Gene Regulation by Drugs Used to Treat Mood Disorders

Xiaoyan Deng

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ABSTRACT

Mood disorders such as Major Depressive Disorder and Bipolar Disorder are devastating burdens upon sufferers, their families and society as a whole. Despite their high prevalence and socioeconomic impact, understanding the aetiology of mood disorders has proved difficult, due in part to the heterogeneity observed in disorders of mental well-being. Antidepressant and mood stabiliser drugs are the main treatment for mood disorders. Although highly efficacious, the mechanisms of action of these drugs are not well understood. This project aimed to establish the effects of such drugs on gene regulation, and in doing so, advance the field by detecting specific transcripts and biochemical pathways involved in drug response. It was anticipated that this knowledge would prove valuable for developing novel therapeutic and treatment options of the future.

Initial work focused upon promoter regions of candidate genes chosen from the literature and previous results from our laboratory. Nineteen constructs were investigated in the rat serotonergic cell line RN46A, using a luciferase reporter assay and a commonly prescribed SSRI antidepressant paroxetine. Although significant differences in expression were noted between drug-exposed and control cell cultures, the system proved inconsistent, despite considerable care in experimental design and execution.

A more direct approach, real-time quantitative PCR (qPCR), was then utilised to detect drug-induced gene expression differences in RN46A cells. Five drugs were used: the antidepressants paroxetine, citalopram and nortriptyline; the antipsychotic haloperidol; and the mood stabiliser sodium valproate. Of five potential reference genes for normalization of qPCR experiments, *Actb*, *G6pd* and *Rnf4* were stably expressed over

differing treatments. I screened thirty-nine candidate genes for expression changes attributable to drug exposure. The most striking result was a reproducible ~1700-fold up-regulation of sepiapterin reductase (*SPR*), by the drug sodium valproate. *SPR* encodes a key enzyme for neurotransmitter synthesis. *Qdpr*, encoding a protein in the same pathway, was also up-regulated by valproate, but to a lesser extent. *SPR* and members of the biopterin biosynthesis pathway have previously been implicated in the biology of depression. Another gene, the serotonin receptor 2A (*Htr2a*) was significantly down-regulated by the SSRIs paroxetine and citalopram.

Having identified that valproate induced the most significant gene expression changes,I further conducted time-course and dose-response experiments to investigate the dynamics and pharmacology of valproate- induced gene expression changes. *Spr* and *Hdac6*, a member of the histone deacetylase gene family, were found to behave differently in these experiments. Subsequently, western blotting confirmed increased SPR protein expression in treated cells, and detected at least three isoforms recognised by a commercially sourced SPR antibody.

Attempts to identify signalling pathways regulated by valproate failed to detect significant changes in the phosphorylation levels of specific kinases using an ELISA array.

Collectively, these data establish SPR and a number of other proteins in the aetiology of mood disorders and their treatment, and provide a basis for further study of the effects of mood stabilisers and antidepressants in a mammalian setting.

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ABBREVIATIONS

5-HT	serotonin
5-HTTLPR	serotonin transporter gene-linked polymorphic region
°C	degree Celsius
ABA	Allen Brain Atlas
AD	antidepressant
Amp	ampicillin
BD	bipolar disorder
BH_4	tetrahydrobiopterin
bp	base pair
BSA	bovine serum albumin
BDNF	brain-derived neurotrophic factor
CBT	cognitive behavioral therapy
cDNA	complementary deoxyribonucleic acid
Cp	crossing point
CREB	cyclic AMP response element binding protein
DA	dopamine
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMI	desipramine
DNA	deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
ERK	extracellular signal regulated kinase
FAM	fluorescein
FBS	fetal bovine serum
GSK-3	glycogen synthase kinase three
GWAS	genome-wide association study
HAT	histone acetyltransferase
HDAC	histone deacetylase
HPA	hypothalamus-pituitary-adrenal
HSV-TK	herpes simplex virus thymidine kinase
ISH	*in situ* hybridization
IPT	interpersonal therapy
kb	kilobase
M	molar
MAOI	monoamine oxidase inhibitor
MARCKS	myristoylated alanine-rich C kinase substrate
MARS	the Munich Antidepressant Response Signature
mg	milligram
min	minutes
miRNA	microRNA
ml	milliliter

mM	millimolar
MDD	major depressive disorder
mRNA	messenger RNA
NA	noradrenaline
NARI	noradrenaline reuptake inhibitors
NaSSA	noradrenergic and specific serotonergic antidepressants
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PKC	phosphokinase C
PLB	passive lysis buffer
qPCR	real-time quantitative PCR
RLU	relative light unit
RNA	ribonucleic acid
RSK	ribosomal S6 kinase
SARI	5-HT_{2A} antagonists/reuptake inhibitors
SAV-HRP	Streptavidin Horseradish Peroxidase
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	second
SEM	standard error of mean
SNP	single nucleotide polymorphism
SNRI	serotonin and noradrenaline reuptake inhibitors
SSRI	selective serotonin reuptake inhibitor
STAR*D	the Sequenced Treatment Alternatives to Relieve Depression
TBS	Tris-buffered saline
TCA	tricyclic antidepressant
TMB	tetramethylbenzidine
TF	transcription factor
UPL	Universal Probe Library
UTR	untranslated region
µg	microgram
µl	microlitres
µM	micromolar
VPA	valproic acid

Chapter 1

Introduction

1.1 Mood disorders

Mood disorders are common, severe afflictions of which the biological origins are poorly understood and the treatment is far from ideal (Murray and Lopez 1996; Kessler et al. 2005). They are ranked as the world's fourth leading cause of disability by the World Health Organization, and have been estimated to become the second by the year 2020 (Lopez and Murray 1998; Mathers et al. 2000). Approximately 15% of the population is affected by mood disorders at any one time (Lanni et al. 2009), with effects on close friends and family meaning the impact of this class of illness is even higher.

In contemporary psychiatry, mood disorders are subdivided into unipolar and bipolar disorders depending on whether or not the patient has experienced a past episode of mania or hypomania (Harwood 2003; Benazzi 2007). Depression, however, is usually common to both subtypes (Benazzi 2007; Ghaemi 2008a). Unipolar disorders are further subdivided into two categories: dysthymia, where no previous major depressive episode has occured; and major depressive disorder (MDD), which is more frequent and characterized by the presence of major depression in the absence of mania or hypomania (Harwood 2003). Bipolar disorder (BD) is characterized by frequent mood swings between depression and mania (Shaltiel et al. 2004; Ghaemi 2008a).

Although few if any genes have been proven to confer susceptibility to mood disorders, there is strong evidence for a genetic component to both MDD and bipolar disorders. A

meta-analysis of five family studies concluded that MDD was a familial disorder, resulting mostly from multiple genes and environmental influences specific to an individual (Craddock and Jones 2001; Harwood 2003; Sullivan et al. 2004). It has been estimated that the first-degree relatives of MDD probands have a two to three times higher risk of developing clinical depression than that of unrelated comparison subjects (Sullivan et al. 2004). The importance of genetic influences in MDD aetiology is also supported by several twin studies which estimated a heritability of 31%- 42% for MDD (Ferreira et al. 2008; Rapoport et al. 2009). The heritability of BD, in comparison, is estimated to be approximately 86-90% (Ferreira et al. 2008; Rapoport et al. 2009) with the risk of first-degree relatives of bipolar probands estimated to be five to ten times that of the general population (Craddock 1999). Furthermore, the lifetime risk of MDD is also increased in first-degree relatives of a proband with BD (Ferreira et al. 2008), which suggests a fundamental similarity between both disorders (Harwood 2003).

1.2 Principles of mood disorders

1.2.1 Pathophysiology of depression

Despite the high prevalence and socioeconomic impact of mood disorders, the underlying pathophysiological mechanisms are not well understood. The heterogeneity of depression and antidepressant response indicates that multiple neural substrates and mechanisms are involved in the aetiology of mood disorders (Manji et al. 2001; Duman 2002; Nestler et al. 2002a). To date, three main hypotheses have been proposed to account for the pathophysiology of both depression and mania: monoaminergic neurotransmission system impairment, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, and reduced neuroplasticity. The following sections will elaborate upon these hypotheses.

1.2.1.1 Monoaminergic neurotransmission imbalance

One of the earliest theories to explain the phenomenon of mental health disorders arose in the 1960s and became known as the monoamine hypothesis (Bunney and Davis 1965; Schildkraut 1965; Rapeport et al. 1983). It suggests reduced availability of monoamine neurotransmitters or functional deficiency in monoamine transmission at key sites in the brain of patients suffering from mood disorders (Hindmarch 2002; Altshuler et al. 2010). The original observation leading to this hypothesis was that certain drugs such as tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) exerted their pharmacological action by altering synaptic levels of monoamine neurotransmitters as determined by measuring the biological fluid from depressed patients (Post et al. 1980; Frazer 1997; Ordway 1998; Bymaster and Felder 2002; Lanni et al. 2009).

Although the monoamine hypothesis has received significant support over the past five decades, it is inadequate in explaining the complex nature of mood disorders and their treatment (Castren 2005; Lanni et al. 2009). Firstly, it does not explain the delayed clinical effects of antidepressants, even though monoamine concentration are rapidly increased in the brain after taking these drugs (Nestler et al. 2002a). Secondly, it does not address the effects of antidepressants in other disorders, such as obsessive-compulsive disorder and panic disorder. Nor does it explain the lack of therapeutic effects of some of the drugs that modify monoaminergic transmission in treating depression, such as amphetamine or cocaine (Castren 2005; Lanni et al. 2009). Despite this, the importance of monoamine hypothesis in understanding the pathophysiology of mood disorders and its treatment cannot be overlooked, and it remains a useful hypothesis in the absence of a more compelling one.

1.2.1.2 Hypothalamic-pituitary-adrenal axis dysregulation

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in

the aetiology of depression, resulting in what is termed the HPA-axis hypothesis to explain onset of depression. Studies have shown that its abnormal activation is detected in approximately fifty percent of depressed patients, and this HPA-axis abnormality can be successfully treated by antidepressants (Sachar and Baron 1979; Arborelius et al. 1999; Holsboer 2001). HPA axis hyperactivity was also documented in bipolar depressed patients, and has been associated with mixed manic states and depression (Manji et al. 2003). Since one of the most important effects of chronic stress, which produces high levels of glucocorticoids, is on cellular morphology (which ultimately leads to atrophy), the highly sensitive CA3 hippocampal neurons have been shown to be particularly sensitive (Sapolsky 2000a; Altshuler et al. 2010). In this way, hyperactivity of the HPA axis may play a role in mediating stress-induced cell death, which is consistent with reduced hippocampal volume observed in depressed patients (Madsen et al. 2000; Malberg et al. 2000; Sapolsky 2000a; Duman 2002).

1.2.1.3 Neuroplasticity hypothesis

One of the more contemporary hypotheses to explain the biology of depression and mood disorders concerns the brain's ability to adapt and evolve neuronal connections for everyday life and functioning. The term neuronal plasticity encompasses three major themes: modification of gene expression, modification of synaptic transmission and neurogenesis. Disruption to any of these processes has emerged as a possible mechanism for the onset of mood disorders (Racagni and Popoli 2008). Structural imaging and post mortem studies have shown reduced hippocampal volume and cell numbers, in the prefrontal cortex and hippocampus of depressed and bipolar patients (Rajkowska et al. 1999; Sheline et al. 1999; Madsen et al. 2000; Duman 2002; Manji et al. 2003; Savitz et al. 2009). This fits well with the suggested role of the hippocampus in modulating emotional response (Kempermann 2002). Studies on the effects of antidepressants on hippocampal neurogenesis also support this model. The first line of evidence that showed the ability of antidepressants to reverse chronic, stress-induced hippocampal neurodegeneration, came from a study on male tree shrews, where the

modified TCA tianeptine counteracted stress-induced changes in hippocampal volume and cell proliferation (Czeh et al. 2001). Another study by Santarelli *et al.* (2003) demonstrated antidepressant-induced hippocampal neurogenesis and its essential role in mediating therapeutic effects of fluoxetine in mice (Santarelli et al. 2003).

1.2.2 Treatment of mood disorders

Antidepressants are the main treatment for several common mood disorders including depression, bipolar disorder and schizophrenia to name but a few (Benmansour et al. 1999; Harlan et al. 2006; Ritter et al. 2006). Although some antidepressants have profound effects on mood for a proportion of patients suffering major depression, between 30% and 50% of patients do not show a response (Ruhe et al. 2006; Schule et al. 2009). Relapse rates are three to six times higher in patients who show a robust response (Tranter et al. 2002).

MAOIs and TCAs became available for treatment in the 1960s, belonging to the first-generation of antidepressants. MAOIs are irreversible inhibitors of the main metabolic enzymes that break down monoamine neurotransmitters such as noradrenaline (NA), serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA). This results in higher levels of these neurotransmitters. Although MAOIs are generally considered the most effective class of antidepressants, their pronounced and potentially serious side effects limit their use (Eisenhofer et al. 2004; Riederer et al. 2004). The most significant risk in side effects of MAOIs is their potential interaction with other drugs or certain foods, resulting in hypertensive crisis such as severe chest pain, severe headache, stiff or sore neck, enlarged pupils, fast or slow heartbeat, increased sensitivity of eyes to light, increased sweating, and nausea and vomiting (Jacob et al. 2005). Other common side effects include blurred vision, mild dizziness, muscle twitching during sleep, nausea, etc. Less common or rare side effects include chills, constipateion, decreased appetite and dryness of mouth

(www.depression-guide.com/maoi-inhibitors.htm)

TCAs work by inhibiting membrane transporters of monoamines, resulting in an elevated extracellular concentration of monoamine neurotransmitters. Despite their higher therapeutic efficacy in treating melancholia and refractory depression, their use has declined dramatically over the last four decades due to a wide profile of adverse effects, such as dry mouth, dry nose, blurry vision, constipation, urinary retention, memory impairment, drowsiness, anxiety, emotional blunting, confusion, akathisia, sexual dysfunction, nausea and vomiting, and rarely, irregular heart rhythms (Blier and de Montigny 1994; Stahl 1998).

Second-generation antidepressants include selective serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), noradrenaline reuptake inhibitors (NARIs), noradrenergic and specific serotonergic antidepressants (NaSSAs) and 5-HT_{2A} antagonists/reuptake inhibitors (SARIs). Their mechanisms of action are all based on affecting monoamine biochemistry with an emphasis on inhibition of monoamine reuptake (Racagni and Popoli 2008). Although they are advantageous in improved tolerability and patient acceptability, they have not shown any improved efficacy over TCAs, in that their usefulness is still limited to approximately 50% of patients, with 10%-20% showing no response to any second generation antidepressants (Ghaemi 2008b; Racagni and Popoli 2008).

Other psychotherapies including cognitive behavioral (CBT) and interpersonal therapies (IPT) are used to treat depression, and it should be noted that they are equally effective as antidepressant drug treatment in treating mild and nonrecurrent depression alone. In addition, they can be used as an adjunct to antidepressants to provide an additive benefit for the treatment of severe and recurrent depression (Cuijpers et al. 2009a; Cuijpers et al. 2009b).

The treatment of Bipolar Disorder is, however, more complex than the treatment of MDD. It usually involves the use of mood stabilisers alone, or in conjuction with antidepressants (Harwood 2003; Harwood and Agam 2003; Rapoport et al. 2009). The most well-known and commonly used mood stabiliser is lithium. It has proven over a period of decades to be an effective anti-manic agent, which also has some weaker antidepressant properties when used for the treatment of recurrent depression (Lenox et al. 1998). Standard anticonvulsants such as valproate and carbamazepine are also commonly used as mood stabilisers to treat acute mania (Marazita et al. 1997; Keck and McElroy 2002). Some reports suggest these anticonvulsants are more effective than lithium in treating acute mixed episodes (Marazita et al. 1997; Keck and McElroy 2002). Other novel anticonvulsants such as lamotrigine and gabapentin and the atypical neuroleptic agents such as clozapine and olanzapine, have shown some evidence of efficacy in preventing bipolar disorders, but are not yet in common use (Muller-Oerlinghausen et al. 2002).

1.2.3 Biological action of antidepressants and mood stabilisers

1.2.3.1 Antidepressant mechanisms

The evolution of hypotheses to explain the pathophysiology of mood disorders in the past four decades has led to the evolution of antidepressants, due in part, to an increased knowledge of intracellular, gene expression and synaptic mechanisms involved in their cellular and biochemical action (Racagni and Popoli 2008). The monoamine hypothesis of depression and mood disorders suggested that one mechanism by which antidepressants exert their effect is to increase the synaptic availability of monoamine neurotransmitters by inhibiting their reuptake or metabolism from the synaptic cleft (Heninger et al. 1996; Stahl 1998; Berton and Nestler 2006). Recently, the serotonin transporter (*SLC6A4*), which is a key target of SSRIs, was found to be regulated by

microRNA-16 (miR-16) (Baudry et al. 2010). Chronic treatment of mice with the SSRI fluoxetine caused a 2.5- fold increase ($p<0.01$) in miR-16 levels in serotonergic raphe nuclei, resulting in a twofold reduction ($p<0.01$) of *Slc6a4* expression. Therefore, miR-16 has been suggested as a new mechanism for the therapeutic action of SSRIs in monoaminergic neurons (Baudry et al. 2010).

Another possible mechanism of antidepressants action has been proposed under an expansion of the monoamine hypothesis, where changes in the sensitization state of ß-adrenoceptor and 5-HT_{1A} receptors as well as increased firing rates of serotonergic neurons are correlated with therapeutic efficacy (Banerjee et al. 1977; Sulser 1989).

In the neuroplasticity hypothesis of the pathophysiology of antidepressants, antidepressants exert their therapeutic effects through slow, adaptive changes in signaling pathways downstream of membrane receptors, which in turn result in changes to gene expression patterns through the activation of transcription factors (Miwa et al. 1992; Hyman and Nestler 1996; Sapolsky 2000a; Sapolsky 2000b; Maya Vetencourt et al. 2008; Savitz et al. 2009). The most thoroughly studied transcription factor proposed to act in this cascade is cAMP-response element binding protein (CREB). Work has shown that chronic antidepressants stimulate CREB function via activity-dependent phosphorylation by Ras-mitogen activated protein (MAP) kinase and the calcium/calmodulin (CaM) kinase (Bito et al. 1996; Kasahara et al. 2001; Tiraboschi et al. 2004). Furthermore, there is evidence showing significant activation of ERK-MAPK and CaM kinase IV cascades and subsequent CREB phosphorylation by chronic fluxotine treatment (Ghosh et al. 1994; Bito et al. 1996; Kasahara et al. 2001; Tiraboschi et al. 2004; Choi et al. 2005; Tardito et al. 2006; Tardito et al. 2007).

BDNF, which is the most comprehensively studied of the CREB-regulated genes with regard to the mechanism of antidepressants, has been implicated in the

pathophysiology of depression and represents a vital component in antidepressants' action (Magill et al.; Duman and Monteggia 2006; Groves 2007; Martinowich et al. 2007; Wu et al. 2008). Synaptic plasticity, which includes all types of neuroplasticity occurring at synapses, has been shown to be altered by stress and restored by antidepressants (Popoli et al. 2002; Mendez and Garcia-Segura 2006; Agid et al. 2007; Pittenger and Duman 2008). Although converging evidence suggests the role of neuroplasticity changes in glutamatergic synapses in the therapeutic action of antidepressants, the cellular and molecular mechanisms involved in these cellular sub-types is still unknown.

Last but not least, changes in neurogenesis, the stimulation and promotion of new nerve cells, is another aspect of neuroplasticity that may act as a potential contributor to the aetiology of depression and mechanism of antidepressants albeit to a relatively minor degree (Cameron et al. 1999; Gould et al. 1999; Gage 2000; Santarelli et al. 2003; Campbell and Macqueen 2004; Duman 2004; Sahay and Hen 2007). This proposal is supported by evidence from pre-clinical studies that showed reduced hippocampal neurogenesis in stress-induced depression, while antidepressants increased neurogenesis (Santarelli et al. 2003; Campbell and Macqueen 2004).

1.2.3.2 Mood stabilizer mechanisms

The mechanistic actions of mood stabilisers in bipolar disorder are not fully understood, although numerous hypotheses have been proposed in the past 50 years. Initial studies implied the involvement of pre-synaptic events of neurotransmitters in the therapeutic actions of mood stabilisers (Stefani et al. 1996; Bacon et al. 2002; Farber et al. 2002; Brunello 2004). One theory proposes that valproate works by inhibiting GABA transaminase, which is the main enzyme responsible for GABA metabolism, leading to increased GABA levels and post-synaptic GABA responses (Loscher 1993). In addition, valproate treatment has been shown to be involved in the blockade of sodium channels,

resulting in a reduction of the glutamate response (Brunello 2004). Lithium treatment has the same effect on these systems (Brunello 2004).

More recent research has focused on the effects of mood stabiliser treatments on post-synaptic intracellular events, especially cellular signal transduction systems, due to the delayed therapeutic efficacy of mood stabilisers (Manji and Lenox 2000b; Li et al. 2002; Gould and Manji 2005; Eden Evins et al. 2006; Du et al. 2007; Ertley et al. 2007; Kim et al. 2007). Among all the hypotheses proposed, the 'myo-inostitol depletion hypothesis' has been studied extensively (Eden Evins et al. 2006). According to this hypothesis, lithium inhibits inositol monophosphatase, resulting in reduced formation of *myo*-inositol, a vital component in neurotransmission (Berridge et al. 1982; Berridge et al. 1989; Hokin and Dixon 1993; Manji and Lenox 2000a). However, direct evidence showing decreased inositol levels following chronic lithium treatment has not been shown, indicating an indirect role of inositol depletion in the mechanisms of action of lithium (Brunello 2004). A further limitation of this hypothesis is that it does not apply to the other common mood stabilisers valproate or carbamazepine (Vadnal and Parthasarathy 1995; Atack 1996). An alternative explanation for the mechanistic actions of mood stabilisers is the glycogen synthase kinase 3 (GSK3) inhibition hypothesis. It proposes GSK-3 as a target of mood stabilisers (Gould and Manji 2005; Schloesser et al. 2008) . It has been shown that both lithium and valproate, but not carbamazepine, inhibit glycogen synthase kinase 3 at therapeutically relevant concentrations through different mechanisms (Van Der Sar and Den Ouden 1976; Harwood and Agam 2003). While lithium treatment exerts its action by increasing inhibitory phosphorylation of GSK-3ß directly, valproate works indirectly, altering gene expression through inhibition of histone deacetylases (Van Der Sar and Den Ouden 1976; Harwood and Agam 2003). Since GSK-3ß and its downstream effector ß-catenin are involved in apoptosis, the GSK-3ß hypothesis may explain the neuroprotective effect of lithium and valproate (Brunello 2004).

Most recent studies have concentrated on how mood stabilisers affect gene expression. They have arisen based on the long-term efficacy of lithium and valproate in treating bipolar disorder, which may indicate a potential role of gene regulation in the therapeutic actions of these mood stabilisers. Altered expression of genes encoding the transcription factors c-*fos* and c-*jun* and increased activator protein 1 (AP-1) DNA binding after lithium and valproate treatment (Asghari et al. 1998), suggesting that lithium and valproate regulate specific gene expression by affecting the corresponding transcription factors.

1.3 Antidepressant gene expression studies

1.3.1 Cell lines

A range of gene expression studies using antidepressants has been carried out in various cell lines. Normalization of HPA axis by antidepressant treatment in depressed patients is suggested to be associated with changes in the efficiency of corticosteroid signal transduction. Therefore, Herr *et al*. (2003) conducted reporter gene assays using the mouse hippocampal cell line HT22 to screen different classes of antidepressants for their effect on glucocorticoid receptor (GR) signaling. Enhanced GR signaling following antidepressant treatment at clinically relevant concentrations in this neuronal cell model system was reported, high-lighting the utility of tissue culture cell model systems to study the mechanism of antidepressant actions (Herr et al. 2003).

In another study using cell lines, the potential regulatory effects of the antidepressant desipramine (desmethylimipramine; DMI), a selective inhibitor of norepinephrine transport, was investigated. Expression of the norepinephrine transporter (NET) was examined in the NET-expressing SK-N-BE(2)M17 cell line (Zhu et al. 2002). Although

chronic treatment with DMI increased the levels of NET mRNA in a concentration-dependent manner, NET protein level was decreased, suggesting that the regulatory mechanisms are complex.

An increase in mRNA and protein levels of the serotonin-synthesizing enzyme tryptophan hydroxylase (TPH), caused by the antidepressant sertraline, was shown in an *in vitro* study using RBL-2H3 cells. This was accompanied by increased TPH enzyme activity and increased levels of total serotonin (Zhu et al. 2002). This finding was supported by *in vivo* studies in which chronic treatment of rats with sertraline increased mRNA and protein levels of TPH, suggesting that sertraline exerts its long-term therapeutic effects by enhancing serotonin synthesis.

The combination of cell line model systems and microarray technology also proved to be promising in understanding the therapeutic mechanism of antidepressants. Baik (2004) carried out a cDNA microarray analysis with RBL-2H3 cells to identify genes involved in the action of fluoxetine (an SSRI). Several genes showed transcriptional changes in the fluoxetine-treated RBL-2H3 cells, including the 14-3-3zeta gene, whose product was suggested to activate TPH in the presence of Ca^{2+}/ calmodulin-dependent protein kinase II *in vitro* (Banik et al. 1997). *TPH* mRNA levels were increased at 72 h in the RBL-2H3 cells too. This indicates a potential role of 14-3-3zeta and TPH genes in the molecular action of fluoxetine.

Previous proteomic analysis in our laboratory, where embryonic stem cell-derived neural cells were treated with paroxetine for 14 days, identified increased expression or modification of several proteins including sepiapterin reductase, heat shock protein 9, RAS and EF-hand domain containing. Decreased expression or modification of proteins included prohibitin, actin, creatin kinase (McHugh et al. 2008b). Some of the genes encoding these proteins will be used in the reporter gene system and real-time

PCR analysis of this study, to identify differences in gene expression upon drug treatment of cell cultures.

1.3.2 Animals

Research on experimental animals has greatly enhanced our understanding of the pathophysiology of depression and the molecular mechanisms of antidepressant action. The established role of the cyclic AMP signal transduction system in antidepressant efficacy is a good example where animal studies were used to help understand the molecular mechanism underlying the therapeutic effect of antidepressants. CREB, which is a transcription factor that mediates gene expression via the post-transcriptional cAMP cascade, was shown to be up-regulated by several different classes of antidepressant treatments in rat hippocampus (Nibuya et al. 1996; Duman et al. 1997; Chen et al. 2001a).

Chen *et al.* (2001) demonstrated that over-expression of CREB in rat hippocampus resulted in an antidepressant effect in two established models used for pharmacological screening of antidepressants: the learned helplessness test and the forced swim test, indicating the potential role of CREB and the cAMP cascade in the pathophysiology of depression and antidepressants action.

Transgenic mice with a cAMP response element (CRE) - LacZ reporter gene construct were utilized to investigate the effects of antidepressants on CRE-mediated gene transcription. These transgenic mice demonstrated a significant increase in CRE-mediated gene transcription in several brain regions known to mediate the action of antidepressants following chronic antidepressant treatment (Thome et al. 2000).

In a recent study by Boer (2007), a transgenic mouse line, in which expression of the

luciferase reporter gene was put under the control of four copies of *CRE* (the CREB binding site), was used to decipher the impact of antidepressant therapy on CRE/CREB gene expression (Boer et al. 2007). The stress-induced increase in CRE/CREB gene expression measured as luciferase activity in several brain regions was reduced to control levels following chronic treatment with the antidepressant imipramine, suggesting that antidepressant effects may be mediated via CRE/CREB-directed gene expression (Holsboer 2001).

One cAMP regulated target gene is *BDNF* (*brain-derived neurotrophic factor*), a member of the neurotrophin family of growth factors. Its role in antidepressant action is supported by several lines of evidence from animal studies (Levine et al. 1990; Thoenen 2000; Poo 2001; Kovalchuk et al. 2002; Wood et al. 2005; Ferreira et al. 2008). BDNF protein has been shown to promote neurogenesis of rat hippocampal neurons and therefore plays an important role in synaptic plasticity in the rat hippocampus (Nibuya et al. 1995; Thoenen 1995). Chronic administration of several classes of antidepressants significantly increased *BDNF* transcription and an antidepressant-like effect of BDNF has been demonstrated in animal models of depression previously (Siuciak et al. 1997). Furthermore, Monteggia *et al.* (2004) developed a conditional knockout mouse in which *BDNF* is deleted selectively in specific regions of the adult brain using the *CRE-Lox* recombinase system. They showed attenuation of antidepressant DMI action in the forced swim test in *BDNF* knockout mice, indicating the role of BDNF in antidepressant response.

The effects of antidepressants on the expression of *c-Fos*, which is an immediate early gene transcription factor, has also been studied in rats (Morinobu et al. 1995). It has been shown that chronic, but not acute administration of electroconvulsive seizures (ECS) and several different classes of antidepressants reversed stress-induced induction of *c-Fos* mRNA levels in rat frontal cortex, suggesting a role for c-Fos in post receptor action of antidepressant treatment (Morinobu et al. 1995).

The possible role of changes in gene expression in the mechanism of antidepressant action is confirmed further by the observation that chronic, but not acute, antidepressant treatment resulted in a differential and region-specific effect on the DNA-binding activities of transcription factors *CRE*, *SP1* and *GRE* (Frechilla et al. 1998). Since CREB, c-Fos, SP1- and GRE- are vital transcription factors that regulate target gene expression by binding to promoter regions of target genes, it is likely that the identification of target genes that are altered by antidepressant treatment in the central nervous system will lead to a better understanding of the effects of antidepressants (Chen et al. 2003).

The relatively recent advances of the genome project and DNA microarray technology have made simultaneous monitoring of expression profiles of thousands of genes after drug manipulation possible (Lockhart and Winzeler 2000; Rockett and Dix 2000). For example, using microarrays, Chen *et al.* (2003) found increased gene expression of six genes and decreased expression of two genes after chronic treatment of rat cultured hippocampal cells with desipramine. One of the upregulated genes is *growth associated protein 43* (*GAP-43*), whose protein product is known to regulate growth of axons and modulate the formation of new neuronal connections (Benowitz and Routtenberg 1997), indicating the potential effect of DMI on neuronal plasticity in the central nervous system.

In addition, multiple kinase pathways appear to play an important role in determining serotonin transporter regulation, and it has been hypothesised that these would show altered expression with chronic treatment by selective serotonin reuptake inhibitors (Rausch et al. 2002). Therefore, this group (Rausch *et al.*) conducted microarray analysis to study whole brain kinase mRNA expression changes in rats treated with SSRIs. Several kinase genes were shown to be downregulated with chronic SSRI

treatment, but not with acute treatment, which is consistent with homeostasis of serotonin transporter function through reduced protein kinase expression.

Region-specific transcriptional changes following three antidepressant treatments, ECT, sleep deprivation, and fluoxetine (an SSRI) were detected in another microarray study (Rosetti et al. 2006). In this study, RNA extracted from seven different brain regions of the rat following the treatments were used to evaluate transcriptional changes (Rosetti et al. 2006). In addition to the observed region-specific gene expression changes, multiple transcripts were found to be regulated by antidepressant treatments used in this study, including *Bdnf,* serum/glucocorticoid-regulated kinase, whose gene expression was found to be changed by antidepressants previously (Rosetti et al. 2006). Several novel genes, such as *homer*, *erg2*, *Hsp27*, *glycerol 3-phosphate dehydrogense* and the proto oncogen *Ret*, were also found to be regulated by several antidepressant treatments, representing novel targets for antidepressant action.

In addition, proteomic studies in our laboratory, where rats were exposed to paroxetine for 12 days and hippocampal proteins were measured by 2-D gel electrophoresis, showed increased or decreased expression or modification of several proteins, including parvalbumin, prohibitin, and complexin 1 (McHugh et al. 2009a). Microarray studies on rat hippocampus chronically treated with paroxetine also identified several genes that are expressed differentially, such as *Ccnd1* and *Hes6* (McHugh et al. 2008a). A selection of these genes will be included in this study.

1.3.3 Human post mortem studies

Human post-mortem studies are another valuable way of exploring the pathophysiology of depression and mechanisms of action of antidepressants. Since dysfunction of brain glutamatergic transmission has been implicated in MDD and its treatment, several post-mortem studies have been conducted to investigate the potential

role of the glutamatergic N-methyl-d-aspartate (NMDA) receptor in MDD. It has been shown that concentrations of proteins associated with NMDA receptor signaling, such as the NR2A subunits of the NMDA receptor as well as post-synaptic density protein-95 (PSD-95), were significantly increased in the amygdala of post-mortem-interval-matched depressed patients compared to psychiatrically healthy controls, suggesting that glutamate signaling at the NMDA receptor in the amygdala is disturbed in depression (Karolewicz et al. 2009).

In support of the HPA hypothesis, one post-mortem study demonstrated elevated concentrations of corticotrophin-releasing factor (CRF), in post mortem locus coeruleus samples from MDD patients, when compared to age and sex matched non-depressed subjects (Bissette et al. 2003). This suggested that CRF may play a role in the pathophysiology of MDD.

The importance of gene regulation in the aetiology and treatment of depression has been implicated in several post-mortem studies. In a study where gene expression pattern was examined in post-mortem brains of normal controls, depressed, bipolar and schizophrenic patients, ATF2, which is a member of the CREB/ATF family of transcription factors, did not show any brain region-specific alterations. However, the level of *pATF2* was increased in untreated compared to treated patients in the depressed group, indicating a possible role of *ATF2* in antidepressant therapeutic action (Laifenfeld et al. 2004).

Post-mortem studies have also shown brain region-specific reduction in the number and density of glia, and a decrease in glia/neuron ratio in MDD patients, which supports the notion that glial changes, which are an important factor in neuroplasticity, may contribute to the aetiology and treatment of depression (Czeh et al. 2006; Wu et al. 2008).

