The Role of Glutathione Metabolism in the Neuroprotective Effect of Mood Stabilizers

by

Clarissa C. Pasiliao

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for the degree of Master of Science

Department of Pharmacology and Toxicology
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Abstract

Several lines of evidence implicate oxidative stress in the pathophysiology of bipolar disorder (BPD). The mood stabilizers lithium and valproate have been shown to protect against oxidative stress-induced cell death. This study examined whether an increase in cellular reductive potential due to glutathione (GSH) synthesis up-regulation underlies this neuroprotective effect. Using primary rat cortical neurons as a model, this study demonstrated that unlike lithium and valproate, carbamazepine and lamotrigine do not exert neuroprotective effects against H_2O_2-induced cell death. Moreover, the level of GSH and the GSH:GSSG ratio in neurons and in rat brain remained unchanged following chronic treatment with either lithium or valproate. Similarly, this study did not find a significant effect of treatment on the expression of genes encoding γ-glutamylcysteine ligase sub-units, *Gclc* and *Gclm*, in both neurons and the rat brain. These findings suggest that other molecular targets of lithium and valproate likely mediate the observed neuroprotective effects.

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ADHD	Attention deficit/ hyperactivity disorder
4HNE	4-Hydroxynonenal
AIF	Apoptosis inducing factor
ANK3	Ankyrin 3
ANOVA	Analysis of variance
AP-1	Activator protein 1
Ara-C	Cytosine arabinoside
ARE	Antioxidant response element
ATP	Adenosine triphosphate
B27	Serum-free supplement
BDNF	Brain-derived neurotrophic factor
BPD	Bipolar disorder
BPD-NOS	Bipolar disorder-not otherwise specified
BSO	Buthionine sulfoximine
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
ChAT	Choline acetyltransferase
CHOP	C/EBP homologous protein
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
COX 2	Cyclooxygenase 2
CREB	cAMP response element binding protein
DAOA	d-amino acid oxidase activator
DGKη	Diacylglycerol kinase eta
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSM-IV TR	Diagnostic and Statistical Manual, Fourth Edition, Text Revision
DTI	Diffusion tensor imaging
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
fMRI	Functional magnetic resonance imaging
GAPDH	Glyceraldehyde-3-phosphase dehydrogenase
GCL	Glutamylcysteine ligase
GCLC	Glutamylcysteine ligase, catalytic subunit
GCLM	Glutamylcysteine ligase, modifier subunit
GFAP	Glial fibrillary acidic protein

GPx	Glutathione peroxidase
GRed	Glutathione reductase
GRK3	G-protein coupled receptor kinase 3
GRP	Glucose regulated protein
GS	Glutathione synthetase
GSH	Reduced glutathione
GSH-T	Total oxidized and reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-Transferase
GSTNB	Glutathione 5-thio-2-nitrobenzoic acid conjugate
HBSS	Hank's buffered salt solution
HMOX-1	Heme-oxygenase 1
HSF	Heat shock factor
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
iPSC	induced pluripotent stem cells
ITPKA	inositol-1,4,5-triphosphate kinase A
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MEK	Mitogen-activated protein kinase kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAA	N-acetylaspartate
NAC	N-acetylcysteine
NBM	Neurobasal media
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartic acid
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 1% Tween
PEI	Polyethyleneimine
PERK	Protein kinase R-like endoplasmic reticulum kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B

PKC	Protein kinase C
PLC	Phospholipase C
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TRPC	Canonical transient receptor potential cation channel
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UPR	Unfolded protein response
WMH	White matter hyperintensities
XBP 1	X-box binding protein 1

1 Introduction

1.1 Overview of bipolar disorder

Bipolar disorder (BPD) is classically defined as a type of mood disorder characterized by manic and depressive episodes interspersed with normal mood. Symptoms of mania as described in the Diagnostic and Statistical Manual IV-TR (DSM-IV TR) include an overly out-going or irritable mood accompanied by increased goal-directed activities, expansive mood that can intensify to thoughts of grandiosity, decreased need for sleep, over-activity, and engagement in risky activities. Meanwhile, loss of interest and persistent feelings of worthlessness, anhedonia, lethargy, sleep disturbance (insomnia or hypersomnia), marked weight fluctuations, and suicidal ideation are cardinal symptoms of a depressive episode (DSM-IV TR). Under the DSM-IV TR, BPD is divided into 4 subtypes. Diagnosis of BPD type I is based on the presentation of a full manic episode that may be followed by bouts of depression. Patients who experience symptoms of hypomania, a milder and less debilitating form of mania, and depression are diagnosed with BPD type II. Cyclothymia is characterized by the presence of mild, brief hypomanic and depressive episodes, while a diagnosis of BPD-Not Otherwise Specified (BPD-NOS) shows BPD symptoms that impair functioning but do not meet the criteria set for the other subtypes (DSM-IV TR).

Besides the aforementioned changes in mood, BPD patients may also exhibit cognitive deficits and a range of psychiatric and medical co-morbidities that further contribute to functional impairment. On neuropsychological tests measuring attention, memory, and facets of executive function, patients with BPD performed worse relative to age-, gender-, and education-matched healthy controls (Malhi et al., 2007; Martinez-Aran et al., 2004). The persistence of these deficits even during euthymia (Martinez-Aran et al., 2004; Mur et al., 2007; Sanchez-Morla et al., 2009; Schretlen et al., 2007) suggests that impairment is a state -independent trait of BPD. Moreover, studies showing a correlation between verbal memory impairment and lower psychosocial function (Martinez-Aran et al., 2007; Sanchez-Morla et al., 2009) highlight the impact of these deficits on overall functioning.

In addition to cognitive impairments, the increased frequency of a concurrent psychiatric disorder has also been noted. In a sample population comprised of patients diagnosed with BPD-I or BPD-II, it was reported that nearly two-thirds of the patients were also diagnosed with substance use disorder, anxiety disorder, or eating disorder during their lifetime (McElroy et al.,

2001). Similarly, other studies have also reported rates of co-morbidity ranging from 30 to 75% (Fajutrao et al., 2009). Among the clinical or DSM-IV Axis I disorders, substance use and anxiety disorders were equally prevalent, with both accounting for majority of the diagnoses (Fajutrao et al., 2009; McElroy et al., 2001). Within paediatric patients, high rates of co-morbid attention-deficit/ hyperactivity disorder (ADHD) have also been reported (Goldberg, 2009).

Another factor contributing to the burden of BPD is the increase in prevalence of medical illnesses. In a bipolar outpatient clinic sample, 1 in 10 patients presented with a disease affecting the circulatory system (Beyer et al., 2005). This finding was further extended by another study demonstrating that patients with BPD-I are five times more likely to develop cardiovascular disease and hypertension at an earlier age relative to age-, race-, and gender-matched controls (Goldstein et al., 2009). In addition, diseases of the respiratory system have also been shown to be highly prevalent, with one study indicating that respiratory illness affected almost three-quarters of patients with BPD-1(Kemp et al., 2009). Other common medical illnesses that have been identified include diseases affecting the nervous system, the endocrine system, and metabolism (Beyer et al., 2009). These co-morbidities, which negatively affect quality of life and functioning, have been associated with poor response to mood stabilizers (Kemp et al., 2009) and worse prognoses (Thompson et al., 2006).

A variety of pharmacological approaches including lithium, anticonvulsants, antipsychotics, and antidepressants have been approved for symptomatic treatment. These interventions are evaluated based on their efficacy in treating mania and bipolar depression as well as in prevention of relapse and maintenance. Serendipitously discovered in 1949 for its efficacy in treating acute mania (Cade, 1949), lithium is a standard mood stabilizer and remains a first-line treatment for acute mania, hypomania, depression, and prevention of relapse (Levine, 2009). A number of anticonvulsants including valproate, carbamazepine, and lamotrigine, have been used as alternatives or adjuncts to lithium. Valproate is prescribed in the treatment of acute mania and hypomania (Kusumakar, 2009) and has also been shown to have moderate efficacy in treating acute depression and in preventing depressive relapse (Malhi et al., 2009a). While carbamazepine has been shown to exhibit antimanic and antidepressant properties in BPD patients, limited data support its efficacy as a prophylactic agent (Yatham, 2009). Another anticonvulsant, lamotrigine, has been shown to have efficacy in hypomania, acute bipolar depression, and prevention of depressive relapse, but not in mania (Yatham, 2009). With regards

to antipsychotics, randomized controlled trials have demonstrated the efficacy of second generation antipsychotics in acute mania and as an adjunct to standard mood stabilizer therapy (Bond, 2009).

Although the number of psychotropic medications indicated for BPD has increased since the discovery of lithium, optimal pharmacotherapy remains a challenge. Side effects associated with medications limit their clinical use. Lithium, which has a narrow therapeutic index, can cause toxicity at serum levels higher than 1.2 mM (Chen et al., 2004b) and has been associated with hypothyroidism and decreased renal function following prolonged treatment (Malhi et al., 2009b). Lithium, valproate and atypical antipsychotics with the exception of ziprasidone and aripiprazole can cause potential weight gain while lamotrigine and carbamazepine have been associated with rashes (Malhi et al., 2009b). Moreover, the achievements in symptom management have not been paralleled by improvements in functional outcome (Tohen, 2005). For example, in a prospective study involving patients experiencing their first manic or mixed episode, less than half of the patients achieved functional recovery compared to nearly three-quarters reporting symptomatic recovery (Tohen et al., 2003). Hence, there is still a need to develop more efficacious pharmacological interventions for BPD.

Affecting 1-1.5% of the human population, BPD I alone has been ranked 6th among the 10 leading causes of disabilities among adults between 19-44 years worldwide (WHO). As mentioned previously, the symptoms and co-morbidities associated with BPD impair functioning and impose a personal burden. In addition to personal suffering, BPD also exacts a high socioeconomic toll. The results of an American study involving family caregivers indicate that BPD puts a strain on the physical health and finances of family members (Perlick et al., 2007). An economic study estimated the prevalence cost of BPD in the United States at $45 billion in 1991 (Wyatt and Henter, 1995). In the UK, the total amount of direct expenses and indirect costs including lack of employment and loss of human capital due to suicide was estimated at £2 billion in 1999/2000 (Das Gupta and Guest, 2002). Together, these epidemiological data position BPD as a major public health concern and underscore the need for improved treatment based on a greater understanding of the pathophysiology of this disorder.

1.2 Neurobiological disturbances in BPD

While the exact causes of BPD have not yet been clearly identified, accumulating evidence suggests that BPD arises from an interaction between environmental and genetic/biological factors. On the one hand, studies have shown that environmental adversity including stressful life events and lack of social support contribute to the onset, progression, and expression of BPD (Alloy et al., 2005). On the other hand, neurobiological disturbances at the systemic, molecular and cellular levels associated with BPD have also been well-documented. It is, however, unclear whether these disturbances are causes or consequences of BPD. While both behavioural and pharmacological interventions could manage the symptoms of the disorder, this study largely focused on the molecular mechanisms that may be involved in the action of mood stabilizers. The goal of assessing the relevance of these mechanisms to the drugs' therapeutic efficacy would be aided by an understanding of the neurobiological disturbances associated with BPD. Hence, the sections hereafter review the current literature on imaging, genetics, and cellular and molecular biology that have contributed towards elucidating the pathophysiology of BPD.

1.2.1 Systemic level

1.2.1.1 Results of functional imaging studies

At the systemic level, advances in neuroimaging have aided the search for neurobiological substrates of mood dysregulation and neurocognitive deficits that accompany BPD. Functional magnetic resonance imaging (fMRI), which involves the measurement of cerebral blood flow, provides a measure of brain activity. Used in conjunction with neuropsychological tests, fMRI has facilitated the inference of neural circuitries based on task-related changes in activity within different brain regions. This approach has been widely used to identify altered neural networks in unipolar depression, and emerging data support its relevance towards elucidating the potential neural circuitry(ies) dysregulated in BPD. Evidence of abnormalities within neural circuits controlling affect regulation arises from reports of altered emotion processing noted in BPD patients. An abnormal pattern characterized by an increase in striatal activity and a decrease in activity within the dorsolateral pre-frontal cortex was detected in BPD patients relative to healthy volunteers in response to emotional facial expressions (Hassel et al., 2008) . Another study examining emotional memory processing found that BPD patients exhibited greater

hippocampal activation in response to emotional stimuli relative to healthy controls and patients with schizophrenia (Whalley et al., 2009). Furthermore, processing of negative emotional stimuli was found to be associated with lower prefrontal cortex activity and increased activation of the left hippocampus and the right amygdala in euthymic patients relative to healthy controls (Lagopoulos and Malhi, 2007).

The integration within a neural network can be further characterized by assessing temporal correlations of regional activities (functional connectivity) and the influence of these activities on other components of the circuit (effective connectivity) (Friston et al., 1997). Using this approach, Foland and coworkers (Foland et al., 2008) reported a decrease in the functional connectivity between the ventrolateral prefrontal cortex and the amygdala in manic patients relative to healthy controls while performing an emotion labelling task. In another study examining the circuitry within the ventromedial system using a similar task, increases in the effective connectivity between the parahippocampal gyrus and the subgenual cingulate gyrus was observed in symptomatically remitted patients relative to healthy controls in euthymic patients. Together, these reports of disturbances within limbic, paralimbic and related cortical regions suggest that abnormalities within the medial prefrontal network likely underlie the impairments in mood regulation.

Complementary to studies on affect regulation, several investigators have also examined the neural correlates of cognitive dysfunction in BPD. For instance, by engaging the neural network associated with executive control using a counting Stroop task, Strakowski and colleagues (Strakowski et al., 2005) observed a decrease in activity within regions of the temporal cortex and the ventrolateral prefrontal cortex. Using the same task, another study reported a decrease in activity within the right inferior and medial frontal gyri—independent of mood symptoms—in patients diagnosed with BPD-I (Roth et al., 2006). More recently, impairments in working memory have been linked to differential activation of the fronto-temporal network based on observations in symptomatically remitted patients performing a delayed non-match-to-sample task (Robinson et al., 2009). Interestingly, the reported decrease in activity within neural networks implicated in executive function may be accompanied by hyperactivity of circuitries associated with affect regulation. In unmedicated euthymic patients performing an attentional task, an increase in activity was detected within the limbic, paralimbic, and ventrolateral prefrontal cortex—regions which form the network associated with emotional arousal

(Strakowski et al., 2004). Collectively, these findings suggest that BPD is associated with imbalances in circuits controlling executive function and emotion.

1.2.1.2 Results of structural imaging studies

Further evidence of neuropathology in BPD arises from reports of structural brain changes in patients diagnosed with this disorder. Previously assessed by computed tomography, brain volume and morphometry are currently measured using MRI, which reduces exposure to ionizing radiation (Na et al., 1991). In addition to safety benefits, MR images also feature improved resolution which facilitates measurement of smaller regions and better discrimination between white and gray matter (Konarski et al., 2007).

