulatory variation impacts gene expression in a cell type-dependent manner. *Science* **325**, 1246-50.

Dobson P.D. & Kell D.B. (2008) Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat Rev Drug Discov* **7**, 205-20.

Dyhrfjeld-Johnsen J., Berdichevsky Y., Swiercz W., Sabolek H. & Staley K.J. (2010) Interictal spikes precede ictal discharges in an organotypic hippocampal slice culture model of epileptogenesis. *J Clin Neurophysiol* **27**, 418-24.

Ertekin-Taner N. (2011) Gene expression endophenotypes: a novel approach for gene discovery in Alzheimer's disease. *Mol Neurodegener* **6**, 31.

Huang Y. & Sadee W. (2006) Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. *Cancer Lett* **239**, 168-82.

Hurle M.R., Yang L., Xie Q., Rajpal D.K., Sanseau P. & Agarwal P. (2013) Computational drug repositioning: from data to therapeutics. *Clin Pharmacol Ther* **93**, 335-41.

Kapur K., Xing Y., Ouyang Z. & Wong W.H. (2007) Exon arrays provide accurate assessments of gene expression. *Genome Biol* **8**, R82.

Kim S., Cho H., Lee D. & Webster M.J. (2012) Association between SNPs and gene expression in multiple regions of the human brain. *Transl Psychiatry* **2**, e113.

Kralic J.E., Ledergerber D.A. & Fritschy J.M. (2005) Disruption of the neurogenic potential of the dentate gyrus in a mouse model of temporal lobe epilepsy with focal seizures. *Eur J Neurosci* **22**, 1916-27.

Lee T.S., Mane S., Eid T., Zhao H., Lin A., Guan Z., Kim J.H., Schweitzer J., King-Stevens D., Weber P., Spencer S.S., Spencer D.D. & de Lanerolle N.C. (2007) Gene expression in temporal lobe epilepsy is consistent with increased release of glutamate by astrocytes. *Mol Med* **13**, 1-13.

Lockstone H.E. (2011) Exon array data analysis using Affymetrix power tools and R statistical software. *Brief Bioinform* **12**, 634-44.

Merico D., Isserlin R., Stueker O., Emili A. & Bader G.D. (2010) Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One* **5**, e13984.

Nakanishi T. (2007) Drug transporters as targets for cancer chemotherapy. *Cancer Genomics Proteomics* **4**, 241-54.

Nicolae D.L., Gamazon E., Zhang W., Duan S., Dolan M.E. & Cox N.J. (2010) Trait-associated SNPs are more likely to be eQTLs: annotation to enhance discovery from GWAS. *PLoS Genet* **6**, e1000888.

Ozbas-Gerceker F., Redeker S., Boer K., Ozguc M., Saygi S., Dalkara T., Soylemezoglu F., Akalan N., Baayen J.C., Gorter J.A. & Aronica E. (2006) Serial analysis of gene expression in the hippocampus of patients with mesial temporal lobe epilepsy. *Neuroscience* **138**, 457-74.

Reverter A., Hudson N.J., Nagaraj S.H., Perez-Enciso M. & Dalrymple B.P. (2010) Regulatory impact factors: unraveling the transcriptional regulation of complex traits from expression data. *Bioinformatics* **26**, 896-904.

Rochat B. (2009) Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents. *Curr Cancer Drug Targets* **9**, 652-74.

Rogawski M.A. (2013) The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. *Epilepsia* **54 Suppl 2**, 33-40.

Rosenbaum S. (2011) *Basic pharmacokinetics and pharmacodynamics : an integrated textbook and computer simulations*. John Wiley & Sons, Hoboken, N.J.

Schadt E.E. (2005) Exploiting naturally occurring DNA variation and molecular profiling data to dissect disease and drug response traits. *Curr Opin Biotechnol* **16**, 647-54.

Schmidt D. & Loscher W. (2009) New developments in antiepileptic drug resistance: an integrative view. *Epilepsy Curr* **9**, 47-52.

Sloviter R.S., Bumanglag A.V., Schwarcz R. & Frotscher M. (2012) Abnormal dentate gyrus network circuitry in temporal lobe epilepsy. In: *Jasper's Basic Mechanisms of the Epilepsies* (eds. by Noebels JL, Avoli M, Rogawski MA, Olsen RW & Delgado-Escueta AV), Bethesda (MD).

Thessen Hedreul M., Moller S., Stridh P., Gupta Y., Gillett A., Daniel Beyeen A., Ockinger J., Flytzani S., Diez M., Olsson T. & Jagodic M. (2013) Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity. *Hum Mol Genet*.

Tranchevent L.C., Barriot R., Yu S., Van Vooren S., Van Loo P., Coessens B., De Moor B., Aerts S. & Moreau Y. (2008) ENDEAVOUR update: a web resource for gene prioritization in multiple species. *Nucleic Acids Res* **36**, W377-84.

van Gassen K.L., de Wit M., Koerkamp M.J., Rensen M.G., van Rijen P.C., Holstege F.C., Lindhout D. & de Graan P.N. (2008) Possible role of the innate immunity in temporal lobe epilepsy. *Epilepsia* **49**, 1055-65.

Venugopal A.K., Sameer Kumar G.S., Mahadevan A., Selvan L.D., Marimuthu A., Dikshit J.B., Tata P., Ramachandra Y., Chaerkady R., Sinha S., Chandramouli B., Arivazhagan A., Satishchandra P., Shankar S. & Pandey A. (2012) Transcriptomic Profiling of Medial Temporal Lobe Epilepsy. *J Proteomics Bioinform* **5**.

Wahab A., Albus K., Gabriel S. & Heinemann U. (2010) In search of models of pharmacoresistant epilepsy. *Epilepsia* **51 Suppl 3**, 154-9.

Xing Y., Ouyang Z., Kapur K., Scott M.P. & Wong W.H. (2007) Assessing the conservation of mammalian gene expression using high-density exon arrays. *Mol Biol Evol* **24**, 1283-5.

Appendix

Appendices can be found in the enclosed compact disc.