1.4 Mood stabiliser gene expression studies

Studies using cell culture and animal models were conducted to explore the effects of mood stabilisers on gene regulation. It has been demonstrated that valproate increased transcription factor AP-1 DNA binding activity time- and concentration-dependently in rat C6 glioma cells and human neuroblastoma cells SH-SY5Y (Chen et al. 1997b; Asghari et al. 1998). Chronic lithium treatment has also been shown to cause increased AP-1 binding activity both *in vitro* and *in vivo* (Ozaki and Chuang 1997; Chen et al. 1998), suggesting that these two structurally dissimilar drugs may regulate patterns of gene expression through the AP-1 family of transcription factors in vital neuronal circuits. This may explain the long-term mood stabilization effects of mood stabilisers (Hughes and Dragunow 1995; Chen et al. 1998). In fact, chronic lithium treatment resulted in elevated levels of tyrosine hydroxylase (*TH*), which is a known AP-1 regulated gene, in rat hippocampus, frontal cortex and striatum as well as human SH-SY5Y neuroblastoma cells (Chen et al. 1998).

Bcl-2, which is known to have anti-apoptotic and neurotrophic effects (Oh et al. 1996; Chen et al. 1997b; Hilton et al. 1997; Kempermann et al. 1997) is another gene that has been studied extensively in gene expression studies of mood stabilisers. Chronic lithium treatment of cerebella granule cells stimulated a concentration-dependent increase in mRNA and protein levels of *Bcl-2*/ Bcl-2 significantly (Chen et al. 1999b). Valproate has also been shown to increase *Bcl-2* gene expression in cultured cells (Yuan et al. 2001; Laeng et al. 2004; Michaelis et al. 2004; Creson et al. 2009) and in specific regions of animal brains (Chen et al. 1999b; Hao et al. 2004; Creson et al. 2009), which in turn promotes Bcl-2 related functions such as neurogenesis and neurite growth (Chen et al. 1999b).

1.5 Genetic analysis of response to drugs used to treat mood disorders

Although antidepressants are the most effective treatment for depression, substantial improvement is still required due to its lack of adequate drug response, undesired side effects and a substantial lag between the onset of treatment and full remission of depressive symptoms (McMahon et al. 2006). Drug response in particular, is influenced by numerous factors including environmental and genetic factors. The role of environment context in drug response has been demonstrated in a study examining the induction of psychostimulant sensitization by the circumstances surrounding drug administration (Crombag et al. 2001). It was shown that the interaction amongst the pharmacological actions of drugs and the circumstances surrounding drug administration plays a pivatol role in psychomotor sensitization. Genetic factors, such as the role of polymorphisms in drug-metabolisming enzymes, contribute significantly to drug response too (Eichelbaum and Evert 1996; Kirchheiner et al. 2004). Due to advances in human genome sequencing the field of pharmacogenetics of antidepressant response has experienced significant development over the past ten years (Rioux et al. 2001; Venter et al. 2001). In addition, the international HapMap project, which generated a haplotype map of common human genetic variants, has provided valuable information on genes or genetic regions that affect health, disease and individual response to drugs (Altshuler et al. 2010).

Most of the pharmacogenetic studies to date were driven by current hypotheses about the mechanism of action of antidepressants and the pathophysiology of depression. Therefore, antidepressant response candidate genes were often selected from those involved in monoaminergic, stress hormone or neurotrophic systems, such as serotonin receptors, serotonin and norepinephrine transporters, metabolizing enzymes, glucocorticoid receptors and chaperones, and brain derived neurotrophic factor

(Holsboer 2000; Nemeroff and Owens 2002; Duman and Monteggia 2006). Although a variety of genes have been associated with mood disorders by pharmacogenetic studies, results are often controversial or non-replicable due to various reasons such as sample sizes, sample stratification, the type of antidepressant treatment used, and outcome measures, which make meaningful comparisons difficult (Horstmann and Binder 2009). However, several genes and polymorphisms are found to be consistently associated despite all the differences mentioned above.

The gene encoding the serotonin transporter (*SLC6A4*) has been studied intensively in antidepressant pharmacogenetics because that it is the main target of SSRIs, TCAs and SNRIs (Lesch et al. 1993). Among all the polymorphisms described for this gene, the serotonin transporter gene-linked polymorphic region (5-HTTLPR) has been the most studied. The short (S) allele was reported to be associated with worse response and remission rate under SSRI treatment compared to the long (L) allele in a meta-analysis of Caucasians and Asian cohorts. However, contradictory results were reported in three studies investigating the association of 5-HTTLPR and remission in response to citalopram in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) sample (McMahon et al. 2006). While one study replicated an association of the S-allele with worse treatment response in white non-Hispanic patients (Mrazek et al. 2009), no association was found between 5-HTTLPR and remission under citalopram treatment in the whole sample and caucasian subsample (Hu et al. 2007; Peters et al. 2009). The inconsistencies between these studies are partly due to different ethnicity groupings and defining criteria for treatment remission (Hu et al. 2007; Mrazek et al. 2009; Peters et al. 2009).

Since antidepressants are suggested to normalise HPA-axis hyperactivity through restoration of the glucocorticoid receptor (GR), genes involved in this system have been studied for association with antidepressant treatment response (Ising et al. 2005). Three SNPs (rs1360780, rs4713916, rs3800373) in *FKBP5*, which regulates GR

sensitivity, was reported to be strongly associated with antidepressant drug response in 280 depressed patients of the Munich Antidepressant Response Signature (MARS) sample independent of the class of antidepressans tested (Horstmann et al. 2010). MARS is project aiming to identify genetic variants that are predictive of treatement outcome. It consists of all inpatients with depressive disorder treated at the hospital of the Max Planck Institute. This finding suggests that FKBP5 brings about treatment response by affecting events downstream of the primary binding of antidepressants (Horstmann and Binder 2009). This positive finding is supported by two further studies in the STAR*D cohort and a German sample (Kirchheiner et al. 2004; Lekman et al. 2008).

Another gene that has been investigated rigorously for antidepressant pharmacogenetics is the post-synaptic *serotonin receptor 2A* (*HTR2A*). Two SNPs, one in exon 1 and the other in the promoter region (rs6313 and rs6311, respectively) were found to be associated with response to antidepressants in numerous small pharmacogenetic studies, indicating a role of *HTR2A* in treatment outcome (Rioux et al. 2001; Cusin et al. 2002; Sato et al. 2002; Peters et al. 2004; Yoshida et al. 2004; Choi et al. 2005; Kato et al. 2006; Lee et al. 2007; Drago et al. 2009; Wilkie et al. 2009). However, it was not replicated in a later study in the MARS sample. In a reduced sample of the STAR*D cohort, 768 SNPs in 68 candidate genes were examined. Only one SNP (rs7997012) was found to be associated with response to citalopram after correction for multiple testing both in sub-samples and in the full STAR*D sample (McMahon et al. 2006; Paddock et al. 2007). However, the opposite allele of rs7997012 was reported to be associated with treatment response in the MARS sample. A third genetic variation, rs17288723, also showed association with treatment outcome in the MARS sample (Horstmann et al. 2010). Taken together, multiple genetic variations in *HTR2A* may be involved in antidepressant treatment response with no single one being more predictive than others (Horstmann and Binder 2009).

Because of our limited understanding of the pathophysiology of depression and the mechanisms of action of antidepressants, unbiased genome-wide association studies (GWAS) have the potential to advance this field. Indeed, several new candidate disease risk genes were identified using this approach (Horstmann and Binder 2009). For example, Ferreira *et al.* (2008) conducted a collaborative genome-wide association study that incorporated three independent GWAS of bipolar disorder, including the Wellcome Trust Case Control Consortium (WTCCC), STEP-UCL, and ED-DUB-STEP2, in an attempt to identify susceptibility loci for bipolar disorder (Corsortium 2007; Ferreira et al. 2008). Among all the 1.8 million SNPs in 4,387 cases and 6,209 controls tested for association with the pathophysiology of bipolar disorder and the mechanisms of action of mood stabilisers, two regions were shown to be strongly associated in three independent studies (Ferreira et al. 2008). Variation in *ANK3*, which encodes a family of proteins found at axon initial segments in the central and peripheral nervous system and has been shown to be involved in the assembly of voltage-gated sodium channels (Kordeli et al. 1995; Poliak and Peles 2003), showed the strongest association. The second strongest association was in *CACNA1C* encoding voltage-gated calcium channels (Green et al. 2010; Wessa et al. 2010). Furthermore, both *ANK3* and the calcium channel showed downregulation upon exposure to lithium (Ferreira et al. 2008), indicating a vital role of ion channels in the aetiology and treatment of bipolar disorder (Gargus 2006).

In summary, no clinically tested predictive genetic variations have been identified so far despite numerous pharmacogenetic studies and GWAS studies, calling for more refined and larger studies to be conducted. In addition, inclusion of environmental factors such as number of depressive episodes, the occurrence of life events and repeated treatment, will be likely to benefit the field of pharmacogenetics of antidepressant response (Holsboer 2008).

1.6 Research Aim

Antidepressants such as SSRIs have been shown to affect not only the serotonin transporter, but also other cellular targets, such as 5-HT2B receptors (Altshuler et al. 2010), 5-HT4 receptors (Licht et al. 2009), RNA metabolism genes (Ericson et al. 2008), genes involved in inhibition of proliferation and induction of apoptosis such as caspase-3 and Bcl2 (Amit et al. 2009; Licht et al. 2009), and neuronal vesicular monoamine transporter 2 (Yasumoto et al. 2009). It has also been shown that a serotonin transporter polymorphism is not directly involved in patients' response to paroxetine in a Japanese study (Yoshimura et al. 2009), indicating the possibility that the action of paroxetine may not depend on serotonin transporter alone. Therefore, this project aims to explore the broader serotonergic as well as non-serotonergic effects of psychoactive drugs on gene regulation.

1.7 Research hypotheses

Despite the urgent need for better therapies, recent efforts to develop novel drugs to treat mood disorders have been rather unproductive mainly due to lack of understanding of the pathophysiology of mood disorders and the mechanisms of action of their treatment (Agid et al. 2007). This project sought to provide a better understanding of the molecular and cellular effects of antidepressants and mood stabilisers, which in turn may provide insights into the limited therapeutic efficacy of these drugs, and suggest ways to address this situation.

The hypotheses to be tested were that:

Genes important to the mechanisms of action of antidepressants and mood stabilisers

will show either up- or down-regulation when neural cells are exposed to these drugs.

Transcriptional effects detected in this study will be mediated by specific intra-cellular signaling pathways.

Genes with regulatory responses specific to antidepressants and mood stabilisers will function in pathways of relevance to normal mood control.

Chapter 2

Materials and Methods

2.1 Reporter gene assays

2.1.1 Cloning

2.1.1.1 Genomic DNA extraction

Genomic DNA was extracted from peripheral blood samples using standard protocols of either guanidium chloride extraction (Ciulla et al. 1988) or sodium chloride precipitation (Lahiri and Nurnberger 1991), followed by isopropanol precipitation.

2.1.1.2 Primer design

Genomic DNA sequences were retrieved from public databases via Ensembl (www.ensembl.org/index.html) and the UCSC Genome Brower (http://genome.ucsc.edu/). The promoter region was operationally defined as the region approximately 1kb-2kb upstream of the relevant transcription start site of each gene, which should encompass the proximal regulatory regions and important transcriptional control elements. Primer pairs for each promoter were designed using DNAMAN version 5.2.2 (Lynnon Corporation, Vaudreuil- Dorion, Quebec, Canada). Self- and primer-primer complementarity was minimised in order to reduce the occurrence of primer self extension and primer-dimer formation, which can reduce the amplification efficiency of target promoters. The 5´untranslated region (5'- UTR) of each transcript was included as part of the putative promoter in order to include downstream regulatory elements (van der Stoep et al. 2002; Ng et al. 2004; Wang et al. 2005; Qin et al. 2006). Restriction enzyme recognition sites were introduced at the 5´end of each primer along with extra bases to allow for efficient digestion by restriction enzymes (*Cleavage close*

to the end of DNA fragements (oligonucleotides) New England Biolabs. 15 Dec 2010. http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_oligonucleotides.asp).

2.1.1.3 Polymerase chain reaction

To obtain the putative promoter regions of candidate genes for this thesis (Table 3.1), human promoters were amplified by polymerase chain reaction (PCR) using primers listed in Table 2.1. All PCR reactions were performed in a volume of 10µl containing 1µl 10 x Platinum *Taq* buffer (Invitrogen), 1µl 2mM dNTPs, 0.3µl 10µM of each primer (Table 2.1), 0.04µl Platinum Taq polymerase (5 U/µl), 1µl of template DNA and an appropriate volume of MPW (purified water; Millipore, MA, USA) to a final volume of 10µl. The reaction conditions for a standard PCR programme were: a denaturation step at 94°C for 2 minutes, 35 cycles involving annealing at 62°C for 30s, extension at 72°C for one minute and denaturation at 94°C for 30s, followed by a final extension at 72°C for 5 minutes. Variations to these conditions are presented in Table 2.1. All reactions were carried out either on a DNA Engine^{TM} Thermal Cycler (PTC-200, MJ Research Inc, USA) or a Mastercycler epgradient S (Eppendorf, Hamburg, Germany).

Table 2.1 PCR primers and reaction conditions for candidate promoter amplification. Amplicons were subsequently cloned for luciferase reporter gene assays.

GENE	Primer name	PCR primers	PCR conditions
ADM	ADMF1 ADMR1	5′ACGTGCTAGCGGCTCTAAGATGGGGACTCGAGA3′ 5′ TAGTAAGCTTCGGACTCACTTCTTTCGAAACTCC3′	94° 2 minutes (94° 30s, 62° 30s, 72° 1 minute) x 34 72° 5 minutes
ARC	ARCF1 ARCR1	5′ACGTAGATCTCTGTGAGTGAGGGCATGAGTGTGT 3′ 5′ TAGTAAGCTTTTCAGCTCGCGCTCCACCTGCTTG 3′	*95° 2 minutes (95° 30s, 63.2° 30s, 72° 1 minute) x 34 72° 5 minutes
CREB1	CREB1-F1 CREB1-R1	5′ACGTAGATCTGCGGAGTCCAGAATCGAACC 3′ 5′TAGTAAGCTTCACCGCGTCAAACTACACCTC 3′	94° 2 minutes (94° 30s, 63° 30s, 72° 2 minutes) x 34 72° 5 minutes
CRH	CRHF1 CRHR1	5′ACGTCTCGAGCAGGCTGACATGAAGCACATTTG 3′ 5′TAGTAGATCTCTTCTTATGACTTGTCCATTG 3′	94° 2 minutes (94° 30s, 62° 30s, 72° 1 minute) x 34 72° 5 minutes
CRHR2	CRHR2F1 CRHR2R1	5′ACGTCTCGAGCAAGGCACTAACAACTGGGTG 3′ 5′TAGTAAGCTTGAGTGGACGCGAGAGTGAGC 3′	94° 2 minutes (94° 30s, 62° 30s, 72° 1 minute) x 34

			72° 5 minutes
GCH1	GCH1-F5-SD GCH1-R4-SD	5′TAGTGGTACCGAGTTCAGTTGGTGAATAGC 3′ 5′ TAGTCTCGAGCGCAACCTGCTTAGATCACA 3′	94° 2 minutes (94° 30s, 62° 30s, 72° 2 minutes) x 34 72° 5 minutes
GCHFR	GCHFR-F4 GCHFR-R2	5′ACGTAGATCTGTGTTGGAACGGCAGCATCC 3′ 5′TAGTAAGCTTGGACTGCGACGCGCAGCTGG 3′	94° 2 minutes (94° 30s, 63° 30s, 72° 2 minutes) x 34 72° 5 minutes
GRIK4	GRIK4F1 GRIK4R1	5′ACGTAGATCTCTGAGCATGGTGGCTTCTCC 3′ 5′TAGTAAGCTTCACACTTACCGATCCTCAAG 3′	94° 2 minutes (94° 30s, 62° 30s, 72° 1 minute) x 34 72° 5 minutes
HTR1A	HTR1AF1 HTR1AR1	5′ACGTAGATCTACTAGCCACAAAGCTATGGG 3′ 5′TAGTAAGCTTGATACCAGTAGTGTTGCCGC 3′	94° 2 minutes (94° 30s, 66.8° 30s, 72° 2 minutes) x 34 72° 5 minutes
PCBD	PCBDF1 PCBDR1	5′ACGTAGATCTTGATCTTGGGTTAGGCCAAG 3′ 5′TAGCAAGCTTAGAGACCCCACTTTCGGAC 3′	94° 4 minutes (94° 30s, 62° 30s, 72° 1minute 30s) x 10 (94° 30s, 62° 30s, 72° 1minute 30s + 5sec/cycle) x 20 72° 5 minutes

PCLO	PCLO-F1 PCLO-R1	5′ACGTACGCGTGTAACAACATTGAGGCTGCAG 3′ 5′TAGTAAGCTTGTCCCGCTCGCAGGTAACGC 3′	96° 3 minutes (96° 20s, 65.2° 45s, 72° 1minute 30s) x 35 72° 5 minutes
PHB	PHB-F1 PHB-R1	5′ACGTAGATCTCATGCCACCACATCCAGGTAC 3′ 5′TAGTAAGCTTGCTTTCCAAGATCCACGCTTC 3′	95° 2 minutes (95° 30s, 62° 30s, 72° 2 minutes) x 34 72° 5 minutes
PTS	PTS-F4 PTS-R2	5′ACGTAGATCTACGAGGGTCTTGCTCTATTG 3′ 5′TAGTAAGCTTCATCTTCCCGGCGCTGTCTGC 3′	94° 2 minutes (94° 30s, 63° 30s, 72° 2 minutes) x 34 72° 5 minutes
PVALB	PVALB-F1 PVALB-R1	5′ACGTACGCGTCATCCTAGGATTCAACACCAC 3′ 5′TAGTCTCGAGCTCGGTAGCTGCCACTCACCTC 3′	94° 2 minutes (94° 30s, 62.5° 30s, 72° 2 minutes) x 34 72° 5 minutes

QDPR	QDPR-SD-F1 QDPR-SD-R1	5´ACGTAGATCTTGTGTCTGCTGTGTACAAGG 3´ 5´TACTAAGCTTCCGAAAAGCCTGCACGCATC 3´	94° 2 minutes (94° 30s, 62.5° 30s, 72° 2 minutes) x 34 72° 5 minutes
SLC6A4	SLC6A4F1 SLC6A4R1	5´ ACGTAGATCTAGCATTCTCCTTGCACCCTA 3´ 5´ TAGTAAGCTTGCCCTCACATGGTCTGATCT 3´	95° 2 minutes (95° 30s, 62° 30s, 72° 2 minutes) x 34 72° 5 minutes
TPH2	TPH2F3 TPH2R3	5´ACGTACGCGTTAGCTGATTAGAGCTGACCC 3´ 5´TAGTCTCGAGAACATCATCATTGCTGGCTG 3´	95° 2 minutes (95° 30s, 62.4° 30s, 72° 2 minutes) x 34 72° 5 minutes

* °: °C

2.1.1.4 Agarose gel electrophoresis

All PCR products were eletrophoresed and visualized on 1-2 % agarose gels. Agarose gels were made by melting DNA grade agarose (AppliChem GmbH, Germany) in 1x TBE containing 0.5 µg/ml ethidium bromide (Sigma Aldrich Corporation St. Louis, MO, USA). The melted agarose solution was cooled to 50-60°C and poured into a DNA gel-casting tray (Easy-Cast™ Electrophoresis system, Owl separation systems, Portsmouth, NH, USA). After the gel was set, 1x TBE was poured into the electrophoresis apparatus until the gel was immersed. Samples were prepared in 0.6ml tubes (Axygen Scientific, Inc, Union City, CA, USA) containing 2µl DNA sample, 6µl MPW and 2µl of 6x Loading Dye Solution (Fermentas International Inc., Burlington, Otario, Canada). Two µl of GeneRuler™ DNA ladder (Fermentas) was run to indicate the molecular weight (MW) of the PCR products. The gel was run at 100 V until the smallest dye front, xylene cyanol, had migrated approximately three quarters of the length of the gel, approximating the migration of a 50 bp DNA fragment. The DNA was visualized under UV light and the image recorded using Quantity One 4.6.1 software (Bio-Rad Laboratories Pty Ltd, NSW, Australia).

2.1.1.5 PCR product purification

PCR products were purified using AxyPrep PCR clean-up kit (Axygen Biosciences, Union City, CA, USA). Briefly, a volume of 100µl DNA binding buffer was added to 20-30µl of PCR product and centrifuged through a PCR column at 12,000 x g in a bench-top centrifuge. A volume of 700µl of PCR wash buffer was added to the column and centrifuged for one minute. This step was repeated with 400µl of PCR wash buffer. The DNA was eluted by adding 30µl of eluent to the column and centrifuging for one minute.

2.1.1.6 Plasmid vector/insert digests and ligation

All the enzymes and buffers used for restriction digestion and ligation were obtained from New England Biolabs (Beverly, Massachusetts, USA). PCR products were cloned into pGL3- Basic reporter vector (Promega Corporation, Madison, Wisonsin, USA), which contains a modified coding region for the firefly (*Photinus pyralis*) luciferase

gene (Figure 3.1). PCR products and plasmids were digested in 20µl reaction mixes containing the appropriate enzymes, buffers and additives. Reaction mixes were incubated at 37°C for 2-3 hours followed by heat inactivation at 65°C for 15 minutes. PCR primers were designed to contain appropriate restriction sites for each gene (Table 2.1). Agarose gel electrophoresis was used to visualize the digested DNA and confirm digestion of the vector. Ligation was accomplished by combining the digested PCR products and plasmid vectors in a 10µl mix containing 1µl T4 DNA ligase and 1 x T4-ligase buffer. Reactions were incubated overnight at 4 °C.

2.1.1.7 Bacterial transformation

Prior to transformation, competent cells were prepared using the calcium chloride method of Sambrook (Sambrook et al. 1989). A volume of 1.5ml of 2YT bacterial culture medium was inoculated with a single colony of *Escherichia coli* (*E. coli*) DH5α and incubated in a shaking incubator at 37°C overnight. 100µl of this overnight culture was diluted into 50ml 2YT media in a 250ml Ehrlenmeyer flask and incubated at 37°C with shaking for 2-3 hours to early log phase of growth. The cells were collected by centrifugation in 50ml Falcon^{TM} conical centrifuge tubes (BD Biosciences, Bedford, Massachusetts, USA) at 4,000 x g for 15-20 minutes in a bench top centrifuge (Thermo scientific, Germany) and subsequently re-suspended in 50ml of ice-cold 100mM $CaCl_2$. After incubation on ice for 20 minutes, cells were collected by centrifugation again as described above. The pellet was re-suspended in 5ml cold 100mM $CaCl_2$, at which point the cells were ready for transformation. For long-term storage, ice-cold sterile 100% glycerol (Merck) was added to the competent cells to a final concentration of 20%. The cells were stored in 1ml aliquots at -80°C after thorough mixing.

For transformation, 100µl competent DH5α cells were mixed gently with 5µl of ligation mix and incubated on ice for 30-60 minutes. The transformation mix was heat shocked at 42°C for 45 seconds without shaking and incubated on ice for a further 2 minutes. A volume of 400µl 2YT media was added to the transformation mix and incubated at 37°C for 45 minutes in a shaking incubator to allow time for the bacteria to begin synthesizing the ß-lactamase enzyme. 100µl transformation mix was spread onto 2YT plates containing 100 µM/ml ampicillin (Amp) and incubated overnight at 37°C.

2.1.1.8 Small scale plasmid preparation

Small scale, rapid plasmid preparations were performed by a boiling lysis method (Lan et al. 2009). Amp resistant colonies were inoculated into 1.5ml Amp containing 2YT and incubated overnight with shaking at 37 °C. Cell pellets were obtained by centrifuging the overnight culture in 1.5ml centrifuge tubes at 12,000 x g for 2 minutes. Supernatant was decanted and cell pellets were re-suspended in residual broth by vortexing. A volume of 200µl STET buffer was added, followed by 10µl of 20 mg/ml lysozyme. The cap of each tube was pierced with a small needle and the tube was placed in a covered boiling water bath for 90 s before centrifugation at 12,000 x g for 10 minutes. The supernatant was decanted into a new set of tubes containing 200µl isopropanol. The contents of each tube were mixed by inversion or vortexing, followed by a further centrifugation at 12,000 x g for 5 minutes. The resulting pellet was rinsed gently with 500µl of 70% ethanol and re-suspended in 100µl MPW containing 1µl boiled 10mg/ml RNase A. Positive clones were verified by restriction digestion and sequencing. The plasmid template and corresponding gene specific primers were premixed in a total volume of 15µl. The concentration of plasmid templates and primers used were 300ng/15µl and 2ng/100bp/15µl respectively. The sequencing mix was sent to Allan Wilson Genome Service at Massey University, which used the BigDye^{TM} Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit from Applied Biosystems Inc for the sequencing reactions. DNAMAN was then used to analyse the sequences to confirm positive clones.

2.1.2 Transfection of mammalian cells

2.1.2.1 Preparation of transfection grade plasmid DNA

Transfection grade plasmid DNA for reporter gene assays was prepared using the AxyPrep^{TM} Plasmid Miniprep kit (Axygen Scientific). A single colony was inoculated into1.5ml 2 x YT bacterial medium containing 100 µl/ml Amp and incubated at 37°C in a shaking incubator overnight. Bacteria were pelletted by centrifugation at 12,000 x g for one minute. The supernatant was aspirated and the bacterial pellet was resuspended in 250µl resuspension buffer containing RNase A. 250µl lysis buffer was added into the homogenate and incubated at room temperature for less than 5 minutes. A volume of 350µl neutralisation buffer was subsequently added, followed by centrifugation at

12,000 x g for 10 minutes to clarify the lysate. The clarified supernatant was then transferred to an AxyPrep DNA binding column and centrifuged at 12,000 x g for one minute. Column-bound plasmid DNA was washed by the addition of 700µl of washing buffer, followed by centrifugation at 12,000 x g for one minute. The filtrate was discarded and a second washing process was used to ensure complete removal of salt. Purified plasmid DNA was eluted by the addition of 60-80µl 2.5 mM Tris buffer (pH 8.5) eluent to the column, followed by a one minute incubation at room temperature, and a one minute centrifugation.

2.1.2.2 Plasmid quantification

A NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Deleware, USA) was used to quantify the purified plasmid DNA for transfection of mammalian cells. Three measurements were taken and averaged to calculate the dilution factor required to make a 50 ng/µl working solution. The concentration of the working solution was confirmed by a further NanoDrop® ND-1000 Spectrophotometer measurement. An average reading between 49 and 51 ng/µl measured over 3 samples for each dilution was considered acceptable for subsequent experiments.

2.1.2.3 Mammalian cell lines

The COS-7 cell line used in this study originates from kidney fibroblast-like cells of an African green monkey. They were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). This line was derived from the CV-1 cell line by transformation with an origin-defective mutant of SV40 which codes for wild type T antigen (Gluzman 1981; Manos and Gluzman 1985). COS-7 cells are routinely used in transfection experiments because they are relatively easy to manipulate and transfect, and are able to express a wide range of genes once transfected.

RN46A cells were a gift from Scott Whittemore (Laboratory of Molecular Neurobiology, Louisville, Kentucky, USA). This is an immortalised rat serotonergic precursor cell line generated by infecting medullary raphe cells, dissociated from embryonic day 13 Sprague-Dawley rat brain, with a retrovirus that encodes the temperature-sensitive mutant of SV40 large T- antigen (White et al. 1994). At

permissive temperature (33ºC), RN46A cells undergo proliferation with a doubling time of nine hours. Although they are neuronally restricted at this stage, they still express serotonergic markers such as the serotonin transporter and HTR1A receptor (White et al. 1994). Temperature alteration to a non-permissive 39ºC forces these cells to cease mitotic division, and thus differentiate constitutively to adopt a full serotonergic phenotype over the course of 8 days (White et al. 1994). This project largely used undifferentiated cells rather than differentiated ones because they are much easier to manipulate and transfect than their differentiated counterparts, while maintaining the largely serotonergic character that these cells have, which was desired for this project.

2.1.2.4 Tissue culture

All tissue culture reagents were purchased from Invitrogen (NZ) Ltd, Auckland, New Zealand. All tissue culture plasticware was purchased from BD Bioscience (Bedford, Massachusetts, USA). COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin (pen/strep) in 25 cm^2 or 75 cm^2 tissue culture flasks in a 37ºC incubator with 5% CO_2. For subculturing, a subcultivation ratio of 1:5 was used. Culture medium was removed and discarded. The cells were treated with trypsin-EDTA solution at 37 ºC for 5 minutes to facilitate complete detachment of cells from the flask surface. Complete growth medium was added and cells were pelleted in a 50ml centrifuge tube at 448 x g for 5 minutes (Global Science, Germany). Cells were then resuspended in fresh medium and appropriate aliquots of the cell suspension were added onto new culture flasks. The same procedure was used to maintain and subculture RN46A cells except that the medium used was DMEM/F12 supplemented with 5% FBS and 250 µg/ml G418 (Geneticin) and cells were propagated at 33 ºC. The medium was renewed 2 to 3 times each week for both cell lines.

For long-term storage of COS-7 or RN46A cells, cells were grown in a 75 cm^2 flask until they were approximately 80% confluent. Cells were trypsinised and resuspended in 10ml freezing medium which consisted of the complete growth medium for each cell type supplemented with 5% (v/v) DMSO. The cell suspension was aliquotted into

cryovials (Nalge Nunc International, Rochester, New York, USA) and placed at -80 ºC in a 5100 Cryo 1ºC Freezing Container. Cells were transferred to liquid nitrogen for long term storage after 24 hours. Prior to use, cells were thawed rapidly and resuspended in 10ml of complete growth medium followed by centrifugation at 796 x g for 5 minutes to collect the cell pellet. The pellet was resuspended in culture medium and cells were seeded onto either a 25 cm^2 or a 75 cm^2 tissue culture flask depending on the amount of cells required.

2.1.2.5 Transient transfection conditions

Transient transfection of RN46A cells was conducted using the lipid transfection reagent Effectene (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Cells were counted by haemocytometer and seeded at a density of 5 x 10^4 cells per well, into 24-well tissue culture plates the day before transfection. On the day of transfection, 0.2 µg DNA was diluted in TE buffer to a total volume of 29 µl. 0.8 µl Enhancer supplied with the kit was added and mixed by vortexing for 1s. The mixture was then incubated at room temperature for 5 minutes before the addition of 2.5 µl Effectene transfection reagent. The DNA-Enhancer-Effectene mixture was mixed by vortexing for 10s and incubated for 10 minutes at room temperature. 400 µl fresh growth medium was added to each well while the complex was forming. Finally, 150 µl growth medium per well was added to the tube containing the transfection complexes followed by gentle trituration. The final mix was then added onto the cells in the 24-well culture plates and again gently agitated.

2.1.2.6 X-gal staining of cells

A lacZ expressing vector (p610ZA; (Ferreira et al. 2008) was used for optimizing transfection conditions. Cells transfected with this vector were stained for lacZ expression by X-gal staining. Culture media was aspirated off before cells were washed with cold PBS once. Cells were fixed with 5ml fix buffer (section 2.6) for 15 minutes. Fixed cells were rinsed with PBS for four minutes three times. 5ml of X-gal staining buffer (section 2.6) was added to each well. Cells were incubated at 37°C overnight. Transfection efficiency was determined by the proportion of blue cells measured by haemocytometer.

2.1.3 Luciferase reporter gene assays

2.1.3.1 Co-transfection

Dual-Luciferase® Reporter Assay System (Promega Corporation) was used in the luciferase assays. Plasmids were purified and quantified as described previously (Section 2.1.2.1 & Section 2.1.2.2 Plasmid quantificationA co-transfection mix was made by adding 5µl of 50 ng/µl pRL-TK, which contains the *Renilla* luciferase, to 95µl of 50 ng/µl pGL3-Basic construct which contains the promoter of interest fused to the firefly luciferase gene. This co-transfection mix was transfected into each cell line according to the protocol described above (Section 2.1.2.5).

2.1.3.2 Harvesting and assaying of cells

After incubation of transfected cells for the appropriate period of time, cells were harvested using the Dual-Luciferase® Reporter Assay System. Growth medium was aspirated from the cultured cells and a sufficient amount of phosphate buffered saline (PBS) was added to wash the surface of the 24-well plates. 100µl of 1x passive lysis buffer (PLB) was dispensed into each well after removal of PBS and incubated at room temperature for 15 minutes on a rocking platform to ensure complete cell lysis. The cell lysate was then transferred to a tube for further analysis. To determine firefly luciferase activity, 5µl of cell lysate was added into a 384-well plate followed by addition of 12µl of Luciferase Assay Reagent II (LAR II). The bio-luminescence was measured with a Wallac VICTOR^{3TM} 1420 multilable counter (Perkin Elmer^{TM}, Singapore). 12µl of Stop&Glo® Reagent was then added into the same well to measure the *Renilla* luciferase generated bio- luminescence. A 9-second delay between the addition of the Dual-Luciferase reagents and measuring luminescence was maintained for each sample.

2.2 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was employed to measure differential gene

expression due to its high sensitivity and wide dynamic range of quantification compared with traditional end-point RT-PCR (Gurvich and Klein 2002; Wilhelm and Pingoud 2003).

2.2.1 Drug treatment

RN46A cells were treated with a range of drugs including SSRI (paroxetine, citalopram), TCA (nortriptyline), antipsychotic (haloperidol) or anticonvulsant (sodium valproate) respectively, usually for 72 hours (unless otherwise specified). Cells were lysed directly in the culture dish by adding 0.4ml of TRIZOL® LS Reagent (Invitrogen) to a 3.5-cm diameter dish.