White Matter

Studies examining white matter have revealed abnormalities within this tissue, which is highly involved in brain connectivity. The coherence and orientation of white matter tracts can be estimated through measures of water diffusion (apparent diffusion coefficient) and directionality of diffusion (anisotropy) obtained through diffusion tensor imaging (DTI). Using this MR-based technique, Adler and colleagues (2004) first reported lower fractional anisotropy in patients with BPD within white matter tracts connecting the prefrontal cortex with subcortical and other cortical regions suggesting disturbances in the organization of these axons. Since then, these findings have been replicated (Bruno et al., 2008) and extended by a number of studies reporting alterations within the corpus callosum (Barnea-Goraly et al., 2009; Chaddock et al., 2009; Wang et al., 2008b; Yurgelun-Todd et al., 2007) as well as in fibre bundles connecting brain regions implicated in emotional, behavioural, and cognitive regulation (Barnea-Goraly et al., 2009; Chaddock et al., 2009; Haznedar et al., 2005; Kafantaris et al., 2009; Mahon et al., 2009; McIntosh et al., 2008; Pavuluri et al., 2009; Sussmann et al., 2009; Versace et al., 2008; Wang et al., 2008a; Wang et al., 2009a; Zanetti et al., 2009)

The presence of these alterations during the early stages of the disorder is supported by reports of changes in paediatric BPD patients (Barnea-Goraly et al., 2009; Frazier et al., 2007; Kafantaris et al., 2009) as well as in medication-naïve adolescents experiencing their first manic episode (Adler et al., 2006) relative to healthy controls. Differences in anisotropy between healthy controls and unaffected relatives of BPD patients (Chaddock et al., 2009; Frazier et al.,

2007) also suggest a possible hereditary influence. Moreover, reports of differences in anisotropy between healthy controls and BPD patients in remission (Wessa et al., 2009; Yurgelun-Todd et al., 2007) suggest that structural changes in white matter are likely to be state-independent. Although speculative, these findings suggest that disruptions in microstructure and organization of white matter may represent an endophenotype of BPD.

In addition to studies using DTI, involvement of white matter abnormalities in the pathophysiology of BPD has also been supported by MRI studies measuring white matter hyperintensities (WMH). Often identified as bright spots in T2-weighted and fluid-attenuated inversion recovery MR images taken of patients diagnosed with cardiovascular disease (Jeerakathil et al., 2004), WMH have been pathologically associated with ischemia-induced damage, interruptions in white matter fibres, and local demyelination. Although a number of studies argue against its association with BPD (Chang et al., 2005; McDonald et al., 2004b; Sassi et al., 2003), findings of increased frequency of white matter hyperintensities in BPD have been confirmed by a recent meta-analysis of 98 independent neuroimaging studies (Kempton et al., 2008). Individual studies further examining this association have noted an increase in the frequency of WMH in brains of patients with BPD relative to patients with unipolar depression (Silverstone et al., 2003b) and schizophrenia (Pillai et al., 2002; Regenold et al., 2008). Moreover, an increase in WMH has also been shown to correlate with a number of traits related to BPD. For instance, Gulseren and colleagues (2006) reported a positive correlation between the number of manic episodes and incidence of WMH. Interestingly, frequency and severity of WMH have also correlated inversely with degree of symptomatic recovery and length of euthymic periods (Moore et al., 2001). Moreover, Regenold and colleagues (2008) also showed a positive correlation between volumes of WMH and treatment resistance as indicated by the number of relapses requiring hospitalization per year over the span of 5 years.

Another focus of MRI studies in BPD has been the analysis of white matter volume. A modest decrease in white matter volumes was observed in patients experiencing their first manic episode relative to healthy controls (Rosso et al., 2007; Strakowski et al., 1993). Moreover, significant reductions in white matter volume were reported within the inferior cingulate cortex (Haznedar et al., 2005) as well as in regions of the frontal (Haznedar et al., 2005; McDonald et al., 2004b), parietal (McDonald et al., 2004b), and temporal (Chen et al., 2004a; McDonald et al., 2004b) lobes of patients with BPD relative to healthy controls. Interestingly, Davis and

colleagues (2004) reported that total cerebral white matter volume was reduced in male euthymic BPD I patients who have a family history of BPD I relative to healthy controls. Additionally, studies comparing brain volumes of twin pairs affected with BPD and healthy control twin pairs showed reduced white matter volume in both the affected twin and the unaffected co-twin (Kieseppa et al., 2003; van der Schot et al., 2009), suggesting that genetic risk may underlie the increased vulnerability of white matter tissue associated with BPD

However, findings of decreased white matter volume have not been consistent. Nugent and colleagues (2006) reported an increase in white matter volume within specific regions of the prefrontal cortex in medicated and unmedicated BPD patients, which may reflect decreases in volume of adjacent gray matter (Nugent et al., 2006). Alternately, increases in white matter volume may also be due to the effect of medication as Jones and colleagues (2009) found that white matter volumes within the temporal lobe were greater in a sub-set of BPD patients taking antipsychotic medication relative to patients who were taking other psychotropic medications. In addition to the reported increases in white matter volume, several studies have also shown no difference in total (Brambilla et al., 2001; Lim et al., 1999; Sassi et al., 2002; Scherk et al., 2008b; Zipursky et al., 1997) or regional (Lopez-Larson et al., 2002; Zipursky et al., 1997) white matter volumes between BPD patients and healthy controls. While differences in image acquisition and data analysis may account for some of the discrepancies, these seemingly conflicting results suggest that white matter abnormalities may only be present in select BPD sub-groups. Studies examining the association between white matter volume and genetic liability for BPD, as well as responsiveness to mood stabilizer treatment, may contribute towards greater understanding of white matter pathology in BPD.

Gray Matter

As with results of studies examining structural changes in white matter, findings regarding the association between BPD and changes within the gray matter have been equivocal. A number of studies have presented evidence suggesting that BPD is linked to a reduction in gray matter volume or density. Consistent with the results of decreased blood flow to the subgenual prefrontal cortex in depressed, medication-naïve patients diagnosed with BPD, patients with BPD had lower gray matter volumes within the subgenual prefrontal cortex, irrespective of mood state (Drevets et al., 1997). Similarly, lower gray matter volumes were observed within

the left and right prefrontal cortex of patients hospitalized for a manic episode (Lopez-Larson et al., 2002). Interestingly, in euthymic patients, a decrease in gray matter volumes only within the right subgenual prefrontal cortex was detected (Sharma et al., 2003). The influence of mood states has also been underscored by findings of decreased gray matter density within left and right prefrontal cortex of depressed patients when compared to euthymic patients (Brooks et al., 2009). In addition to the prefrontal cortex, gray matter loss has also been observed in other brain regions including the cingulate (Doris et al., 2004; Farrow et al., 2005; Koo et al., 2008; Lyoo et al., 2004; McDonald et al., 2004a; Sassi et al., 2004), the hippocampus (Moorhead et al., 2007), the parietal lobe (Adler et al., 2005; Doris et al., 2004; Frazier et al., 2005; Ha et al., 2009; Haldane et al., 2008; Zipursky et al., 1997), and the temporal lobe (Chen et al., 2007b; Doris et al., 2004; Farrow et al., 2005; Frazier et al., 2005; Ha et al., 2009; Lochhead et al., 2004; Nugent et al., 2006; Zipursky et al., 1997).

Several studies have presented evidence suggesting that these reductions may be evident during the early stages of BPD. Smaller gray matter volumes have been detected within the portions of the cingulate (Kaur et al., 2005; Wilke et al., 2004), dorsolateral prefrontal cortex (Dickstein et al., 2005), and medial temporal lobe (Wilke et al., 2004) of paediatric patients, suggesting that reductions are not solely due to the influence of normal aging on brain volume. To examine the progression of volumetric decline in gray matter over the course of illness, Koo and colleagues (2008) conducted a cross-sectional and longitudinal study comparing healthy controls with patients with first-episode affective psychosis, most of who were in the manic phase of BPD. Initial scans revealed bilateral reductions in gray matter volume within the subgenual cingulate gyrus of BPD patients which progressed over 1.5 years at a rate greater than that observed in healthy controls (Koo et al., 2008). A faster rate of gray matter loss within the hippocampus, fusiform gyrus, and cerebellum of patients with BPD I has also been reported by a 4-year longitudinal study (Moorhead et al., 2007).

The notion that loss of gray matter is involved in the pathophysiology of BPD is contested by reports of comparable gray matter in patients and healthy controls. In a paediatric sample, similar gray matter volumes were observed in BPD patients and healthy controls (Chang et al., 2005). Similarly, brain morphometric analyses in adults suggest that BPD is not linked with changes in gray matter (Beyer et al., 2009; Brambilla et al., 2001; Caetano et al., 2001; Jones et al., 2009; McDonald et al., 2005; Scherk et al., 2008b; Zipursky et al., 1997). Moreover,

increases in gray matter volume in various sub-regions of the cortex (Adler et al., 2005; Frazier et al., 2005; Lochhead et al., 2004; Najt et al., 2007), the cingulate (Adler et al., 2005), the limbic system (Haldane et al., 2008), and the cerebellum (Kempton et al., 2008) have also been observed. These reported differences may be attributed to medication status. For example, treatment of patients with BPD I or II with lithium for 4 weeks resulted in an increase in total and regional gray matter (Moore et al., 2009). Similar effects of lithium treatment were also observed by Sassi and colleagues (2002) who reported greater intracranial volumes and total gray matter in patient on lithium monotherapy relative to healthy controls and unmedicated patients.

1.2.2 Cellular level

Although the BPD brain does not exhibit the neuropathological hallmarks of classical neurodegenerative disorders (Rajkowska, 2002), reports of alterations in gray and white matter tissue suggest that cellular changes are also present in this mood disorder. As discussed below, multiple lines of evidence arising from *in vivo* imaging, neuropathological examinations, and *in vitro* studies support the notion that a decrease in cellular resilience—defined as the ability of cells to adapt to environmental and/or cellular stresses (Hunsberger et al., 2009)—may be involved in the pathophysiology of BPD.

In addition to choline, changes in the concentration of N-acetylaspartate (NAA), an amino acid found at high concentrations in neurons (Urenjak et al., 1993), have also been the focus of ^{1}H-MRS studies in BPD. It has previously been shown that a loss of neuronal integrity is accompanied by decreased NAA levels in the absence of changes in choline levels (Guimaraes et al., 1995; Howe et al., 1993). Hence, numerous studies on the neuropathology of BPD have used this metabolite as a marker of neuronal function. In a sample of euthymic patients diagnosed with either BPD I or II, Winsberg and colleagues (2000) observed a bilateral decrease in the proportion of NAA to creatine relative to healthy controls within the dorsolateral prefrontal cortex. Consistent with these results, more recent studies have also reported lower NAA levels within the prefrontal cortex (Molina et al., 2007) and the hippocampus (Atmaca et al., 2006; Deicken et al., 2003; Scherk et al., 2008a) of BPD patients relative to age- and gender-matched healthy controls. The affected regions, however, may vary depending on BPD sub-type. For instance, in a mixed sample of paediatric BPD patients, lower levels of NAA were detected within the left dorsolateral prefrontal cortex (Olvera et al., 2007; Sassi et al., 2005). On the contrary, Chang and colleagues (Chang et al., 2003c) reported a decrease in NAA levels within the right dorsolateral prefrontal cortex of young patients with a family history of BPD, suggesting a separate pathophysiology for familial BPD. Collectively, these reports of decreased NAA suggest that selective neuronal loss is associated with BPD.

The findings, however, have not been consistent. A number of studies have reported a lack of change in NAA levels between patients and healthy subjects (Amaral et al., 2006; Colla et al., 2009; Michael et al., 2009; Ohara et al., 1998). This difference may be due in part to medication

status as chronic treatment with lithium (Moore et al., 2000; Silverstone et al., 2003a) and valproate (Atmaca et al., 2007) have been associated with elevated NAA levels.

Results of neuropathological studies also present evidence indicating that changes in cell number and morphology are involved in the pathophysiology of BPD. In a post-mortem study, Ongur and colleagues (1998) observed reductions in glial density in patients with a family history of BPD within the subgenual prefrontal cortex when compared against non-psychiatrically ill controls. Employing a 3-dimensional cell counting technique, Rajkowska and colleagues (2001) also noted a decrease in glial density attributed to fewer mid-sized glia within the dorsolateral prefrontal cortex. These observations, however, may be region-specific as differences in glial density between patients with BPD and controls have not been detected in other brain regions including the supragenual anterior cingulate cortex (Benes et al., 2001), the amygdala (Bezchlibnyk et al., 2007; Hamidi et al., 2004), and Heschl's gyrus (Cotter et al., 2001).

In conjunction with glial abnormalities, a significant decrease in neuronal somal size within the anterior cingulate cortex was observed in patients with BPD relative to non-psychiatrically ill controls (Chana et al., 2003). Similar decreases in neuronal somal size were also found within specific amygdalar nuclei, namely the lateral amygdalar nucleus and the accessory basal parvocellular nucleus (Bezchlibnyk et al., 2007). Moreover, lamina-specific decreases in neuronal density have also been reported within the layers III and Va of dorsolateral prefrontal cortex (Rajkowska et al., 2001) and non-pyramidal cells within layer II of the anterior cingulate cortex (Benes et al., 2001) of patients with BPD relative to non-psychiatrically ill controls. Collectively, the results of these post-mortem studies suggest that abnormalities leading to neuronal atrophy are associated with BPD.

Recently, the hypothesis that impairments in cellular resilience are involved in BPD has garnered support from results of in vitro studies showing BPD-related alterations in cellular function. For instance, McCurdy and colleagues (2006) reported a significant increase in cell death in cultures of differentiated neurons obtained from biopsied olfactory mucosa of patients with BPD I relative to cultures established from healthy subjects. Further profiling of gene expression revealed that BPD is associated with decreased expression of genes encoding neuronal kinases involved in inositol phosphate metabolism and phosphatidyl inositol signaling,

including inositol-1,4,5-triphosphate kinase A (ITPKA) (McCurdy et al., 2006). This enzyme has been shown to regulate intracellular calcium homeostasis and structural plasticity by terminating IP3 signaling (Irvine et al., 2006) and bundling actin (Johnson and Schell, 2009), respectively. Hence, a decrease in the levels of ITPKA could disrupt cellular function and impair the ability of neurons to adapt to environmental stresses. That BPD is associated with disturbances in regulatory pathways influencing neuronal viability is also supported by the findings of altered expression of pro- and anti-apoptotic genes. The pro-apoptotic factors found by Benes and colleagues (2006) to be upregulated in unmedicated patients include the FAS ligand, RIP, BID, TRAF 1, FADD, MDM-2, caspase-2, p53, and c-myc. This increase was also paralleled by a downregulation in the expression of anti-apoptotic genes including IAP2, NF-κB-p105, and poly (ADP-ribose) polymerase (PARP) (Benes et al., 2006). This imbalance is also evident in the results of a recent post-morten study which revealed that the decrease in brain derived neurotrophic factor (BDNF) and anti-apoptotic Bcl-2 is accompanied by an increase in pro-apoptotic proteins including BAX, BAD, and caspase 3/9 (Kim et al., 2010).

The hypothesis of increased cellular vulnerability to cellular stressors in BPD was elegantly tested by Naydenov and colleagues (2007) using lymphocytes established from patients diagnosed with BPD I or II and healthy controls. This study examined changes in gene expression in response to energy stress induced by glucose deprivation. Under normal glucose conditions, no difference in gene expression was observed between BPD and control lymphocytes; however, under low-glucose conditions, the upregulation of mitochondrion and electron chain-related transcripts in healthy controls was absent in BPD lymphocytes (Naydenov et al., 2007). In fact, the reverse was observed: the electron chain-related transcripts that were elevated in controls were downregulated in BPD lymphocytes (Naydenov et al., 2007). This differential response to stress may reflect a disruption in the ability of some cell types from BPD patients to adapt to changes within their local environment. The resulting decrease in resilience could predispose cells to stress-induced damage which may underlie the pathophysiology of BPD.

1.2.3 Molecular level

Some of the factors that have been suggested to account for heightened cellular vulnerability include (1) genetic predisposition, (2) aberrant signal transduction mechanisms, (3) abnormalities in calcium dynamics, (4) disruptions in unfolded protein response, and (5) mitochondrial dysfunction.

1.2.3.1 Genetic predisposition

Results of family, twin, and adoption studies, which show an increased risk of developing BPD in first-degree relatives of affected patients, allude to a genetic component of this disorder (Craddock and Jones, 1999). Heritability of BPD has also been examined through molecular genetics. Linkage studies have implicated a number of chromosomal regions. For example, fine-mapping of 21q22.3 revealed a significant association between BPD and a locus containing genes encoding C21ORF29/TSPEAR and transient receptor potential melastatin type 2 (TRPM2) (McQuillin et al., 2006), a calcium-permeable ion channel highly expressed in the brain (Nagamine et al., 1998). Interestingly, further studies have revealed an association between BPD and genetic variants of TRPM2 ((McQuillin et al., 2006; Xu et al., 2009; Xu et al., 2006a). Meanwhile studies using the candidate gene approach have provided support that mutations in d-amino acid oxidase activator (*DAOA*) and *BDNF* confer susceptibility to BPD (Craddock, et. al. 2005). Recent advances in genotyping technology coupled with greater understanding of linkage disequilibrium patterns have allowed for genome-wide association studies (Hirschhorn and Daly, 2005). Using this novel approach, studies have identified several risk alleles that are associated with BPD, including single nucleotide polymorphisms (SNPs) within genes coding for diacylglycerol kinase eta (DGK η) (Baum, et. al. 2007), ankyrin 3 (ANK3; Ferreira, et. al. 2008; Smith et. al. 2009; Schulze et. al. 2009; Scott, et. al. 2009), and CACNA1C (Ferreira, et. al. 2008), a subunit of the L-type voltage regulated calcium channel. Interestingly, the A/G variant in CACNA1C previously identified by a genome-wide association study in BPD, was recently found to be associated with decreases in total gray matter volume in healthy subjects (Kempton et al., 2009), providing further evidence for the role of this single nucleotide polymorphism in determining neuronal viability. Given the array of genes that have been implicated, the results of these genetic studies stress the polygenic nature of BPD.

Moreover, the proteins encoded by these putative disease-associated genes may contribute to disruptions in cellular mechanisms highlighted in the next sections.

1.2.3.2 Aberrant signal transduction mechanisms

Signaling mechanisms are responsible for transduction of environmental stimuli into intracellular responses. Thus, the integrity of these systems is important in ensuring proper adaptation to changes in the extracellular milieu. Associations between disturbances in these signaling cascades and BPD have been well-documented. Studies in post-mortem brain and peripheral tissues, namely leukocytes and platelets have reported that BPD is associated with higher levels of $G_{s\alpha}$ (Friedman and Wang, 1996; Manji et al., 1995; Mitchell et al., 1997; Young et al., 1994; Young et al., 1993; Young et al., 1991) and $G_{\alpha q/11}$ (Mathews et al., 1997). In addition, increased receptor-G protein coupling following stimulation with serotonin and thrombin has been observed in platelets from BPD patients but not in healthy controls (Hahn et al., 2005). More recently, Rao and colleagues (Rao et al., 2009) also reported that the levels of G-protein coupled receptor kinase 3 (GRK3), a kinase that influences receptor-G-protein coupling by regulating the desensitization of the agonist-stimulated receptor, are lower in the frontal cortex of BPD patients relative to healthy controls.

The involvement of aberrant G-protein signaling in the pathophysiology of BPD is also indirectly supported by results of molecular pharmacological studies suggesting that the efficacy of mood stabilizers may partly be related to the drugs' effect on the signaling cascade. A decrease in receptor-G protein coupling have been observed following chronic treatment with lithium (Avissar et al., 1988; Hahn et al., 2005), valproate (Hahn et al., 2005), and carbamazepine (Avissar et al., 1990). Also, chronic lithium treatment has been shown to lower the mRNA levels of Gαs (Li et al., 1993) and $G_{\alpha i1/2}$ (Colin et al., 1991; Li et al., 1991; Li et al., 1993) in the rat cortex. In combination with carbamazepine, this mood stabilizer has also been shown to increase the levels of GRK3 in rat frontal cortex (Ertley et al., 2007). Further evidence for the ability of mood stabilizers to attenuate G-protein signalling is derived from clinical studies in BPD patients showing an association between long-term lithium treatment and lower levels of $G_{\alpha s}$ (Karege et al., 1999) and $G_{\alpha q/11}$ (Manji et al., 1995; Mathews et al., 1997).

Consistent with reports of altered G-protein function, changes downstream have also been associated with BPD. Early evidence has been provided by findings of increased protein

phosphorylation in BPD platelets relative to controls following cAMP stimulation (Perez et al., 1995). Further support for the involvement of cAMP signaling has also arisen from studies characterizing cAMP-dependent kinases in BPD. A decrease in the levels of the regulatory sub-units of these kinases has been suggested by reports of lower cytosolic [^{3}H]cAMP binding in brain tissue of BPD patients relative to controls (Rahman et al., 1997). Meanwhile, another study has also found an increase in the immunoreactive levels of the catalytic sub-unit of protein kinase A (PKA), a cAMP-dependent kinase, in platelets of depressed and manic BPD patients but not in platelets of euthymic patients and healthy subjects (Perez et al., 1999). Similarly, Chang and colleagues (Chang et al., 2003a) reported higher levels of catalytic and regulatory sub-units of PKA within frontal and temporal cortices of post-mortem brain from BPD patients relative to matched non-neurological, non-psychiatric controls. Interestingly, this BPD-associated increase in protein levels is not reflected by differences in mRNA levels within post-mortem brains of BPD patients and healthy controls (Chang et al., 2003b) suggesting that post-translational mechanisms may be involved in regulating PKA levels within the brain. That BPD is associated with changes in kinase activity is directly supported by findings of higher PKA activity in BPD platelets (Tardito et al., 2003) and post-mortem brain (Chang et al., 2003a) relative to controls.

In addition to alterations in cAMP signaling, BPD-associated changes in the phosphatidylinositol bisphosphate second messenger system have also been reported. Relative to healthy controls, a decrease in phospholipase C (PLC) activity was observed in platelets of BPD patients (Pandey et al., 2002). Furthermore, abnormalities in the levels of its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2) have also been reported. In lymphoblatoid cells, Banks and colleagues (1990) found that incorporation of [^{3}H] inositol was lower in BPD patients relative to controls despite the lack of difference in the quantity of intracellular inositol between the two groups. The latter finding however is contested by reports of lower levels of free inositol in the pre-frontal cortex (Shimon et al., 1997) and lymphocyte-derived cells (Belmaker et al., 2002) of BPD patients relative to controls. This apparent inconsistency may be attributed to the difference in measuring inositol as Banks and colleagues used thin layer chromatography while gas chromatography was employed in the latter two studies. Interestingly, higher levels of PIP2 have been reported in platelets of manic (Brown et al., 1993) and depressed (Soares et al., 2001) BPD patients. Although further studies comparing PIP2

levels across mood states are needed, these preliminary findings suggest that an increase in PIP2 synthesis may be characteristic of the acute phases of BPD. Downstream, increased membrane association and heightened response to serotonin stimulation of protein kinase C, a target of diacylglycerol, have been documented in the frontal cortex (Friedman and Wang, 1996; Wang and Friedman, 1996) and platelets (Friedman et al., 1993; Wang et al., 1999a) of BPD patients relative to controls. Together, these findings suggest that a hyperactive phosphatidylinositol signaling system may be involved in the pathophysiology of BPD.

The imbalances in G-proteins, effectors, and second messengers have led investigators to examine whether disturbances in downstream targets of affected signal transduction systems at the level of transcription are involved in BPD pathophysiology. The report of an increase in NF-κB accompanied by decreases in nuclear c-fos and c-jun N kinase (JNK) within leukocytes of depressed BPD patients relative to healthy controls (Spiliotaki et al., 2006) provides direct evidence for the involvement of altered transcription factor activity. However, with regards to cAMP response element binding protein (CREB), an established target for PKA, a postmortem study did not detect a significant difference in brain levels of active phosphorylated CREB (pCREB) between healthy controls and patients with BPD (Young et al., 2004). Nevertheless, these signaling cascades abnormalities represent disruption in the communication between environmental signals and intracellular processes that may hamper a cell's ability to properly respond to stimuli.

1.2.3.3 Abnormalities in calcium dynamics

Fluctuations in cytosolic calcium can initiate a wide array of cellular processes. Within neurons, changes in the levels of intracellular calcium can influence action potentials, neurotransmitter release, synaptic remodeling, neurite outgrowth, and cell death (Kandel, 2000). Hence, disturbances in calcium homeostasis may alter cellular sensitivity to environmental stressors. For example, calcium dysregulation has been shown to potentiate cell death in traumatic brain injury (Limbrick et al., 1995) Conversely, enhanced calcium buffering capacity has been shown to decrease the neurotoxicity of oxygen-glucose deprivation (Abdel-Hamid and Tymianski, 1997).

Studies of peripheral cell models have reported higher levels of basal calcium in platelets (Dubovsky et al., 1989), white blood cells (Dubovsky et al., 1992), lymphocytes (Eckert et al.,

1994) and lymphoblasts (Emamghoreishi et al., 1997) of patients with BPD. In addition, several studies have also demonstrated that calcium responses following stimulation with agonists, including thrombin (Kusumi et al., 1992), serotonin (Kusumi et al., 1994; Okamoto et al., 1995; Yamawaki et al., 1996), and thapsigargin (Hough et al., 1999; Kato et al., 2003), are elevated in samples derived from BPD patients relative to healthy controls. Furthermore, BPD has also been associated with changes in proteins that directly mediate calcium flux including the calcium-ATPase pump (Bowden et al., 1988), Na/K ATPase (Kassir and Meltzer, 1991; Li and El-Mallakh, 2004; Wood et al., 1991), and the calcium channel TRPM2 (Yoon et al., 2001). In conjunction with results of genetic studies implicating TRPM2 (McQuillin et al., 2006; Xu et al., 2009; Xu et al., 2006a) and CACNA1C (Ferreira et al., 2008), these findings support the hypothesis that altered cellular vulnerability due to disruptions in calcium homeostasis are relevant to the pathophysiology of BPD.

1.2.3.4 Impairment in the unfolded protein response

Homeostatic calcium regulation and protein and lipid biosynthesis are in part governed by the endoplasmic reticulum (ER). Under conditions that disrupt ER homeostasis, the accumulation of misfolded proteins triggers an adaptive mechanism referred to as the unfolded protein response (UPR) to restore normal ER function (Kaufmann, 1999). The importance of this pathway in maintaining cell viability under stress conditions is highlighted by results of a study by Gavilan and colleagues (2009) in aged rats. By inducing stress through proteasome inhibition, they demonstrated that anomalies in the initiation and downstream signaling of the UPR in older animals promoted neuronal loss due to accumulation of ubiquitinated proteins. By comparison, neurodegeneration was delayed by 3 days in younger animals, where activation of IRE1α, ATF6α, and protein kinase-like endoplasmic reticulum kinase (PERK) followed proteasome inhibition (Gavilan et al., 2009).

Indirect support for the involvement of the UPR in the pathophysiology of BPD has been gained from molecular pharmacology. Studies examining the mechanisms of action of mood stabilizers have demonstrated that chronic valproate treatment enhances the expression of genes encoding ER resident chaperone proteins glucose regulated protein 78 (GRP78), GRP94, and calreticulin in rat glioma C6 cells (Bown et al., 2000a). Similarly, elevated levels of these chaperone proteins in PC12 cells (Hiroi et al., 2005) and primary rat cortical neurons (Shao et al., 2006) have also been attributed to chronic lithium treatment. These data highlight the significance of

the UPR in the mechanism of action of mood stabilizers and point to the possibility that disturbances in this stress-response may be associated with BPD. Indeed, an early twin study reported lower ER stress induction of *XBP1*, a gene encoding a UPR regulatory protein, in affected twins relative to their monozygotic siblings (Kakiuchi et al., 2003). More recently, So and colleagues (2007) have reported UPR abnormalities in lymphoblasts established from patients diagnosed with BPD type I. Following induction of UPR with thapsigargin and tunicamycin, they observed lower expression of genes enconding X-box binding protein 1 (XBP1) and C/EBP homologous protein (CHOP) in patients relative to age- and gender-matched healthy controls (So et al., 2007). This finding has since been replicated in a larger sample by a study that also found lower induction of GRP94 in BPD (Hayashi et al., 2009). Taken together, these results suggest that disturbances in adaptive ER stress signaling may contribute to impairments in cellular resiliency associated with BPD.

1.2.3.5 Mitochondrial dysfunction

The mitochondrion is a multifunctional organelle that plays crucial roles in ATP synthesis, calcium homeostasis, and apoptosis regulation. Thus, disturbances in this organelle can have profound impacts on cellular viability. Reductions in mitochondrial metabolism have been reported for BPD. As mentioned earlier (Section 1.2.2), BPD has been associated with reduced levels of NAA, a putative indicator of mitochondrial function. Another indirect evidence for metabolic alterations is the high lactate concentrations in cerebrospinal fluid (Regenold et al., 2009) of patients diagnosed with BPD. Further support for the involvement of mitochondrial dysfunction has been derived from studies profiling gene expression. Reductions in nuclear mRNA coding for mitochondrial proteins that were involved in oxidative phosphorylation have been reported in the hippocampus (Konradi et al., 2004) and the frontal cortex (Sun et al., 2006). In addition, mutations in mitochondrial DNA that may influence intracellular redox status and calcium regulation have also been detected (Kato, 2008).

Pharmacological studies have also contributed to the growing literature supporting the hypothesis of mitochondrial dysfunction in BPD. Lithium, at therapeutically relevant concentrations, has been shown to increase the activity of mitochondrial complexes I and II isolated from human frontal cortex homogenates (Maurer et al., 2009). Also, Bachmann and colleagues (2009) have demonstrated that prolonged treatment with lithium and valproate increased respiration and mitochondrial oxidation as well as improved mitochondrial membrane

potential in human neuroblastoma SH-SY5Y cells. Moreover, chronic treatment with lithium and valproate has also been shown to attenuate the pro-apoptotic changes induced by methamphetamine including decreased Bcl-2/Bax ratio and increased cytosolic translocation of cytochrome c (Bachmann et al., 2009). Taken together, these findings, which identify the mitochondria as downstream targets of established mood stabilizers, indirectly suggest that disturbances in this organelle are relevant in the pathophysiology of BPD.

1.3 Oxidative stress in BPD

It has been recognized that the majority of intracellular reactive oxygen species (ROS) originates from the series of enzyme complexes mediating electron transport within the mitochondria (Adam-Vizi, 2005; Taylor and Crack, 2004). Under physiological conditions, small amounts of ROS —H_2O_2 and superoxide and hydroxyl radicals—are necessary for maintenance of a cellular redox environment that is conducive to proper protein folding and activation of kinases and phosphatases (Maher, 2006). Aside from its influence on cellular oxidative status, it is also widely accepted that ROS can directly regulate the activity of transcription factors and gene expression (Maher, 2006). The intracellular level of ROS is tightly regulated by a network of anti-oxidant consisting of enzymes and compounds that neutralize ROS. Intracellular anti-oxidants include thiols, ascorbic acid, and α-tocopherol which readily reduce ROS while enzymatic defences are comprised of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GRed), thiol-specific peroxidase, methionine sulfoxide reductase, and catalase (CAT). When the amount of ROS exceeds the antioxidant capacity of the cell, oxidative stress occurs (Sies, 1997). Reduced O_2 can readily react with DNA, protein, and lipids to form by-products listed in Table 1.1, induce cell damage, and under extreme conditions, trigger cell death.

The hypothesis that oxidative stress, possibly due to mitochondrial impairments, plays a role in the pathophysiology of BPD is supported by increasing evidence of elevated stress markers in patients diagnosed with the disorder. An examination of the anterior cingulate cortex of patients with major depressive disorder, schizophrenia, or BPD revealed that the brains of patients with BPD exhibited the highest levels of 4-hydroxynonenal (4HNE), a product of lipid peroxidation, relative to controls (Wang et al., 2009b). Consistent with these results, increased levels of thiobarbituric acid reactive substances (TBARS) were also reported in blood samples obatained

from BPD patients relative to samples from healthy controls (Andreazza et al., 2007a; Kunz et al., 2008). In addition to products of lipid peroxidation, there is a greater frequency of DNA fragmentation in blood samples collected from BPD patients relative to healthy controls (Andreazza et al., 2007b).