2.2.2 RNA isolation

RNA was isolated using TRIZOL® LS Reagent according to the manufacturer's instructions. Briefly, the homogenized samples were incubated at room temperature for 5 minutes before adding 0.2ml of chloroform per 0.75ml of TRIZOL® LS Reagent used. The mixture was mixed well by shaking tubes vigorously by hand for 15 s and incubated at room temperature for 15 minutes before centrifugation at 12,000 x g for 15 minutes. The upper aqueous phase was transferred to a clean tube and RNA was precipitated by adding 0.5ml of isopropyl alcohol per 0.75ml TRIZOL® LS Reagent used. The sample was then incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes. The supernatant was removed and the RNA pellet was washed by mixing with 75% ethanol and centrifuged at 7,500 x g for 5 minutes. The RNA pellet was air-dried briefly and redissolved in DEPC water before incubating at 55°C for 10 minutes. RNA was quantified spectrophotometrically on a Nanodrop. The quality and quantity of RNA was then assessed by running RNA samples on a TBE agarose gel. RNA was estimated visually to be of acceptable quality provided 28S and 18S rRNA bands were apparent and in the correct molar ratios.

2.2.3 cDNA synthesis

First-strand cDNA synthesis was carried out using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Five µg of total RNA was treated with 1µl of DNase at room temperature for 15 minutes. A combination of anchored-oligo $(dT)_{12-18}$ (Invitrogen) and random hexamers (25µM final concentration), 1µl dNTP mix was added subsequently. The reaction mix was incubated at 65°C for 10 minutes, and placed on ice for at least 1 minute. The combination primers were used to avoid bias towards mRNAs with intact polyA tail during cDNA synthesis. This also circumvents the problem with oligo (dT) that the reverse transcription may not reach the far 5´ end of long transcripts (Klasens et al. 1999; Zhang and Byrne 1999; Stangegaard et al. 2006; Armour et al. 2009). A cDNA Synthesis Mix containing 4µl First-strand RT buffer, 1µl DTT, 1µl RNaseOut and 1µl Superscript™ III Reverse Transcriptase (Invitrogen) was added and mixed gently. The reaction mix was incubated at 25°C for 5 minutes, followed by 50 minutes at 50°C. The reaction was terminated at 85°C for 5 min and chilled on ice. 1µl of RNaseH was added to each sample and incubated at 37°C for 20 minutes. The resultant cDNA was stored undiluted in aliquots at -20°C.

2.2.4 Relative quantification

The "crossing point" (Cp) of a sample is normally used in a quantification analysis to determine the initial concentration of DNA in the sample. In a PCR reaction, Cp is the cycle at which the fluorescence of a sample increases above the background fluorescence. The lower the intial concentration of target DNA a sample contains, the more amplification cycles are required to reach the Cp, resulting in a higher Cp, and vice versa. The Cp values for all gene transcripts of interest in each sample were determined by real-time PCR on the LightCycler 480 System (Roche) with Universal ProbeLibrary (UPL) probes (Roche). UPL consists of 165 pre-validated real-time PCR hydrolysis probes that provide optimal coverage of all transcripts in a given transcriptome (Please refer to Chapter 4 for detailed description of UPL system). It combines the flexibility, convenience and cost-effectiveness of SYBR Green I assay with the specificity of probe-based PCR assays (Rein et al. 2006; Christoph Leucht and Bally-Culf 2007; Ina Horst and Peterhansel 2007)

Figure 2.1 shows the layout of the 384-well plate format used in qPCR. Nine samples plus the common calibrator, which was used to normalise inter-run variations, and a negative control, were analyzed on one plate. For one plate run, eight test genes and three reference genes were included. Three technical replicates for each of the six biological replicates were conducted for each sample. A biological replicate corresponds to an independent experimental sample, which was cultured RN46A cells

Table 2.2 Two-step RT-PCR cDNA synthesis protocol.

5 µg	Total RNA
1µl	DNase
1µl	DNase buffer
15 minutes	Room temperature
1µl	EDTA
10 minutes	65°C
1µl	Anchored oligo(dT)
1µl	Random hexamer primer
1µl	dNTP mix
2µl	PCR-grade water
5 minutes	65°C, then on ice for 1 minute
4µl	First-strand RT buffer
1µl	DTT
1µl	RNaseOut
1µl	SuperScrpit™ III Reverse Transcriptase
10 minutes	25°C
50 minutes	50°C
5 minutes	85°C, then on ice for 1 minute
1µl	RNaseH
20 minutes	37°C

from a single cell thaw that underwent indicated time of drug exposure.

The JANUS Automated Workstation was used to set up each 384-well plate. Ten master mixes were prepared containing the cDNA for nine test samples and the calibrator respectively for each plate. A negative “no template” control was prepared also. Eight microlitres of master mix were added into each well of a 384-well plate. Primer probe mixes for each target gene were preloaded into a skirted 96-well PCR microplate (Axygen). Two microlitres of this primer probe mix was subsequently added into wells containing master mixes to make up to a total volume of 10 microlitres.

Data normalisation using one or several reference genes is a key aspect of relative quantification of mRNA (Pfaffl 2001; Tricarico et al. 2002; Vandesompele et al. 2002). Since it is difficult to find a gene that expresses uniformally across five drug treatments throughout the entire sample population, several reference genes were measured and the most suitable ones were used for later analysis. This work is described in Section 4.3.1.

Relative expression values of each target gene were calculated by determining the ratio between each target and the three references genes, which was then normalised against a common calibrator cDNA using LightCycler® 480 Software 1.5. The calibrator is a positive sample used to correct for differences in detection sensitivities between target and reference genes due to differences in probe annealing, FRET efficiency, or dye extinction coefficients within one run and between several PCR runs. The common calibrator cDNA was synthesized from an untreated RN46A cell RNA sample and used for the whole qPCR study. Inter-run variability was corrected using LightCycler® 480 Multiple Plate Analysis Software.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	A1	A1	A1	R1	R1	R1	A1	A1	A1	A1	A1	A1	R1	R1	R1	A1	A1	A1	A1	A1	A1	R1	A1	A1
B	A1	A1	A1	R1	R1	R1	A1	A1	A1	A1	A1	A1	R1	R1	R1	A1	A1	A1	A1	A1	A1	R1	A1	A1
C	A2	A2	A2	R2	R2	R2	A2	A2	A2	A2	A2	A2	R2	R2	R2	A2	A2	A2	A2	A2	A2	R2	A2	A2
D	A2	A2	A2	R2	R2	R2	A2	A2	A2	A2	A2	A2	R2	R2	R2	A2	A2	A2	A2	A2	A2	R2	A2	A2
E	A3	A3	A3	R3	R3	R3	A3	A3	A3	A3	A3	A3	R3	R3	R3	A3	A3	A3	A3	A3	A3	R3	A3	A3
F	A3	A3	A3	R3	R3	R3	A3	A3	A3	A3	A3	A3	R3	R3	R3	A3	A3	A3	A3	A3	A3	R3	A3	A3
G	A4	A4	A4				A4	A4	A4	A4	A4	A4				A4	A4	A4	A4	A4	A4		A4	A4
H	A4	A4	A4				A4	A4	A4	A4	A4	A4				A4	A4	A4	A4	A4	A4		A4	A4
I	A5	A5	A5				A5	A5	A5	A5	A5	A5				A5	A5	A5	A5	A5	A5		A5	A5
J	A5	A5	A5				A5	A5	A5	A5	A5	A5				A5	A5	A5	A5	A5	A5		A5	A5
K	A6	A6	A6	R1	R1	R1	A6	A6	A6	A6	A6	A6	R1	R1	R1	A6	A6	A6	A6	A6	A6	R1	A6	A6
L	A6	A6	A6	R1	R1	R1	A6	A6	A6	A6	A6	A6	R1	R1	R1	A6	A6	A6	A6	A6	A6	R1	A6	A6
M	A7	A7	A7	R2	R2	R2	A7	A7	A7	A7	A7	A7	R2	R2	R2	A7	A7	A7	A7	A7	A7	R2	A7	A7
N	A7	A7	A7	R2	R2	R2	A7	A7	A7	A7	A7	A7	R2	R2	R2	A7	A7	A7	A7	A7	A7	R2	A7	A7
O	A8	A8	A8	R3	R3	R3	A8	A8	A8	A8	A8	A8	R3	R3	R3	A8	A8	A8	A8	A8	A8	R3	A8	A8
P	A8	A8	A8	R3	R3	R3	A8	A8	A8	A8	A8	A8	R3	R3	R3	A8	A8	A8	A8	A8	A8	R3	A8	A8

A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	R1										
B	A2	R2										
C	A3	R3										
D	A4											
E	A5											
F	A6											
G	A7											
H	A8											

B)

Figure 2.1 Layout of qPCR reaction. A) 384-well plates and B) Axygen (Union City, CA, USA) 96-well PCR microplates containing primer and probe mixes. A1-A8: Target gene assays 1-8 with each number representing a different target gene of interest, R1-R3: Reference gene assays 1 to 3. Target and reference gene assays are coloured yellow and pink respectively. Calibrator samples and negative control samples are coloured blue and green respectively.

2.3 Protein analysis

2.3.1 Cell lysis

After exposure of cultured RN46A cells to valproate, DMEM/F12 medium was removed followed by washes in ice-cold PBS. PBS was then aspirated and cells were lysed in 500µl of RIPA buffer per well of six-well tissue culture plates.

2.3.2 Protein assay

Protein concentration was determined using Bio-Rad DC Protein Assay kit (Bio-Rad) according to the manufacturer's instructions. Briefly, bovine serum albumin (BSA) was used as a protein standard to create standard curves. Five dilutions of BSA containing from 0 µg/ml to 10 µg/ml proteins were set out in duplicate in a clean, dry 96-well microtiter plate. Cell lysate samples were also set out in duplicate. 25µl of reagent A was mixed with an alkaline copper tartrate solution, and 200µl of reagent B (a dilute Folin Reagent), were added into both cell lysate samples and to the standards sequentially. The plate was incubated for 15 minutes at room temperature. Absorbance was measured at 750nm with Spectramax 190 plate reader using Softmax Pro V5.3 (Molecular Devices, VIC, Australia). A standard curve was generated with the absorbance against the protein concentration and the concentration of cell lysate samples was extrapolated from the standard curve.

2.3.3 Preparing cell lysate for western blotting

10-20µg of cell lysate was loaded for western blotting according to the concentration determined by the protein assay. Cell lysate samples were mixed with loading buffer in a 1:6 ratio and boiled at 100°C for 2 minutes. The mix was then left on ice until used for the experiment.

2.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated using SDS-PAGE. Gel electrophoresis equipment (Bio-Rad) was cleaned with 70% ethanol and assembled according to manufacturer's instructions. A 10% polyacrylamide resolving gel was prepared in a casting tray, which was overlaid with distilled water until set. A 10% stacking gel was applied on top of the resolving gel, after removing excess water, and a comb was inserted. The gel was set for 30 minutes and the comb removed. Gels were transferred to an electrophoresis tank, and immersed in running buffer (section 2.6). A pre-stained SDS-PAGE protein size standard (Bio-Rad) and cell lysate samples were subsequently loaded into wells in the gel, followed by electrophoresis at a constant current of 150 volts for one hour.

2.3.5 Protein transfer

Proteins separated by electrophoresis were transferred from the gel onto a PVDF membrane (Amersham Hybond-P, GE Healthcare) using a Bio-Rad Trans-Blot cell. The PVDF membrane was cut to the size of the gel and pre-wetted in 100% methanol briefly. To assemble the transfer sandwich, one sponge was placed on the transfer cassette followed by two sheets of Whatman filter paper. The gel was placed on top of the filter paper and the pre-wetted PVDF membrane on top of the gel. Two sheets of Whatman filter paper were then placed on top of the PVDF membrane followed by one sponge to complete the sandwich. The transfer cassette was clamped together and immersed in transfer buffer (section 2.6). Protein transfer was conducted at a constant current of 100 volts for one hour.

2.3.6 Blocking and immunodetection of transferred proteins

Blocking of non-specific binding was achieved by incubating the PVDF membrane in blocking buffer (5% skimmed milk in TBST) for 20 minutes at room temperature following transfer of proteins. The primary antibody was diluted in TBST containing

3% skimmed milk at 1:1000 to 1:3000 depending on the abundance of proteins in the samples, and was incubated with the PVDF membrane on a rocking platform at 4°C overnight. Following incubation, the membrane was washed in TBST for 5 minutes four times under gentle shaking at room temperature. A dilute solution of secondary antibody (generally between 1:3000 and 1:10000), which is directed at a species-specific portion of the primary antibody, was incubated in TBST with 5% skimmed milk, with the membrane for two hours at room temperature with agitation. After rinsing the membrane four times for 5 minutes in TBST to remove unbound secondary antibodies, the western blots were detected by ECL+ western blotting detection system (GE Healthcare) according to the manufacturer's instructions. PVDF membranes were visualized on ChemiDoc XRS (Bio-Rad) using Gel-Doc software.

2.4 Multi-kinase ELISA Array

The multi-kinase ELISA array (Symansis, Timaru, New Zealand) was used to analyse activation of multiple protein kinases simultaneously. The kit was kindly gifted by Professor Peter Shepherd, Department of Molecular Medicine, University of Auckland.

2.4.1 Cell lysate preparation

After valproate exposure, cultured cells were treated with Trypsin-EDTA solution at 37ºC for 5 minutes to facilitate complete detachment of cells from the flask surface after removing DMEM/F12 medium. Complete growth medium was then added and cells were pelleted in 50ml Falcon centrifuge tubes at 448 x g for 5 minutes. Cells were washed with PBS twice and resuspended in 0.5ml of Cell Lysis Buffer containing protease/phosphatase inhibitors and PMSF. After 10 minutes at room temperature, the cell suspension was mixed by vortexing and then microcentrifuged at 14,000 x g for 10 minutes at 4 ºC. The clear cell lysate was collected and stored at -80ºC.

2.4.2 Assay procedure

The multi-kinase ELISA assay was performed according to the manufacturer's protocol. Briefly, antibody-coated microwell strips were placed in the microwell strip holder and were allowed to equilibrate to room temperature before use. 100 µg/well of cell lysate was diluted in 1x Sample Dilution Buffer to a total volume of 100µl and was added into the appropriate well. 100µl of the provided positive control and negative control (1x Sample Dilution Buffer alone) were added to corresponding wells. The plate was sealed with a plate cover and incubated for 2 hours at room temperature. The plate cover was removed gently and solution was aspirated from wells. 0.3ml of 1x Wash buffer was added into each well, and this process was repeated 3 times before adding 100µl of the appropriate Biotin-Conjugated Detection Antibody, which also binds specifically to its corresponding antigen, to each well. The plate was sealed with a plate cover again, and the reaction mixture was incubated at room temperature for 2 hours. The washing process was repeated 3 times as described previously at the end of incubation step. 100µl Streptavidin Horseradish Peroxidase (SAV-HRP) was equilibrated to room temperature before it was added to each microwell. The plate was sealed and incubated for 30 minutes at room temperature. Cells were washed 3 times again followed by 20 minutes incubation with 100µl of tetrametylbenzidine (TMB) substrate solution at room temperature. Colour development reaction was stopped with 50µl Stop solution and the absorbance at 450 nm was measured with the Spectramax 190 plate reader using Softmax Pro V5.3 (Molecular Devices, VIC, Australia) within 30 minutes of stopping the reaction.

2.5 Bioinformatic analysis tools, databases and websites

MatInspector: *www.genomatix.de*

A software tool designed to search for transcription factor binding sites utilizing a large library of matrix descriptions for each target binding site

TF-search: *http://www.cbrc.jp/research/db/TFSEARCH.html*

This program predicts DNA transcription factor binding sites by searching for matched sequence fragment in TFMATRIX transcription factor binding site profile database.

National Centre for Biotechnology Information (NCBI): *www.ncbi.nlm.nih.gov*

NCBI is a resource for molecular biology information that supports and distributes a variety of databases including GenBank DNA sequence database. It also provides citations for biomedical literature from various sources via PubMed.

UCSC Genome Bioinformatics: *genome.ucsc.edu/*

UCSC Genome Brower is a website that contains the reference sequences for a large collection of genomes. It integrates numerous genomic resources and datasets and displays them in one coordinated graphic output.

European Bioinformatics Institute & Sanger Centre (ENSEMBL): *www.ensembl.org*

Ensembl have databases for vertebrates and other eukaryotes.

Mouse Genome Informatics (MGI): *www.informatics.jax.org*

MGI provides information on the genetics and genomics of laboratory mouse.

Rat Genome Database (RGD): *rgd.mcw.edu/wg*

RGD provides access to data on the genetics and genomics of rats.

Allen Brain Atlas: *www.brain-map.org*

The Allen brain atlas is an interactive, genome-wide image database that combines genomics with neuroanatomy to provide gene expression information for the mouse or human brain.

ArrayExpress: www.ebi.ac.uk/arrayexpress

The ArrayExpress is a public database that stores microarray data, which contains Minimum Information About a Microarray Experiment (MIAME) in accordance with Microarray and Gene Expression Data (MGED) society recommendations.

NetAffx™ Analysis Centre: www.affymetrix.com/analysis/index.affx

The NetAffx™ Analysis Centre is a public database that enables correlation of GeneChip® array results with array design and annotation information

Microarray data analysis:

The microarray data (McHugh et al. 2009a) were submitted to the European Bioinformatics Institute (www.ebi.ac.uk) and loaded in to ArrayExpress (Experiment name: GSFL-RN46A-acute paroxetine exposure; ArrayExpress accession: E-MEXP-1582), a public repository for microarray data, which stores Minimum Information About a Microarray Experiment (MIAME)-compliant data in accordance with Microarray and Gene Experession Data (MGED) society recommendations. Gene expression data of untreated RN46A cells werer retrieved from ArrayExpress, and were compared with gene expression levels of untreated cell samples measured in qPCR. A Welch two sample t-test (Welch 1947) was used for statistical analysis.

2.6 Stocks, solutions and reagents

Lysis buffer for western blotting:

50mM HEPES pH7.5

150mM NaCl

10mM EDTA

1% NP40

+ Phosphatase inhibitors

+ Protease inhibitors

Stored at 4°C

Deoxynucleoside triphosphates (dNTPs) 2mM stock: 250µl each of 100mM dNTP (Roche Diagnostics) (dATP, dCTP, dGTP and dTTP) was added to 11.5ml MPW, aliquoted and stored at -20°C

Diethyl pyrocarbonate (DEPC) water, 0.1%: 2ml DEPC (Sigma Aldrich Corporation, St. Louis, MO, USA) was added into MPW to a final volume of 2L, shaken vigorously and incubated overnight at 37°C. The solution was then autoclaved to remove residual DEPC before use.

DNA loading dye:

3ml glycerol

200µl 2.5% bromophenol blue stock

200µl 2.5% xylene cyanol stock

Added to 6.6ml MPW, aliquoted and stored at -20°C

Ethidium bromide, 10mg/ml: 1 tablet (100mg) (Sigma) was dissolved in 10ml MPW and stored at 4°C away from light.

PBS: 1 tablet (Oxoid Australia Pty Ltd, South Australia) was dissolved in 100ml MPW

RNA loading dye:

500µl glycerol (BDH)

2µl 0.5M EDTA

4 mg bromophenol blue (Sigma)

100µl ethidium bromide (10mg/ml)

Added to DEPC water to a final volume of 1ml, aliquoted and stored at -20°C

2YT broth:

16g bactotryptone (BD Biosciences)

10g yeast extract (Invitrogen)

5g NaCl (Sigma)

Made up to a final volume of 1L with MPW and autoclaved before use

2YT agar: 1L 2YT broth +7.5g bacteriological agar (Invitrogen), autoclave before use.

2YT broth Amp$^+$: 1L 2YT broth + 2ml 100mg/ml Amp

2YT agar Amp$^+$: 1L 2YT agar + 2ml 100mg/ml Amp

STET buffer:

8g Sucrose (Sigma)

5ml Triton X-100 (Merck)

10ml 0.5M EDTA (Sigma)

5ml 1M tris-HCl

Made up to 100ml with MPW and autoclaved before use

TBE, 10x:

121g Tris (Merck)

62g orthoboric acid (BDH)

7.4g EDTA

Made up to 1L with MPW

TBE, 1x:

200ml 10x TBE

20µl ethidium bromide (10mg/ml)

1.8 L MPW

X-gal staining buffer:

Solution 1

88ml PBS

0.1ml 1M MgCl2

2ml 2% X-gal made up in DMF

Solution 2

210mg potassium ferrocyanide dissolved in 5ml MPW

165mg potassium ferricyanide dissolved in 5ml MPW

Mix 9ml solution 1 with 1ml solution 2

X-gal fix buffer:

0.1M phosphate buffer pH 7.3

5mM EGTA (Sigma) pH 7.3

2mM MgCl2

0.2% glutareldahyde (Sigma)

Primary antibody solution: 1:1000 dilution of primary antibody in TBST containing 3% skim milk

Secondary antibody solution: 1:5000 dilution of HRP-conjugated secondary antibody in TBST containing 3% skim milk

Running buffer (5x):

25mM Tris-HCl

250mM pH8.3 Glycine

0.1% SDS

TBS (Tris-buffered saline) (10x):

80g NaCl

30g Tris-HCl

Made up to 1L with MPW

Adjusted to pH7.6

TBST: 1x Tris buffered saline supplemented with 0.05% Tween-20

10% resolving gel (2 gels):

4.89ml H_2O

3.00ml Acrylamide (40% 37.5:1)

2ml 2M Tris-HCl, pH8.8

50µl 2% SDS

50µl APS (ammonium persulfate)

15µl TEMED (tetramethylethylenediamine)

Stacking gel (2 gels):

3.19ml H_2O

0.5ml Acrylamide (40% 37.5:1)

1.25ml 0.5M Tris-HCl, pH6.8

25µl 20% SDS

25µl APS

8µl TEMED

Western blot buffer 10x:

30.2g Tris

144.4g glycine

Made up to 1L with MPW

Cell Lysis Buffer for ELISA:

Stock lysis buffer solution: 50ml

Urea 6M (18g)

HEPES 50mM (2.5ml from 1M stock)

NaCl 150mM (1.5ml from 5M stock)

EDTA 10mM, pH 8.0 (1.0ml from 0.5M stock)

$Na_2P_2O_7$ 10mM (5ml from 0.1M stock)

Vanadate 2mM, pH 10 (1ml from 0.1M stock)

NaF 100mM (5ml from 1M stock)

NP40 1% (5ml from 10% stock)

To make 50ml buffer, the measured amount of urea was added in to a 50ml Falcon tube containing 35ml of autoclaved H_2O. The solution was left on ice after adding HEPES, NaCl and EDTA. The rest of the compounds were added and sterile H_2O was used to make up the volume to 50ml. Protease inhibitors and PMSF were added just before use to the stock lysis buffer.

1x ELISA wash buffer:

25ml 20x wash buffer concentrate was diluted to distilled water to a total volume of 500ml. The diluted wash buffer is stored at 4°C for up to 14 days.

1x ELISA sample diluted buffer:

25ml 5x sample dilution buffer concentrate was diluted to distilled water to a total volume of 125ml. The diluted Sample Buffer is stored at 4°C for up to 14 days

Table 2.3 Oligonucleotide primers and UPL probes for used for quantitative PCR

Gene	Gene ID	Primer name	qPCR oligonucleotide sequence	UPL probe
Actb	81822	ACTB F-QPCR ACTB R-QPCR	5'CCCGCGAGTACAACCTTCT 3′ 5'CGTCATCCATGGCGAACT 3′	17
Adm	25026	ADM F1-QPCR ADM R1-QPCR	5'CGCAGTTCCGAAAGAAGTG 3′ 5'TCGGGACTGTCTTCTCATCA 3′	120
Ank3	361833	ANK3F2-QPCR ANK3R2-QPCR	5'GTCACAGCTAATGCATCATGG 3′ 5'GCAATGCTGTTTCTCCTCTCA 3′	119
Arc	54323	ARC F1-QPCR ARC R1-QPCR	5'GCTGAAGCAGCAGACCTGA 3′ 5'TTCACTGGTATGAATCACTGCTG 3′	79
Ascl1	64186	ASCL1 F-QPCR ASCL1 R-QPCR	5'CTGGGAATGGACTTTGGAAG 3' 5'TGACGTCGTTGTCAAGAAACA 3'	121
Bdnf	24225	BDNF F1-QPCR BDNF R1-QPCR	5'AGCGCAGGTGTGTTAGTGGT 3′ 5'GCAATTGTTTGCCTCTTTTTCT 3′	92
Cacna1a	25398	CACNA1F2-QPCR CACNA1R2-QPCR	5'AGGACCAGCTTGCTGACATC 3′ 5'TCCAGCTTGGCACTTTTGAT 3′	21
Crhr2	64680	CRHR2 F1-QPCR CRHR2 R1-QPCR	5'TGAACCCATTTTGGATGACA 3′ 5'GTTGATGAGGGCGATTC 3′	6
Creb1	81646	CREB1 F1-QPCR CREB1 R1-QPCR	5'CTAGTGCCCAGCAACCAAGT 3′ 5'GGAGGACGCCATAACAACTC 3′	9
Dazap1	362836	DAZAP1 F-QPCR DAZAP1 R-QPCR	5'TCGAGGACGAACAATCAGTG 3′ 5'AGCCCGTTTAACCTCCACTT 3′	64
Fkbp5	361810	FKBP5 F1-QPCR FKBP5 R1-QPCR	5'CTCAAACCCCAATGAAGGAG 3′ 5'GCAGTCAAACACCCTTCCAG 3′	66
Gch1	29244	GCH1 F1-QPCR GCH1 R1-QPCR	5'CGCCTTACCAAACAGATTGC 3′ 5'ACCTCGCATGACCATACACA 3′	40
Gchfr	171128	GCHFR F1-QPCR GCHFR R1-QPCR	5'GGACAGAGCAGGGAGTAACG 3′ 5'GAGTGCTCATCACCCACCA 3′	114
Gnb3	60449	GNB3 F1-QPCR GNB3 R1-QPCR	5'TGGCTATGATGACTTCAACTGC 3′ 5'GTCATGGCCAGAAAGAACG 3′	95
Grik4	24406	GRIK4 F1-QPCR GRIK4 R1-QPCR	5'TCATCTGTGCCAAAGCAGAA 3′ 5'TAAGGAATTGCCGGAGCA 3′	80
G6pd	24377	G6PD F-QPCR G6PD R-QPCR	5'TGCAGCAGCTGTCCTCTATG 3′ 5'ACTTCAGCTTTGCGCTCATT 3′	58
Hdac1	297893	HDAC1 F1-QPCR HDAC1 R1-QPCR	5'CCGGTATTTGATGGCTTGTT 3′ 5'TCAGACTTCTTCGCATGGTG 3′	21
Hdac3	84578	HDAC3 F1-QPCR HDAC3 R1-QPCR	5'ATCATGCCAAGAAGTTTGAGG 3′ 5'GCACCCGAGGGTGGTACT 3′	1
Hdac4	363287	HDAC4 F1-QPCR HDAC4 R1-QPCR	5'TTCTAGGAAAGGCCAGGATG 3′ 5'GCCAGAAAGTCCATCTGAGG 3′	82
Hdac6	84581	HDAC6 F1-QPCR HDAC6 R1-QPCR	5'AGATTCGGAATGGCATGG 3′ 5'CATCCATAAGACTGCGCTGA 3′	79
Hdac7	84582	HDAC7a F1-QPCR HDAC7a R1-QPCR	5'TACCTGGCTGCTTTCAGGAT 3′ 5'ACCAGGTCTGGAGCAAACTC 3′	9
Htr1a	24473	Htr1aF2-QPCR Htr1aR2-QPCR	5'GGCACCTTCATCCTCTGCT 3′ 5'GTGGCAGCTGCTTTCACAG 3′	21
Htr2a	29595	HTR2A F-QPCR HTR2A R-QPCR	5'TGATGTCACTTGCCATAGCTG 3' 5'TCGCACAGAGCTTGCTAGG 3'	3
Htr2b	29581	HTR2B F1-QPCR HTR2B R1-QPCR	5'TGCCGATTGCTCTCTTAACAA 3′ 5'CAGGGAAATGGCAGAGAGAT 3′	16

Htr2c	25187	HTR2C F1-QPCR HTR2C R1-QPCR	5'CCGAGTCCGTTTCTCGTCTA 3′ 5'CTATGCTTGCAGGTAATAGTTGAC A 3′	69
Lmx1b	114501	LMX1B F- QPCR LMX1B R-QPCR	5'TGCAAGGGTGACTACGAGAA 3' 5'TGTCTCCATCTTCATCCTCACTC 3'	73
Gar1	499709	GAR1 F-QPCR GAR1 R-QPCR	5'TGTCAGAAAACATGAAGGCATC 3′ 5'GCCGAGGCAGAAACCTCT 3′	74
Maoa	29253	MAOA F-QPCR MAOA R-QPCR	5'TGGTATCATGACCCAGTATGGA 3' 5'TGTGCCTGCAAAGTAAATCCT 3'	20
Ntrk2	25054	Ntrk2F2-QPCR Ntrk2R2-QPCR	5'CGTGTGGTCCCACTACCC 3′ 5'TCGCGCACGAAGTAATAGC 3′	26
Olfml2b	304960	Olfml2b F-QPCR Olfml2b R-QPCR	5'CGGACAACCAGGAGAACG 3′ 5'CCTCAGACACAGCCTTGACC 3′	3
P2rx7	29665	P2RX7 F-QPCR P2RX7 R-QPCR	5'AGGAAGTGTGAGCCCATCGT 3' 5'GCTCGTCCACAAAGGACAC 3'	117
Pcbd	29700	PCBD F-QPCR PCBD R-QPCR	5'GACCAGCTGCTGCCAAAC 3' 5'TCTTGTCATGAAGCCAAAAGC 3'	97
Phb	25344	PHB F2-QPCR PHB R2-QPCR	5'CACAGTACGCATTCTCTTCTGG 3′ 5'GCCAATGCTGGTGTAGATACG 3′	20
Pts	29498	PTS F1-QPCR PTS R1-QPCR	5'GCTGCACAGCCCATCTCT 3′ 5'TCCATGAATTGTCACCACAAC 3′	73
Pvalb	25269	PVALB F2-QPCR PVALB R2-QPCR	5'TTCTGGACAAAGACAAAAGTGG 3′ 5'CTGAGGAGAAGCCCTTCAGA 3′	49
Pclo	56768	PCLO F1-QPCR PCLO R1-QPCR	5'GCCCAATCTACTTCCCATCC 3′ 5'TTCCTGGCTGTTTCACAGG 3′	21
Qdpr	64192	QDPR F1-QPCR QDPR R1-QPCR	5'CAGCTCCTGGACACCCTTAG 3′ 5'TGTTCCCAGTGATCCAGTCA 3′	22
Rnf4	29274	Rnf4 F-QPCR Rnf4 R-QPCR	5'TTGGTGTAGATCACTTCCTATTCTGT 3′ 5'GCCGCTTTCTTTGAGGATTT 3′	74
Slc6a2	83511	SLC6A2 F1-QPCR SLC6A2 R1-QPCR	5'AGATTGAGGATGTCGCCACT 3′ 5'TGGCTTCTGGATACAGGACA 3′	17
Slc6a4	25553	SLC6A4 F1-QPCR SLC6A4 R1-QPCR	5'AAAGGCGTCAAAACATCTGG 3′ 5'AGCAGGACAGAGAGGACAATG 3′	69
Spr	29270	SPR QPCR-F1 SPR QPCR-R1	5'TGTGAGGGTGCTGAGCTATG 3′ 5'GGGTCCATGGAGGTTTCC 3′	130
Th	25085	TH F2-QPCR TH R2-QPCR	5'TCTCCCTGAGGGGTACAAAA 3′ 5'GAATTTTGGCTTCAAATGTCTCA 3′	64
Tph2	317675	TPH2 F1-QPCR TPH2 R1-QPCR	5'TACGGCACCGAGCTTGAC 3′ 5'TGGCCACATCCACAAAATAC 3′	69

Chapter 3

Detection of antidepressant drug induced gene expression changes using the luciferase reporter gene system

3.1 Introduction

The aim of this project was to explore the effects of antidepressants on gene regulation using model systems. Both animal and cell culture models have been utilised for studying antidepressant function. For my project I sought to establish a simplified and consistent model system in which gene expression changes elicited by drug exposure could be robustly demonstrated and then studied. For this purpose, a cell culture model seemed preferable over an animal model. The cell line I elected to use for these studies was RNA46A, which is a rat serotonergic cell line. Although other cell lines such as C6 which is a rat glioma cell line (Meller et al. 2002; Sullivan et al. 2004; Hisaoka et al. 2008) and SH-SY5Y which is a human neuroblastoma cell line (Laifenfeld et al. 2002; Dziedzicka-Wasylewska and Solich 2004; Donnici et al. 2008; Hisaoka et al. 2008; Vik-Mo et al. 2009), have been commonly used for studying depression and antidepressant treatment response, RN46A was chosen specifically because its serotonergic nature makes it a highly relevant cell type in which to study antidepressant pharmacogenetics.