Table 1.1 By-products of oxidative stress

Oxidative stress markers
DNA damage
DNA lesions (8-hydroxy-2-deoxyguanosine)
Strand breaks
DNA-protein cross-links
Protein carbonyls
Lipid hydroperoxides
Alkanes
Isoprostanes
Aldehydes
4-hydroxynonenal (4HNE)
Malondialdehyde (MDA)
Thiobarbituric acid reactive substances (TBARS)

Moreover, studies examining the expression of gene encoding antioxidant enzymes suggest that BPD is associated with lower cellular antioxidant capacities. Within the dorsolateral prefrontal cortex of patients with BPD, Sun and colleagues (Sun et al., 2006) found that expression of genes encoding SOD1, GPx4, and GSTπ1 was decreased by 80 to 90% of controls. A similar downregulation of expression of genes encoding antioxidants including GPx, glutathione synthetase (GS), GST A2, M5 and omega isoforms, catalase, and SOD was also observed in the hippocampus of patients with BPD relative to controls, regardless of mood stabilizer treatment (Benes et al., 2006). Further evidence for disruptions in the regulation of oxidant-antioxidant balance in BPD is presented by studies reporting changes in the activity of antioxidant enzymes. However, as shown in Table 1.2, the direction of change has not been consistent. In contrast to findings of increased SOD (Andreazza et al., 2007a; Kuloglu et al., 2002; Machado-Vieira et al., 2007; Savas et al., 2006), GPx (Andreazza et al., 2007a) and catalase (Andreazza et al., 2007a) activities, an equal number of studies have reported the reverse. Studies examining manic (Gergerlioglu et al., 2007) and depressed (Selek et al., 2008) patients have detected a decrease in SOD activity relative to healthy controls. Similarly, a decrease in catalase activity has also been reported in a sample of manic, depressed, and euthymic patients (Andreazza et al., 2007a; Ranjekar et al., 2003). Some of the factors that may potentially contribute to the differences in results are mood states and duration of illness. In a comparison of antioxidant enzyme activities in manic, depressive, and euthymic patients, Andreazza and colleague (2007a) identified mood-related changes in the activity of SOD, CAT and GPx relative to control. More recently, an increase in the activity of glutathione-related enzymes specific to patients affected with BPD for at least 10 years has also been observed (Andreazza et al., 2009), suggesting that increases may represent an adaptive response to chronic oxidative stress. Collectively, increased oxidative stress markers and disruption in enzymatic antioxidant defences in patients with BPD strongly suggest that oxidative stress is involved in the pathophysiology of this mood disorder.

Table 1.2 Activities of blood antioxidant enzymes in patients with BPD

Reference	P/C	Patients with BPD			SOD	CAT	GST	GRed	GPx
		M/D/E	Duration (yrs)	Unmedicated					
(Andreazza et al., 2009)	60/60	NA	2.1 ± 2.9; 13.9 ±5.1	0	NA	NA	↑[1]	↑[1]	↔
(Kunz et al., 2008)	82/ 32	32/19/31	NA	0	↑	NA	NA	NA	NA
(Selek et al., 2008)	30/30	0/30/0	NA	0	↓	NA	NA	NA	NA
(Machado-Vieira et al., 2007)	45/30	45/0/0	NA	30	↑[2]	↑[2]	NA	NA	NA
(Gergerlioglu et al., 2007)	29/30	29/0/0	NA	0	↓	NA	NA	NA	NA
(Andreazza et al., 2007a)	84/32	32/21/31	22.1±10.9	0	↑[3]	↑[4]	NA	NA	↑[5]
(Savas et al., 2006)	20/20	0/0/20	NA	0	↑	NA	NA	NA	NA
(Ranjekar et al., 2003)	10/10	NA	NA	0	↓	↓	NA	↔	NA
(Kuloglu et al., 2002)	23/20	NA	NA	0	↑	NA	NA	NA	↔
(Abdalla et al., 1986)	20/58	NA	NA	0	↑	NA	NA	NA	↔

P/C: Patients/ Controls, M/D/E: Manic/ Depressive/ Euthymic

[1] An increase was observed only in patients with BPD for more than 10 years.

[2] Only in unmedicated patients compared to controls.

[3] Increase only observed in manic and depressed, but not euthymic, patients

[4] Observed only in manic and euthymic, but not depressive, patients

[5] Observed only in euthymic patients

1.4 Protective effects of mood stabilizers against oxidative stress

1.4.1 Evidence for the neuroprotective effects of lithium and valproate

As discussed in the previous sections, numerous lines of evidence implicate neuronal/glial loss and altered responsitivity to cellular stress are involved in the pathophysiology of BPD. This has led to the hypothesis that neuroprotection may mediate the therapeutic efficacy of mood stabilizers. Early studies have demonstrated that treatment with lithium or valproate in vitro reduces the neurotoxicity of

- NMDA receptor agonists including glutamate (Chalecka-Franaszek and Chuang, 1999; Chen and Chuang, 1999; Chen et al., 2003; Hashimoto et al., 2003; Hashimoto et al., 2002; Leng and Chuang, 2006; Nonaka et al., 1998; Shao et al., 2005) and quinolinic acid (Senatorov et al., 2004; Wei et al., 2001);
- inhibitors of cellular respiration including malonate (Morland et al., 2004) and rotenone (Lai et al., 2006);
- stressors that stimulate ROS generation including H_2O_2 (Lai et al., 2006) and $FeCl_3$ (Wang et al., 2003); and
- culture media deprived of potassium (Mora et al., 1999), oxygen and glucose (Costa et al., 2006), or trophic support (Jin et al., 2005) .

In addition, these mood stabilizers have also been shown to exert neuroprotective effects against various other insults including ouabain (Hennion et al., 2002), lipopolysaccharide (Chen et al., 2007a), amyloid-beta (Rametti et al., 2008), thapsigargin (Hiroi et al., 2005), and ethanol (Zhong et al., 2006). As well, lithium and valproate have been shown to decrease neuronal damage induced by kainate (Caldero et al., 2010), ibotenic acid (Eleuteri et al., 2009), and ischemia (Han et al., 2008; Kim et al., 2007a; Ma and Zhang, 2003; Xu et al., 2003; Xu et al., 2006b) in vivo. As summarized in Table 1.3, the results of these studies have attributed the neuroprotective effects of lithium and/ or valproate to various, albeit complimentary, pathways including (1) the attenuation of stress-induced kinase activity, (2) the inhibition of apoptosis, (3) the enhancement of pro-survival signalling, and (4) the induction of pro-survival genes. However, a number of these regulatory effects were evident only in specific cell lines (Lai et al.,

2006), after a short period of treatment (Costa et al., 2006; Han et al., 2008; Hennion et al., 2002; Jin et al., 2005; Kim et al., 2007a), or at drug concentrations higher than the therapeutic serum levels in BPD patients (Caldero et al., 2010; Chalecka-Franaszek and Chuang, 1999; Hiroi et al., 2005; Jin et al., 2005). Hence, a number of these mechanisms are not likely to be involved in the therapeutic efficacy of lithium and valproate. Greater understanding of the mechanism of action of lithium and valproate would be derived from studies examining the effects of these mood stabilizers at therapeutic levels in disease–relevant models.

Table 1.3 Molecular mechanisms implicated in the neuroprotective effect of mood stabilizers

Molecular mechanisms	Mood stabilizer	Reference
Deactivation of NMDA receptors	*Lithium*	(Ma and Zhang, 2003);(Hashimoto et al., 2002);(Nonaka et al., 1998)
Inhibition of voltage-gated ion channels	*Carbamazepine*	(Lakics et al., 1995);(Costa et al., 2006)
	Lamotigine	(Shuaib et al., 1995)
Increased Akt/PKB activity	*Lithium*	(Chalecka-Franaszek and Chuang, 1999); (Mora et al., 1999); (Kopnisky et al., 2003); (Beaulieu et al., 2004); (Yazlovitskaya et al., 2006); (Sasaki et al., 2006); (Chakraborty et al., 2008) ; (Liang et al., 2008) ; (Xia et al., 2008); (Aubry et al., 2009) ; (Tajes et al., 2009)
	Valproate	(Pan et al., 2005); (Aubry et al., 2009)
Extra-cellular regulated kinases/ Mitogen-activated protein kinase kinase (ERK/MEK) activation	*Lithium*	(Mai et al., 2002); (Einat et al., 2003) ; (Di Daniel et al., 2005) ; (Michaelis et al., 2006) ; (Yan et al., 2007); (Nielsen et al., 2008); (Xia et al., 2008); (Young et al., 2008); (Straiko et al., 2009)
	Valproate	(Yuan et al., 2001); (Einat et al., 2003); (Hao et al., 2004) ; (Di Daniel et al., 2005); (Pan et al., 2005) ; (Creson et al., 2009); (Biermann et al., 2010)
	Carbamazepine	(Mai et al., 2002);
JNK and p38 inhibition	*Lithium*	(Chen et al., 2003); (Hongisto et al., 2003);
Altered AP-1 binding	*Lithium*	(Ozaki and Chuang, 1997); (Asghari et al., 1998); (Chen et al., 2003); (Hongisto et al., 2003); (Hiroi et al., 2005)
	Valproate	(Rao et al., 2007)

Molecular mechanisms	Mood stabilizer	Reference
CREB	*Lithium*	(Chen et al., 1999a); (Grimes and Jope, 2001) ; (Mai et al., 2002) ; (Einat et al., 2003) ; (Kopnisky et al., 2003) ; (Omata et al., 2008)
	Valproate	(Einat et al., 2003) ; (Rouaux et al., 2007) ; (Biermann et al., 2010)
	Carbamazepine	(Mai et al., 2002)
HSF-1	*Lithium*	(Senatorov et al., 2004)
Tcf/Lef-1-β-catenin complex	*Lithium*	(Stambolic et al., 1996); (Gould et al., 2004)
Histone deacetylase inhibition	*Valproate*	(Jeong et al., 2003); (Ren et al., 2004); (Leng and Chuang, 2006); (Kim et al., 2007a); (Shi et al., 2007); (Sinn et al., 2007) ; (Marinova et al., 2009)
Increased BDNF	*Lithium*	(Fukumoto et al., 2001); (Hashimoto et al., 2002); (Einat et al., 2003); Frey Andreazza Life sci 2006; (Hammonds et al., 2007) ; (Tseng et al., 2008) ; (Yasuda et al., 2009)
	Valproate	(Einat et al., 2003); (Castro et al., 2005) ; (Chen et al., 2006a); (Frey et al., 2006a); (Jeon et al., 2006); (Casu et al., 2007) ; (Wu et al., 2008) ; (Fukuchi et al., 2009) ; (Yasuda et al., 2009)
	Carbamazepine	(Chang et al., 2009)
Induction of ER stress proteins	*Lithium*	(Hiroi et al., 2005); (Shao et al., 2006)
	Valproate	(Wang et al., 1999b); (Bown et al., 2000b); (Chen et al., 2000); (Wang et al., 2001); (Kim et al., 2005); (Shi et al., 2007)
HSP70 induction	*Lithium*	(Bijur and Jope, 2000); (Ren et al., 2003)
	Valproate	(Ren et al., 2003); (Ren et al., 2004) ; (Pan et al., 2005) ; (Marinova et al., 2009)

Molecular mechanisms	Mood stabilizer	Reference
Induction of Bcl-2	*Lithium*	(Chen and Chuang, 1999); (Wei et al., 2001); (Senatorov et al., 2004) ; (Hiroi et al., 2005); (Lai et al., 2006) ; (Yazlovitskaya et al., 2006) ; (Bachmann et al., 2009)
	Valproate	(Wlodarczyk et al., 1996); (Chen et al., 1999b) ; (Yuan et al., 2001) ; (Corson et al., 2004) ; (Hao et al., 2004); (Laeng et al., 2004); (Pan et al., 2005) ; (Lai et al., 2006); (Michaelis et al., 2006) ; (Li et al., 2008) ; (Tsai et al., 2008); (Bachmann et al., 2009) ; (Creson et al., 2009)
	Carbamazepine	(Chang et al., 2009)
	Lamotrigine	(Chang et al., 2009)
Induction of GST isoforms	*Lithium*	(Wang et al., 2004); (Bhalla and Dhawan, 2009)
	Valproate	(Wang et al., 2004)
	Lamotrigine	(Bakare et al., 2009)
Increased GPx activity	*Lithium*	(de Vasconcellos et al., 2006)
GCLC induction	*Lithium*	(Cui et al., 2007)
	Valproate	(Cui et al., 2007)
	Carbamazepine	(Cui et al., 2007)
	Lamotrigine	(Cui et al., 2007)

1.4.2 Antioxidant properties of lithium and valproate

That oxidative stress has been implicated in neuronal death induced by several stressors mentioned above [glutamate (Miyamoto et al., 1989; Murphy et al., 1989; Penugonda et al., 2005; Pereira and Oliveira, 1997); potassium deprivation (Ramiro-Cortes and Moran, 2009; Samhan-Arias et al., 2004; Schulz et al., 1996); trophic deprivation (Ferrari et al., 1995; Niidome et al., 2006); ouabain (Riegel et al., 2010); amyloid beta (Behl et al., 1994; Butterfield, 2002) ; thapsigargin (Hsieh et al., 2007); ethanol (Chen et al., 2008; Haorah et al., 2008; Maffi et al., 2008; Ramachandran et al., 2003; Sheth et al., 2009)] raises the possibility that the neuroprotective effects of lithium and valproate may also be mediated, in part, by the drugs' antioxidant effects. In primary rat cortical neurons, Shao and colleagues (Shao et al., 2005) reported that the neuroprotective effect of lithium and valproate against glutamate was associated with decreased levels of the oxidative stress markers malondialdehyde and protein carbonyl derivatives. These findings are also consistent with the results of an earlier study which demonstrated that chronic treatment with valproate effectively reduced lipid peroxidation and protein carbonyl formation attributed to ferric chloride (Wang et al., 2003), suggesting that the protection is not specific to the oxidant. These findings are extended in vivo by the results of studies using the amphetamine-model of mania. Pre-treatment with lithium or valproate has been shown to decrease transient DNA damage induced by amphetamine treatment (Andreazza et al., 2008). Moreover, Frey and colleagues (2006b) demonstrated that treatment with lithium or valproate led to decreases in amphetamine-induced TBARS formation which, interestingly, correlated with reductions in hyperactivity. Taken together, the results of these pre-clinical studies suggest that the neuroprotective effects of lithium and valproate are closely related to their ability to reduce oxidant damage.

In conjunction with results suggesting that oxidative stress is involved in the pathophysiology of BPD (Section 3), these findings also raise the possibility that the antioxidant properties of lithium and valproate may also be associated with their therapeutic efficacy. This notion is supported by results of clinical studies examining the effect of drug treatment on the oxidative status of patients diagnosed with BPD. Machado-Vieira and colleagues (Machado-Vieira et al., 2007) conducted a cross-sectional study comparing the oxidative stress parameters of medicated and unmedicated patients experiencing their second manic episode against healthy controls. The

study showed that while higher levels of TBARS were detected in unmedicated patients relative to controls, this difference was not observed in patients who had received lithium 2 weeks prior to testing (Machado-Vieira et al., 2007). In line with these findings, a similar effect of lithium treatment on oxidative status was also reflected in the results of a longitudinal study by Aliyazicioglu and colleagues (Aliyazicioglu et al., 2007), which measured the levels of TBARS, antioxidant enzyme activities, and total plasma antioxidant status in during the course of lithium and olanzapine treatment. During the first phase of the study, patients were taking a combination treatment of lithium and olanzapine which was followed by lithium monotherapy initiated during the second phase of the study. Relative to measures at baseline, levels of TBARS decreased at the end of the first and second phases of the study. Furthermore, the findings of increased total antioxidant capacity following lithium monotherapy support the antioxidant properties of this mood stabilizer (Aliyazicioglu et al., 2007).