This study utilizes the reporter gene assay to determine whether specific candidate genes are affected by antidepressant drugs. This assay is one approach for the functional analysis of gene promoter regions, providing straightforward information about the impact of drug exposure on gene expression in a simple model system. It

utilizes the firefly luciferase reporter gene, so that candidate gene promoters can be cloned into a vector to drive luciferase transcription. To achieve this, the promoter regions of selected genes were coupled to the reporter gene vector by bacterial cloning. The cloned promoter constructs are introduced into cultured cells and either treated with drug or untreated. After drug exposure, promoter activity was measured (Figure 3.1). Gene expression is regulated at both transcriptional and translational levels. The former includes mechanisms such as transcriptional initiation, chromatin condensation, DNA methylation, alternative splicing of RNA and mRNA stability (Wray et al. 2003). Transcriptional initiation, which is suggested to play a major role in determining the overall mRNA gene expression profile, is mediated by the interaction between cis-acting DNA regulatory sequences and trans-acting proteins (Lemon and Tjian 2000; Park 2001; Malo et al. 2003; Wray et al. 2003; Malo et al. 2006; Schneider-Stock and Ocker 2007). Reporter gene assays are commonly used to examine the activity of promoters and other *cis*-acting regulatory sequences under different experimental conditions. Although there are several available reporter systems, luciferase was chosen as it is sensitive and has a wide dynamic range of detection, and it is widely used and was already well established in our laboratory (Prost and Moore 1986; MacGregor and Caskey 1989; Mitchell and Tjian 1989; Chalfie et al. 1994; Park 2001; Lin et al. 2007; Zhou et al. 2009).

Luciferase reporter gene assays are based on the ability of firefly luciferase to catalyse the oxidation of beetle luciferin in the presence of ATP, Mg^{2+}, O_2 and Coenzyme A (CoA), which generates bio-luminescence (Figure. 3.2 A). In order to achieve this, a promoter of interest is introduced directly upstream of a reporter plasmid containing the firefly luciferase gene. This is accomplished using one of a variety of reporter plasmids, such as pGL3-basic (Promega). Since firefly luciferase does not require post-translational modification for enzymatic activity, the light produced in luciferase assays is directly proportional to the activity of the promoter controlling the expression of the luciferase gene.

Renilla reniformis (Sea pansy) luciferase, in addition, is often used as an internal control to minimise experimental variability caused by differences in cell viability, transfection efficiency, pipetting errors, cell lysis efficiency and assay efficiency. It is incorporated into the experiment by co-transfection of the firefly luciferase construct with a reporter plasmid containing the *Renilla* luciferase gene under the control of a constitutively expressed mammalian promoter pRL-TK (Promega Corporation). *Renilla* luciferase catalyses the oxidation of coelenterazine to coelenteramide. This event also results in light production (Figure 3.2 B). Since firefly and *Renilla* luciferase have different enzyme structures and substrates due to their distinct evolutionary origins, the level of promoter activity and thus the amount of enzyme produced can be measured independently. The promoter activity generated in this study was expressed as a ratio of firefly luciferase and *Renilla* luciferase.

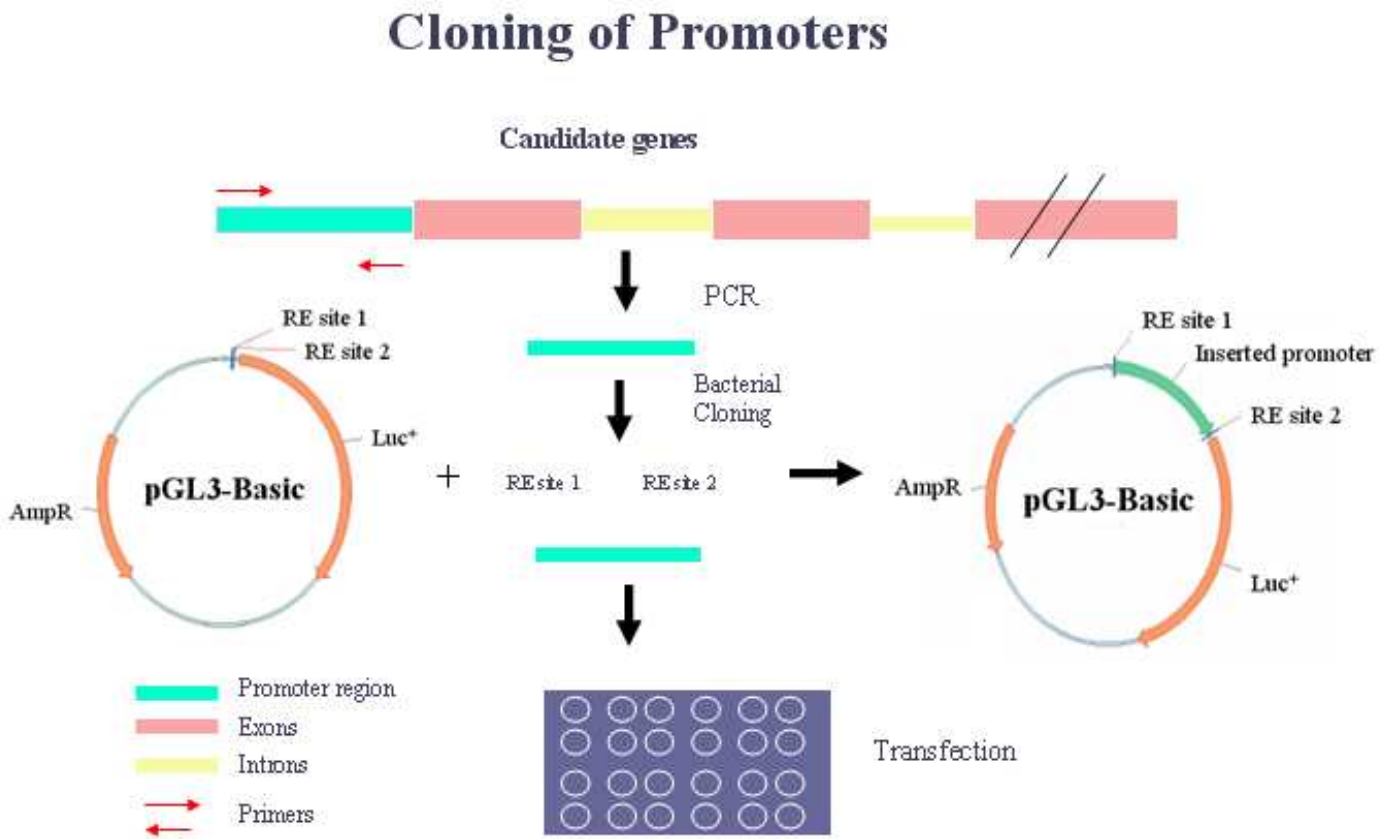


Figure 3.1 Outline of bacterial cloning of candidate gene promoters and screening for basal promoter activity. Promoters were amplified by PCR. Both PCR products and the pGL3-Basic plasmid were digested by restriction enzymes followed by ligation.RE: restriction enzyme. This figure is adapted from Promega Technical Manual of pGL3 Luciferase Reporter Vectors.

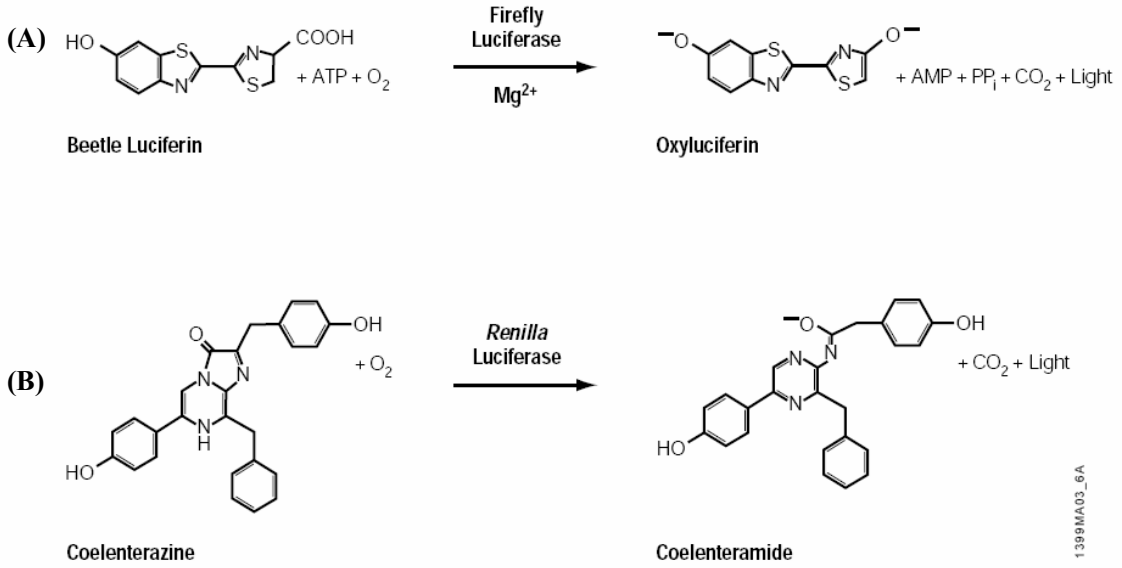


Figure 3.2 Bioluminescent reactions catalysed by (A) firefly and (B) *Renilla* luciferase. This figure is adapted from Promega Technical Manual of Dual-Luciferase® Reporter Assay System.

3.2 Results

3.2.1 Candidate genes and reporter gene construct cloning

A total of eighteen candidate human upstream regulatory regions (promoters; Table 3.1) were introduced into the luciferase expression plasmid pGL3-Basic. These regulatory regions were operationally defined as approximately 1kb-2kb upstream of the relevant translation start site of each gene, which was estimated to encompass the proximal regulatory regions and important transcriptional control elements of each candidate gene.

Candidates were drawn from two main sources: (a) an analysis of the existing literature in the field and (b) prior experimental work from our laboratory, where microarray and proteomic analysis had identified genes exhibiting altered expression profiles following exposure of either cell lines or rats to antidepressants (Table 3.1). The justification for

choosing each candidate gene is as follows. *ARC* is an immediate early gene implicated in consolidation of synaptic plasticity and long-term memory formation (McIntyre et al. 2005). It has been shown that chronic treatment of rats with antidepressant drugs caused *Arc* gene upregulation in specific regions across the rat forebrain, indicating its potential role in antidepressant action (Pei et al. 2003). *CREB1* gene encodes cAMP responsive element binding protein 1, which is a CREB transcription factor belonging to the leucine zipper family of DNA binding proteins (Hoeffler et al. 1988). It has been repeatedly implicated in behavioral models of depression (Hebda-Bauer et al. 2004; Wallace et al. 2004; Hettema et al. 2009), and antidepressant response (Chen et al. 2001a; Lai et al. 2003; Blendy 2006; Matsumoto et al. 2008; Dong et al. 2009; Drago et al. 2009). Corticotrophin releasing hormone (CRH) is a hormone and neurotransmitter and plays an important role in stress response. It has been suggested that dysfunctional stress hormone regulation caused by impaired corticosteroid receptor function is involved in the pathogenesis of depression. Since the combined dexamethasone/CRH test has been proposed to be a suitable biomarker for the predication of antidepressant treatment response (Ising et al. 2005; Schule et al. 2009; Paslakis et al. 2010), it is reasonable to include *CRH* as a candidate gene in this study. Corticotrophin releasing hormone receptor 2 (*CRHR2*), which is an essential component of the CRH system, has also been implicated in animal models of depression (Sheng et al. 2008; Frisch et al. 2010). *GRIK4* encodes a kainate receptor that belongs to the glutamate-gated ionic channel family (Szpirer et al. 1994). A single nucleotide polymorphism (rs1954787) in the *GRIK4* gene has been shown to be associated with outcome of antidepressant treatment in both the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study (Paddock et al. 2007) and the Munich Antidepressant Response Signature (MARS) study (Horstmann et al. 2010). The serotonin receptor 1A gene (*HTR1A*) encodes a subtype of serotonin receptor that binds the endogenous neurotransmitter serotonin. Converging evidence from pharmacological, post-mortem, position emission tomography, and genetic studies support the potential role of *HTR1A* in the pathogenesis of depression and treatment response, making it a strong candidate as an antidepressant responsive gene (Kato et al. 2009; Savitz et al. 2009; Kato and Serretti 2010). *PCLO* (piccolo) encodes a presynaptic protein which affects monoamine neurotransmitter release (Cases-Langhoff et al. 1996). It has been suggested that piccolo may be involved in antidepressant treatment response (Schuhmacher et al. 2010). Various genome-wide association studies have found an association between

PCLO and MDD as well as HPA system response to antidepressant treatment (Sullivan et al. 2004; Bochdanovits et al. 2009; Hek et al. 2010; Schuhmacher et al. 2010). The involvement of *SLC6A4*, which encodes serotonin transporter, in depression and antidepressant response has been demonstrated by several studies. In particular, a polymorphism in the promoter region of *SLC6A4* called serotonin transporter linked polymorphic region (5-HTTLPR) has been found to be associated with efficacy of serotonin reuptake inhibitors (SSRIs) (van Gijn et al. 2001; Lenze et al. 2005; Lotrich and Pollock 2005; Alessandro and Kato 2008; Drago et al. 2009; Fukuchi et al. 2009). Another serotonin-related gene *TPH2*, which encodes a rate-limiting enzyme in serotonin synthesis in the brain, is also included in this study because an association between polymorphisms of human *TPH2* gene and treatment response to fluoxetine has been demonstrated (Peters et al. 2004). Studies on rats also showed changed *TPH2* gene expression in the brain after fluoxetine treatment, indicating the potential role of *TPH2* in depression and fluoxetine action (Dygalo et al. 2006).

In addition to these genes, a number of genes uncovered by prior work in this laboratory were examined. *ADM* was found to be associated with response to paroxetine in a differentiated RN46A cell culture system (Glubb et al. 2010). Three genes (*PVALB*, *PHB*, and *SPR*) came from proteomic studies in two model systems, a differentiated embryonic stem (ES) cell model exposed to paroxetine (McHugh et al. 2008b) and rats chronically treated with paroxetine (McHugh et al. 2009a). *PHB* was identified in both studies; *PVALB* was found in rat hippocampus; and *SPR* was discovered in the differentiated ES cell model. *SPR* encodes sepiapterin reductase, which is a homodimer protein that catalyses the biosynthesis of tetrahydrobiopterin (BH_4) (Smith 1987; Levine et al. 1990; Katoh et al. 1992; Katoh et al. 1994). BH_4 is an essential cofactor for several rate-limiting enzymes in the biosynthesis of neurotransmitters (Nagatsu and Ichinose 1999; Matsumoto et al. 2008), making the BH_4 biosynthesis pathway an interesting candidate for further study. All of the other selected genes are drawn from the BH_4 pathway.

Table 3.1 Candidate genes investigated in the reporter gene assays of this study.

Gene[1]	Gene name	Key references
Literature		
ARC	activity regulated cytoskeletal- associated	(Pei et al. 2003; McIntyre et al. 2005)
CREB1	cAMP responsive element binding protein 1	(Dong et al. 2009; Drago et al. 2009; Hettema et al. 2009)
CRH	corticotrophin releasing hormone	(Ising et al. 2007; Schule et al. 2009; Paslakis et al. 2010)
CRHR2	corticotrophin releasing hormone receptor 2	(Sheng et al. 2008; Frisch et al. 2010)
GRIK4	glutamate receptor, ionotropic kainate 4	(Paddock et al. 2007; Horstmann et al. 2010)
HTR1A	5-hydroxytryptamine receptor 1A	(Kato et al. 2009; Savitz et al. 2009; Kato and Serretti 2010)
PCLO	Piccolo	(Sullivan et al. 2004; Bochdanovits et al. 2009; Hek et al. 2010; Schuhmacher et al. 2010)
SLC6A4	sodium-dependent serotonin transporter	(van Gijn et al. 2001; Lenze et al. 2005; Lotrich and Pollock 2005; Alessandro and Kato 2008; Drago et al. 2009; Fukuchi et al. 2009)
TPH2	tryptophan 5- hydroxylase 2	(Dygalo et al. 2006; Chen et al. 2008)
Prior work		
ADM	Adrenomedullin precursor	(Glubb et al. 2010)
GCH1	GTP cyclohydrolase 1 precursor	(McHugh et al. 2009b; McHugh et al. 2010a)
GCHFR	GTP cyclohydrolase 1 feedback regulatory	(McHugh et al. 2009b; McHugh et al. 2010a)
PCBD	6-pyruvoyl-tetrahydrobiopterin	(McHugh et al. 2009b; McHugh et al. 2010a)
PHB	Prohibitin	(McHugh et al. 2008b)
PTS	6-pyruvoyl-tetrahydrobiopterin synthase	(McHugh et al. 2009b; McHugh et al. 2010a)
PVALB	Parvalbumin	(McHugh et al. 2010b)
QDPR	dihydropteridine reductase	(McHugh et al. 2009b; McHugh et al. 2010a)
SPR	sepiapterin reductase	(McHugh et al. 2008b; McHugh et al. 2009b)

[1] Candidate genes were selected from two sources: (A) the literature pertaining to this project and (B) from prior lab work in cell culture and proteomic studies.

The promoters for each candidate gene were amplified from human genomic DNA by PCR, using specifically designed oligonucleotides with relevant restriction endonuclease recognition sites for each gene. Plasmid pGL3-Basic was digested with the appropriate restriction enzymes for the corresponding promoter inserts. This facilitated directional cloning to enhance the generation of plasmid constructs containing correctly oriented promoters. Samples of undigested and digested plasmids and promoter inserts were separated by electrophoresis on a 1% agarose gel. Gels were stained with ethidium bromide and photographed. Figure 3.3 is one example of a restriction enzyme digested PCR product and pGL3-Basic plasmid. In this example, a PCR product for the *GCHFR* promoter region, and the pGL3-Basic vector, were digested with the enzymes BglII and HindIII. The purified, digested PCR inserts were ligated to purified, digested pGL3-Basic plasmid overnight at 4°C, and transformed

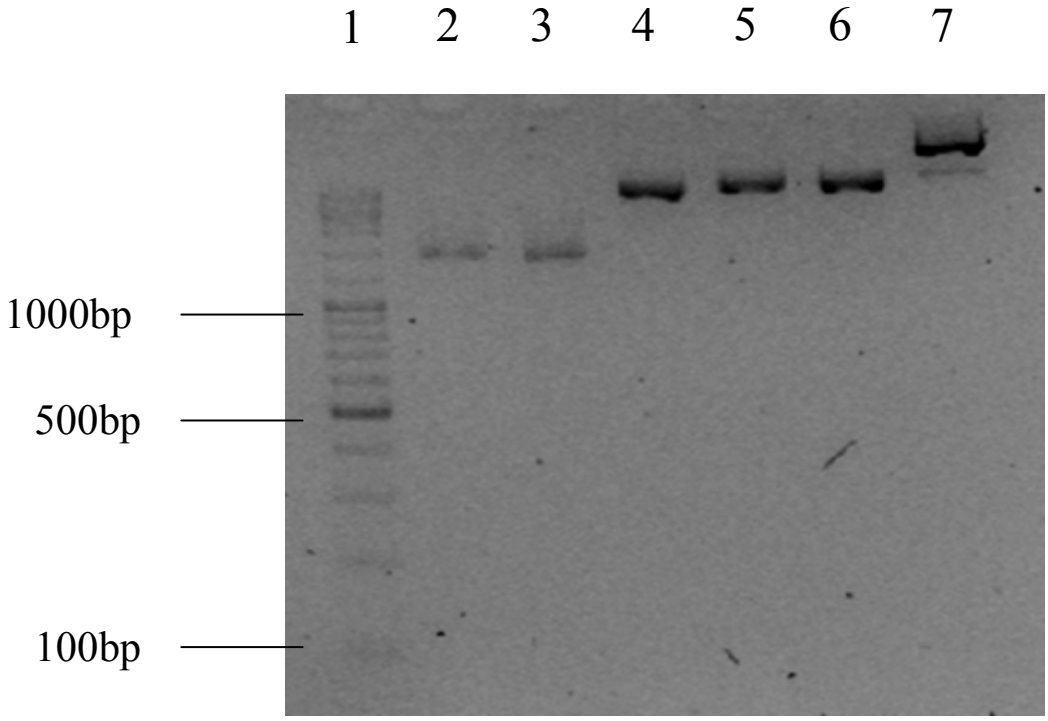


Figure 3.3 *GCHFR* promoter cloning. GCHFR promoter was amplified by PCR and digested by restriction enzymes BglI and HindIII. pGL3-basic plasmid was digested by the same enzymes. The digested products were run on agarose gel along with undigested controls. Lane 1, GeneRuler^{TM} 100 bp plus DNA ladder (Fermentas); Lane 2, BglII + HindIII digested GCHFR sample 1 insert; Lane 3, BglII + HindIII digested GCHFR sample 2 insert; Lane 4, BglII + HindIII digested pGL3-Basic; Lane 5, BglII digested pGL3-Basic; Lane 6, HindIII digested pGL3-Basic; Lane 7, undigested pGL3-Basic.

into *E.coli* by standard methods (section 2.1.1.7). Transformation efficiencies for

different constructs were between 7.3 x 10^3 and 8.4 x 10^4 cfu/µg DNA. Amp resistant *E.coli* colonies were screened for successful construct ligation by selecting twelve Amp-resistant colonies which were picked and grown in a rich culture medium overnight. Plasmid DNA was isolated by a boiling lysis method (section 2.1.1.8). The resultant plasmid DNA was digested with relevant restriction enzymes, and run on an agarose gel to confirm the existence of the promoter insert (Figure3.4). Six clones (lanes 5, 6, 7, 12, 13 and 14) contained an insert of the expected size (1420bp). The identity of promoter inserts was subsequently verified by DNA sequencing.

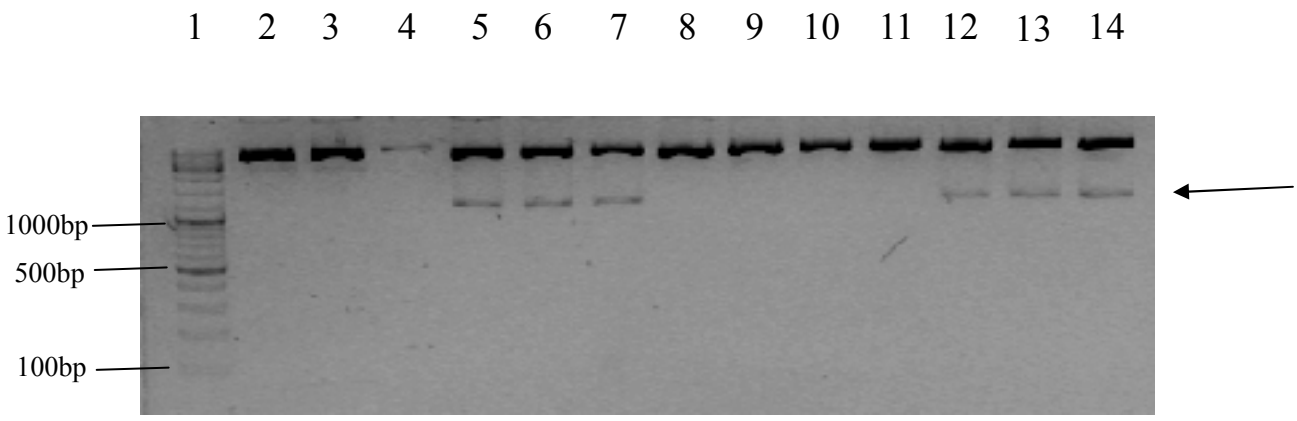


Figure 3.4 Screening positive clones for *GCHFR* promoter inserts. Plasmid DNA was isolated from 13 independent Amp resistant colonies and digested with relevant restriction enzymes. Lane 1, GeneRuler^{TM} 100 bp plus DNA ladder (Fermentas); Lane 5, 6, 7, 12, 13 and 14 show positive clones that contain the *GCHFR* promoter insert (arrowed).

3.2.2 Optimization of transient transfection

Three transfection reagents were used to optimise transient transfection of constructs into the two mammalian cell lines used (COS-7 and RN46A). These reagents were Effectene (Qiagen), Lipofectamine™ LTX and Plus reagent (Invitrogen), and Fugene® HD transfection reagent (Roche). ß-galactosidase expressing plasmid vector p610ZA was used for monitoring transfection efficiencies of both COS-7 and RN46A cells. Since ß-galactosidase is a reporter enzyme that produces a blue colour when reacting with its substrate X-gal, the proportion of blue cells measured by haemocytometer was used to compare transfection efficiencies after transient transfections (section 2.1.2.6). Table 3.2 illustrates the results of transfection optimisation. Fugene® HD transfection reagent gave the highest transfection efficiency in COS-7 cells (82%) while Effectene was most effective at transfecting RN46A cells (78%). For simplicity I chose to use one transfection reagent, and since the key target cells in my experiments were RN46A, Effectene, which is a reagent that suits them better, was selected for all luciferase reporter gene assays.

Table 3.2 Optimization of transfection conditions. Transfection efficiency was measured histochemically following transient transfection with p610ZA, and is expressed as the proportion of blue cells observed amongst all cells. These results are the mean of two independent experiments.

	COS-7	RN46A
Effectene	57%	78%
Fugene	82%	53%
Lipofectamine	55%	43%

3.2.3 Basal activity screening

Each promoter construct was tested for basal activity in COS-7 and RN46A cells respectively prior to drug exposure experiments (Table 3.3). The COS-7 cells were used in addition to RN46A cells because they are relatively easy to grow and transfect, and are able to express a wide range of transfected mammalian genes. They also serve as a way to detect the expression status of transfected promoters in a cell line other than

RN46A cells. This can be useful, for example, to add confidence that the promoter construct is actually functional, even when not expressed in RN46A.

Table 3.3 Gene expression levels produced by the promoter region of candidate genes

Promoter	Insert Size (bp)	COS-7 Expression *	RN46A Expression *
ARC	1802	+	-
CREB1	1487	++	+
CRH	934	+	-
CRHR2	907	+	+
GRIK4	970	+	-
HTR1A	1474	+	-
PCLO	1401	+	-
PHB	1328	++	++
PVALB	1394	+++	+++
SLC6A4	1401	+	-
TPH2	1650	+	-
ADM	1460	+++	++++
GCH1	960	++	+
GCHFR	1420	++	++
PCBD	1485	++	++
PTS	545	++	+
QDPR	1062	++	-
SPR	1270	+	+

* With increasing levels of gene expression expressed as relative light unit (RLU).

– represents less than 1 RLU, + represents 1- 20 RLU, ++ represents 20- 50 RLU, +++ represents 50-100 RLU, ++++ represents 100- 200 RLU.

Equal concentrations of each reporter gene construct were transfected into COS-7 and RN46A cells as described previously. A negative control, which was empty pGL3-basic plasmid, was included in all the luciferase reporter assays. The pRL-TK vector, which contains the gene encoding *Renilla* luciferase, was used as an internal positive control, in combination with all candidate reporter constructs, to co-transfect either COS-7 or RN46A cells at a 20:1 molar ratio. The vector pRL-TK contains the herpes simplex virus thymidine kinase (HSV-TK) promoter, driving low to moderate levels of *Renilla* luciferase expression which can be measured by chemiluminescence (Lorenz et al. 1991). Gene expression results were measured as relative light units

(RLU), which was a ratio of firefly luciferase activity to *Renilla* luciferase activity. In this way, data were normalised for variabilities caused by differences in transfection efficiency between different samples of transfected cells (Matuszyk et al. 2002; Malo et al. 2003). Three transfections were conducted for each reporter construct, and a negative control was included for each experiment. This process was independently repeated twice for each construct.

All reporter constructs were successfully expressed in COS-7 cells (Table 3.3), which indicated that each putative promoter or regulatory region was capable of driving transcription. Two of the reporter constructs tested was highly expressed in COS-7: *PVALB* (50- 100 RLU) and *ADM* (50- 100 RLU). The remaining majority of promoter constructs generated moderate levels of gene expression, ranging from 20 to 50 RLU, such as *CREB1, PHB, GCH1, GCHFR, PCBD, PTS* and *QDPR*. Nine promoters demonstrated low levels of gene expression, with RLU only slightly higher than the background level (1- 20 RLU). These promoters were *ARC, CRH, CRHR2, GRIK4*, *HTR1A, PCLO, SLC6A4, TPH2* and *SPR*.

However, candidate promoter driven luciferase expression was different in this system when using the serotonergic cells RN46A. Several promoter constructs failed to show detectable gene expression. *ADM*, which showed the highest expression in COS-7, was similarly highly expressed in RN46A cells with RLU of 100 to 200. The expression level of *PVALB* in RN46A was slightly lower than in COS-7 (30- 50 RLU). *PHB*, *GCHFR* and *PCBD* were moderately expressed, as they were in COS-7 (20-50 RLU). In contrast, *CREB1*, *GCH1* and *PTS*, expressed poorly in the RN46A cells (1-10 RLU). Interestingly, eight constructs, which were expressed in COS-7, did not have detectable expression in RN46A with RLU of less than 1. They were *ARC*, *CRH*, *GRIK4*, *HTR1A*, *PCLO*, *SLC6A4*, *TPH* and *QDPR*. Further experiments concentrated solely on those genes for which promoter constructs were shown to express in RN46A cells.

3.2.4 SSRI drug exposure of RN46A cells

3.2.4.1 Paroxetine and fluoxetine exposure

To test the effects of SSRI antidepressants on gene regulation, RN46A cell line was chosen specifically because of its serotonergic nature, which makes it a highly relevant cell line to study antidepressant response. COS7 cells were not included for further studies due to their lack of relevance in antidepressant response studies. Cells were exposed to 0.5μM either paroxetine or fluoxetine for 14 days with a single passage on day 6 (Figure 3.5). 0.5μM was chosen because it is physiologically relevant (Kim et al. 2002; Zhu et al. 2002; Chen et al. 2003; Wood et al. 2005; McHugh et al. 2008b) and is routinely used in our lab (McHugh et al. 2008b). A 14-day exposure time period was selected because it was a balance between the longevity of healthy cultured cells and the minimum time required for a therapeutic response in the clinical setting (McHugh et al. 2008b). Forty-eight hours after transfection with plasmid-promoter constructs, cells were lysed and Dual-Luciferase^{TM} reporter assays were conducted to measure the changes in gene expression following drug treatment.

Initial results showed significant expression changes of two of the genes tested. As shown in Figure 3.6A, *SPR* was upregulated significantly by paroxetine, although the fold change was small (1.2-fold increase). In contrast, *PCBD* showed significant down-regulation when RN46A cells were exposed to paroxetine (a 0.27-fold decrease).

However, results were distinctively different when the same experiment was conducted on a second day (day 2, Figure 3.6B). There was a significant difference in expression of *SPR* gene, but it was in the opposite direction compared with the day 1 experiment (Figure 3.4A). Instead of increased gene expression, *SPR* was downregulated. The results of *PCBD* expression were inconsistent too. The basal expression of *PCBD* in the first experiment was approximately 12 RLU whereas it was about 4 RLU in the second

experiment. Moreover, no change in gene expression was observed in the second experiment whereas significant decrease in gene expression of *PCBD* was detected previously.

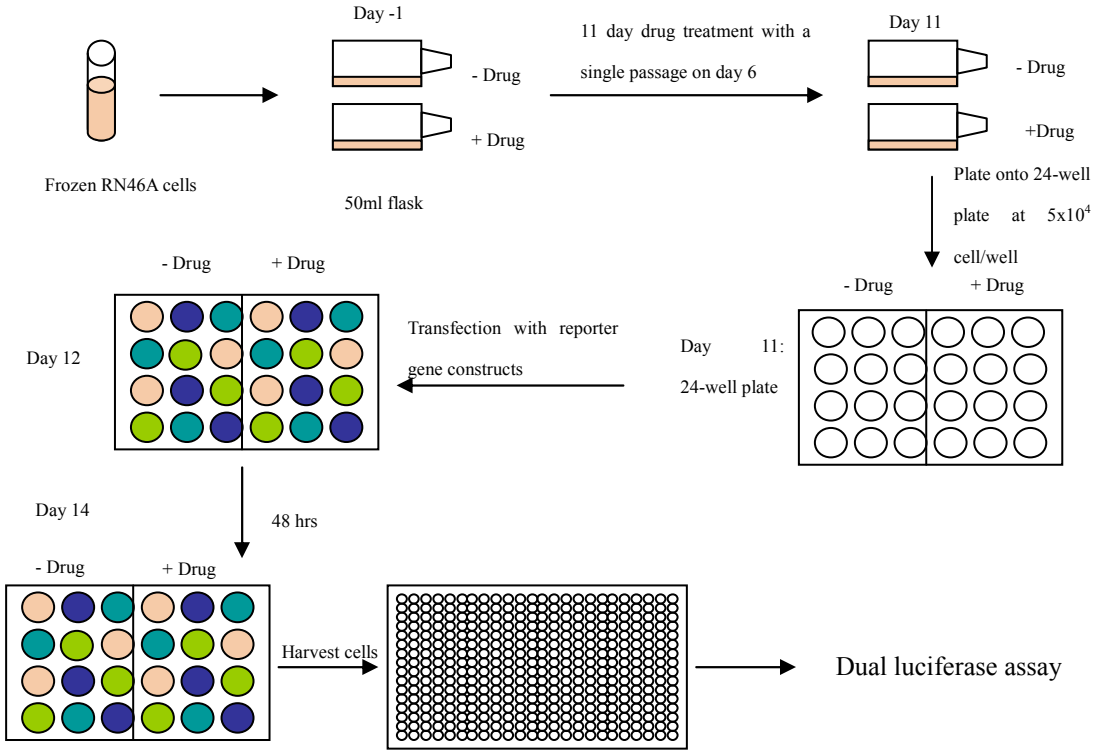


Figure 3.5 Outline of drug exposure experiments. RN46A cells were exposed to several drugs for a period of time ranging from 48 hours to 14 days prior to transfection. 48 hours after transfection with plasmid-promoter constructs, cells were lysed and the Dual-luciferase™ Reporter Assay System was conducted to measure the changes in gene expression following drug treatment. Replicates were randomly assigned to wells in order to minimise variations caused by the position of wells in which promoter constructs were added. Different colors in 24-well plates indicate different promoter constructs.