Interestingly, a recent clinical trial reported the beneficial effects of antioxidant therapy on management of BPD symptoms (Berk et al., 2008). In this randomized, double-blind clinical trial, N-acetylcysteine, a glutathione precursor, was introduced as an adjunct to maintenance therapy with mood stabilizers. While significant improvements in symptoms and functioning associated with N-acetylcysteine (NAC) were evident after 24 weeks of treatment, these effects were no longer observed 4 weeks after discontinuation of treatment (Berk et al., 2008). These results further suggest that the antioxidant-like effects of mood stabilizers, although transient, may be involved in their therapeutic efficacy.

1.4.3 Neuroprotective effects of carbamazepine and lamotrigine

In contrast to the large body of data supporting the neuroprotective effects of lithium and valproate, a limited amount of evidence suggests that this property is shared by two other mood stabilizers: carbamazepine and lamotrigine. As listed in Table 1.3, Carbamazepine in vitro has been shown to protect neurons against veratridine, an alkaloid that activates sodium channels (Lakics et al., 1995) and ischemia (Costa et al., 2006; Dong et al., 1994), an experimental paradigm that involves excessive release of excitatory amino acids and increased ion channel activation. Similarly, studies have also found an association between lamotrigine treatment and protection against ischemia-induced neuronal death (Crumrine et al., 1997; Papazisis et al., 2008; Shuaib et al., 1995; Smith and Meldrum, 1995; Wiard et al., 1995; Yi et al., 2008) and the

mitochondrial toxins, MPTP/ MPP+ (Arpin et al., 2009; Kim et al., 2007b; Lagrue et al., 2007) and rotenone (Kim et al., 2007b). In these studies, the neuroprotective effects of these drugs, which were administered shortly before or immediately after insult, were examined in the context of ion channel inhibition. Interestingly, Serteser and colleagues (2002) reported that acute treatment with lamotrigine decreased the levels of malondialdehyde, a product of lipid peroxidation, following middle cerebral artery occlusion. Because of the short exposure to lamotrigine, the observed effects, however, are more likely due to the reduction of ischemia-induced glutamate release (Papazisis et al., 2008) rather than direct inhibition of oxidative stress-induced damage. Thus, it is still not clear whether the antioxidant mechanisms are involved in the neuroprotective effects of lamotrigine and carbamazepine.

1.5 Glutathione metabolism as a target of mood stabilizers

As mentioned above, over the past 2 decades, studies examining the action of mood stabilizers have uncovered a number of molecular mechanisms that underlie the observed neuroprotective effect of these agents, particularly lithium and valproate. In understanding the molecular mechanisms mediating the neuroprotective properties of mood stabilizers against oxidative stress, an examination of the drugs' effects on the metabolism of glutathione (GSH), a major contributor to the cellular defense against oxidative stress, is crucial. GSH, a non-protein thiol preset in mM levels within cells (Meister, 1988), is a multi-functional tripeptide that acts as a cysteine reservoir and regulates the apoptotic cascade in addition to neutralizing excessive levels of ROS, (Dringen, 2000a; Franco and Cidlowski, 2009). As an antioxidant, GSH (1) readily reacts with and neutralizes radicals; (2) assists in the breakdown of H_2O_2 catalyzed by GPx; and (3) conjugates with electrophiles through a reaction catalyzed by GST (Aoyama et al., 2008). The importance of this tripeptide as an antioxidant is further underscored by a variety of studies showing that GSH depletion sensitizes cells to oxidative damage (Bains and Shaw, 1997). Hence, maintenance of the intracellular GSH pool is important for limiting oxidative stress-induced neuronal injury.

Intracellular GSH is replenished through 2 pathways. On the one hand, GSH can be regenerated via the reduction of GSSG, which is catalyzed by GSH reductase (GRed). On the other hand, GSH is synthesized from its components: glutamate, cysteine, and glycine. Of the 2 pathways, de novo synthesis (Figure 1.1) has a greater influence on intracellular levels of GSH (Dickinson et al., 2004). GSH synthesis is initiated by glutamylcysteine ligase (GCL) which catalyzes the conjugation of glutamate and cysteine (Dringen, 2000b). This step is then followed by the addition of glycine to the γGluCys dipeptide catalyzed by GS. The first reaction catalyzed by GCL represents the rate-limiting step in the synthesis of GSH (Anderson and Meister, 1983). This rate-limiting enzyme is composed of a catalytic sub-unit (GCLC) and a modifier sub-unit (GCLM). While GCLC alone can sufficiently synthesize γGluCys, binding of GCLM to form a holoenzyme enhances the sub-unit's enzymatic activity (Yang et al., 2002). With regards to the relative abundance of these sub-units, Chen and colleagues (2005) have demonstrated that within the brain, GCLM is present in limited amount. Thus, the amount of GSH within the brain is in part dependent on levels of both GCLC and GCLM.

Results of recent studies suggest that enhancement of glutathione metabolism may mediate the neuroprotective effect of mood stabilizers against oxidative stress. Expression of GST in primary rat cortical neurons has been shown to increase following treatment with lithium and valproate (Wang et al., 2004) as well as lamotrigine (Bakare et al., 2009), suggesting that an increase in ROS neutralization through GSH conjugation may underlie the antioxidant-like effects of these drugs. In addition to enhancing GST levels, Cui and colleagues (Cui et al., 2007) demonstrated that chronic treatment with lithium and valproate also led to the induction of GCLC and elevation of GSH levels in primary rat cortical neurons. Moreover, similar results were obtained in SH-SY5Y cells following chronic 7-day treatment with carbamazepine and lamotrigine, suggesting that GSH synthesis may be a common target of mood stabilizers (Cui et al., 2007). Together, these findings warrant further investigation into the role of GSH metabolism in the mechanism of action of mood stabilizers.

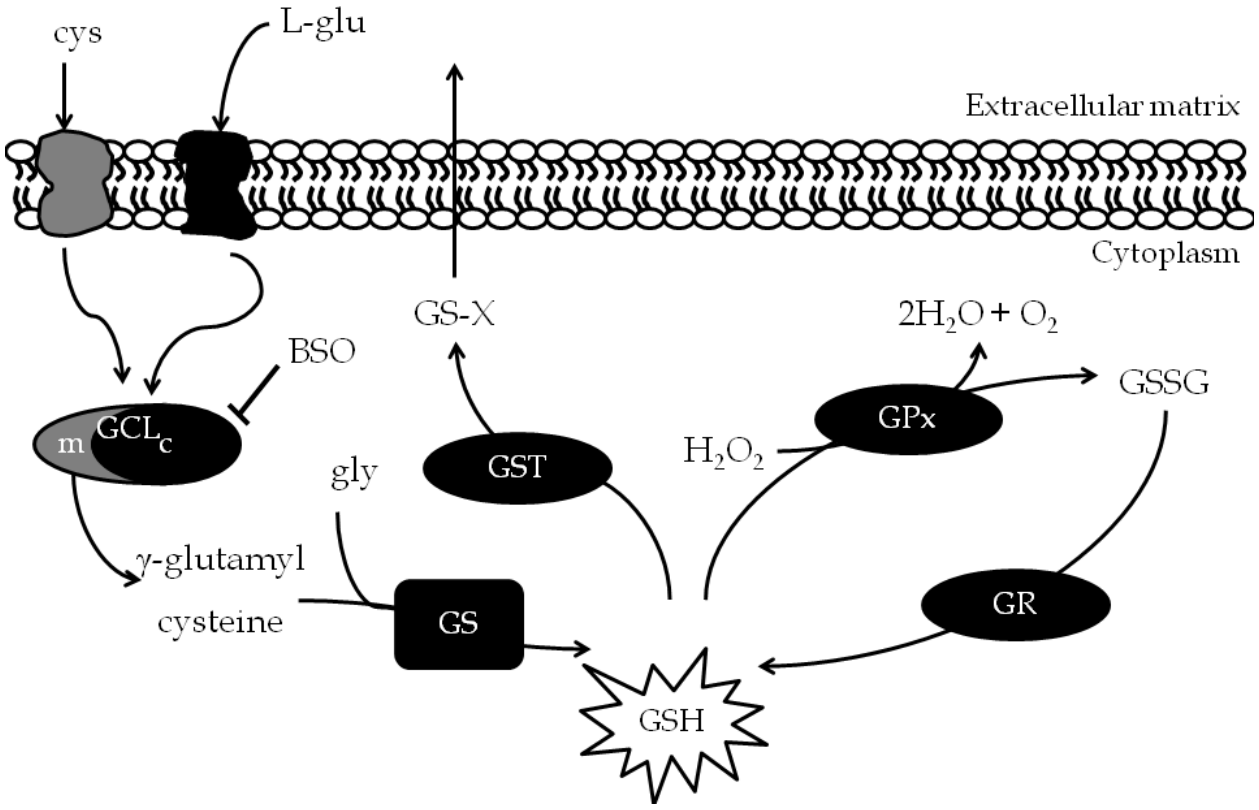

Figure 1.1 Schematic of GSH metabolism. GSH is produced through (1) the 2-step de novo synthesis catalyzed by glutamylcysteine ligase (GCL) and glutathione synthetase (GS) and (2) GSSG recycling catalyzed by glutathione reductase (GR). GSH acts as a co-factor in the detoxifying reactions catalyzed by glutathione-s-transferase (GST) and glutathione peroxidase (GPx).

1.6 Objectives and hypotheses

Accumulating evidence has implicated oxidative stress consequent to ER and/or mitochondrial disturbances and impairments in cellular resilience in the pathophysiology of BPD. Lithium and valproate, two well-studied mood stabilizers, have been shown to exert neuroprotective effects against oxidative stress. As discussed earlier, the results of a study by Cui and colleagues (Cui et al., 2007) suggest that an increase in the levels of GCLC and the subsequent rise in GSH underlie the observed effects of lithium and valproate. Moreover, the study also demonstrated that chronic treatment with carbamazepine and lamotrigine also up-regulates GSH synthesis in SH-SY5Y cells (Cui et al., 2007). While these findings may imply that this mechanism is common to these four mood stabilizers, their study has a number of caveats.

First, the study did not examine whether carbamazepine and lamotrigine exert neuroprotective effects against oxidative stress. In addition, the effects of carbamazepine and lamotrigine on levels of GCLC and GSH were examined in human neuroblastoma SH-SY5Y cells rather than in primary rat cortical neurons. Although the easily accessible SH-SY5Y cell line may exhibit a phenotype similar to mature neurons, the use of this model is not without limitations. Some of the disadvantages include (1) greater propensity for developing mutations with increasing passages (Falkenburger and Schulz, 2006) and (2) altered susceptibility to stressors and neuroprotective agents depending on the differentiating agent used (Xie et al., 2010). The differential effect of mood stabilizers in different cell models is highlighted by results of a study demonstrating that lithium activated the ERK/MAPK cascade in rat cortical neurons but not in SH-SY5Y cells (Di Daniel et al., 2005). These findings raise the need for examining the effects of pharmacological interventions within the same system. Therefore, the first objective of the present study was

- To establish whether chronic treatment with therapeutically relevant concentrations of carbamazepine and lamotrigine—like lithium and valproate—would exert neuroprotective effects against oxidative stress.

Second, the earlier study (Cui et al., 2007) only measured total GSH (GSH-T) levels and did not distinguish between the oxidized and reduced form, GSH and GSSG, respectively. Higher levels of GSH-T may be accounted for by an increase in the synthesis of GSH, the predominant

species within the cell. However, lithium and valproate (Wang et al., 2004), as well as lamotrigine (Bakare et al., 2009), have been shown to induce GST, suggesting that other pathways in GSH metabolism may also influence the levels of GSH and GSSG. Furthermore, the level of GSH-T alone is not a reliable indicator of intracellular redox status. Since the GSH:GSSG ratio would more accurately reflect the cellular reducing potential and resistance against oxidative damage, the second objective of the present study was

- To examine the effect of chronic mood stabilizer treatment on GSH and GSSG levels

Third, the earlier study by Cui and colleagues (2007) only evaluated the effect of the mood stabilizers on the protein levels of GCLC. In light of evidence demonstrating that chronic treatment with mood stabilizers influences second messenger signaling and gene expression, it would be of interest to examine whether these drugs regulate GSH synthesis by inducing the genes coding for GCL. Recent evidence also suggests that GCLM, in addition to GCLC, plays a crucial role in controlling GSH synthesis. Hence, the third objective of the present study was

- To determine whether chronic treatment with mood stabilizers at therapeutically relevant concentrations alters the expression of *Gclc* and *Gclm*

While enriched neuronal preparations provide a relatively simple platform for biochemical analyses, the conditions in vitro do not simulate the intercellular interaction matrix present in vivo. For instance, glial support that is available within the tissue matrix is absent in neuron-enriched cultures. With regards to neuronal GSH, several studies have demonstrated the crucial role of astrocytes in supplying precursors necessary for the synthesis of the tripeptide (Dringen et al., 1999; Wang and Cynader, 2000). Moreover, treatment with mood stabilizers has also been shown to influence the interaction between neurons and glia through changes in glia-derived neurotrophic factors (Angelucci et al., 2003; Chen et al., 2006a; Wu et al., 2008). Hence, the fourth objective was

- To evaluate the effect of chronic mood stabilizer treatment on GSH metabolism in rat brain, especially in regions previously implicated in BPD

In the present study, the following hypotheses were tested:

1. Chronic treatment with mood stabilizers including lithium, valproate, carbamazepine or lamotrigine attenuates oxidative stress-induced death in primary rat cortical neurons.

2. The neuroprotective effect of mood stabilizers is associated with an increase in cellular reductive capacity as reflected by increases in the levels of GSH and in the GSH:GSSG ratio.

3. The increase in GSH is mediated by the up-regulation of *Gclc* and *Gclm* in primary rat cortical neurons following chronic mood stabilizer treatment.

4. Chronic treatment with mood stabilizers at therapeutically relevant doses increases GSH and GSH:GSSG ratio as well as up-regulates *Gclc* and *Gclm* expression in the rat hippocampus and pre-frontal cortex.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and drugs

Lithium chloride, sodium valproate, carbamazepine, lamotrigine, buthionine sulfoximine (BSO), 2-vinylpyridine, triethanolamine, metaphosphoric acid, paraformaldehyde, propidium iodide, dimethylsulfoxide (DMSO) and hydrogen peroxide were purchased from Sigma-Aldrich (Oakville, ON). Both phosphate buffered saline (PBS) and Hank's buffered salt solution (HBSS) with and without calcium and magnesium were obtained from Gibco/ Invitrogen (Burlington, ON).

2.1.2 Animals

Pregnant Sprague-Dawley rats at gestation day 13 and male Sprague-Dawley rats weighing 225-250 g were obtained from Charles River Laboratories. The animals were maintained in a 12-hour light:dark cycle at 23^{o}C. Chow and normal drinking water were available ad libitum. In addition, hypertonic saline (1.5%) was also available to animals receiving lithium. The female rats were allowed to acclimatize for 5 days prior to sacrifice. The male rats were housed in pairs and acclimatized for 7 days prior to experiments. The control chow and chow containing lithium carbonate (2.4 g/kg) and valproate (20 g/kg) were prepared by Bioserve Ltd. (Frenchtown, NJ)

2.1.3 Cell culture reagent

Neurobasal media (NBM), B27 serum-free supplement with and without antioxidants, and heat-inactivated horse serum were purchased from Gibco (Burlington, ON) while penicillin/ streptomycin, L-glutamine, and cytosine arabinoside were obtained from Sigma-Aldrich (Oakville, ON). Serum-based media were comprised of 88% NBM, 1% horse serum, 1% penicillin/streptomycin, and 1% L-glutamine. The serum-free media were supplemented with B27 serum-free supplement with or without antioxidants. The serum-free media were composed of 96% NBM, 2% B27 supplement, 1% penicillin/ streptomycin, and 1% L-glutamine. Media were freshly prepared and warmed to 37^{o}C prior to use.