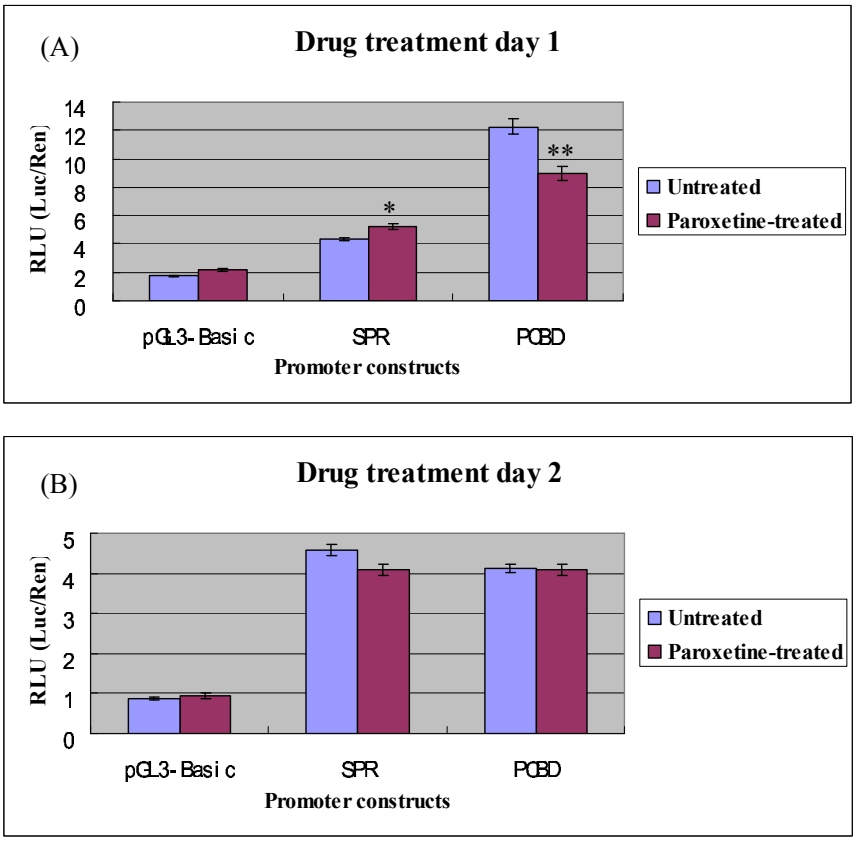


Figure 3.6 Initial drug treatment experiments. A) Day 1 and B) day 2 are luciferase reporter assay results obtained with identical experimental conditions, on two independent days one week apart. RN46A cells were exposed to 0.5µM paroxetine for 14 days. Results are expressed as the mean ± standard error (SEM) of three independent experiments, each with triplicates. *$p<0.05$, **$p<0.01$ indicate results differing from untreated control significantly (Tukey's t-test).

The same drug exposure experiments were repeated several times. Unfortunately, with extended experimentation I found that observed differences in expression of reporter constructs between treated and untreated RN46A cells were not robust or consistently reproducible, with significant day to day variability in outcomes despite considerable care in experimental design and execution.

To illustrate these difficulties, Figure 3.7 is compiled from a subset of experiments for ten genes. For each gene I have amalgamated the data from six independent experiments, each of which showed significant expression changes for paroxetine.

The observed fold-changes were subtle, ranging from 1.2 to 1.8 fold increases. Although this pattern is suggestive of consistent drug-induced gene expression changes, several replicates showed either no significant change, or significant change in the opposite direction, and these were not included in this analysis. In addition, for several genes, no consistent patterns were seen (not included in Figure 3.7). Despite the variability in these data, two genes, *GCHFR* and *ADM,* were selected for subsequent drug treatment experiments. *GCHFR* and *ADM* represent two apparently different types of promoter construct because *GCHFR* was significantly upregulated by both paroxetine and fluoxetine whereas *ADM* only responded to paroxetine. In addition, both of these genes were the subject of prior work in this laboratory.

3.2.4.2 Time-course experiment

Examining the effects of prior drug exposure (pre-conditioning) on gene expression may be an informative way of understanding the drug's mechanisms of action. A time course experiment was conducted to establish how the duration of paroxetine pre-conditioning in RN46A cells might affect reporter gene expression, and to optimize this assay for use as a model system with which to explore drug induced expression effects. To achieve this, RN46A cells were exposed to paroxetine for time periods ranging from 48 hours to 14 days prior to transfection with two representative reporter constructs, *ADM* and *GCHFR*. Results (Figure 3.8) showed that paroxetine pre-treatment led to an increased luciferase level for both constructs as early as 48 hours after initial treatment, which peaked at 72 hours. The drug's effect decreased over time until day 14, when it reached similar levels to that seen at 48 hours post treatment. Because luciferase activity peaked at 72 hours following drug exposure, this treatment time point was used for all subsequent experiments.

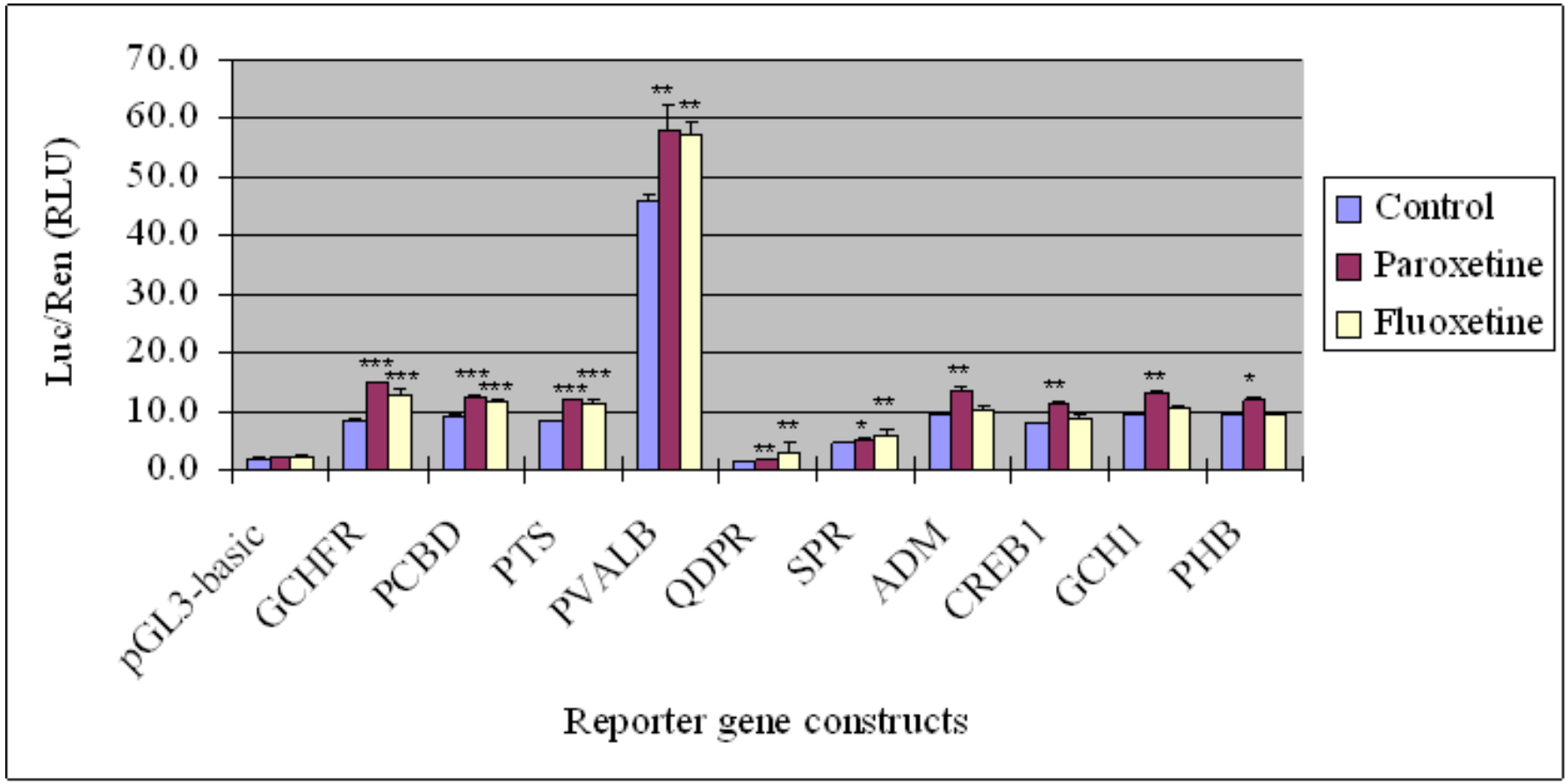


Figure 3.7 Gene expression levels of promoters following a 14 day 0.5μM paroxetine or fluoxetine treatment in RN46A cells. Levels of firefly luciferase activity are standardized to *Renilla* luciferase activity (relative light units). Promoter construct names are indicated on the X-axis. The negative control is the pGL3-basic plasmid with no promoter inserted. Transfections were randomized across the plate and results are expressed as the mean ± SEM of six independent experiments, each with triplicates. *p<0.05, **p<0.01, ***p<0.001 indicate results differing from untreated control significantly (Tukey's t-test).

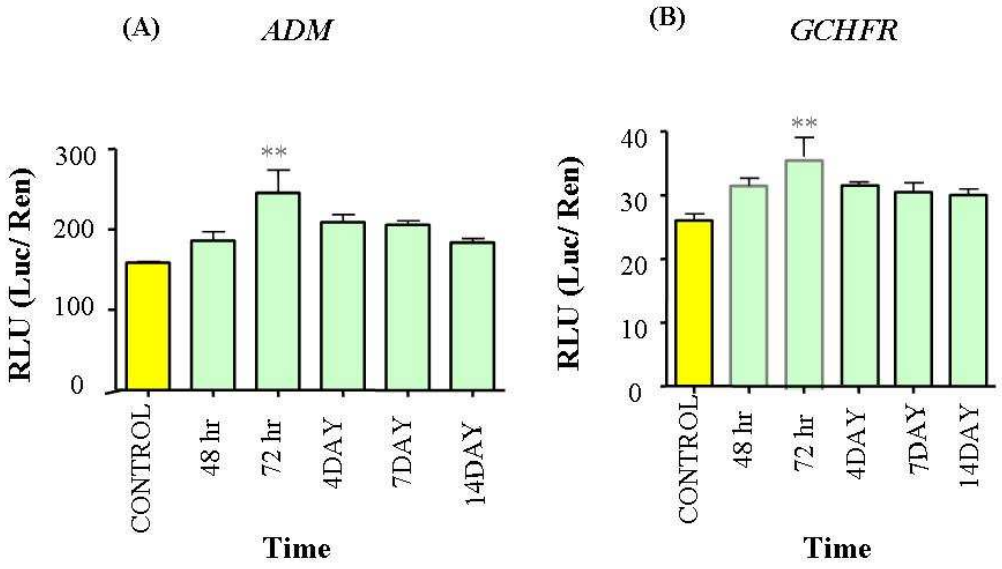


Figure 3.8 Time-course of gene activation in paroxetine-treated RN46A cells. Cells were exposed to 0.5µM paroxetine for 48 hours, 72 hours, 4, 7 and 14 days respectively. The expression of *ADM* and *GCHFR* was measured by the Dual-Luciferase™ Assay. The results represent the mean ± SEM values of six independent experiments, each performed in triplicate. **p<0.01 indicates results differing from the untreated control significantly (Tukey's t-test).

3.2.4.3 Dose-response experiment

Having established an optimal pre-treatment duration, experiments were directed at understanding the concentration-dependent effects of paroxetine on *ADM* and *GCHFR* promoter activities. RN46A cells were treated with concentrations of paroxetine from 0.05µM to 10µM for 72 hours prior to transfection. Expression levels from the construct containing the putative promoter of *ADM* showed increased expression at 0.05µM, with up-regulation peaking at 0.5µM and then decreasing with higher concentrations of paroxetine. However, none of these changes reached statistical significance (Figure 3.9).

Figure 3.9 displays a gradual increase in *GCHFR* gene expression with increasing concentrations of paroxetine which reached statistical significance at 10µM. However,

significant levels of cell death were visible at this concentration (Figure 3.10).

In summary, these dose-response experiments were not very informative. Paroxetine did not affect the expression of *ADM*. Neither did it change expression of *GCHFR* at physiologically relevant concentrations. Nevertheless, 0.5μM was selected as the concentration for future drug exposure experiments as it approximates a physiologically appropriate concentration that has been routinely used in previous studies (Kim et al. 2002; Zhu et al. 2002; Chen et al. 2003; Wood et al. 2005; McHugh et al. 2008b). There was a trend of increased expression for both *ADM* and *GCHFR* at this concentration, and unlike at some higher concentrations, it was not found to be cytotoxic.

3.2.4.4 Drug specificity experiment

Despite the variable nature and small size of putative expression changes observed, it was considered worth testing the effect of several drugs in the system. Drugs tested included paroxetine (a SSRI antidepressant), fluoxetine (a SSRI antidepressant), citalopram (a SSRI antidepressant), nortriptyline (a tricyclic antidepressant); sodium valproate (an anticonvulsant and mood stabilizer) and haloperidol (an antipsychotic drug) (Figure 3.11). Our results showed that pre-treatment of RN46A cells with paroxetine, haloperidol and sodium valproate for 72 hours prior to transfection of the luciferase constructs caused a slight increase in *ADM* expression whereas fluoxetine, citalopram and nortriptyline resulted in a slight decrease in *ADM* expression relative to the negative control. However, these results did not replicate previous findings, in that paroxetine upregulation of *ADM* did not reach statistical significance in this experiment. *GCHFR*, on the other hand, behaved differently. It showed increased gene expression following all the treatments, with the biggest change seen with sodium valproate exposure (Figure3.11).

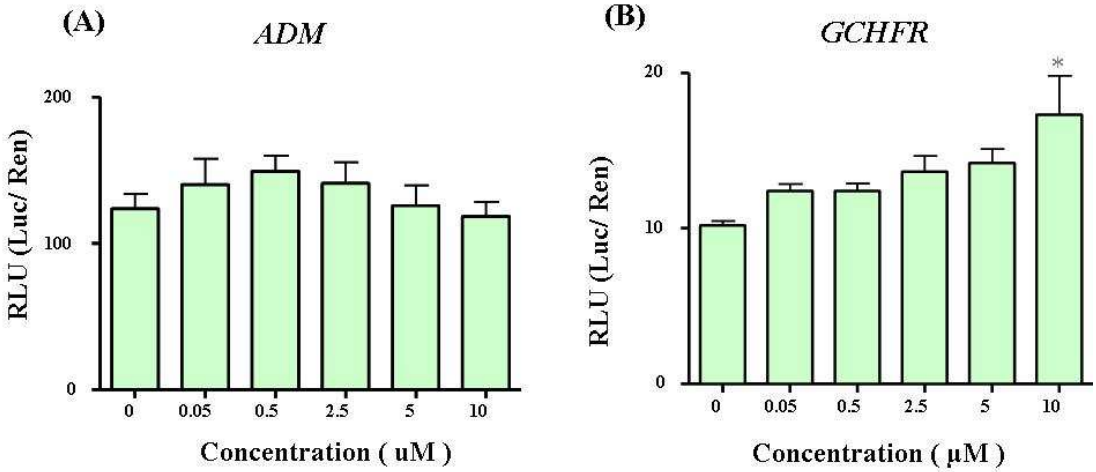


Figure 3.9 Dose-response of *ADM* and *GCHFR* by paroxetine exposure in RN46A cells. RN46A cells were treated with the indicated concentrations of paroxetine for 72 hours. Changes in expression of A) *ADM* and B) *GCHFR* were measured using the Dual--Luciferase™ Assay. The results represent the mean ± SEM values of six independent experiments, each performed in triplicate. *$p<0.05$ indicates results differing from the untreated control significantly (Tukey's t-test).

(A) **(B)**

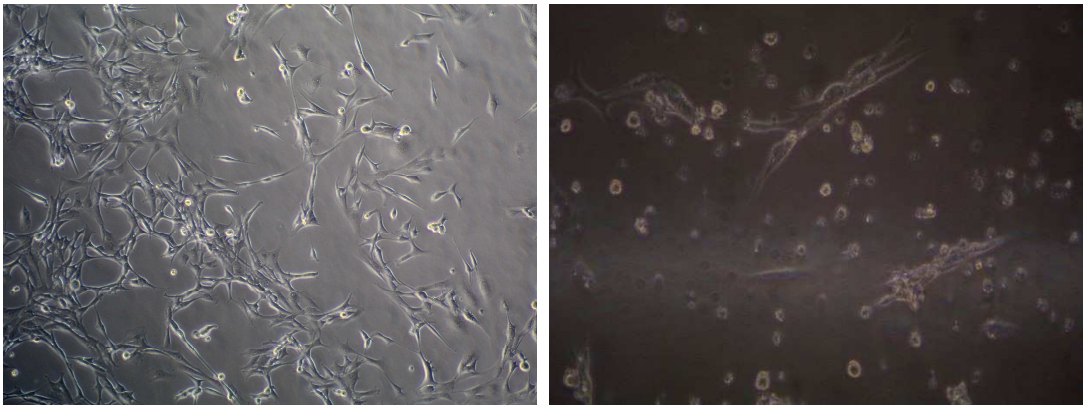


Figure 3.10 RN46 cells exposed to paroxetine for 72 hours at A) 0.5µM or B) 10µM. Cell death was apparent when cells were exposed to 10µM.

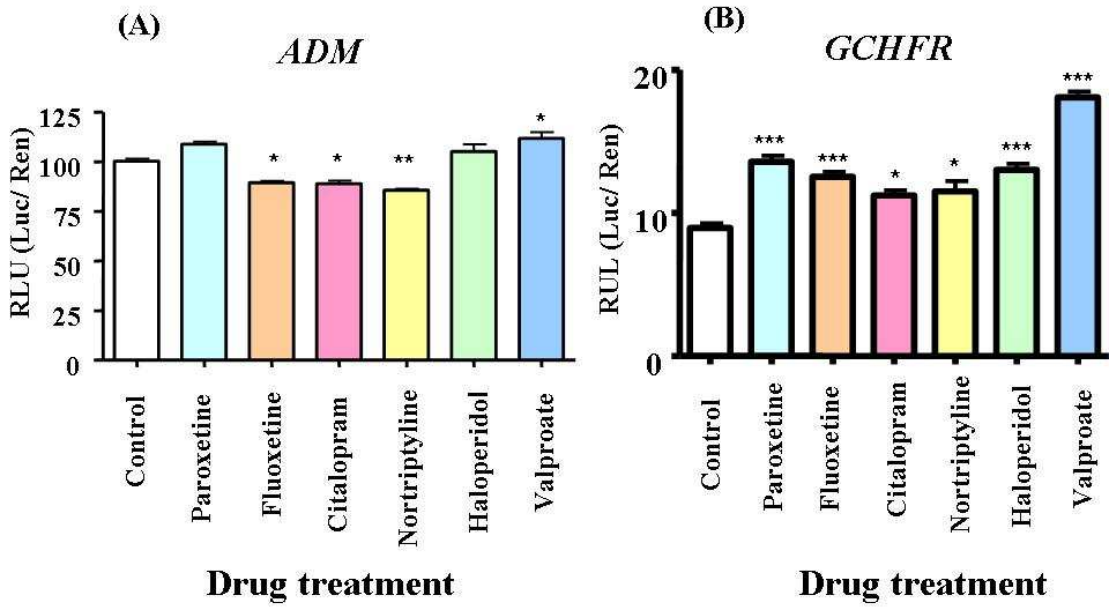


Figure 3.11 Effects of various drugs on gene expression in RN46A cells. RN46A cells were treated with 0.5μM paroxetine, fluoxetine, citalopram, nortriptyline, haloperidol or 1mM sodium valproate for 72 hours prior to transfection with luciferase constructs. Expression of a) *ADM* and b) *GCHFR* was measured using Dual-Luciferase™ Assay. The results represent the mean ± SEM values of three independent experiments, each performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 indicate results significantly different from untreated control (Tukey's t-test).

3.2.4.5 Inconsistent findings

Several subsequent experiments were conducted utilising findings from previous time-course and dose- response experiments, where RN46A cells were exposed to 0.5μM paroxetine for 72 hours. However, experimental results were often inconsistent, failing to replicate across experiments on different days (data not shown). *ADM* and *GCHFR* were shown to be significantly upregulated by paroxetine under optimised conditions only about 50% of the time. Furthermore, statistical significance was not reached in a proportion of experiments; so findings went from significant to insignificant from experiment to experiment. On some occasions, opposite findings were observed where *ADM* and *GCHFR* were downregulated by paroxetine, although not statistically significant. These inconsistencies led us to abandon this approach, and move on to a

more direct method of assessing gene regulation by drugs.

3.3 Discussion

3.3.1 Variation in basal activity between cell lines

Nineteen promoters were cloned into a luciferase reporter gene system and analysed for drug-induced gene expression changes. All of them were expressed in COS-7 cells whereas some did not show expression in RN46A cells. The majority of the reporter gene constructs showed moderate levels of gene expression ranging from approximately 20 to 50 RLU. However, *ADM* gene expression levels failed to reliably reach the basal expression level obtained previously in RN46A cells (Table 3.3).

The expression differences seen between cell lines may be attributed to two main factors. The first factor may be tissue and cell type specificity. Extensive evidence has suggested tissue specific expression between species under the same regulatory elements (Hochheimer and Tjian 2003; Steinhoff et al. 2009). RN46A cells are derived from rat brain and resemble the environment of raphe serotonergic neurons, and are quite different to the kidney fibroblast derived COS-7 cells, which may provide a partial explanation for the difference in gene expression levels I saw between the two cell lines.

Species-specific transcriptional control may be another factor that caused differential basal gene expression between cell lines. Comparisons of mouse and human embryonic stem cells factors provide the first evidence of large-scale divergence in mammalian transcription factor (TF) binding (Odom et al. 2007; Tesar et al. 2007; Wilson et al. 2008; Wilson and Odom 2009). These studies showed that most TF binding events in mammals are species-specific. In addition, TF binding divergence in yeast and mammals has been confirmed by direct genome-wide experimental evidence (Borneman et al. 2007). It has been shown that motif mutations for a specific TF could account for most losses of TF binding. Conserved motifs, however, did not guarantee TF binding because many shared TF binding events were lost from one of

three related yeast species examined, even though they share a conserved motif. Therefore, it is likely that transcription factor binding events in a primate-derived cell line such as COS-7 may be different from those in a rodent-derived cell line such as RN46A cells. Moreover, human promoters were used throughout my project, and this species difference may have differentially impacted on promoter activities in the different cell lines.

3.3.2 Time-course experiments

In the time-course experiment, paroxetine induced gene upregulation in both *ADM* and *GCHFR* rather acutely (48 hours). This upregulation peaked at 72 hours for both genes. The result of this experiment demonstrated that instead of exposing RN46A cells to paroxetine for 14 days as described in other studies (McHugh et al. 2008b; McHugh et al. 2009a; McHugh et al. 2009b; McHugh et al. 2010a), a period of 72 hours was determined to be an optimal treatment time required to observe the largest gene activation in our model system. It also provides an advantage over the longer 14 day drug treatment as the cells at 72 hours pre-conditioning are likely to be healthier than those exposed to culture conditions for the longer time. Prior work suggested a 14 day treatment was the maximal time that cells survive and remain healthy under tissue culturing conditions (McHugh et al. 2008b). Therefore, the expression changes I detected are likely to be more physiologically relevant to drug exposure and unaffected by cell survival or death mechanisms that may be activated when cells are under stress. In addition, paroxetine has been shown to be metabolized by cytochrome P4502D6 (Bloomer et al. 1992), which is an inducible enzyme responsible for the metabolism of several antidepressants (Crewe et al. 1992; Brosen 1993; Laine et al. 2001; Skinner et al. 2003). Prolonged exposure of the cells to paroxetine might lead to induced drug metabolism, which may provide explanation for the observed pattern of drug-induced gene expression changes. The initial increase in *ADM* and *GCHFR* expression induced by paroxetine may be offset by induced drug metabolism by P4502D6 over time, resulting in decreased concentration of drugs in the cell and reduced induction of genes.

3.3.3 Dose-response experiments

According to the results of dose response experiments, exposing RN46A cells to

0.5μM paroxetine seemed to be the optimal condition to induce changes in *ADM* and *GCHFR* gene expression. This concentration is also physiologically relevant because it is close to the steady-state brain concentrations of antidepressant drugs predicted from clinical studies (Kim et al. 2002; Zhu et al. 2002; Chen et al. 2003; Wood et al. 2005; McHugh et al. 2008b). Although *GCHFR* showed the largest increase in gene expression following 10μM paroxetine exposure, cell death was observed, which is an indication that cytotoxicity ocurs at higher concentration of paroxetine. Previous studies have also demonstrated cytotoxicity of paroxetine at higher concentrations. For example, in a study that examined the cytotoxic activity of paroxetine against tumor cells (Rosetti et al. 2006), paroxetine was found to inhibit the growth of a human lung cancer cell line completely at 5μM. Other primary murine cancer cells, such as ChaGo-K1, CAEP cells and MRFibS cells exhibited complete inhition at 10μM. Differential cytotoxicity was maximized at a concentration range from 5 to 10μM between cancer and non cancerous fibroblasts.

3.3.4 Drug specificity of RN46A cells

To determine whether paroxetine-induced *ADM* and *GCHFR* gene expression changes also occur with either other antidepressant drugs or non-antidepressant psychotropic drugs, several other drugs were tested in the reporter system. No drug specificity was detected for either gene in our model system. *ADM* was either up or down regulated depending on the drug treatment used. There was no apparent consistent pattern in gene expression changes. Change in expression of *ADM* brought about by paroxetine was not a phenomenon specific to SSRIs such as paroxetine and citalopram, nor was it specific to antidepressants. Expression of *GHCFR* was increased by treatment with various classes of antidepressants including serotonin and norepinephrine reuptake inhibitors, which have different acute actions. However, this effect does not seem to be pharmacologically specific to antidepressant drugs because non-antidepressant psychotropic drugs (haloperidol and sodium valproate) also caused significant increase in *GCHFR* gene expression. These results do not provide evidence to support the importance of *ADM* and *GCHFR* gene expression changes resulting from exposure to paroxetine, in the context of this transient luciferase assay system.

3.3.5 Issues with this model system

Although the luciferase reporter system has been commonly used in gene expression studies, it appeared to have limited value for detecting gene expression changes caused by antidepressants. The major limitation was inability to obtain consistent data. With extended experimentation we found that observed differences in expression of reporter constructs between treated and untreated RN46A cells were not robust or consistently reproducible, with significant day to day variability in outcomes despite considerable care in experimental design and execution involving multiple biological and technical replicates. One possible explanation for this inconsistency is that the luciferase reporter system is an artificial system which involves various manipulations and depends on several assumptions, not all of which may be correct. Firstly, we assumed that the promoter region that we cloned into each reporter construct contained an adequate amount of the *cis*-regulatory elements required for transcription, which may not be true. Secondly, we assumed that the internal control reporter plasmid used to normalise errors due to variable transfection efficiency is unaffected by any cellular and experimental factors. However, this assumption is not always true (Lowry et al. 1951; Ibrahim et al. 2000; Matuszyk et al. 2002; Mulholland et al. 2004; Vesuna et al. 2005; Malo et al. 2006). It has been shown that co-transfection with vectors expressing orphan nuclear receptors (the Nur77 family) enhanced the expression of the *Renilla* luciferase encoded by the internal control pRL-TK plasmid (Matuszyk et al. 2002). Another study by Ho and Strauss also reported increased *Renilla* luciferase gene expression of pRL-TK when co-transfected with plasmids expressing GATA-4 or GATA-6 transcription factor (Ho and Strauss 2004). Therefore, transfection efficiency normalisation using pRL-TK may lead to misinterpretations of the reporter gene activity of interest. Finding an internal control plasmid that shows a constant level of transcription regardless of experimental conditions used, may improve the consistency, accuracy and/or sensitivity of this system. A plasmid that does not contain a promoter, such as the promoter-less plasmid phRG-B (Malo et al. 2006), may be useful for this purpose. Thirdly, we assumed that the process of transfection does not affect gene expression. However, it is conceivable that chemically introducing promoter constructs into tissue culture cells by transfection may initiate gene expression changes due to the chemicals used to promote plasmid uptake by cells.

The other vital issue associated with our model system is its apparent lack of sensitivity in detecting gene expression changes. This may be partly due to antidepressants not tending to cause large changes in the expression of a particular gene (Ising et al. 2009; Horstmann et al. 2010). Instead, they exert their effects through the action of multiple genes working together to bring about their therapeutic effects (Ising et al. 2009; Horstmann et al. 2010). Together with the issues discussed above, it is possible that this model system is not capable of detecting subtle changes in gene expression consistently.

Thirdly, the RN46A cell line we chose to use is a rat cell line that closely resembles serotonergic neurons. Although we viewed this as a useful and relevant cell line in which to test the luciferase assay as a model system for exploring gene regulatory effects of antidepressants, it is possible that other types of neural cell lines may have given quite different results.

The inconsistent findings from these reporter gene experiments led us to seek another approach capable of providing the consistency and sensitivity required for our goal of developing a model in which to explore gene expression changes induced by antidepressant drugs., Among methods available for gene expression studies, qPCR was chosen as it allows a more direct interrogation of gene expression changes in response to drug treatments.

Chapter 4

Detection of antidepressant drug-induced gene expression changes by real-time quantitative PCR

4.1 Introduction

Following the inconsistent findings established in the reporter gene experiments, this part of my work focussed on a more direct approach to detect gene expression changes in cells following drug treatment. Over several years, real-time quantitative PCR (qPCR) has become a powerful tool for DNA and RNA quantification (Provenzano and Mocellin 2007; Romanowski et al. 2007; VanGuilder et al. 2008). It has been used extensively for gene expression analysis and is now considered the highest quality technique for the precise and accurate detection of transcripts. This is achieved due to its high sensitivity and dynamic range of detection, spanning six to eight orders of magnitude. Furthermore, it reduces the risk of introducing errors and contamination by combining amplification and detection in a single tube, thus obviating post-PCR manipulations (Provenzano and Mocellin 2007; Romanowski et al. 2007; VanGuilder et al. 2008).

Although qPCR is a robust and powerful technique, caution must be taken when interpreting the final quantification results because they can be influenced by several key elements in the workflow (Bustin et al. 2005; Demeke and Jenkins 2010). These elements include the quality of the input RNA and cDNA template, as the sequence integrity and absence of inhibitors can affet reaction viability; the PCR assay itself, such as specificity, efficiency and the limits of detection within each assay. Finally and

probably most importantly, normalisation approaches, such as the choice and validation of reference genes, with which to compare transcripts. All these factors need to be closely controlled in order to enhance the reliability of the analysis (Bustin et al. 2005; VanGuilder et al. 2008).

4.2 Experimental approach

Due to the nature of qPCR, which is carried out in 96 or 384 well plates, I was able to expand the number of drugs tested for gene expression changes. As in experiments conducted in Chapter 3, cultured, undifferentiated RN46A cells were exposed to paroxetine, as well as the drugs citalopram, nortriptyline, haloperidol all at 0.5µM, or 1mM sodium valproate, depending on the specific experiment. Total RNA was isolated using TRIZOL® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantification of RNA was analysed spectrophotometrically by NanoDrop analysis (Thermo Scientific). The RNA quality was visually assessed for degradation status on agarose gels (Figure 4.1). All RNAs contained two discrete bands representing the 28S and 18S RNAs, indicating good and comparable RNA quality across all the samples with a concentration between 754 ng/µl and 1295 ng/µl (Figure 4.1). Two-step RT-PCR was conducted using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA). A total of 36 cDNA samples were synthesized using RNA from cells treated with the five drugs and a vehicle control, with six biological replicates for each group. The resulting cDNA was stored in frozen aliquots for future qPCR experiments.

Relative quantification (Pabinger et al. 2009; Sirakov et al. 2009; Jarosova and Kundu 2010; Pernot et al. 2010; Regier and Frey 2010), rather than absolute quantification

Figure 4.1 RNA extracted from drug-treated and control RN46A cells. Undifferentiated RN46A cells were exposed to paroxetine, as well as the drugs citalopram, nortriptyline, haloperidol all at 0.5μM, or 1mM sodium valproate for 72 hours. Total RNA was isolated using TRIZOL® reagent. The RNA quality was visually assessed for degradation status on agarose gels. Lanes 1 to 11 represents independent samples.

was chosen as the analysis technique, because it enables the comparison of gene expression levels of a target gene under at least two conditions (for example, treated or untreated cells), which is the major aim of this gene expression study. The Roche Universal ProbeLibrary System (UPL, Roche) was employed for relative quantification due to its flexibility, availability, convenience and specificity of hydrolysis probes (Wenzel et al. 2009). Since rat RNA was isolated from the rat serotonergic cell line RN46A, the UPL system specific for rat genome was used. The UPL system consists of 165 pre-validated short real-time PCR probes, which are 5' labeled with fluorescein (FAM) and have a dark quencher dye at the 3′ end. The 165 UPL probes are 8-9 nucleotides in length, with probe sequences selected to cover the most frequently occurring 8-9 mers across the entire rat transcriptome, ensuring extensive transcript coverage. Although each probe is capable of binding to approximately 7000 transcripts with a specific transcriptome, only one particular transcript is detected at a time in a given PCR assay. This specificity is achieved by selecting gene- specific primers which can be developed using software to match any one probe to a target gene sequence of choice (Mauritz 2005; Mouritzen 2005; Rein et al. 2006).

The web-based software, ProbeFinder (available at the Assay Design Center, Roche) was used for this study to generate candidate gene primer pairs (Mouritzen 2005). The software designs qPCR assays by combining a matching UPL probe with a set of PCR primer pairs specific for a given target of interest. ProbeFinder software is based on the software package Primer3, and all primer pairs designed are checked by an *in silico* PCR algorithm in order to minimise the risk of false positive assays from genomic DNA, unrelated transcripts formed by splice variants or gene family members with homology to the target gene. The *in silico* algorithm is also capable of avoiding assays that detect pseudogenes. Furthermore, qPCR assays were designed to make the amplicon span an intron in order to eliminate false positive signals from contaminating genomic DNA (Mauritz 2005; Mouritzen 2005; Rein et al. 2006; Christoph Leucht and Bally-Culf 2007).

Expression differences of 39 candidate genes, which were deemed relevant to the treatment of mood disorders including the previously investigated genes of the luciferase reporter assays (Table 2.3), were determined by qPCR on the LightCycler® 480 system. Candidate genes were drawn either from the existing literature (Knable et al. 2004; McMahon et al. 2006; Drago et al. 2009; Peters et al. 2009), or from prior experimental work in our laboratory (McHugh et al. 2009a; McHugh et al. 2009b). Reactions were set up in 384-well plates with the aid of a JANUS® Automated Workstation. This automated liquid handling system allowed precise, faster and high-throughput processing of samples. Nine cDNA samples plus the common calibrator cDNA, which was a common cDNA sample used to normalise inter-run variation, and a negative control, were analyzed on one plate. To complete the measurement of 36 cDNA samples for each of the 39 target genes in this study, twenty-four 384-well plates were required in total.

4.3 Results

4.3.1 Reference gene assays

Data normalisation using one or several reference genes is a key aspect of relative quantification of mRNA (Pfaffl 2001; Tricarico et al. 2002; Vandesompele et al. 2002; Bustin et al. 2005). Since it is difficult to find a gene that expresses uniformly across five drug treatments throughout the entire 36 cDNA samples, several reference genes were measured and the most suitable ones were used for later analysis. In this study, five possible reference genes were tested, with the aim that three genes could be identified and used for data normalisation. Two of them (*Actb* and *G6pd*) are routinely used by other groups as housekeeping genes in real-time PCR studies (Kwon et al. 2009; Passmore et al. 2009; Sirakov et al. 2009). The other three genes (*Dazap1*, *Olfml2b* and *Rnf4*) showed minimum changes in microarray analysis in previous studies from our laboratory, where RN46A cells were exposed to paroxetine for 36 hours (Glubb et al. 2010).

Testing of the five candidate reference genes was conducted by qPCR on a LightCycler® 480 (Roche) and analysed by LightCycler® 480 software 1.5 (Roche). Cells were exposed to the drugs paroxetine, citalopram, nortriptyline, sodium valproate and haloperidol for 72 hours. This experiment was repeated independently six times, each with three replicates. The results were exported into NormFinder software (Andersen et al. 2004), where a stability value was generated for each gene. The lower the stability value, the less variable the gene expression is upon different drug treatments. Although *Dazap1* had a lower stability value than *G6pd*, it proved difficult to amplify in this system due to its low expression level. The transcript *G6pd*, on the

other hand, was highly and stably expressed with a stability value similar to that of *Dazap1*. Therefore, *Actb*, *G6pd* and *Rnf4* were chosen as the three reference genes for subsequent experiments to normalise samples for differences in quality and quantity under different drug exposure experiments (Table 4.1).

Table 4.1 Assessment of putative reference gene expression stabilities for five drug exposure experiments. RN46A cells were exposed to paroxetine, as well as the drugs citalopram, nortriptyline, haloperidol all at 0.5μM, or 1mM sodium valproate for 72 hours. Gene expression levels of five candidate reference genes *Actb, Rnf4, Dazap1, G6pd* and *Olfml2b* were tested in NormFinder.

Stability rank	Gene Name	Stability Value
1	*Actb*	0.269
2	*Rnf4*	0.272
3	*Dazap1*	0.345
4	*G6pd*	0.368
5	*Olfml2b*	0.534

4.3.2 Relative quantification of gene expression changes using qPCR

Relative expression values of each target gene were calculated by determining the ratio between the target and the three reference genes. These values were normalised against a common calibrator cDNA using LightCycler® 480 Software 1.5. Inter-run variability was corrected using LightCycler® 480 Multiple Plate Analysis Software.

Among the 39 candidate genes examined in the qPCR system, 23 failed to express in this system with the software-selected probes and primers (Table 4.2). Among the 16 target genes that were successfully expressed, seven showed significant changes upon exposure of RN46A cells to various drugs (Table 4.3). The most noteworthy gene expression change observed was with a gene called sepiapterin reductase (*Spr*), which

was significantly up-regulated by sodium valproate (1755 ± 413). Also upregulated by sodium valproate was histone deacetylase 6 (*Hdac6*), but to a much lesser extent (6.03 ± 1.40). Three other transcripts *Hdac1*, *Hdac3* and *Qdpr,* showed enhanced expression in response to sodium valproate by 1.9, 1.7 and 1.4 fold respectively. The *Hdac1* transcript was also up-regulated with exposure to paroxetine (3.26 ± 0.73). The serotonin receptor 2a (*Htr2a*) was down-regulated upon exposure to the two SSRIs paroxetine (0.57 ± 0.06) and citalopram (0.71 ± 0.07). Another histone deacetylase, this time *Hdac2,* was found to be down-regulated two fold upon exposure to all the drugs tested apart from sodium valproate. However, only expression in paroxetine and citalopram treated cells reached statistical significance.

Table 4.2 Candidate genes examined in qPCR. RN46A cells were exposed to paroxetine, as well as the drugs citalopram, nortriptyline, haloperidol all at 0.5µM, or 1mM sodium valproate for 72 hours. Expression levels of candidate genes were tested in qPCR. This table shows the mean Cp values of candidate genes in untreated RN46A cells.

Gene name	Mean Cp value[1]	Gene name	Mean Cp value
Adm	~ 19	*Cacna1a*	N/E
Bdnf	~ 30	*Comt*	N/E
Creb1	~ 19	*Crhr2*	N/E
Fkbp5	~ 28	*Grik4*	N/E
Gap43	~ 30	*Hdac4*	N/E
Gata2	~ 28	*Htr1a*	N/E
Gch1	~ 25	*Htr2b*	N/E
Gchfr	~ 29	*Htr2c*	N/E
Hdac1	~ 24	*Lmx1b*	N/E
Hdac3	~ 26	*Maoa*	N/E
Hdac6	~ 28	*Ntrk2*	N/E
Hdac7	~ 29	*Pts*	N/E
Htr2a	~ 27	*Pvalb*	N/E
Phb	~ 19	*Pclo*	N/E
Qdpr	~ 27	*P2rx7*	N/E
Spr	~ 27	*Slc6a2*	N/E
Ank3	N/E[2]	*Slc6a4*	N/E
Arc	N/E	*Th*	N/E
Ascl1	N/E	*Tph2*	N/E
Bcl2	N/E		

[1] Cp is the cycle at which the fluorescence of a sample increases above the background fluorescence.

[2] N/E: no amplification detected after 45 cycles of amplification.

Table 4.3 Drug - induced gene expression changes detected by qPCR. RN46A cells were exposed to paroxetine, as well as the drugs citalopram, nortriptyline, haloperidol all at 0.5μM, or 1mM sodium valproate for 72 hours. Expression levels of candidate genes were tested in drug-treated and untreated cells in qPCR.

Gene	Treatment	Relative expression value (Mean±SEM[1])*	p-value (df^2=5)
Spr	Sodium valproate	1755±413	0.013
Htr2a	Paroxetine	0.57±0.06	0.001
Htr2a	Citalopram	0.71±0.06	0.007
Hdac1	Paroxetine	3.26±0.73	0.036
Hdac1	Sodium valproate	1.89±0.19	0.006
Hdac2	Paroxetine	0.58±0.14	0.034
Hdac2	Citalopram	0.51±0.13	0.012
Hdac3	Sodium valproate	1.71±0.20	0.017
Hdac6	Sodium valproate	6.03±1.40	0.016
Qdpr	Sodium valproate	1.40±0.08	0.007

* Relative expression values of each target transcript are expressed as a ratio between the target gene and reference genes, which is further normalised to that of a calibrator. The one-sample t-test was used for statistical analysis. [1] SEM.: standard error of mean. [2] df: degree of freedom

4.4 Discussion

4.4.1 Evaluation of reference genes for data normalisation

Real-time quantitative PCR (qPCR) is considered the highest standard for gene expression analysis due to its high sensitivity, wide dynamic range of quantification and decreased risk of contamination compared with traditional end-point RT-PCR (Gurvich and Klein 2002; Wilhelm and Pingoud 2003). Data normalisation using one or more reference genes to account for differences in quantity and quality between samples is a key aspect of relative quantification of mRNA (Pfaffl 2001; Tricarico et al. 2002; Bustin et al. 2005). Since it is difficult to find a gene that is expressed at a stable level regardless of the experimental conditions under investigation (Tricarico et al. 2002; Dheda et al. 2004; Radonic et al. 2004; Jain et al. 2006), normalisation using the geometric means of more than one reference gene is believed to be an alternative approach to generate reliable and biologically relevant results (Vandesompele et al.

2002; Kirchheiner et al. 2004; Hellemans et al. 2007). One of the aims of this study was to experimentally select three reference genes for normalising qPCR data generated from drug-treated RN46A cells. In order to achieve this, qPCR was conducted on cDNA derived from RN46A cells treated with five drugs to measure the expression of five possible reference genes, carefully selected from either the literature or previous microarray studies in our laboratory. When NormFinder was used to assess the stability of the five reference genes, *Olfml2b*, which had previously not shown significant gene expression changes in a microarray study using RN46A cells exposed to paroxetine (Glubb et al. 2010), was found to have the least stable expression under the five treatments across all samples. However, *Actb* and *G6pd*, which were shown to vary significantly in different tissue types or under different experimental conditions by other groups (MacFarlane et al. 2005; Jorgensen et al. 2006), did not show significant changes in gene expression in this study. Our findings highlight the importance of evaluating the stability of chosen reference genes for qPCR normalisation in specific experimental conditions.

Ideally, the expression of reference genes should not be influenced by the experimental conditions under investigation (Schmittgen and Zakrajsek 2000). However, it seems unrealistic to identify a gene that is stably expressed in all cell types under different experimental conditions (Radonic et al. 2004). This study successfully identified three reference genes that are at least minimally regulated under exposure of RN46A cells to various drugs, and allowed the normalisation of data for differences in quality and quantity of cDNA in individual samples, and thus ensured the relative accuracy of gene expression analysis results presented in this study.

4.4.2 Gene expression changes detected by qPCR

4.4.2.1 Gene expression pattern analysis

A total of 39 candidate genes were examined in the qPCR system. Of these, 23 genes failed to exhibit detectable signals (Table 4.2). There are several possible explanations for these failures. One obvious reason was that the candidate transcript of interest was not expressed, or poorly expressed, in cultured RN46A cells under our experimental

conditions. Poor PCR primers or probes may be another reason for lack of detection. However, this is considered unlikely because each pair of primers was tested in various PCR conditions in order to optimise primer performance. For those that did not amplify after optimisation, a second pair of primers was tested. All genes failing to show amplification with an initial primer pair failed to detect a transcript with the second pair of primers.

In order to better understand the reasons for failure to amplify so many of the candidate genes, I examined a microarray gene expression dataset previously generated in our laboratory on differentiated, paroxetine exposed as well as untreated RN46A cells, and submitted to the AffyExpress database (McHugh et al. 2009a). Comparison between the qPCR data of untreated RN46A cells presented here (Table 4.2) and the AffyExpress array data of untreated RN46A cells (McHugh et al. 2009a) (Table 4.4) was revealing. The data showed great consistency given that they utilised two different methods (qPCR versus microarrays) by two different operators, under somewhat different conditions (undifferentiated vs. differentiated cells). Six of the six highest expressing genes in the AffyExpress arrays were expressed in this qPCR system (*Gchfr, Phb, Hdac1, Qdpr, Hdac3* and *Hdac6*). Fifteen of the genes which failed to show detectable gene expression in qPCR were found to be among the lowest expressing genes in the array dataset. They were *Ank3, Ascl1, Bcl2, Cacna1a, Crhr2, Grik4, Htr1a, Htr2b, Htr2c, Ntrk2, Pvalb, Pclo, P2rx7, Slc6a2* and *Slc6a4*. Two genes undetected in qPCR (*Lmx1b* and *Tph2*) also failed to give detectable signals in the microarray study of Glubb (2009). However, there were also some inconsistencies between the two systems. Six genes (*Maoa, Arc, Comt, Th, Pts,* and *Hdac4)* undetected in my qPCR system would have been predicted to give expression results based on the array system. This may indicate that the failures in qPCR were not solely because these genes were not expressed. Other factors such as primer and/or probe failure, alternative transcripts, or reaction failure may contribute to obtaining detectable signals in qPCR.

Table 4.4 Comparison of candidate gene expression levels measured in qPCR with expression levels detected in microarray and dorsal raphe nuclei.

Gene name	Mean Cp value	Expression in microarray[1]	Expression level in dorsal raphe nuclei [2]
Adm	~ 19	487	N/E[4]
Bdnf	~ 30	319	~ 180
Creb1	~ 19	401	N/E
Fkbp5	~ 28	1337	N/E
Gap43	~ 30	172	~ 230
Gata2	~ 28	409	~ 185
Gch1	~ 25	817	~ 215
Gchfr	~ 29	8403	~ 195
Hdac1	~ 24	3430	N/E
Hdac3	~ 26	2139	~ 195
Hdac6	~ 28	1399	~ 190
Hdac7	~ 29	624	~ 180
Htr2a	~ 27	375	~ 175
Phb	~ 19	5327	~ 200
Qdpr	~ 27	2493	~ 195
Spr	~ 27	834	~ 170
Not expressed (N/E)			
Ank3	N/E	261	~ 190
Arc	N/E	497	N/E
Ascl1	N/E	206	~ 200
Bcl2	N/E	298	N/E
Cacna1a	N/E	256	~ 200
Comt	N/E	444	N/E
Crhr2	N/E	285	~ 185
Grik4	N/E	324	N/E
Hdac4	N/E	425	N/E
Htr1a	N/E	266	~ 195
Htr2b	N/E	173	N/E
Htr2c	N/E	292	N/E
Lmx1b	N/E	N/E	~ 170
Maoa	N/E	1817	~ 165
Ntrk2	N/E	209	N/E

Pts	N/E	348	N/E
Pvalb	N/E	222	~ 195
Pclo	N/E	175	N/E
P2rx7	N/E	274	N/E
Slc6a2	N/E	235	~ 146
Slc6a4	N/E	270	~ 210
Th	N/E	382	~ 205
Tph2	N/E	N/E	~ 210

[1] Microarray data generated when differentiated RN46A cells were exposed to vehicle for 36 hours. The microarray data were previously generated in this laboratory by Dr Dylan Glubb, and submitted to the European Bioinformatics Institute (www.ebi.ac.uk) and loaded in to ArrayExpress (Experiment name: GSFL-RN46A-acute paroxetine exposure; ArrayExpress accession: E-MEXP-1582).

[2] Mouse brain ISH data according to Allen Brain Atlas (www.brain-map.org)

A Welch two sample t-test (Welch 1947) was conducted to look at the differences in expression levels between genes with a zero and non-zero qPCR Cp value (Figure 4.2). There was strong evidence ($p < 0.0002$) that genes detected by qPCR had nearly four times higher, on average, microarray expression levels than genes that were not detected by qPCR (95% CI 1.9 times, 6.9 times). This suggests that a low expression level of RN46A cell genes was a large factor influencing the non-amplification of target transcripts in these qPCR experiments.

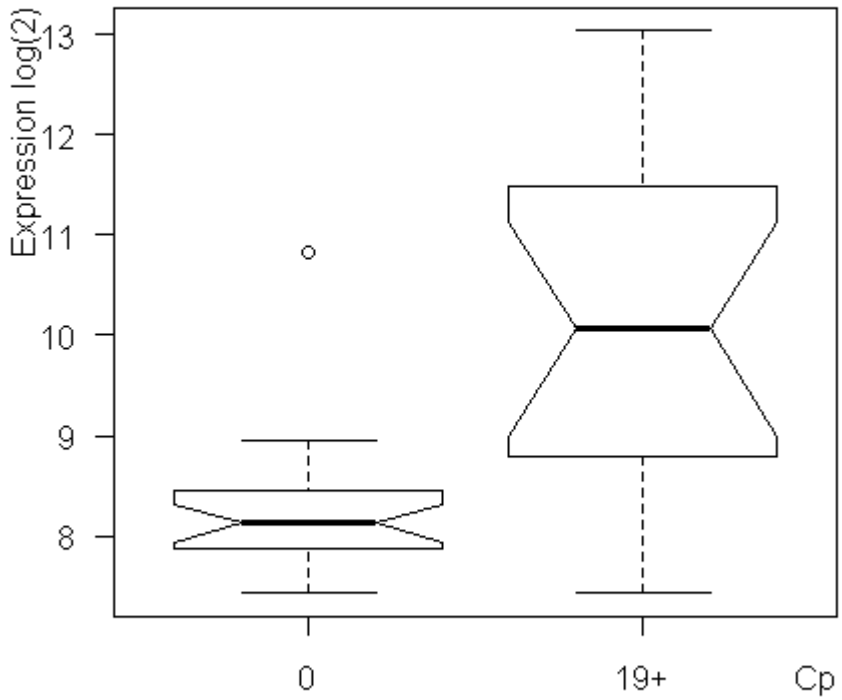


Figure 4.2 Comparison of qPCR results with microarray data. A Welch two sample t-test was conducted to test that qPCR Cp values and microarray expression are positively correlated. Note that this estimate is based only on those genes which were tested and does not reflect the distribution of all genes on the array. Cp value of 19 was chosen because it was the lowest Cp value obtained in qPCR experiments. The circle symbol is an outlier.

Since RN46A cells are derived from the rat dorsal raphe nuclei, a useful question would be how closely does gene expression in this cell line recapitulate endogenous gene expression in the rodent dorsal raphe tissue? To answer this queston I compared my qPCR data with mouse brain *in situ* hybridisation (ISH) data from the Allen Brain Atlas

(ABA) (Table 4.4). This atlas is an ISH image database of mouse gene expression which correlates anatomic structure with gene expression patterns in mouse brain. According to the mouse ABA (equivalent data are not available for the rat), five of the expressed genes in my qPCR system are not detected in the mouse dorsal raphe nuclei (*Adm, Creb1, Fkbp5, Hdac1* and *Hdac2*). One possible reason is that qPCR is a more sensitive technique than ISH (Biedermann et al. 2004). Eleven of the non-expressing genes in qPCR (*Ank3, Ascl1, Cacna1a, Crhr2, Htr1a, Lmx1b, Maoa, Pvalb, Slc6a2, Slc6a4, Th,* and *Tph2*) showed various levels of gene expression in mouse dorsal raphe nuclei of the ABA. Primer failure or PCR reaction failure are possible factors that caused non-amplification of these genes in qPCR. There was no obvious correlation between gene expression levels measured by qPCR and ISH data in mouse ABA. Discrepancies in gene expression patterns can be partly explained by differences in experimental techniques. Another difference to note is the different species examined between the two methods. RN46A cells are derived from rat whereas the ISH data are generated in mouse tissue. Taken together, these data suggest that the endogenous patterns of gene expression seen in RN46A cells, at least for the genes under study here, do not strongly correlate with endogenous expression in the dorsal raphe nucleus.

4.4.2.2 Up-regulation of *Spr* by sodium valproate

The most striking of all the findings in this analysis was that *Spr*, which encodes sepiapterin reductase, is strongly and specifically upregulated when RN46A cells were exposed to sodium valproate. Sepiapterin reductase is a homodimer protein that catalyses the biosynthesis of tetrahydrobiopterin (BH_4), (Smith 1987; Levine et al. 1990; Katoh et al. 1992; Katoh et al. 1994). It acts as an essential cofactor for several rate-limiting enzymes in the biosynthesis of neurotransmitters such as dopamine, noradrenaline, adrenaline, and serotonin (Nagatsu and Ichinose 1999; Matsumoto et al. 2008) (Figure 4.2).

Although the possible involvement of SPR and the BH_4 pathway in the aetiology of neuropsychiatric disorders, such as major depression, schizophrenia and bipolar disorder, is not well recognised in psychiatry, it has been previously implicated (Bonafe et al. 2001; Steinberger et al. 2004; McHugh et al. 2009a; McHugh et al. 2009b). Since

catecholamines and indolamine neurotransmitters play an important role in the pathophysiology of several neuropsychiatric disorders, it is reasonable to postulate that SPR may influence tyrosine and tryptophan hydroxylase activity indirectly through its effect on BH_4 synthesis, which in turn affects the levels of catecholamines and serotonin. In fact, it has been shown that mutations in the human *SPR* gene result in a serious neurotransmitter deficiency, causing depression, among other symptoms (Bonafe et al. 2001; Steinberger et al. 2004). In addition, increased expression or modification of *Spr* was observed when embryonic stem cell-derived neural cells were exposed to paroxetine chronically (McHugh et al. 2009a), supporting the hypothesis that *Spr* is a plausible candidate for normal mood control and drug action. This finding is further supported by a follow-up study, where the association of *SPR* promoter polymorphism with antidepressant response was examined in a family study of mood disorders (McHugh et al. 2009b). A statistically significant association of a particular pair of *SPR* haplotypes with bipolar I disorder was found, indicating *SPR* polymorphisms may influence susceptibility to bipolar disorder through a biological process that involves BH_4-mediated neurotransmitter production.

More indirect evidence supporting the involvement of SPR in mood dysregulation and control comes from various studies on BH_4 pathway and mood disorders. Treatment of depressed patients with BH_4 resulted in an obvious improvement in mood (Curtius et al. 1983). It was also reported that BH_4 deficiency may be linked to clinical symptoms of depression by several studies (Coppen et al. 1989; Hashimoto et al. 1990a; Hashimoto et al. 1990b; Bottiglieri et al. 1992; Hashimoto et al. 1994; Abou-Saleh et al. 1995; Blair et al. 2006). Moreover, Miura *et al.,* (2004) provided additional evidence for the role of SPR in mechanisms of drug actions used to treat mood disorders. In rodent models of depression, BH_4 levels in the mouse hippocampus was found to be regulated by the SSRI fluvoxamine, and the action of BH_4 levels was suggested to bring about the behavioual changes seen in the rodents (Miura et al. 2004; Miura et al. 2005). Last but not least, another BH_4 pathway protein GTP cyclohydrolase I feedback regulator (GFRP), which is the rate-limiting enzyme in BH_4 biosynthesis, was shown to influence antidepressant response in patients with major depression, providing evidence for the pivotal role of this pathway in mood control (McHugh et al. 2010a). Taken together, compelling evidence supports the involvement of BH_4 level in mood control, making

our observation of increased *Spr* gene expression after exposure of RN46A cells to the mood stabiliser sodium valproate very interesting.

4.4.2.3 Up-regulation of *Hdac6* by sodium valproate

Another intriguing finding of this part of the project was that *Hdac6*, which encodes the enzyme histone deacetylase 6, was significantly up-regulated (more than 6 fold) by sodium valproate. It is the second largest gene expression change observed in our experiments.

Histone deacetylases (HDACs) are a class of enzyme that catalyse the removal of acetyl groups from lysines on a histone, whose action is opposite to that of histone acetyltransferase (HAT), (de Ruijter et al. 2003). Since some aspects of gene expression are controlled by the coiling and uncoiling of DNA around histones, HAT contributes to this process by acetylating the lysine residue in core histones. Acetylation neutralizes the positively charged amine groups on histone tails by changing amines into amides, which impairs the ability of histones to bind to negatively charged phosphate groups on the DNA backbone. As a result, gene transcription is activated due to a less compact and more transcriptionally active chromatin. HDACs, on the other hand, repress transcription by removing acetyl groups, which leads to increased positive charges on histone tails and hence more condensed and transcriptionally silenced chromatin (Yang and Seto 2007). Classified as a Class II HDAC, HDAC6 is a cytoplasmic enzyme that is involved in many vital biological processes, including cell migration and cell morphology regulation (Valenzuela-Fernandez et al. 2008). The various functions of HDAC6 indicate that it may be a potential therapeutic target for the treatment of a variety of diseases. Sodium valproate is a commonly used anticonvulsant and mood stabilizer. It has been used primarily in the treatment of epilepsy, bipolar disorder, and, to a lesser extent, major depressive disorder. Interestingly, sodium valproate functions as a histone deacetylase inhibitor (HDAC), (Thiagalingam et al. 2003; Yang and Seto 2007; Marinova et al. 2009; Nalivaeva et al. 2009; Slattery et al. 2009). Although the exact mechanisms of action of sodium valproate are not well characterized, it has been suggested that it

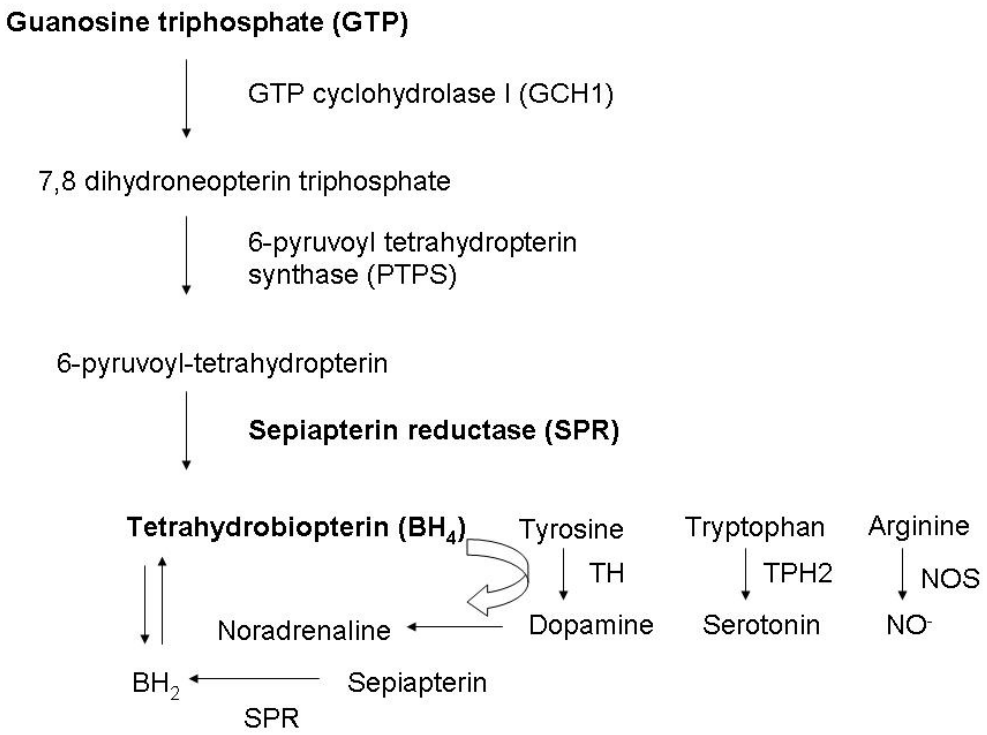


Figure 4.2 Tetrahydrobiopterin (BH_4) biosynthesis and salvage pathways. This figure was adapted from McHugh *et al.* (2010a). TH = tyrosine hydroxylase; TPH2 = tryptophan hydroxylase; NOS = nitric oxide synthase.

exerts its function by binding to the zinc-containing catalytic site of HDACs (Drummond et al. 2005). Therefore, the up-regulation of *Hdac6* gene expression brought about by sodium valproate in this study may be explained by a feedback mechanism caused by reduced function of HDACs. Since histone acetylation plays a vital role in the regulation of gene expression (Sengupta and Seto 2004; Zupkovitz et al. 2006), the degree of histone acetylation must be strictly regulated. The possibility that sodium valproate may bind to HDAC6 indicates that valproate may impair the function of HDAC6, and in turn, affect the downstream biological processes involving this protein. Cells may up-regulate the expression of *HDAC6* as a way to counteract the reduced function of HDAC6 that is required to maintain the homeostasis of histone's acetylation state.

The biological significance of this increased gene expression of *Hdac6* is unclear. Increased histone deacetylase activity caused by *Hdac6* up-regulation may result in hypoacetylated histones that lead to transcriptionally silenced chromatin (Sengupta and Seto 2004; Zupkovitz et al. 2006). The expression of genes that may be involved in mood disorders and their treatment may be repressed as a result. Furthermore, *Hdac6* is found to be highly and specifically expressed in the mouse dorsal raphe nucleus (DRN) demonstrated by *in situ* hybridisation and published in the Allen Brain Atlas (Figure 4.3), (Lein et al. 2007). Since RN46A cells are derived specifically from the medullary raphe cells, this finding seems to be especially relevant physiologically, because cells of the raphe nucleus, which has the most abundant serotonin, form a key component of the brain's serotonergic system (Michelsen et al. 2008). Since the serotonergic system has been strongly implicated in the pathogenesis of major depression and its treatment outcome (Papolos et al. 1996), the DRN has received considerable attention in studies on brain dysregulation, especially mood disorders such as depression (Michelsen et al. 2008). *Hdac6* expression in RN46A presumably reflects the raphe nucleus origin of this cell line, and perhaps the altered expression as a result of valproate treatment reflects a process that could occur *in vivo.* This finding would warrant further examination in rodent models exposed to valproate to establish its biological relevance.

Figure 4.3 In situ hybridisation (ISH) images from the Allen Mouse Brain Atlas showing *Hdac6* gene expression levels in mouse dorsal raphe nuclei. Dorsal raphe nuclei are indicated by the red arrows. The signals detecting the expressed gene (*Hdac6*) are the dark blue spots.

4.4.2.4 Down-regulation of *Htr2a* by SSRIs

These qPCR experiments not only revealed *Spr* and *Hdac6* as highly responsive to valproate treatment, they also revealed that serotonin receptor 2A (*Htr2a*) expression is significantly and specifically down-regulated by the two SSRIs paroxetine and citalopram. The gene *Htr2a* encodes one subtype of the serotonin receptor (Cook et al. 1994). Extensive data demonstrated an important role of *Htr2a* in antidepressant drug action (Choi et al. 2005; McMahon et al. 2006; Kato 2007; Lee et al. 2007; Kishi et al. 2009; Lin et al. 2009; Peters et al. 2009; Wilkie et al. 2009; Zhang et al. 2009; Zhou et al. 2009). It has been shown that *Htr2a* expression is down-regulated by different classes of antidepressants, including citalopram, in rodent and primary forebrain, which is similar to treatment response in humans (Peremans et al. 2005; Strome et al. 2005; Savitz et al. 2009). In a mouse learned helplessness model of depression, *Htr2a* antagonism using antisense oligonucleotides was shown to be effective in regulating depressive-like behaviour (Papolos et al. 1996).

Since several lines of evidence have demonstrated that patients' response to antidepressant drugs are influenced by genetic factors, numerous studies have been conducted to examine the role of *Htr2a* polymorphisms in the modulation of antidepressant response The impact of a -1438A/G *Htr2a* promoter polymorphism on

patients' response to SSRIs was examined (Choi et al. 2005; Kato et al. 2006). In a study conducted in a Korean population with MDD, the G allele of the -1438A/G polymorphism is significantly associated with MDD and patients' response to citalopram treatment, where patients who are homozygous for the A allele have a better response to citalopram compared to patients with other genotypes (Choi et al. 2005). This finding was replicated in another Japanese study where the influence of *Htr2a* gene on clinical response to two other SSRIs (paroxetine and fluoxamine) was investigated (Kato et al. 2006). It was found that the -1438G/G genotype was associated with both a better clinical response to SSRIs and severe adverse drug reactions (Kato et al. 2006).

In addition, the important role of *Htr2a* in the mechanism of antidepressant action was demonstrated in the Sequenced Treatment Alternatives for Depression (STAR*D) study (McMahon et al. 2006). In this study, 1,953 MDD patients were treated with citalopram and treatment outcomes were prospectively assessed. Among the 768 SNPs chosen from a selection of 68 candidate genes, two SNPs in the *Htr2a* gene (rs7997012 and rs1928040) was found to be significantly and reproducibly associated with treatment outcome (McMahon et al. 2006). These two SNPs were later analysed in two independent Caucasian samples (n=637) of patients with MDD for association with treatment outcome. It was found that SNP rs7997012 was significantly associated with remission, which replicated the initial finding of the STAR*D project (Lucae et al. 2010)

Although the precise mechanism by which *Htr2a* affects antidepressant treatment response is not well understood, the new genetic findings discovered here replicated previous neurobiological and genetic data, and provide further evidence for a role of *Htr2a* in the mechanism of action of drugs used to treat mood disorders.

Chapter 5

Effects of valproate on gene expression and intracellular signalling

5.1 Introduction

Although now one of the most commonly used mood stabilisers, the anticonvulsant sodium valproate was fortuitously found to be therapeutically effective in the treatment of mood disorders, especially bipolar disorder, in France in 1962 (Meunier et al. 1963; Perucca 2002; Citrome 2003; Shaltiel et al. 2004). Our lack of understanding of the pathophysiology of bipolar disorder makes it difficult to understand precisely how this drug exerts its therapeutic actions (Nestler et al. 2002b). My observation that valproate has a profound effect on the expression of the gene for sepiapterin reductase in RN46A cells, and the key role of this enzyme in the production of BH_4 and the functioning of multiple neurotransmitter systems, suggests that this pathway may be an important component in mediating the therapeutic action of this drug. In order to understand this observation in more detail, I sought to establish how valproate brings about this strong upregulation of *Spr*. Several cellular signaling pathways have been invoked to explain the actions of valproate (Duman et al. 2000; Manji et al. 2001), and the three main ones are introduced below.

5.1.1 ERK signalling pathway

One of the first intracellular signalling pathways implicated in the action of valproate was the extracellular signal regulated kinase (ERK) pathway (Figure 5.1); (Yuan et al. 2001; Einat et al. 2003; Hao et al. 2004; Michaelis et al. 2004; Di Daniel et al. 2006; Creson et al. 2009). These studies suggest that valproate-mediated neuronal protection may result from the activation of ERK signalling (Coyle and Duman 2003; Hao et al. 2004; Boeckeler et al. 2006), a pathway involved in processes such as neuronal differentiation, survival and long-term plasticity (Huang and Reichardt 2001; Marinissen and Gutkind 2001; Dawson and Ginty 2002; Xu et al. 2007).