2.1.4 Kits

The bicinchoninic acid (BCA) protein assay kit used to determine protein concentrations was purchased from ThermoFisher (Nepean, ON). The amounts of total and oxidized glutathione were measured using the Glutathione Assay Kit II purchased from Calbiochem (San Diego, CA). The ECLPlus kit was obtained from GE Healthcare (Baie d'Urfe, Quebec). The RNeasy Plus Mini kits used to extract RNA were purchased from Qiagen (Mississauga, ON). DNA concentration was determined using the Quant-iT High-Sensitivity DNA assay kit purchased from Invitrogen (Burlington, ON).

2.1.5 Antibodies

The polyclonal antibody raised in rabbit against the GCLC was purchased from Thermo Scientific (Fremont, CA). The polyclonal antibody against β-actin was raised in rabbit and was purchased from Cell Signaling Technology (Beverly, MA). The secondary horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody was obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies against microtubule-associated protein 2 (MAP-2), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and glial fibrillary acidic protein (GFAP) were purchased from Abcam (Cambridge, MA). Alexa-conjugated antibodies were obtained from Invitrogen (Burlington, ON).

2.2 Cell culture and drug treatment

2.2.1 Establishment of primary rat cortical neuron culture

2.2.1.1 Preparation of 96-well plates, Petri dishes, and coverslips

On the day of dissection, 96-well plates, 6-cm dishes, and coverslips were coated with polyethyleneimine (PEI). The plates and dishes were incubated with 0.25 mg/ml PEI in PBS with calcium and magnesium for 1 hour at 37^{o}C. The PEI solution was freshly prepared from a 25mg/ml stock in PBS with $CaCl_2$ (0.901 mM) and $MgCl_2$ (0.493 mM), which was stored at 4^{o}C for 1 month. Following incubation, the PEI solution was replaced with serum-based media. The plates and dishes were then kept at 37^{o}C under 5% CO_2/ 95% air until seeding.

2.2.1.2 Dissection of rat embryonic day 18 (E18) brain

Pregnant rats were killed on prenatal day 18 by cervical dislocation following induction of anaesthesia using isoflurane. The uterine horns were then dissected and placed on a Petri dish containing sterile cold HBSS with $CaCl_2$ (1.26 mM) and magnesium ($MgCl_2$-$6H_2O$, 0.493 mM; $MgCl_2$ -$7H_2O$, 0.407 mM). Embryos were removed and transferred onto a separate Petri dish with cold HBSS. The brains were dissected from decapitated embryos and collected in another Petri dish containing cold HBSS with calcium and magnesium. Under a stereomicroscope, the cerebral cortices were isolated, removing the meninges and carefully excising the hippocampi and striata. The dissected cortices were collected in a 15 ml Eppendorf tube containing cold HBSS with calcium and magnesium.

2.2.1.3 Preparation of cell culture

The isolated cerebral cortices were triturated several times using a fire-polished glass pipette to achieve a homogenous mixture. The resulting cell suspension was then subjected to centrifugation at 800 x g for 10 minutes. The supernatant was discarded and the remaining pellet was resuspended in HBSS with calcium ($CaCl_2$, 1.26 mM) and magnesium ($MgCl_2$-$6H_2O$, 0.493 mM, $MgCl_2$ -$7H_2O$, 0.407 mM) at room temperature. The number of cells was determined using a hemocytometer. The cells were then seeded at densities of 60,000 cells per well of a 96-well plate or 3.5 x 10^6 cells per 6 cm Petri dish.

2.2.1.4 Maintenance of neuronal culture

The neuronal cultures were grown at 37°C in an atmosphere of 5% CO_2/ 95% air and maintained in a chemically-defined media under conditions shown to select for cells of neuronal phenotype (Brewer et al., 1993). On the day after plating (first day in vitro, 1st DIV), half of the media was replaced with serum-free media. From the 2nd to the 4th DIV, serum-free media supplemented with B27 with antioxidant was used for feeding. To inhibit the proliferation of non-neuronal cells, half of the media were replaced with media containing 10 μM cytosine arabinoside (Ara-C) on the 3rd DIV. Following incubation with the mitotic inhibitor for 20 to 24 hours, the entire media were refreshed. After the 6th DIV, serum-free media supplemented with B27 minus antioxidants was used for feeding. The schedule of media changes is summarized in Figure 2.1. To assess the composition of the culture, immunocytochemistry was performed on the 10th DIV.

2.2.1.5 Drug treatment

Drug treatment was initiated on the 7th DIV. Half of the media were replaced with media containing 2 mM lithium, 1.2 mM valproate, 0.12 mM carbamazepine, or 0.2 mM lamotrigine. Half of the media were replaced every other day for 7 days. The media used for feeding drug-treated neurons contained 1mM lithium, 0.6 mM valproate, 0.06 mM carbamazepine, or 0.1 mM lamotrigine to maintain the desired concentrations in culture. On the 14th DIV, both vehicle- and drug-treated neurons were exposed to vehicle, 50 μM H_2O_2 and/or 1 mM BSO, a non-competitive glutamylcysteine ligase inhibitor, and were further incubated for 24 hours. Measurement of cell death, RNA isolation, and glutathione extraction were completed on the 15th DIV. The treatment protocol is illustrated in Figure 2.1.

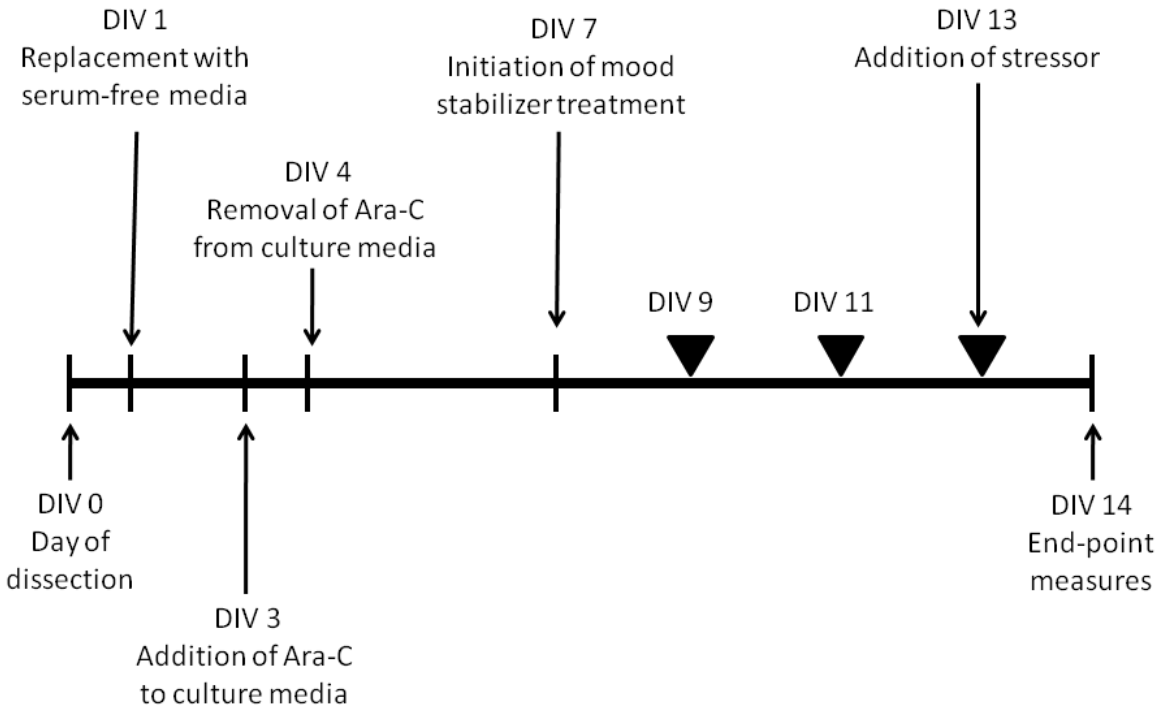

Figure 2.1 Schedule of drug treatment of rat cortical neurons. Primary rat cortical neurons were cultured for 14 days and treated with 1 mM lithium, 0.6 mM valproate, 0.06 mM carbamazepine, or 0.10 mM lamotrigine during the last 7 days. Triangles (▼) represent days when media were replenished. Stressors, namely H_2O_2 and/or BSO, were added on the 13th DIV. End-point measures including, cell death, GSH levels and gene expression, were determined on neuronal preparations collected on the 14th DIV.

2.3 Animal drug treatment

Male Sprague-Dawley rats weighing 288-336 g were fed with control, lithium (2.4 g/kg) or valproate (20 g/kg) chow for 30 days. These amounts of lithium and valproate have been shown to result in plasma concentrations within the therapeutic levels attained in humans (Gould et al., 2004). During the treatment period, animals were weighed every other day.

At the end of the experiments, the animals were killed by decapitation. Trunk blood was collected in heparin-free tubes for measurement of serum levels of lithium and valproate. The brain was rapidly removed and rinsed in ice cold saline. The pre-frontal cortices and hippocampi were excised on ice following demarcations indicated by Dr. John Chambers, an experienced scientist. Tissues were kept on dry ice and stored at -80^{o}C until use. GSH levels and protein levels were quantified from the hippocampi of 5 to 10 animals while gene expression was assessed in the hippocampi and pre-frontal cortices of 9 to 10 animals.

2.4 Methods

2.4.1 Double immunocytochemistry

Immunocytochemistry was performed with a modified protocol as previously described (Marchenko and Flanagan, 2007). On the 10th DIV, cultures grown on coverslips were incubated for 15 minutes in 4% paraformaldehyde fixative [4% w/v paraformaldehyde, 4% w/v sucrose, 2% Triton-X, 5mM magnesium chloride, 10mM EGTA in PBS] pre-warmed to 37^{o}C. Following 3 rinses and 3 washes at 5 minutes each, the coverslips were saturated with ImageEnhancer (Invitrogen, Burlington, ON) for 30 minutes at room temperature. The coverslips were then incubated with blocking solution [5% w/v non-fat milk, 2% goat serum (Invitrogen, Burlington, ON) in PBS] for 1 hour at room temperature. To each coverslip, antibodies against the neuronal marker, MAP2 (1:300 in blocking solution) and the oligodendrocyte marker, CNPase (1:200 in blocking solution) or astrocytic marker, GFAP (1:100 in blocking solution) were added. The coverslips were then wrapped in parafilm and kept at 4^{o}C overnight. After incubation with the primary antibodies, the coverslips were rinsed 3 times and washed 3 times for 5 minutes each with PBS. Secondary antibodies conjugated to Alexa-488 and Alexa-594 dyes were then simultaneously added to the pre-washed coverslips, which were then kept in the dark at room temperature. After 2 hours, excess secondary antibodies were removed by rinsing the coverslips 3 times and washing them 3 times for 5 minutes each. The coverslips were then mounted onto glass slides with Permount and allowed to dry in the dark at room temperature. The edges of the mount were sealed with nail polish. The slides were viewed under an LSM510 Zeiss confocal microscope (Zeiss, Munich, Germany). As revealed by immunocytochemistry, the culture conditions described above (Section 2.2.1.4) resulted in mostly neurons with little or no oligodendrocytes and astrocytes in culture. Representative culture images are shown in Figure 2.2.

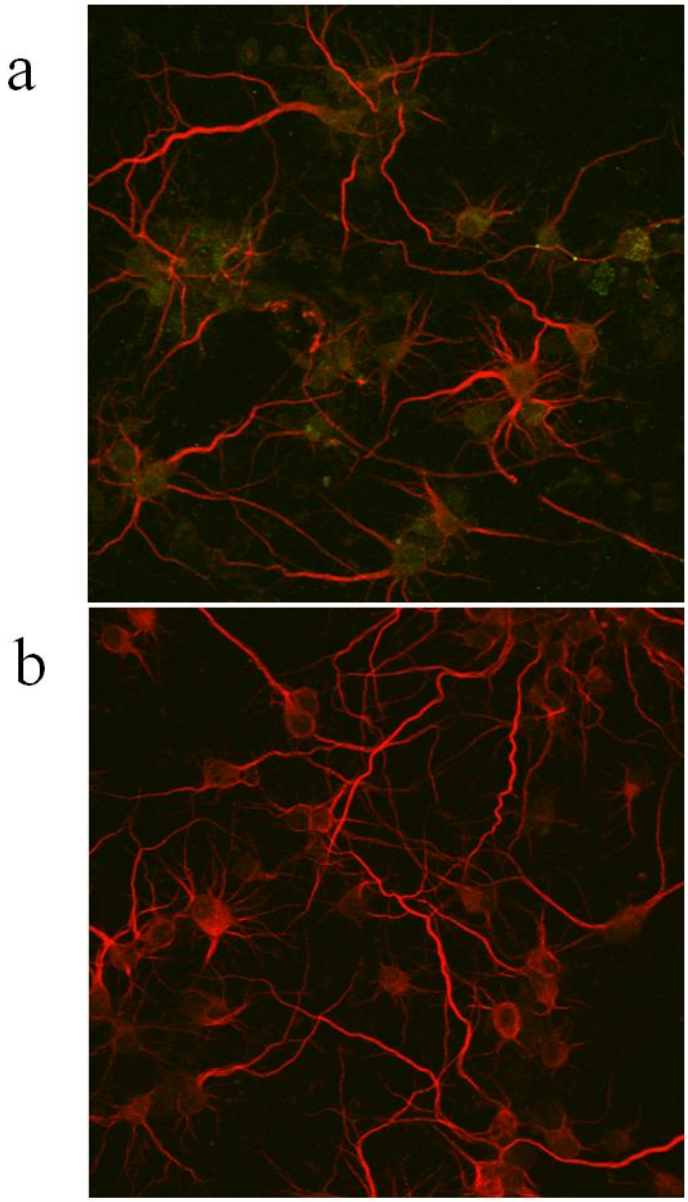

Figure 2.2 Representative images of primary rat cortical neuron culture (10 DIV) immunostained for MAP2 and CNPase (a) and MAP2 and GFAP (b). The primary antibody against MAP2 was detected using a secondary antibody conjugated to Alexa-594 (red) while an Alexa-488-conjugated secondary antibody was used to detect primary antibodies against CNPase and GFAP. The presence of diffuse green signals in (a) is due to excess background.

2.4.2 Measurement of cell death

Propidium iodide fluorescence was used as an indicator of cell death. Propidium iodide, a cell-impermeant dye, fluoresces upon intercalating with the DNA of damaged cells. Hence an increase in propidium iodide fluorescence is proportional to the amount of dead or damaged cells (Rudolph et al., 1997). On the 15th DIV, following the 24 hour-incubation with stressors, the media in 96-well plates were replaced with 50 μM PI in HBSS calcium ($CaCl_2$, 1.26 mM) and magnesium ($MgCl_2$-$6H_2O$, 0.493 mM, $MgCl_2$ -$7H_2O$, 0.407 mM). The neurons were incubated with the dye for 10 minutes at 37^{o}C in an atmosphere of 5% CO_2/ 95% air. Fluorescence was read at an excitation of 485nm and an emission of 519 nm using a Fluoroskan Ascent miniplate reader (ThermoScientific, Fremont, CA). Values are expressed as relative fluorescence units corrected for blank propidium iodide fluorescence.