Sodium valproate activates the ERK pathway in both cultured cells and in the brain (Yuan et al. 2001; Hao et al. 2004; Michaelis et al. 2004; Creson et al. 2009). Activation of ERK phosphorylates one of its substrates, ribosomal S6 kinase (RSK), which in turn directly phosphorylates cAMP response element-binding protein (CREB) and increases *CREB* transcriptional activity. CREB regulates the expression of *BCL-2*, which has been implicated in brain development (Shacka and Roth 2005), neuronal process growth and regeneration (Chen et al. 1997a), and adult hippocampal neurogenesis (Kuhn et al. 2005). CREB also affects the expression of brain-derived nerve growth factor (BDNF) (Mai et al. 2002), which has been shown to promote synaptic strength, survival and mature neuron growth (Duman et al. 2000; Thome et al. 2000; Chen et al. 2001b). Accumulating evidence has demonstrated that valproate treatment causes *BCL-2* upregulation (Chen et al. 1999b; Yuan et al. 2001; Laeng et al. 2004; Michaelis et al. 2004; Sugai et al. 2004; Blair et al. 2006) and promotes BCL-2 function in processes such as neurite growth, neurogenesis and anti-apoptosis (Chen et al. 1999b; Chuang 2005). Given the potential role of BCL-2 in the pathogenesis and treatment of BD, the effects of valproate on ERK signalling may be quite relevant to the therapeutic actions of valproate, which makes it sensible to examine the ERK pathway in our neuronal cell culture model system.

5.1.2 Wnt/GSK-3 signalling pathway

A second target of valproate that has been proposed as underlying its efficacious treatment of bipolar disorder is the enzyme glycogen synthase kinase three (GSK-3) (Figure5.1). GSK-3 is a Wnt (wingless) pathway enzyme that has been implicated in mood disorders previously (Pacheco and Jope 1999; Gould and Manji 2002b). It is negatively regulated by Wnt. Several studies have shown that valproate changes Wnt/ß-catenin signaling by inhibiting GSK-3 directly or indirectly (Chen et al. 1999a; De Sarno et al. 2002; Gould and Manji 2002b; Gould et al. 2004a; Gould et al. 2004b). Since GSK-3 catalyses the phosphorylation of ß-catenin, leading to its degradation, inhibition of GSK-3 results in an accumulation of ß-catenin, which has anti-apoptotic effects, and promotes axon growth through activation of the *Tcf/Lef-1* promoter (Coyle

and Duman 2003). Valproate exerts its anti-apoptoic effects by inhibiting GSK-3 and/or inducing ß-catenin (Phiel et al. 2001).Given the anti-apoptotic effects of ß-catenin and its role in promoting axon growth, it is not surprising that valproate has been suggested to protect against apoptosis and promote neuronal plasticity through these mechanisms (Phiel et al. 2001)

5.1.3 Inositol and phosphatidylinositol signalling pathway

One of the latest proposals to explain how valproate may exert its action is the 'inositol depletion theory', which has previously been proposed for lithium's efficacy (Berridge et al. 1989), but which has now been suggested to explain the action of valproate (Figure 5.1). The theory suggests that lithium acts as a non-competitive inhibitor of inositol monophophatase (Leech et al. 1993) and polyphosphatase (York et al. 1995) to reduce *de novo* biosynthesis of myoinositol, which in turn reduces inositol content. Valproate, like lithium, has been shown to inhibit the inositol-1,4,5-triphosphate ($InsP_3$) signalling cascade by decreasing the amount of inositol (Li et al. 1993; O'Donnell et al. 2003). Evidence to support these ideas has been seen in human, where chronic treatment with valproate and lithium was able to normalise altered $InsP_3$ signalling pathway in patients with bipolar disorder (Silverstone et al. 2002).

The role of inositol depletion in valproate action was further supported by the findings that the effects of inositol depletion on the level of phosphatidylinositol seems to have an impact on phosphokinase C (PKC), phosphoinositide 3-kinase (PI3K) and ERK pathways (Gould and Manji 2002a). For example, chronic valproate treatment resulted in decreased expression of one of PKC's substrates, myristoylated alanine-rich C kinase substrate (MARCKS), in the hippocampus, and enhanced neurite extension in neuronal cultures (Manji and Lenox 1999). Since valproate has been found to affect PKC (Blaheta and Cinatl 2002; Gurvich and Klein 2002; Rosenberg 2007), PI3K (De Sarno et al. 2002) and ERK pathways (Yuan et al. 2001; Hao et al. 2004; Michaelis et al. 2004; Creson et al. 2009), crosstalk between these signalling pathways elicited by inositol depletion complements the effect of valproate on any single signalling pathway.

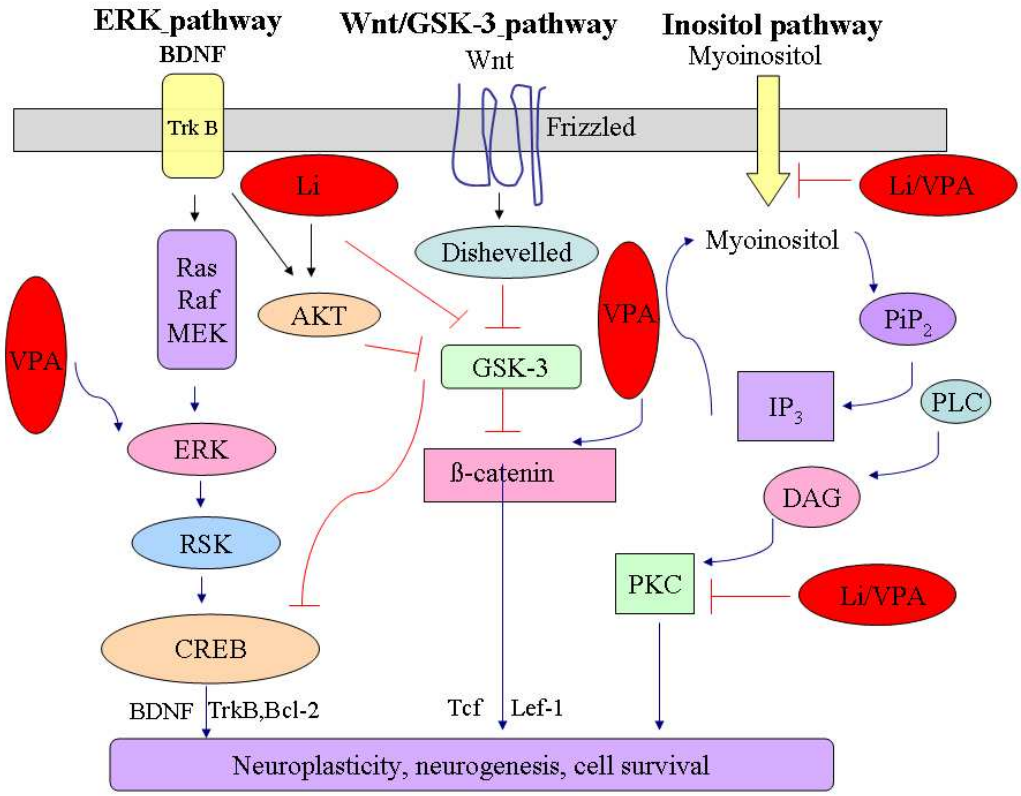


Figure 5.1 Simplified diagram illustrating intracellular pathways affected by mood stabilisers lithium and valproate. Activation →; Inhibition ⊣. (Gould and Manji 2002b; O'Donnell et al. 2003; Boeckeler et al. 2006)

5.2 Experimental approach

Experiments outlined in Chapter 4 showed that the most significant gene expression changes observed by qPCR were caused by valproate exposure in RN46A cells. Previously the focus of this work was directed at antidepressant drug-induced gene expression effects, but with these results, I decided to focus on examining the pattern of valproate-induced gene expression changes. To this end, I re-examined time-course and dose-response experiments with valproate trying to understand the pharmacology of the drug. Time-course experiments could reveal how quickly the gene expression change occurs, which has important functional ramifications. Dose-response analysis may uncover valuable information about the pharmacology of the drug. It could answer questions like: do increasing concentrations of drug lead to greater response, or does the system saturate at some point, which may mean a transporter is involved. In addition to

the primary goal of understanding the pharmacology of the drug, these experiments also allowed optimization of drug exposure conditions in our cell culture system. The genes *Spr* and *Hdac6*, which showed the most significant expression changes in the previous section (section 4.3.2), were examined in the established qPCR system. The same reference genes (*Actb, G6pd* and *Rnf4*) were applied in time-course and dose-response experiments because the experimental conditions were unchanged apart from the drug used for cell culture treatment, and the empirical selection of reference genes (Section 4.3.1) included valproate as one of the treatments. I also examined the effects of valproate on levels of Spr protein. Western blots were employed to test whether valproate-mediated gene expression changes detected at the mRNA level of gene regulation, could also be identified at the protein level.

To dissect further the mechanistic actions of valproate, ELISA assays was performed to explore potential signalling pathways activated by the drug. The goal of these experiments was to provide data on the possible mechanisms underlying gene expression changes in response to valproate, and then to explore whether these mechanisms accounted for the expression changes seen in *Spr* and *Hdac6*.

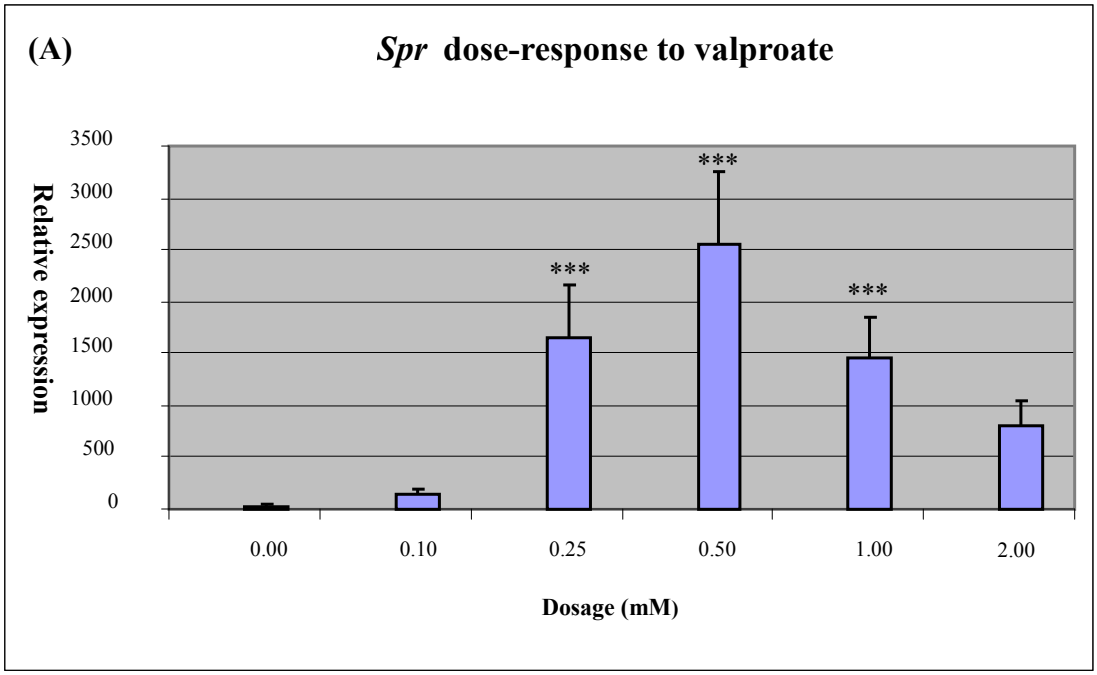
5.3 Results

5.3.1 Dose-response analysis of *Spr* and *Hdac6* gene expression

RN46A cells were exposed to valproic acid at six concentrations: 0, 0.1, 0.25, 0.5, 1 and 2mM for a period of 72 hours, before gene expression changes were measured by qPCR. The culture length of 72 hours drug exposure was chosen as I had previously demonstrated in antidepressant experiments (Section 3.2.4.2) that this time period induced measurable expression changes. Figure 5.2A illustrates that valproate induced an increase in *Spr* expression at as low as 0.1mM, with a maximum activation at 0.5mM valproate, showing an approximately 2540-fold increase. At concentrations of valproate greater than 0.5mM, the expression level of *Spr* appeared to progressively decrease.

This pattern differed for *Hdac6*, where expression levels peaked at 0.1mM valproate (Figure 5.2B), and progressively decreased as the concentration of valproate increased. At a concentration of 2.0mM, the level of *Hdac6* expression had reduced to a level very close to background seen at 0mM valproate. One interesting observation worth noting was that the expression level of *Hdac6* (<5) was many-fold lower than *Spr* (<2600).

The dose-response analysis of *Spr* and *Hdac6* expression demonstrated that they were induced maximally at different concentrations. We chose 0.5mM for subsequent expression analysis experiments because it induced significant upregulation of both genes and is within the therapeutically effective concentration as suggested by previous studies (Rapeport et al. 1983; Yuan et al. 2001; Sztajnkrycer 2002).



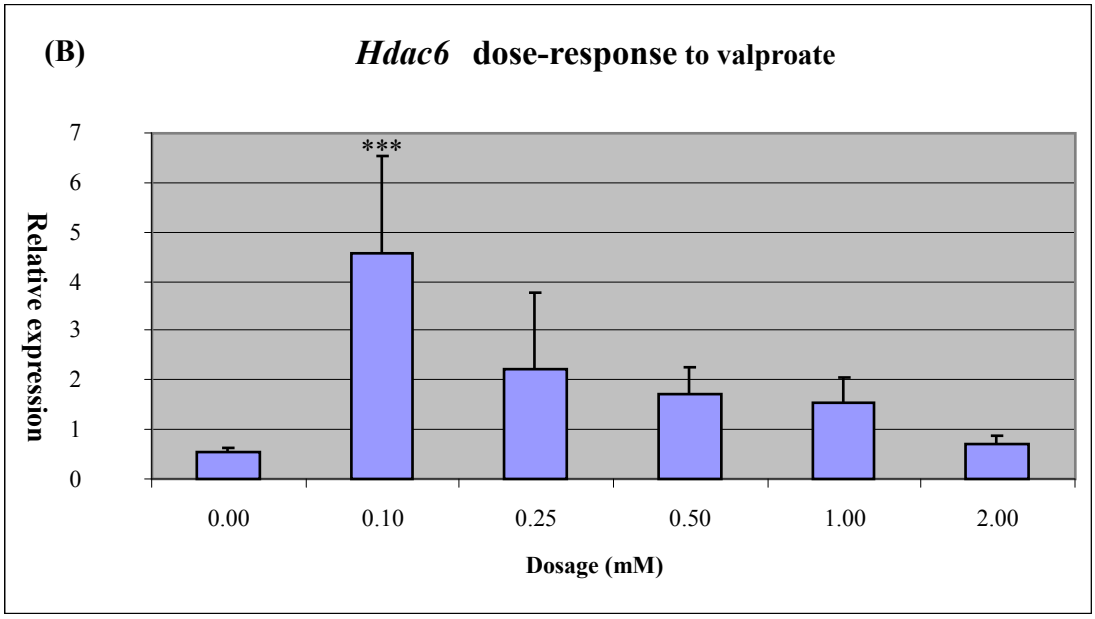


Figure 5.2 Dose-response experiments of RN46A cells treated with valproate for 72 hours. RN46A cells were treated with different concentrations of valproate ranging from 0 to 2mM for 72 hours. Changes in expression of A) *Spr* and B) *Hdac6* were measured using qPCR. The results represent the mean ± SEM values of six independent experiments, each performed in triplicate. Experimental replicates were randomized and data are the average of six independent experiments, each with triplicates. Note: Y axis scales are different for the two genes. ***$p<0.001$ indicate results significantly different from untreated control (Tukey's t-test).

5.3.2 Time-course analysis of *Spr* and *Hdac6* gene expression

The dose response experiments were carried out with a 72 hour exposure, but to better understand the nature of valproate effects on *Spr* and *Hdac6*, it was important to explore the time-course pattern of gene induction. RN46A cells were exposed to 0.5mM valproate for time periods ranging from 12 to 96 hours and RNA was harvested and reverse transcribed to examine gene expression changes of *Spr* and *Hdac6* using qPCR. The results showed different patterns of gene activation for the genes tested. Using *Spr* specific primers and a UPL probe with preparations of cDNA from the six time points, gene expression activation was detected after 24 hours of valproate exposure (Figure 5.3A). This effect was seen to peak at 48 hours, and had decreased significantly by 72 hours, returning to baseline by 96 hours. This was not the situation seen in *Hdac6* induction by valproate (Figure 5.3B). Treatment resulted in a significant increase in *Hdac6* gene expression at 12 hours, returning to baseline gradually by 96 hours.

To summarize these experiments examining the effects of valproate dose and duration of exposure, the two genes were affected quite differently under each condition. *Spr* showed massive but relatively slow induction at higher doses of the drug, while *Hdac6* showed rapid but modest induction at relatively low doses of valproate. This may have implications for future experiments directed at understanding the mechanisms of the drug upon these genes. For instance, are these gene products acting in concert, but at different points in a biochemical pathway, or are they affected individually by valproate, and their different expression over time and dose reflects a direct and independent effect of valproate on these genes?

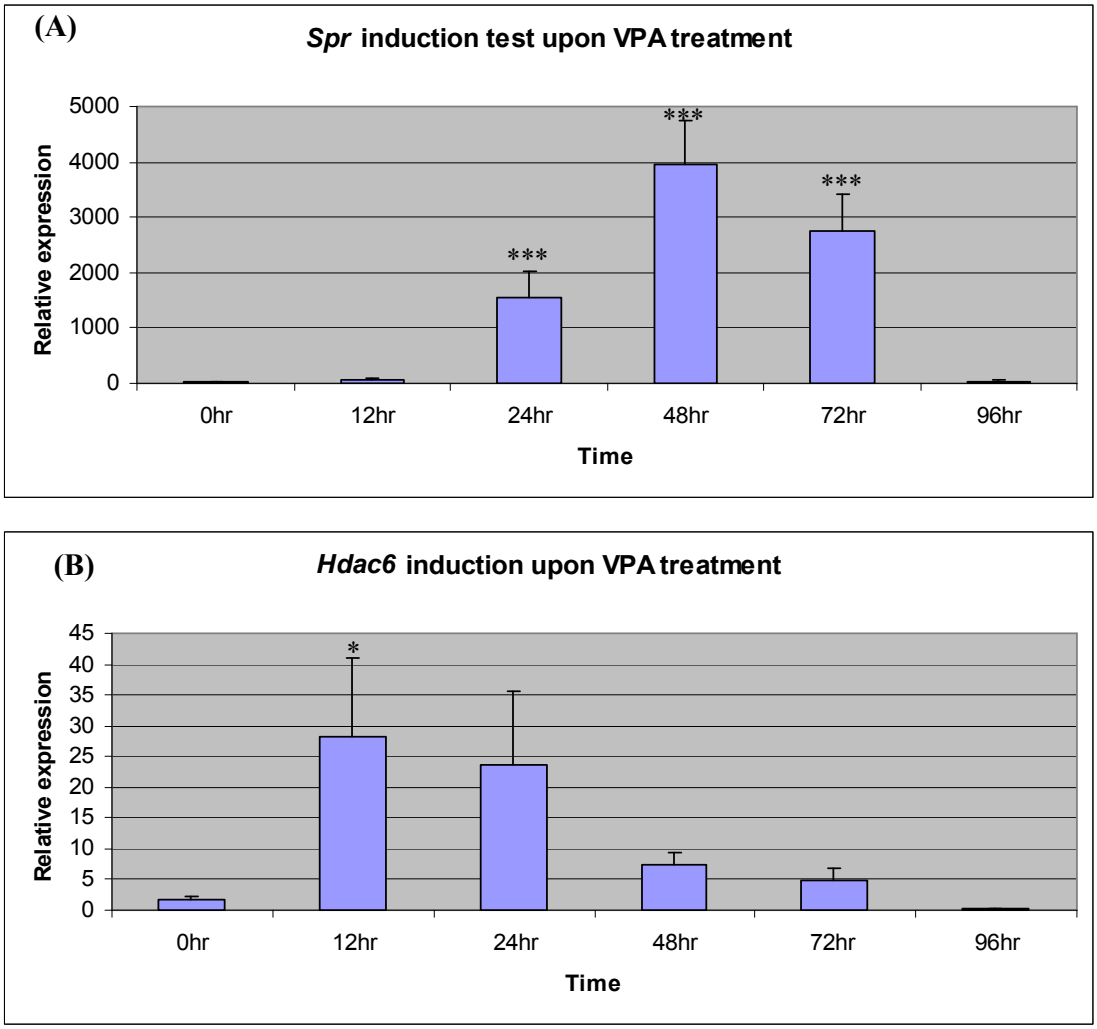


Figure 5.3 Time-course of *Spr* and *Hdac6* induction. RN46A cells were exposed to 0.5mM valproate for 12 hours to 96 hours. The expression of A) *Spr* and B) *Hdac6* was measured using qPCR. Experimental replicates were randomized and data are the average of six independent experiments, each with triplicates. Note: Y axis scales are different for the two genes. *$p<0.05$, ***$p<0.001$ indicate results significantly different from untreated control (Tukey's t-test).

5.3.3 Western blot analysis of Spr protein

The experiments described above indicate quite significant gene induction effects that may be relevant in the therapeutic actions of valproate. These experiments, however, are entirely based around analysis of the mRNA from candidate genes, and it is important to establish whether these effects are also manifested at the protein level. Although both Spr and Hdac6 are interesting candidates, with limited time and resources it was possible to only pursue these experiments for one of the two genes. I

decided to pursue Spr because it is involved in the biosynthesis of neurotransmitters, and because it showed the largest induction at the transcriptional level among all the genes tested.

5.3.3.1 Protein preparation for western blot analysis

RN46A cells were grown and exposed to 0.5mM valproate for 72 hours. Protein lysates were isolated from either valproate-treated RN46A cells or untreated controls using RIPA buffer. Three biological replicates each for either valproate treated or non-treated control samples were pooled together in order to maximise the amount of protein obtained while minimising variations between biological replicates. Bio-Rad *DC* protein assay (Bio-Rad laboratories, Hercules, CA) was used for protein quantification because it is compatible with SDS, the denaturing reagent used for protein extraction. It is also more advantageous than the Lowry assay, which has been routinely used for protein measurement (Lowry et al. 1951), because of its faster colour development and longer colour preservation.

5.3.3.2 Western blot analysis

Approximately 20-50µg of cell lysate was loaded for each protein sample. Three samples were loaded: valproate treated, untreated, and the rat hippocampus protein lysate sample. Samples were run under standard conditions in a 10% polyacrylamide resolving gel, chosen to enable detection of our expected protein (SPR) size of 28 kDa. A pre-stained SDS-PAGE protein size standard was run alongside to determine the appropriate size. Dilution factors for primary and secondary antibodies for SPR were determined to be 1:1000 and 1:2500 respectively, which are within the range suggested by manufacturer (data not shown).

Two primary antibodies directed against SPR protein were tested initially. They were SPR mouse polyclonal antibody (A01) raised against a partial recombinant SPR with GST tag (Cat# H00006697-A01) (Abnova, Taiwan), and whole serum SPR mouse clonal antibody (ab72620) (Abcam, MA, USA). The secondary antibody coupled with SPR (H00006697-A01) was Goat anti-mouse IgG (H&L)-HRP conjugate secondary

antibody (Cat# PAB0096) (Abnova, Taiwan). Rabbit polyclonal secondary antibody to mouse IgG-H&L (HRP) (ab6728) (Abcam, MA, USA) was used to couple the primary antibody SPR (ab72620). After considerable optimisation efforts, only SPR (A01) gave a distinct band on the blots. SPR (A01) and its corresponding secondary antibody PAB0096 was used for subsequent western blot analysis.

Figure 5.4 details the western blot analysis of SPR levels between valproate-treated and untreated RN46A cell samples (lanes 1 and 2). An antibody to ß-actin was used as a loading control. Both valproate-treated and untreated samples contained similar amounts of ß-actin antibody signal, suggesting that the established protein concentrations were correct. Two western blots were analyzed followed by densitometry. Cells exposed to 0.5mM valproate for 72 hours showed an approximately 1.6 fold increase in SPR protein expression when compared with untreated samples, which is consistent with the direction of transcriptional change in the previous chapter. The very large difference in fold-change of gene expression measured at transcriptional versus translational level will be discussed later.

One other interesting finding in this experiment was that the expected size of SPR is different from the observed size for the cultured RN46A cells. The observed size for SPR was 45-55 kDa in cultured RN46A cells. However the expected size for the SPR monomer is approximately 28 kDa. This experiment also highlighted three bands with SPR antibody signal in the brain cell lysate. One of these was of the cell culture derived size seen in lane 1 and 2, but the remaining signals are of 28 kDa and 70-80 kDa. These size discrepancies in mass will be explored further in the following discussion.

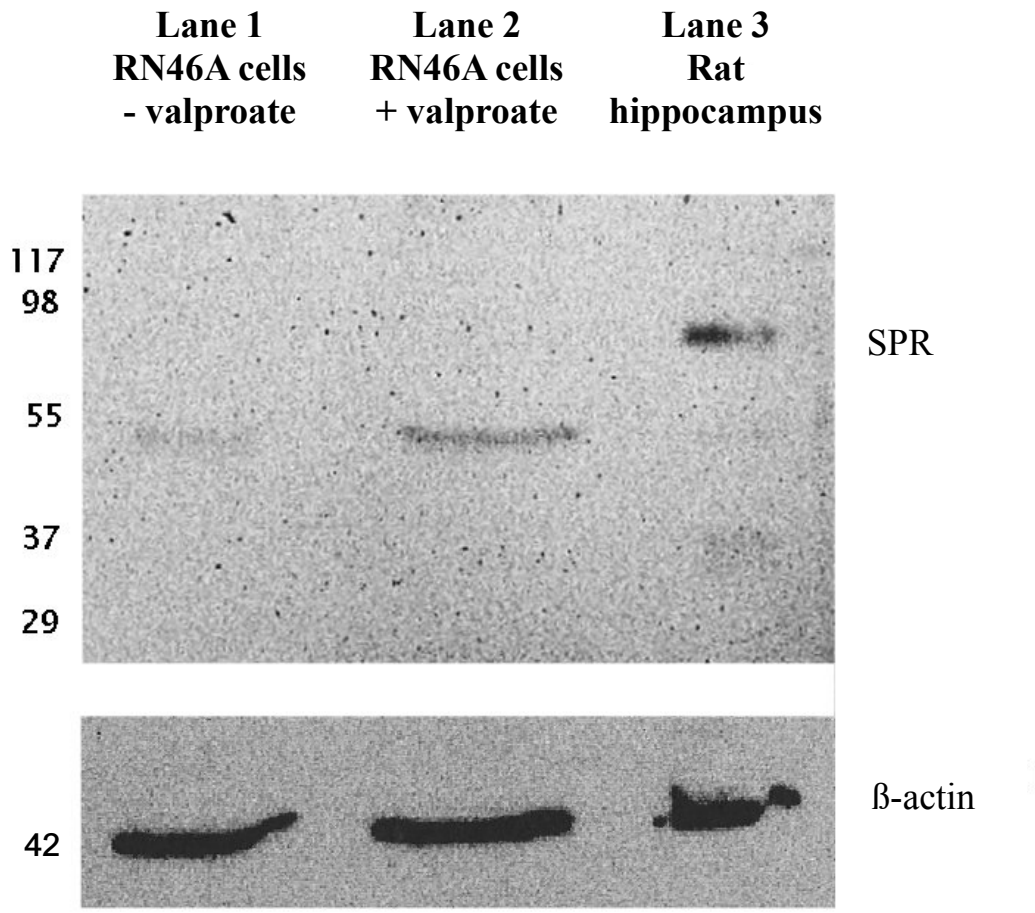


Figure 5.4 Western blot of SPR protein after valproate treatment of RN46A. RN46A cells were treated with 0.5mM valproate for 72 hours. Proteins were extracted and run on SDS-PAGE. An antibody specific to SPR protein was used to detect SPR on western blots. Rat hippocampus tissue sample was used as a positive control. ß-Actin was used as a loading control. The western blots were exposed for 10 minutes and 20 sec for SPR and ß-actin respectively, and were visualized on ChemiDoc XRS using Gel-Doc software. Results are based on two independent western blots, only one of which is shown.

5.3.4 Are common signalling pathways activated by valproic acid?

One way to investigate the possible signalling pathways involved in the regulation of SPR by valproate is to analyse the activation of key kinases following exposure of neuronal cells to valproate. The phosphorylation state of kinases involved in several signalling pathways was investigated using a multi-kinase ELISA array (Symansis, Timaru, New Zealand). Protein kinases analysed in this system include Akt1, ERK1, ERK2, GSK3α, GSK3ß and p38α, which form key components of well-characterised intracellular signalling pathways including the ERK pathway, the canonical Wnt

signalling pathway and the inositol/phosphatidylinositol pathway.

Cultured RN46A cells were treated with 0.5mM valproate for 72 hours. Cell lysates were prepared, and used in the ELISA assays to measure the phosphorylation states of key kinases. Figure 5.5 illustrates the changes in phosphorylation state between valproate-treated and untreated RN46A cell samples of the six protein kinases involved in three major signalling pathways. No kinases showed significant changes in phosphorylation levels when RN46A cells were exposed to valproate. Although p38α showed a slight increase in absorbance at 450 nm upon valproate exposure, it was not statistically significant, because this experiment could only be carried out in duplicate, rather than with several replicates.

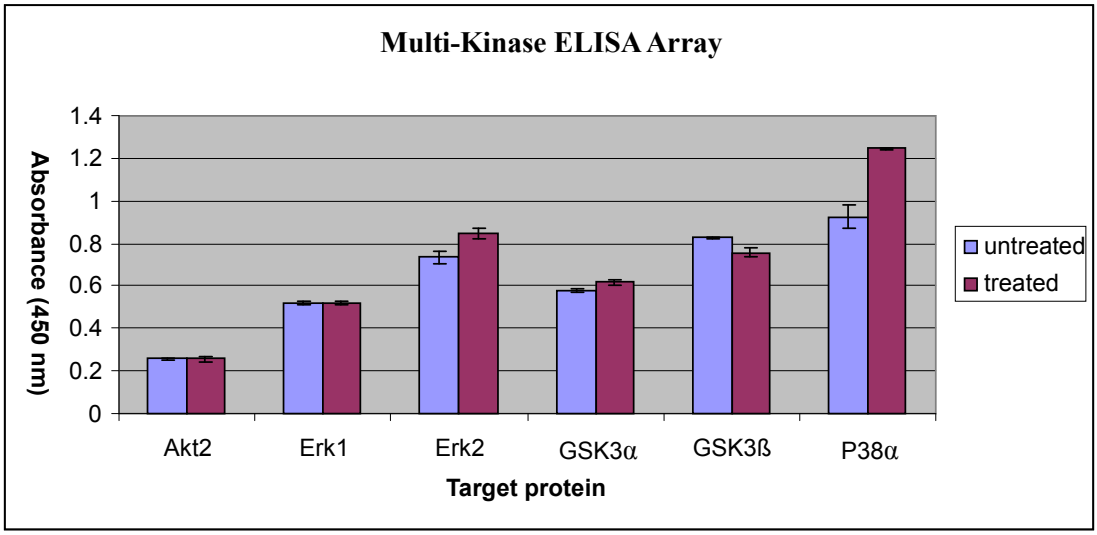


Figure 5.5 Multi-kinase ELISA array results. RN46A cells were exposed to 0.5mM valproate for 72 hours. Cell lysates were prepared and tested for phosphorylation levels of major protein kinases in several signalling pathways using a multi-kinase ELISA array kit (Symansis). Duplicate assays were conducted for each sample on each array and this array was repeated once. Results are shown as mean±SEM. The data should be regarded as preliminary.

5.3.5 Valproate, the *SPR* promoter and revisiting reporter gene assays

Chapter 3 described efforts to detect SSRI antidepressant-induced gene expression changes in a reporter gene system, without much evidence of strong effects. Having now established that valproate exerts a large influence on expression of *Spr*, Idecided to

test the effects of this drug in our original reporter gene system with the *SPR* promoter. To achieve this, the human *SPR* promoter construct cloned in Chapter 3, which spans 1270 bp of sequence upstream from the transcriptional start site of *SPR* (Table 3.3), was utilised. The cloned promoter construct was transfected into cultured RN46A cells and exposed to 0.5mM valproate for 72 hours. The expression level difference is presented in Figure 5.6, where *SPR* expression was found to be 1.6 times higher ($p<0.001$) when RN46A cells were exposed to valproate prior to transfection, compared to untreated cells.