2.4.3 Measurement of total, reduced, and oxidized glutathione

The amounts of GSH-T and GSSG were extracted and determined using the Glutathione Assay Kit II. The amount of GSH was obtained by subtraction of GSSG from GSH-T. The commercially available kit relies on the oxidation of GSH to a mixed disulfide containing 5-thio-2-nitrobenzoic acid (GSTNB) and the subsequent production of TNB in a reverse reaction catalyzed by glutathione reductase. A schematic of the reactions is shown in Figure 2.3.

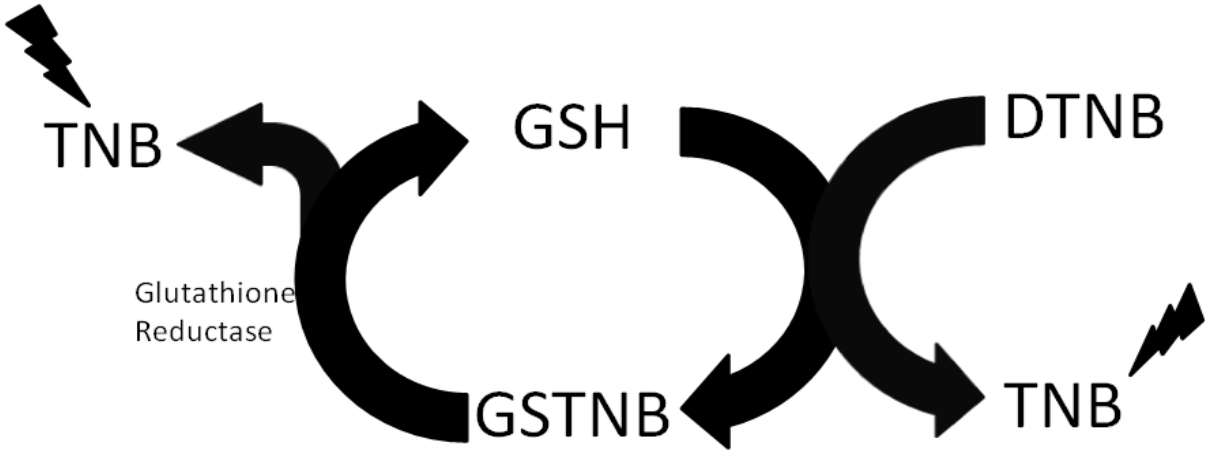

Figure 2.3 Measurement of TNB to estimate levels of GSH and GSSG. GSH reacts with one molecule of dithiobis-2-nitrobenzoic acid, DTNB, to form TNB and GSTNB. The reduction of this latter complex, which is catalyzed by glutathione reductase, leads to the production of TNB. The amount of GSH and GSSG in a sample is determined by measuring the absorbance of TNB at 405 or 414 nm.

2.4.4 Immunoblotting

Hippocampi and pre-frontal cortices were homogenized in lysis buffer [316 mM NaCl, 20 mM Tris, 0.2% SDS, 2% v/v Triton-X 100, 2% sodium deoxycholate, 1x protease inhibitor cocktail (Roche Applied Scientific, Mannheim, Germany)] by ultrasonication for 50 sec at 4^{o}C. The lysates were then subjected to centrifugation at 100,000 x g (Beckman TL-100 centrifuge) for 1 hour at 4^{o}C. The supernatant, which represents the cytosolic fraction, was collected and stored at -80^{o}C until use. The protein concentration of each sample was determined using the colorimetric BCA protein assay kit with BSA as the standard. Absorbance readings and analyses were taken using the UVmax microplate reader and SOFTmax PRO (Molecular Devices, Sunnyvale, CA).

Protein samples were diluted in sample buffer [62.5mM Tris, pH 6.8, 3% SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue] and boiled at 100^{o}C for 3 minutes. Proteins were loaded at an amount of 15 μg per well and separated on a 10% acrylamide/ 27% bisacrylamide SDS gel at a voltage of 200V until the dye front reached the end of the gel. The proteins were then transferred on to 0.45μm nitrocellulose membrane in transfer buffer at a current of 150 mA overnight. Following transfer, the membrane was cut in half to allow for simultaneous probing of GCLC and β-actin. Non-specific binding sites were saturated by incubating the halves of the membrane separately in blocking buffer [5% (w/v) non-fat milk in PBS with 1% Tween, PBST] for 90 minutes. After blocking, the halves were incubated with their respective primary antibodies overnight at 4^{o}C. The top half was probed with the antibody against GCLC [1:2000 in blocking buffer] while the bottom half was incubated with the antibody against β-actin [1:1000 in blocking buffer]. Membranes were rinsed with PBST 3 times followed by 3 washes at 5 minutes each. The membranes were then incubated with the secondary HRP-conjugated goat anti-rabbit IgG [1:10 000 in PBST] for 1 hour. The membranes were then rinsed 3 times with PBST followed by 5 washes at 5 minutes each. The secondary antibody was detected by enhanced chemiluminescence using the ECLPlus kit and was visualized using STORM 860. Band intensity was quantified using ImageQuant 5.2 (Molecular Dynamics). To control for interblot variability, a 4 to 6-point standard curve comprised of pooled rat cortical lysates was included in each gel. A representative blot and the corresponding standard curve are shown in Figure 2.4.

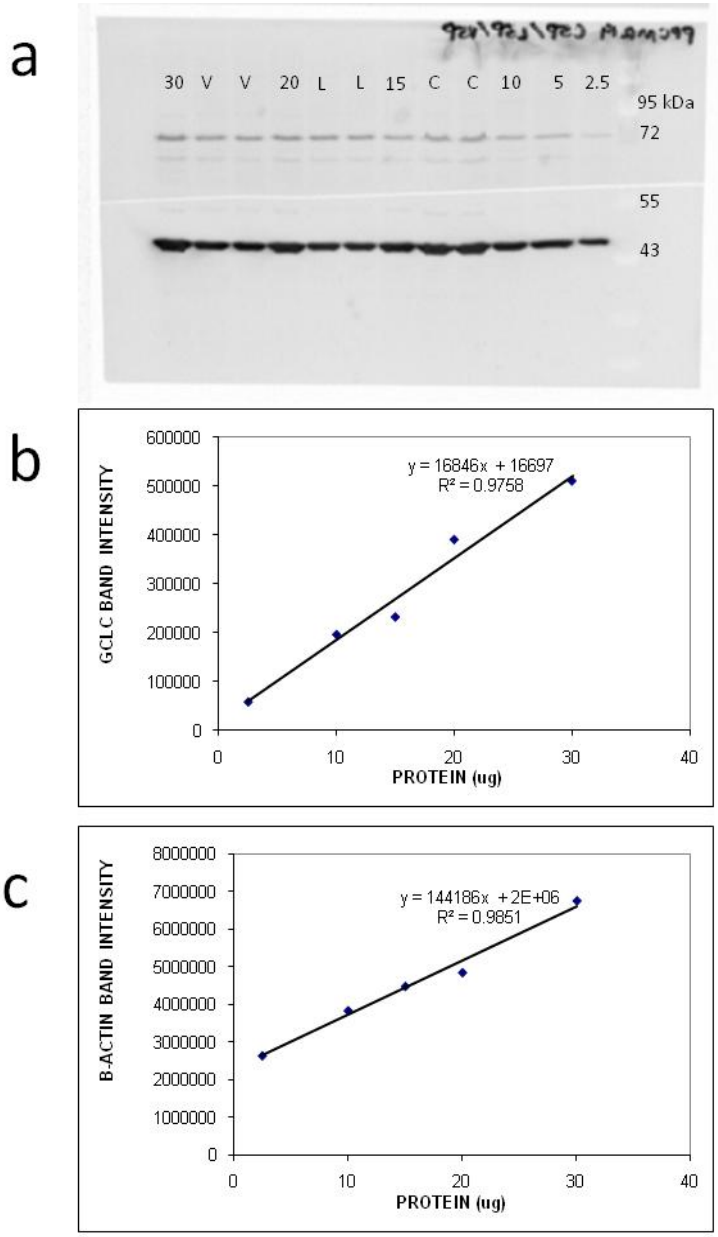

Figure 2.4 Representative immunoblot of GCLC in rat pre-frontal cortex. (a) Numbers represent the amount (µg) of pooled protein loaded per well. Samples from control (C), lithium (L), and valproate (V) treated rats were loaded in duplicate. (b,c) The standard curves for GCLC and β-actin were established from the pooled protein samples. Standard curves with slopes greater than 0.85 were used for quantification.

2.4.5 Determination of serum drug levels

Animal sera were sent to the Diagnostics Laboratory of the Centre for Addiction and Mental Health (Toronto, ON) for analysis of lithium and valproate levels. Lithium concentrations were measured using a colourimetric technique based on the formation of a non-covalent binary complex with a patented compound and analyzed using the Siemens Xpand Chemistry Analyzer (Gruson et al., 2004). Valproate levels were measured using a technique involving a homogeneous particle-enhanced turbidimetric immunoassay performed on the Siemens Dimension RXL platform (Siemens Healthcare Diagnostics).

2.4.6 RNA isolation

Total RNA from neurons (~ 3.5 x 10^6 cells), hippocampal (20-30 mg), and pre-frontal cortical (10-15 mg) tissue was extracted following the protocol in the RNeasy Plus Mini kit, which employs a silica-membrane technology. On-column DNA digestion was accomplished by adding deoxyribonuclease (DNase) I (Qiagen, Mississauga, ON) to each column in the middle of extraction, as prescribed in the protocol. RNA purity as indicated by A260/280 and concentration were determined using the NanoDrop Spectrophotometer (ThermoScientific, Wilmington, DE).

2.4.7 First strand cDNA synthesis

All of the incubations and reactions were performed in a MyCycler Thermal Cycler (Bio-rad, Mississauga, ON). DNA-free RNA was reverse transcribed to synthesize cDNA. To a 5µl sample of RNA, 15µl of Mastermix I [1µl of 250ng/µl random hexamers (Qiagen, Mississauga, ON), 1µl of 10mM dNTP mix (Invitrogen, Burlington, ON), and 13µl RNase-free water] were added for a total volume of 20µl. The reaction tube was incubated at 65^{o}C for 5 minutes and then chilled on ice for at least 1 minute. To each sample, 7 µl of Mastermix II [1µl 5x first-strand buffer (Invitrogen, Burlington, ON), 1 µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, Burlington, ON), and 1µl Superscript III reverse transcriptase (Invitrogen, Burlington, ON)] were added for a final reaction volume of 27 µl. The reaction tubes were then incubated at 25^{o}C for 5 minutes, 50^{o}C for 50 minutes, and 70^{o}C for 15 minutes. The cDNA products were aliquoted and stored at -30^{o}C until further use. cDNA concentration was

determined using the DNA QuantIt kit. Fluorescence at excitation of 485 nm and emission of 518 nm were measured using the Fluoroskan Ascent miniplate reader (ThermoScientific, Fremont, CA).

2.4.8 Primer design

Forward and reverse primers for rat *Gclc* and glyceraldehydes 3-phosphate dehydrogenase (*Gapdh*) were designed using Primer Express 2.0 (Applied Biosystems, Streetsville, ON) based on the sequence from GenBank. Sequences of the forward and reverse primers for rat *Gclm* (Woo et al., 2008) and heme oxygenase-1 (*Hmox-1)* (Martin et al., 2004) were obtained from earlier publications. The selected primers were specific for their target genes as confirmed by an nBLAST search using the National Centre for Biotechnology Information (NCBI). In addition, the PCR products of the selected primer pairs were predicted to cross intron-exon junctions, as verified by Primer-BLAST search using NCBI. Primers for *gapdh* were obtained from ATCG (Toronto, ON). The rest of the primers were synthesized by The Centre for Applied Genomics (Toronto, ON). The accession numbers, primer sequences and predicted amplicon size of each target gene are listed in Table 2.1. Primers were reconstituted in DNase, RNase-free water to a concentration of 100 μM and further diluted to working concentrations of 5 μM and 15 μM.

Table 2.1 Gene accession number and sequences of primers for *Gclc*, *Gclm*, *Hmox-1,* and *Gapdh*

Gene	Accession number	Sequence (5'→3')	Product length (bp)
Gclc	NM_012815	Sense CATCCTCCAGTTCCTGCACAT Antisense ACATCGCCGCCATTCAGTA	144
Gclm	NM_017305	Sense CTGACATTGAAGCCCAGGAG Antisense ACATTGCCAAACCACCACA	270
Hmox-1	NM_012580	Sense GCCTGCTAGCCTGGTTCAAG Antisense AGCGGTGTCTGGGATGAACTA	87
Gapdh	NM_017008	Sense GACTCTACCCACGGCAAGTTCA Antisense TCGCTCCTGGAAGATGGTGAT	93

2.4.9 Quantitative real-time RT-PCR

To monitor gene expression, quantitative real-time polymerase chain reactions were performed using the ABI7300 Real-Time PCR systems (Applied Biosystems, Streetsville, ON). Amplification was detected through SYBR green™ (Applied Biosystems, Streetsville, ON), a fluorescent DNA-binding dye. A reaction volume of 25 µl was comprised of 12.5 µl SYBR green™ mixture, 5 µl of cDNA template, 1-1.5µl each of forward and reverse primers, and 4.5-5.5 µl of DNase/RNase-free water. The thermal cycling conditions were 95^{o}C for 10 minutes, 40 cycles of 95^{o}C for 15 seconds, and 60^{o}C for 1 minute. After verifying that the efficiencies of the target amplicons were similar to the reference amplicon, the comparative threshold (C_T) method was used and the expression of *Gclc*, *Gclm*, and *Hmox-1* were quantified relative to the expression of *Gapdh* (Schmittgen and Livak, 2008).

2.5 Data analysis

Statistical analyses were performed with SPSS 16.0 software (Chicago, Illinois). Propidium fluorescence readings corrected for blank propidium iodide fluorescence were analyzed using repeated measures ANOVA followed by Bonferroni post-hoc analysis. Quantitative RT-PCR data are presented as $2^{-\Delta CT}$, where ΔC_T = (C_T of (*Gclc,Gclm*, or *Hmox-1*) - C_T of *Gapdh*). Differences in levels of GSH-T levels and GSH:GSSG ratios in vitro were analyzed using repeated measures ANOVA to account for data correlated within each experimental run (Lew, 2007) followed by Bonferroni post-hoc analysis. In the animal study, one-way ANOVA, followed by Dunnett post-hoc analysis, was used to analyze differences in animal weight, gene expression, GSH-T levels, and GSH:GSSG ratios among treatment groups. Values are expressed as mean $\pm$ SEM. Differences with $p < 0.05$ were considered statistically significant.