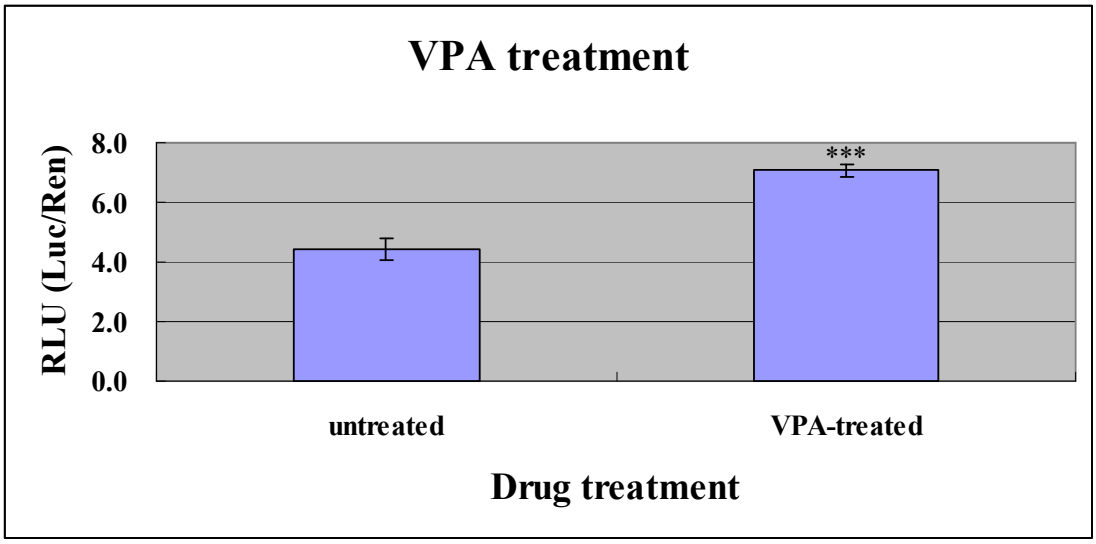


Figure 5.6 Valproate treatment experiments. Reporter gene expression levels of *SPR* following 72 hours treatment with 0.5mM sodium valproate in RN46A cells. The levels of firefly luciferase activity are standardized to *Renilla* luciferase activity (relative light units). Drug treatment is indicated on the X-axis. Transfections were randomised across the plate and results are expressed as mean ± SEM of three independent experiments, each with triplicates. ***$p<0.001$ indicates results differing from the untreated control significantly (Student t-test).

5.4 Discussion

5.4.1 Dose-response experiments of RN46A cells exposed to valproate

According to the results of dose response experiments, exposing RN46A cells to 0.5mM valproate was the optimal condition to induce the largest changes in *Spr* gene expression (Figure 5.2A). It is reassuring that this level is within the therapeutic range of valproate treatment in humans(Gottlicher et al. 2001; Phiel et al. 2001;

Duenas-Gonzalez et al. 2008; Geddes et al. 2010; Gould et al. 2010; Nieoczym et al. 2010; Purrucker et al. 2010; Rui et al. 2010; Wu et al. 2010; Visudtibhan et al. 2011), suggesting that this observed gene expression effect occurs at realistic doses of the drug.

The pattern of gene expression change was different for *Hdac6*. It appeared that *Hdac6* has a much lower basal expression in RN46A cells (Figure 5.2B). Exposing RN46A cells to 0.1mM valproate induced expression of *Hdac6*. However, the degree of upregulation decreased with increasing concentrations of valproate.

The biological relevance of the observed different patterns of dose-response is unclear. While *Hdac6* seemed to be more sensitive to valproate exposure, *Spr* required higher doses of valproate to achieve a similar increase. This suggests that the regulatory processes for each gene are differentially affected by valproate.

5.4.2 Time-course experiments of RN46A cells exposed to valproate

The time-course patterns of gene activation of the two genes are different (Figure 5.3). *Hdac6* showed the biggest increase in gene expression as early as 12 hours after the initiation of valproate exposure whereas *Spr* only started to show significant gene activation after 24 hours of valproate treatment and peaked at 48 hours. This observation may indicate different pathways by which valproate exerts its therapeutic action. *Hdac6* seems to be an early response gene that was activated soon after valproate was administrated. Since *Hdac6* removes acetyl groups in the histone tail, leading to a closed chromatin structure (Berger 2002; Geiman and Robertson 2002), the upregulation of *Hdac6* could serve as a means of widespread gene regulation, where genes controlled by *Hdac6* directly will be repressed. *Spr*, on the contrary, may be a late response gene that could, for example, function downstream of *Hdac6*.

Although only two genes were tested in the dose-response and time-course experiments, the data obtained suggest that the qPCR approach, in RN46A cells exposed to drugs,

could form a useful model system for ongoing investigation of the mechanisms of action of drugs used in mood disorders.

5.4.3 Sepiapterin reductase protein detection

5.4.3.1 Sepiapterin reductase up-regulation by valproate

Western blotting was employed to examine the effects of valproate on SPR protein levels in the cell line RN46A. This experiment detected an approximately 1.6-fold upregulation of SPR after exposure to valproate for 72 hours. This finding complements the transcriptional upregulation of SPR seen in the previous chapter, where *Spr* mRNA level was significantly increased in cultured cells exposed to valproate. Although western blotting is semi-quantitative, it enables direct visual identification of changes in protein expression. The differences detected at both gene and protein level adds confidence to these results. Taken together, the knowledge gained from both qPCR and western blotting studies suggests that valproate upregulates SPR at both gene and protein level in RN46A cells.

However, there is a large difference in the magnitude of fold changes detected at the gene and protein level (1755-fold versus 1.6-fold upregulation). Although it is not clear why there is such a difference, poor correlations between the level of mRNA and the level of protein has been reported and well documented in the literature. A comparison of 19 selected mRNA and protein abundances conducted in human liver revealed a correlation coefficient of 0.48 (Anderson and Seilhamer 1997). Another study on the matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase 1 in human prostate cancers showed no significant relationship between mRNA and protein levels (Lichtinghagen et al. 2002). An study in lung adenocarcinomas also demonstreated discordant protein and mRNA expression levels in a large proportion of genes studied (Chen et al. 2002).

Three possible explanations to account for the observed difference in protein and mRNA expression are proposed here. One of the most plausible explanations is post-transcriptional regulatory mechanisms. These mechanisms, in particular, mRNA transport and microRNA (miRNA)-mediated translational repression, control the

deployment of proteins in both spatial and temporal dimension (Lipscombe 2005; Bramham and Wells 2007; Bushati and Cohen 2007), and play an important role in establishing precise neuronal connectivity in neural cells (Loya et al. 2010). It has been suggested that mRNAs are often packaged and transported in ribonucleoprotein particles (RNPs), which is normally referred to as RNA granules (Ainger et al. 1993; Knowles et al. 1996; Kohrmann et al. 1999; Kiebler and DesGroseillers 2000; Kiebler and Bassell 2006). Protein levels are controlled during the transportation process by RNA-binding proteins (RBPs) and/or miRNAs, which inhibit mRNA translation reversibly (Ule and Darnell 2006; Schratt 2009).

Another possibility that may result in the observed poor noncorrelation is rapid mRNA degradation. In eukaryotic cells, there is a balance between mRNA degradation and translation (Parker and Sheth 2007). The lifetime of an mRNA species is strictly controlled to regulate protein synthesis rapidly in response to changing environmental conditions and needs. It is possible that the majority of *Spr* mRNA was degraded once transcribed in response to cellular needs. Several mechanisms that lead to the destruction of mRNA have been demonstrated. Removal of the poly (A) tail, mediated by specialized exonucleases is thought to promote mRNA degradation, by either the exosome complex (Chen et al. 2001c) or the decapping complex (Fenger-Gron et al. 2005). The other potential factor that may cause mRNA degradation is the presence of AU-rich elements in mRNAs, which have a tendency to destabilise transcripts by stimulating poly (A) tail removal (Shaw and Kamen 1986; Chen and Shyu 1995). Nonsense mediated decay (NMD) occurs when a premature stop codon is detected in the message, which in turn triggers mRNA degradation by 5' decapping, 3' poly (A) tail removal, or endonucleolytic cleavage (Isken and Maquat 2007). In addition, small interfering RNAs (siRNA); (Obbard et al. 2009) and microRNA (miRNA); (Brennecke et al. 2005; Eulalio et al. 2009) are possible mechanisms that mRNA can be degraded. There are several putative miRNA sites in SPR 3' UTR, such as the highly conserved has-miR-940, and the poorly conserved has-miR-671-5p and has-miR-873, as detected by TargetScanHuman 5.2 (http://www.targetscan.org/vert_50/). The effects of these miRNA sites on SPR mRNA and protein levels could be studied in the future.

Thirdly, post-translational modification, such as protein degradation, may affect the level of protein detected, resulting in different levels of mRNA and protein (Basle et al. 2010; Eisenhaber and Eisenhaber 2010).

5.4.3.2 Protein size disparities

The observed size of SPR protein extracted from RN46A cells was approximately two times larger than the predicted size of SPR monomer (~ 55 kDa versus ~ 28 kDa) (Figure 5.4). In addition, three bands were detected in rat hippocampus tissue, indicating that there may be other isoforms of SPR present in the more complex brain tissue sample.

Although there is no obvious explanation for the observed size discrepancy for SPR either in the literature or *in silico*, several possibilities could be considered. One explanation could be that several isoforms of SPR exist, and different cell types or tissues express different forms of SPR. The observation that rat hippocampus expresses three forms of SPR of different size (~ 28 kDa, ~ 56 kDa and ~ 80 kDa) supports the idea that there are different forms of SPR expressed in different cell and tissue types. An additional band of ~ 56 kDa size was also reported in an Spr preparation from rat erythrocyte cell lysate (Smith 1987). The ~ 80 kDa band identified in Fig 5.4 corresponds to the size observed in prior studies from this laboratory, in mouse ES cell-derived neuronal cells (McHugh et al. 2008b). In that study, proteins were separated by two dimensional gel electrophoresis. Specific spots were analysed by mass spectrometry in order to identify the protein of interest. The observed size of SPR in the proteomic study was ~ 70-80 kDa (McHugh et al. 2008b), which is similar to the biggest band identified in my study. Since the ES-cell derived neuronal culture model contains a complex mix of neural cell types, it crudely resembles the cellular environment of rat hippocampus tissue. These observations are consistent with multiple SPR forms.

Another plausible explanation for size discrepancies is post transcriptional

modification (PTM). In fact, PTMs such as glycosylation, phosphorylation and sulfation were suggested to have a remarkable effect on protein size (Gasnier et al. 2004). However, PTMs alone may be insufficient to account for the two fold differences between expected and observed size in our study.

The molecular phenomena of domain swapping may provide a third possible justification for the observed size discrepancies. Domain swapping happens as a result of a mechanism for normal enzyme activation (Larsen et al. 1998). Domain swapping can also happen in protein homodimers. Schonenberg *et al* (1995) has shown that when two copies of a gene are expressed and each is mutated in a different domain, domain swapping takes place to minimise the effects of loss of function mutations in important proteins (Schoneberg et al. 1995). SPR functions as a homodimer and domain swapping is a possible explanation for the apparent dimer observed on SDS-PAGE gels.

5.4.4 Signalling pathway analysis by multi-kinase ELISA assay

A multi-kinase ELISA array was utilised to explore whether key signalling pathways previously implicated in valproate action, were modified in this cell culture model. I did not detect significant changes in the phosphorylation levels of key protein kinases from three signalling pathways examined. Although mounting evidence has suggested that valproate activates the ERK pathway in cultured cells and in the brain (Yuan et al. 2001; Einat et al. 2003; Hao et al. 2004; Michaelis et al. 2004; Di Daniel et al. 2006; Jung et al. 2008; Creson et al. 2009), this does not appear to be the case in RN46A cells. The other three pathway proteins GSK3α, GSK3ß and Akt1, also showed little alteration in phosphorylation levels, indicating that Wnt/GSK3 and PI3K/Akt signalling pathways may not be affected by valproate exposure in RN46A cells, regardless of previous evidence showing their involvement in valproate action (Chen et al. 1999a; De Sarno et al. 2002; Gould and Manji 2002b; Gould et al. 2004a; Gould et al. 2004b). The inability to detect phorphorylation modifications in major signalling pathway proteins may be explained by differing experimental conditions between this study and previous work. For example, MAP kinase was found to be activated when

SH-SY5Y cells was incubated with 1mM valproate for 24 hours in a previous study (Yuan et al. 2001). The valproate-induced phosphorylation of Akt and GSK3ß was most evident after 12 hours of valproate treatment of SH-SY5Y cells at 5mM (De Sarno et al. 2002). The drug exposure conditions used in this study, however, were 72 hours of valproate treatment at 1mM concentration. But equally, it may be that valproate does not affect phosphorylation of these signalling pathway proteins, and acts through another novel or untested pathway. Alternatively, it may act upon this pathway, but not via phosphorylation.

5.4.5 Reporter gene assays

As a common approach for gene expression analysis, the reporter gene system was revisited to examine whether valproate-induced *SPR* gene expression changes were triggered through the putative promoter sequence of *SPR*. A modest increase in gene expression was caused by valproate, with treated cells delivering a 1.6 fold increase in luciferase activity in comparison to untreated cells. This was significantly less than the increase detected in our qPCR systems, but was of particular note, as it may point to a small effect of valproate upon the promoter region of *SPR*, thus meaning that the mechanism of valproate may, in part, be at the transcriptional regulation of *SPR*. This result indicates that the reporter gene system is unlikely to be sensitive enough to detect minor gene expression changes. However, it could be used as a crude method for initial screening of major gene expression changes.

5.5 Potential future work

The dose-response and time-course experiments yielded useful information that shed light on possible mechanisms underlying gene expression changes induced by valproate. More comprehensive dose-response experiments that cover concentrations from 2mM to up to 10mM, or time-course experiments that investigate the acute effects of valproate (0 to 12 hours), will provide additional information on the pattern of expression when RN46A cells are exposed to different doses of valproate for various periods of time.

Another question that is worth investigating is what the pattern of expression would be for genes other than *Spr* and *Hdac6*. It is possible that the pattern of expression of other genes would be different from that observed for *Spr* and *Hdac6*.

Preliminary results from the protein expression data demonstrated that SPR levels were increased significantly when RN46A cells were exposed to 0.5mM valproate for 72 hours. The biological significance of this preliminary finding can be further investigated by various means. For example, it would be interesting to examine whether the observed protein changes occur in brain tissues of animals at either mRNA or protein level by techniques such as *in situ* hybridization or immunohistochemistry. This work is being undertaken in our laboratory.

The multi-kinase ELISA array failed to identify specific signaling pathways affected by valproate. Nevertheless,we cannot exclude the possibility that other kinases in these three signaling pathways may be affected by valproate and play a role in mediating the therapeutic effects of valproate in RN46A cells. Therefore, a more detailed examination of pathway proteins using either western blot or ELISA arrays is warranted, to give a more complete picture of the mechanisms of action of valproate. If a specific pathway was identified, inhibitors or siRNA that target the pathway specifically could be administered, which may allow further testing of whether that pathway mediated *Spr* gene expression changes upon exposure of cells to valproate.

Last but not least, although I did not pursue HDAC6 in the protein analysis due to limited time and resources, its biological functions makes it a highly relevant and interesting target that warrants further investigation. The experiments suggested above for *Spr* and other genes could usefully be applied to *Hdac6* too.

Chapter 6

Discussion

6.1 Thesis summary

In order to investigate the broad effects, both serotonergic and non-serotonergic, of drugs used to treat mood disorders, two approaches, i.e. reporter gene assay and real-time quantitative PCR, were utilised. Each system has its own strengths and weaknesses. Although the reporter gene assay did not yield consistent results, its usefulness for gene expression analysis cannot be fully denied, and could perhaps be improved further with specific modifications, which will be discussed in the following section. The qPCR analysis, in contrast, proved to be a robust, sensitive and reproducible method for gene expression studies when conducted according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR experiments) guidelines (Bustin et al. 2005). Nevertheless, the significant findings discovered in the study by qPCR should be replicated in future work or undergo validation using another approach, such as western blotting.

6.1.1 Reporter gene assay model system

In an attempt to establish a simplified and consistent cell culture model system in which gene expression changes upon drug exposure could be examined, reporter gene assays were designed for 18 candidate antidepressant - responsive genes. Of the 18 reporter gene constructs cloned and examined, ten were expressed in the serotonergic RN46A cell line, while the remainder failed to show detectable expression. Gene expression changes in these ten genes were then measured when RN46A cells were exposed to 0.5μM paroxetine or fluoxetine for 14 days. Although significant gene expression changes were observed for some of the genes on some occasions, it proved difficult to obtain consistently reproducible results in this model system. However, time-course and dose-response experiments identified 72 hours paroxetine treatment at 0.5μM as

the optimal conditions for observing gene expression changes induced by paroxetine, at least for the two promoters tested in this way (*ADM* and *GCHFR*). The observed changes in gene expression caused by paroxetine did not seem to be specific to paroxetine or SSRI (Figure 3.11). Both paroxetine and valproate increased *ADM* expression, and expression of *GCHFR* was increased by all the drugs tested (section 3.2.2.4). These results, taken together, indicated that paroxetine-induced *ADM* and *GCHFR* gene expression changes may not be involved in the therapeutic action of paroxetine. It is possible that these genes respond whenever cells are confronted with any kind of chemical insult.

In summary, the luciferase reporter gene system did not seem to be well suited for exploring gene regulatory effects of antidepressants. Although extreme care was taken with the assay (for example, replicates were randomized across the plate to minimise technical errors, multiple technical and biological replicates were done to increase the statistical power), the system proved to lack both sensitivity and consistency in obtaining reproducible results. The lack of effects may result from several possible situations. Firstly, as only a limited repertoire of genes were selected for testing in the reporter gene system, it is possible that none of these genes were actually antidepressant responsive. Secondly, preconditioning of cells with antidepressants may be insufficient to trigger expression changes. Thirdly, the use of undifferentiated RN46A cells throughout the project may have been an issue. Early reports suggested that these cells do express the serotonin transporter (White et al. 1994; Bethea et al. 2003). However, microarray data has shown that *Slc6a4* is not highly expressed in undifferentiated RN46A cell grown in this laboratory (McHugh et al. 2009a). QPCR analysis in my study also demonstrated that undifferentiated cells express the serotonin transporter poorly if at all (Table 4.2). Although it has been shown that the serotonin transporter is not the sole target of SSRIs (Zhang et al.; Pei et al. 2003; Schuster et al. 2007; Ericson et al. 2008; Amit et al. 2009; Licht et al. 2009; Yasumoto et al. 2009), it may be the primary target of paroxetine that affects other genes downstream. Therefore, low levels of serotonin transporter expression in the system may have made detection of gene expression changes in other downstream candidate transcripts difficult. Fourthly, the problem may lie with the reporter gene system itself. Various *cis*- and *trans*-regulatory elements involved in transcriptional regulation have been suggested

(Altshuler et al. 2010; Georges et al. 2010; Piechota et al. 2010). The segment of promoter that was cloned into reporter gene plasmid may not contain the site of antidepressant action. For example, it is plausible that antidepressants work by changing the chromatin structure of genes leading to changes in gene activity (Wilkinson et al. 2009; Perisic et al. 2010), and a system that only examines the linear structure of a promoter is unlikely to be sensitive to such processes .

Development of the reporter gene system would have benefited from including a good positive control reporter construct. However, I was unaware of any promoter that could be used as such a positive control in the antidepressant exposure experiments. The numerous candidate promoters that were analysed were all selected from potentially antidepressant-responsive genes, but none of these demonstrated robust expression changes in the assay. Although no antidepressant responsive control construct was available, once discovering the effect of valproate on *Spr* by qPCR analysis (section 4.3.2), I was subsequently able to demonstrate that the *SPR* promoter was responsive to valproate in the luciferase system (section 5.3.5). This experiment demonstrated that a drug induced gene expression change could be detected and replicated in this system, although the absence of reporter construct that behaves in a similar way after antidepressant exposure would be an ongoing issue for this experimental system.

6.1.2 The qPCR model system

As an alternative, more direct approach to detect gene expression changes in RN46A cells following drug treatment than the reporter gene system, qPCR was employed. The high-throughput nature of qPCR enabled the analysis of more drugs in this part of the work than for the reporter gene assays.

RN46A cells were exposed to 0.5µM paroxetine, citalopram, nortriptyline, haloperidol or 1mM sodium valproate for 72 hours. Five candidate reference genes were tested to normalise samples for differences in quality and quantity. *Actb*, *G6pd* and *Rnf4* were chosen as the reference genes because they were stably expressed across all five drug treatments throughout the entire 36 cDNA samples. Expression differences of 39

candidate target genes were determined by qPCR, with 23 of them failing to be expressed in this system. Comparison of qPCR Cp values with prior microarray data provided strong evidence that the majority of genes failing to amplify in qPCR had low levels of expression in RN46A cells. No amplification of the serotonin transporter transcript in qPCR and its low expression detected in microarray experiments confirmed the absence or lack of expression of the serotonin reporter in undifferentiated RN46A cells (McHugh et al. 2009a).

Among the 16 target genes that were successfully amplified in this approach, seven showed significant expression changes upon exposure of RN46A cells to different drugs. Treatment with sodium valproate increased the expression of *Spr* (1755±413), *Hdac6* (6.03±1.40), *Hdac1* (1.89±0.19), *Hdac3* (1.71±0.20) and *Qdpr* (1.40±0.08). The *Htr2a* transcript was down-regulated upon exposure to two SSRIs: paroxetine (0.57±0.06) and citalopram (0.71±0.06). *Hdac2* was down-regulated two fold upon exposure to all the drugs tested except sodium valproate. In contrast, *Hdac1* showed up-regulation upon paroxetine exposure of RN46A cells (3.26±0.73).

In comparison to the luciferase reporter gene system I used initially, qPCR proved to be a more sensitive method to detect subtle gene expression changes caused by antidepressant drugs. The technique was more advantageous than the reporter gene system in that it is more direct and involves fewer manipulations, such as transfection, lysis and luciferase measurement. Although it is well known that using one reference gene for data normalisation is insufficient (Vandesompele et al. 2002; Bustin et al. 2005), many studies still use one reference gene for qPCR analysis. Large gene expression changes were detected in some of the single reference gene studies (Lee et al. 2007; Zaravinos et al. 2009), but the reliability of these findings may be questionable, and should be validated in experiments using more than one reference gene. Given the attention paid to stable reference genes, and the large number of repeated assays used here, I am confident that the gene expression changes reported above are robust and highly reproducible.

6.1.3 Sodium valproate experiments

Although the initial focus of this project was on antidepressants, significant gene expression changes were observed serendipitously when sodium valproate was used as a control for drug specificity. This occurred when a nearly 1800 fold increase (1755 ± 413) in *Spr* gene expression was detected with cells treated with valproate. As an important drug in the treatment of mood disorders, and in addition a robust positive control for this kind of experiment, it seemed reasonable to explore sodium valproate pharmacogenetics further in this model system. The biological significance of the *Spr* gene, on which valproate had such a marked effect, made this an intriguing finding. Since *Spr* is an essential enzyme involved in the biosynthesis of neurotransmitters, and has been strongly implicated in mood disorders, it may be a highly relevant and interesting target for psychoactive drugs.

The relevance of the *Spr* transcriptional finding to SPR protein levels was studied using western blotting. Two independent experiments were carried out to measure whether the SPR protein was also up-regulated in RN46A cells. I confirmed SPR was significantly increased in valproate-treated RN46A cells by 1.6 fold. Although this difference was much less than observed by qPCR, this result confirmed that the large increase in transcript resulted in an elevated protein concentration, which may have a strong bearing on valproate's mechanism of action.

In order to explore the signaling pathways that are affected by valproate exposure of RN46A cells, a commercially available multi-kinase ELISA array was utilized that interrogates protein phosphorylation changes in the ERK pathway, the canonical Wnt signalling pathway and the inositol/phosphatidylinositol pathway. No significant alterations in the three signalling pathways were detected. This preliminary analysis suggests valproate does not act through any of these pathways in RN46A, and the observed gene expression changes must be brough about by other means.

In summary, antidepressants did not elicit large gene expression effects in this study; neither did they cause big changes in previous microarray studies conducted in our

laboratory (McHugh et al. 2009a). Since large gene expression changes were detected when valproate was administered, it is possibly in the nature of antidepressant drugs that they only have small effects on multiple genes; or effects on limited number of genes which were not targeted by this candidate gene approach.

6.2 Future work

This thesis has led to several directions for future research. They may prove to be rewarding in exploring the molecular pathways through which sodium valproate acts to upregulate *Spr*. Changes that could be made to improve the model system will also be suggested. However, due to the constraints of time and resources, these suggested experiments were not pursued.

6.2.1 Reporter gene assays

Several alterations to the experimental design of the luciferase reporter gene system could be made to enhance the chance of detecting gene expression changes caused by antidepressants. The first may be to test a wider range of gene promoters to increase the chance of detecting antidepressant-induced gene expression changes. Future studies could also use the pGL4 luciferase reporter vector rather than pGL3-Basic, as it is better optimised for expression in mammalian cells. More importantly, it has been improved to reduce consensus TF binding sites and reduce background transcription, which has been shown to be a problem with pGL3-basic (Dougherty and Sanders 2005). We could also insert larger fragments of each promoter region into the reporter vector to increase the opportunity of covering major *cis*-regulatory elements of candidate genes (Li et al. 1999; Gehrke et al. 2003; Martinelli and De Simone 2005; Ogawa et al. 2007). Since antidepressants may act via regulatory elements other than the promoter, this would increase the chance of detecting antidepressant induced gene expression changes, although the size of constructs will always limit this approach.

6.2.2 Functional study

Since *Spr* gene upregulation occurred at the translational level *in vitro*, it would be informative to examine the effects of sodium valproate on *Spr* gene and protein levels in animal brain, to begin exploring the physiological significance of this finding. This work could initially be carried out in rodents or other model species, through *in situ* hybridisation and immunohistochemistry repectively (Levine et al. 1990). The involvement of SPR in BH_4 biosynthesis suggests that measurement of BH_4 might also be a useful line of investigation for the effects of valproate.

6.2.3 Behavioural study

Though exploring the function of the *Spr* gene and protein at the cellular and molecular level may be important for understanding the action of valproate, investigating *Spr*'s impact on specific rodent behaviour could further assist understanding of the role of *SPR* in valproate response and/or mood disorder aetiology. For example, knockout or knockdown models of the *Spr* gene in animal behavioral models relevant to mood disorders (Chen et al. 2006; Takazawa et al. 2008; Yan et al. 2010), such as the forced swim test or the tail suspension test, which model aspects of depression, can be utilised to initiate behavioral studies. The information obtained from such behavioral studies could be used to assess the physiological relevance of *SPR* in drug response and disease aetiology.

6.2.4 Association study

The clinical relevance of the *SPR* findings could also be investigated in association studies. In order to achieve this, polymorphisms in the upstream promoter region or the entire length of the gene can be screened by sequencing. SNPs identified can then be analyzed for associations with mood disorders and valproate/antidepressants response. For example, McHugh et al (2009) discovered polymorphisms in the *SPR* gene that were associated with bipolar disorder I in a family study of mood disorders (McHugh et al. 2009b). A SNP in the GTP-cyclohydrolase I feedback regulator gene, which encodes an enzyme critical in the BH_4 pathway, was also found to be associated with

antidepressant response (McHugh et al. 2010a).

6.2.5 New cell lines

The lack of major antidepressant-induced gene expression changes in the model system employed here does not mean that antidepressant drugs do not cause gene expression changes. Several factors could be altered to improve the system. The use of RN46A cells, in particular, could be the limiting factor in our exploration of candidate antidepressant responsive genes. Poor expression of serotonin transporter in undifferentiated RN46A cells may be an important factor that prevented us from detecting gene expression changes caused by antidepressants, especially paroxetine. Therefore, it would be useful to carry out antidepressant - induced gene expression analysis in other serotonergic cell lines such as CA77 and 44-2C (Clark et al. 1995) or in differentiated RN46A cells which have been shown to express detectable levels of serotonin transporter (McHugh et al. 2009a). However, serotonergic cell lines may not be the only choice for studying antidepressant pharmacogenomics because antidepressants have been shown to exert their effects through targets other than serotonin transporters (Zhang et al.; Pei et al. 2003; Schuster et al. 2007; Ericson et al. 2008; Amit et al. 2009; Licht et al. 2009; Yasumoto et al. 2009; Yoshimura et al. 2009). Furthermore, instead of using the rat serotonergic cell line used in this study, the use of human cell lines such as human neuronal cell-1 (HCN-1), (Poltorak et al. 1992) or NT2 cell line, a neuronally committed human teratocarcinoma cell line expressing serotonin (White et al. 1994), may yield more relevant results because different species have evolved different transcription factor binding complexes to which genes and drugs may interact with in a species dependent manner.

6.3 Concluding remarks

This thesis examined the effects of drugs used to treat mood disorders on gene regulation using two tools, the luciferase reporter gene system and real-time qPCR. The project sought to provide new knowledge about the therapeutic response to drugs used to treat mood disorders, and perhaps identify genes relevant to mood disorders. The

qPCR approach successfully demonstrated that sepiapterin reductase (Spr) was massively and rapidly upregulated by sodium valproate. The SPR protein levels in RN46A were measured semi-quantitatively by western blot and found to be significantly increased. However, the physiological and clinical relevance of this finding has still to be demonstrated. Nonetheless, this study has offered some insights into gene regulation by mood drugs, which might lead to improved understanding of their molecular actions. The goal of antidepressant or mood stabiliser pharmacogenetics is to shed light on novel markers of drug response, with a possible distant consequence leading to some form of what is currently termed personalized medicine. However, it is evident from this thesis and the literature that considerable work needs to be done before we can achieve these goals (Rasmussen-Torvik and McAlpine 2007; Ferreira et al. 2008; Zhou et al. 2008; Perlis et al. 2009; Kato 2010).

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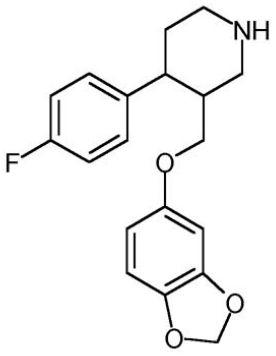
Appendix

Paroxetine



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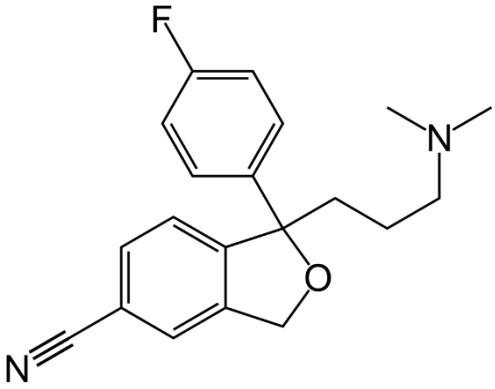

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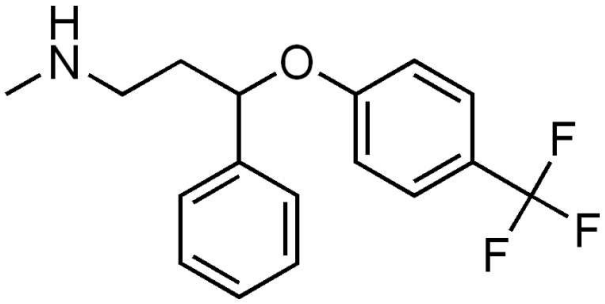

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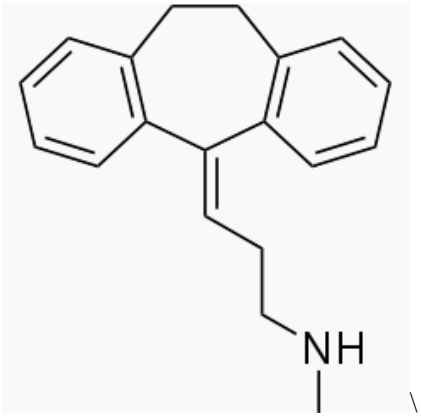
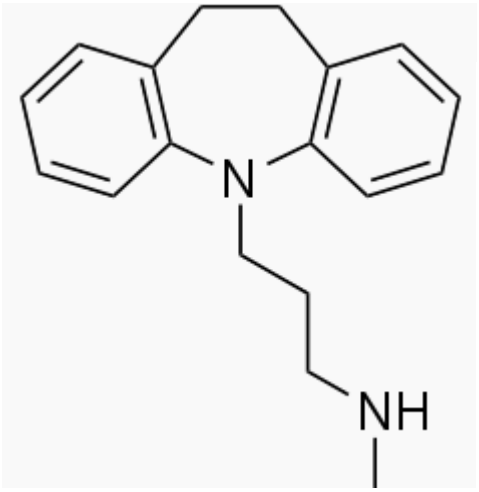


Desipramine



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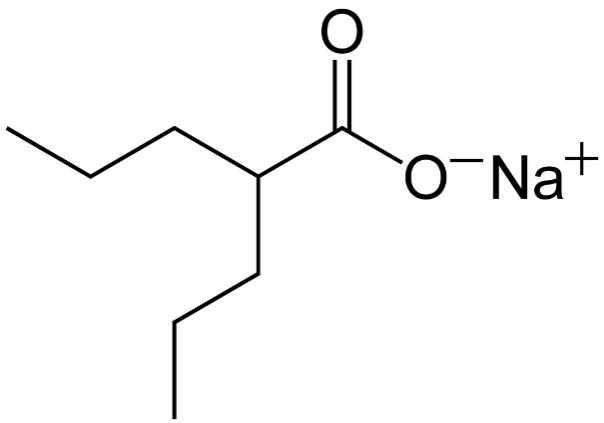
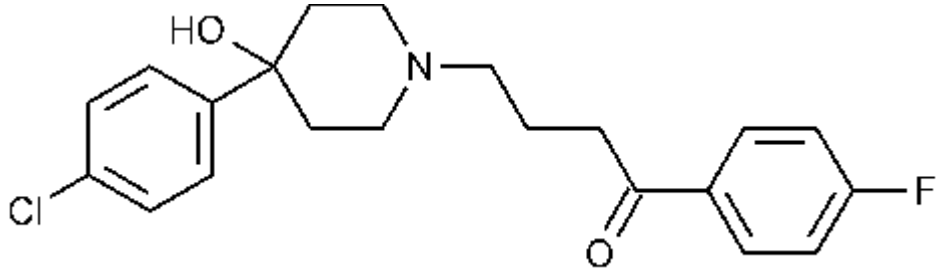


Haloperidol



Appendix 1. Chemical structures of key drugs used. Figures are adapted from Wikepedia (www.wikepedia.com).