3 Results

3.1 Optimal conditions for real-time RT-PCR

Preliminary experiments were conducted to establish the parameters for each primer pair. Primer specificity was evaluated based on the amplification of a single species as indicated by dissociation curves and agarose gel electrophoresis shown in Figure 3.1 and 3.2, respectively. Optimal primer concentrations were established by measuring the amplification of 1 ng cDNA template using concentrations ranging from 50 nM to 750 nM. Following identification of optimal concentrations, the efficiency of each primer pair was determined by examining the amplification of cDNA templates ranging from 0.1 ng to 10 ng. The efficiency curves of *Gclc, Gclm, and Hmox-1* relative to the reference, *Gapdh* are given in Figure 3.3. Optimal primer concentrations and amounts of template used in the reactions are given in Table 3.1

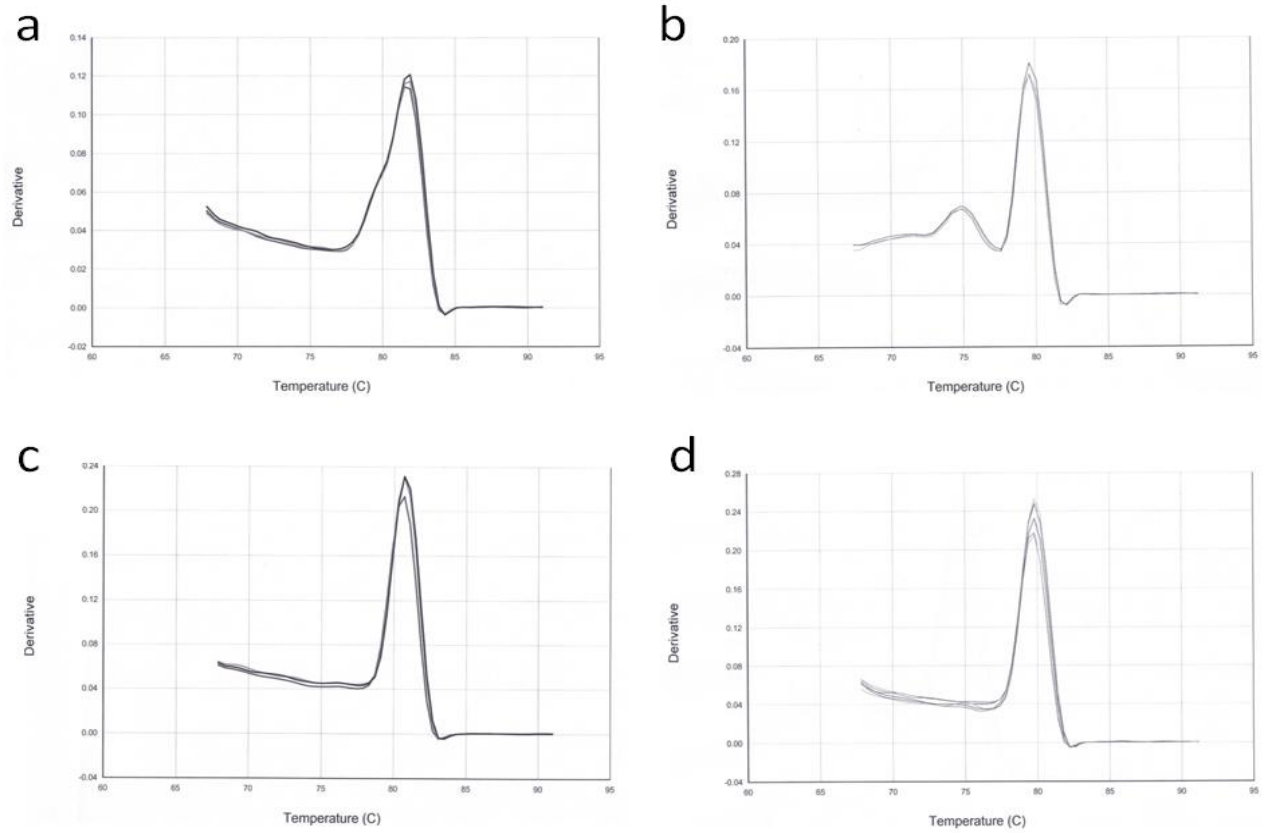

Figure 3.1 Derivative melting curves for *Gclc* (a), *Gclm* (b), *Gapdh* (c), and *Hmox-1* (d). The average melting temperatures for *Gclc*, *Gclm*, *Gapdh*, and *Hmox-1* are 81.9^{o}C, 79.9^{o}C, 80.7^{o}C, and 74.7^{o}C, respectively. The presence of a sharp peak at the melting temperature of each amplicon is indicative of primer specificity.

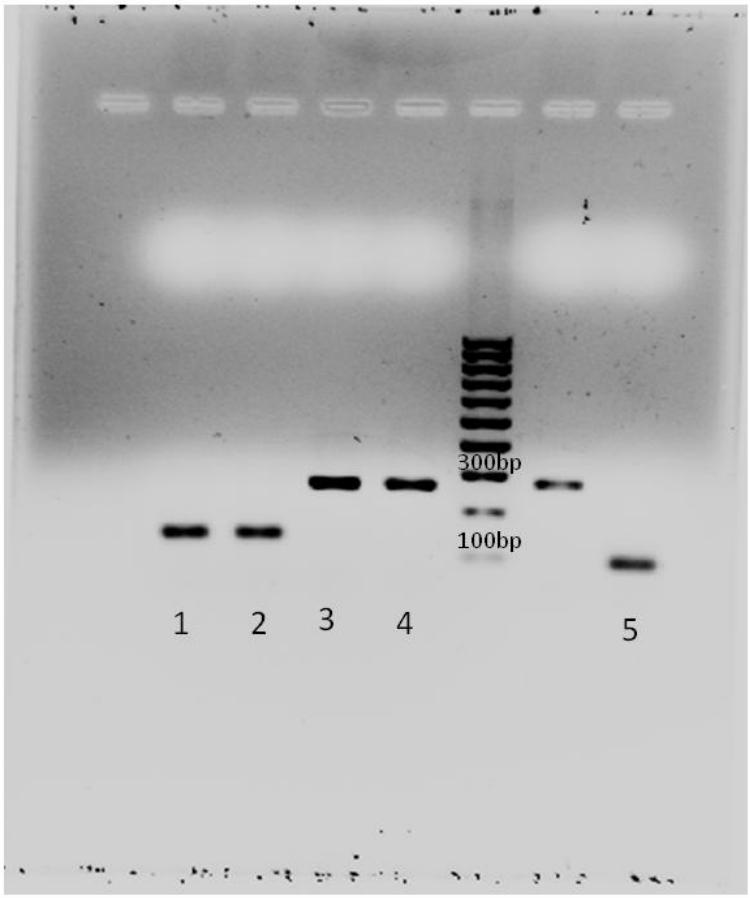

Figure 3.2 DNA agarose gel showing amplicons of *Gclc*, 15 µl (1,2); *Gclm*, 15 µl (3); *Gclm*, 10 µl (4); and *Gapdh*, 15 µl (5). Amplicons were prepared in RNA gel loading buffer and loaded into wells of a 1% agarose gel at the volumes indicated. The predicted sizes for *Gclc, Gclm, and Gapdh* amplicons are 144 bp, 270 bp, and 93 bp, respectively. The gel was visualized using ChemDoc following a 1s exposure. The presence of a single band with predicted size is indicative of primer specificity.

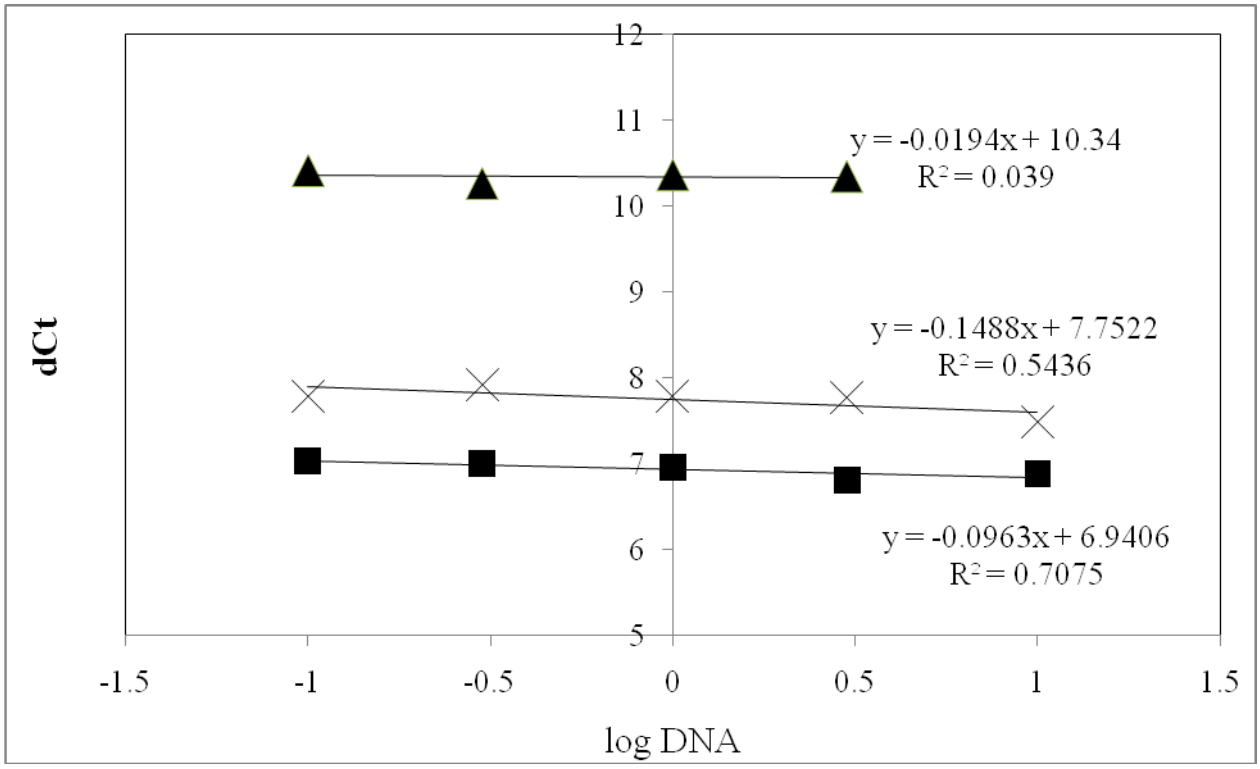

Figure 3.3 Relative efficiency curves of *Hmox-1* (▲), *Gclc* (x), and *Gclm* (■). The amplification efficiencies of *Hmox-1*, *Gclc*, and *Gclm* were compared against the amplification efficiency of the reference gene, *Gapdh*. A horizontal line with a slope < 0.1 (absolute value) is indicative of similar dynamic ranges.

Table 3.1 Optimal primer concentration and template for quantitative RT-PCR

Gene	Primer (nM)	cDNA (ng)
Gclc	300	1.0
Gclm	300	1.0
Hmox-1	600	3.0
Gapdh	600	1.0

3.2 In vitro experiments

3.2.1 Examination of putative neuroprotective effects against H_2O_2-induced cell death

A pilot study was conducted to characterize cell death in response to H_2O_2. As shown in Figure 3.4a, incubation with H_2O_2 caused a dose-dependent increase in cell death in primary rat cortical neurons. At the highest concentration of 1 mM H_2O_2, which corresponded to maximum (100%) cell death, a 5-fold increase in propidium iodide fluorescence was observed, indicating an increase in the uptake and DNA incorporation of the membrane-impermeant dye. Since exposure to 100 μM H_2O_2 caused a 70% increase in cell death (Figure 3.4b) that was within the linear range of the assay, this concentration was used to examine the potential neuroprotective effect of lithium, valproate, carbamazepine, and lamotrigine against H_2O_2-induced cell death.

To examine the neuroprotective effect of these mood stabilizers, cultured neurons were pre-treated with lithium (1 mM), valproate (0.6 mM), carbamazepine (0.06 mM), or lamotrigine (0.1 mM) for 6 days and exposed to 100 μM H_2O_2 for 24 h in the continuous presence of the various mood stabilizing drugs. Treatment with lithium, valproate, carbamazepine, or lamotrigine alone did not significantly affect propidium iodide uptake (p's = 0.60 – 1.00). Similar to results obtained in the preceding experiments, a significant increase in propidium iodide uptake relative to vehicle was detected 24 hours after addition of 100 μM H_2O_2 to untreated controls (p's = 0.001- 0.003). In neurons pre-treated with lithium or valproate, the H_2O_2-induced rise in propidium iodide uptake was significantly reduced by 25% (p = 0.01, Figure 3.5a) and 28% (p = 0.04, Figure 3.5b), respectively. In contrast, 7- day pre-treatment with either carbamazepine (p = 1.00, Figure 3.6a) or lamotrigine (p = 1.00, Figure 3.6b) did not lead to a significant reduction in the propidium iodide uptake following H_2O_2 exposure.

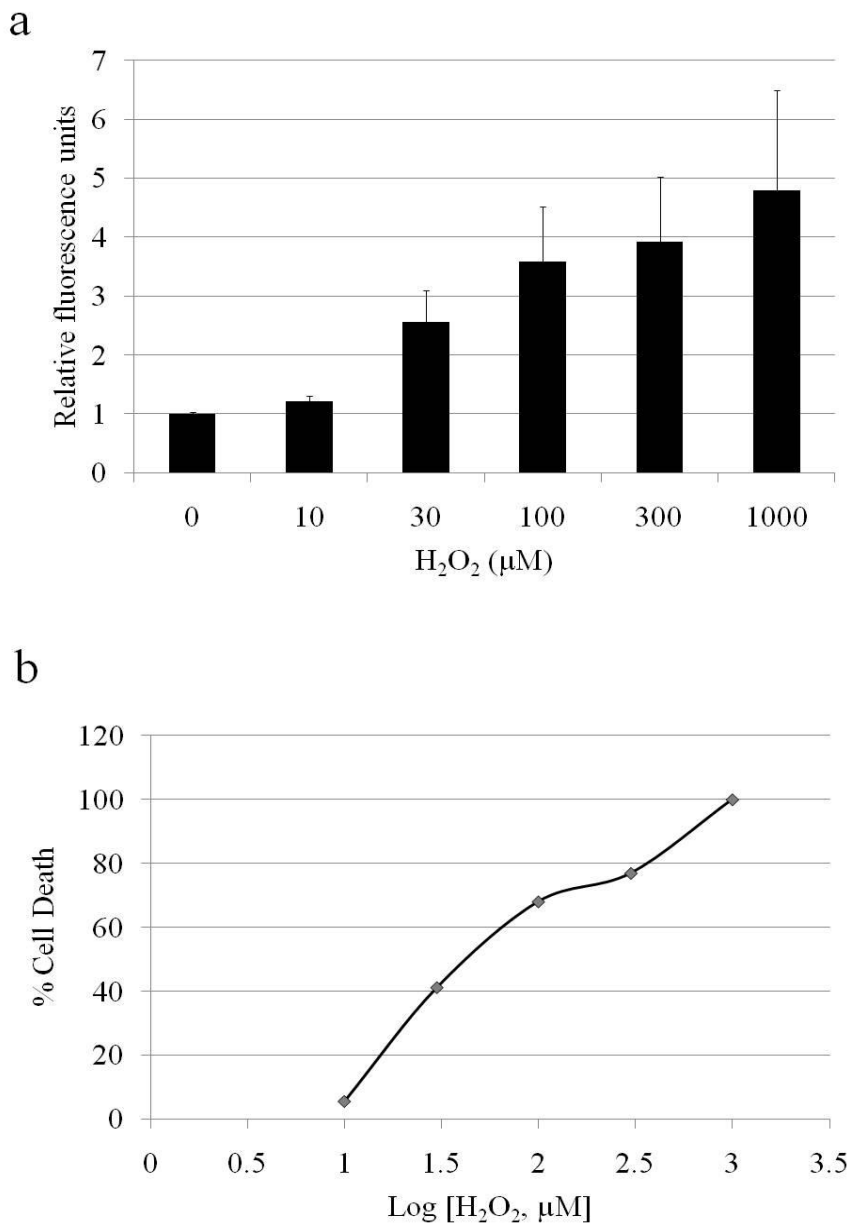

Figure 3.4 H_2O_2-dependent increase in neuronal death. (a) Levels of propidium iodide fluorescence in primary rat cortical neurons relative to vehicle-treated controls. Error bars represent SEM of 3 independent experiments. (b) Neuronal death expressed as percentage relative to maximum cell death (1000 μM H_2O_2) observed following incubation with 10 μM to 1000 μM H_2O_2.

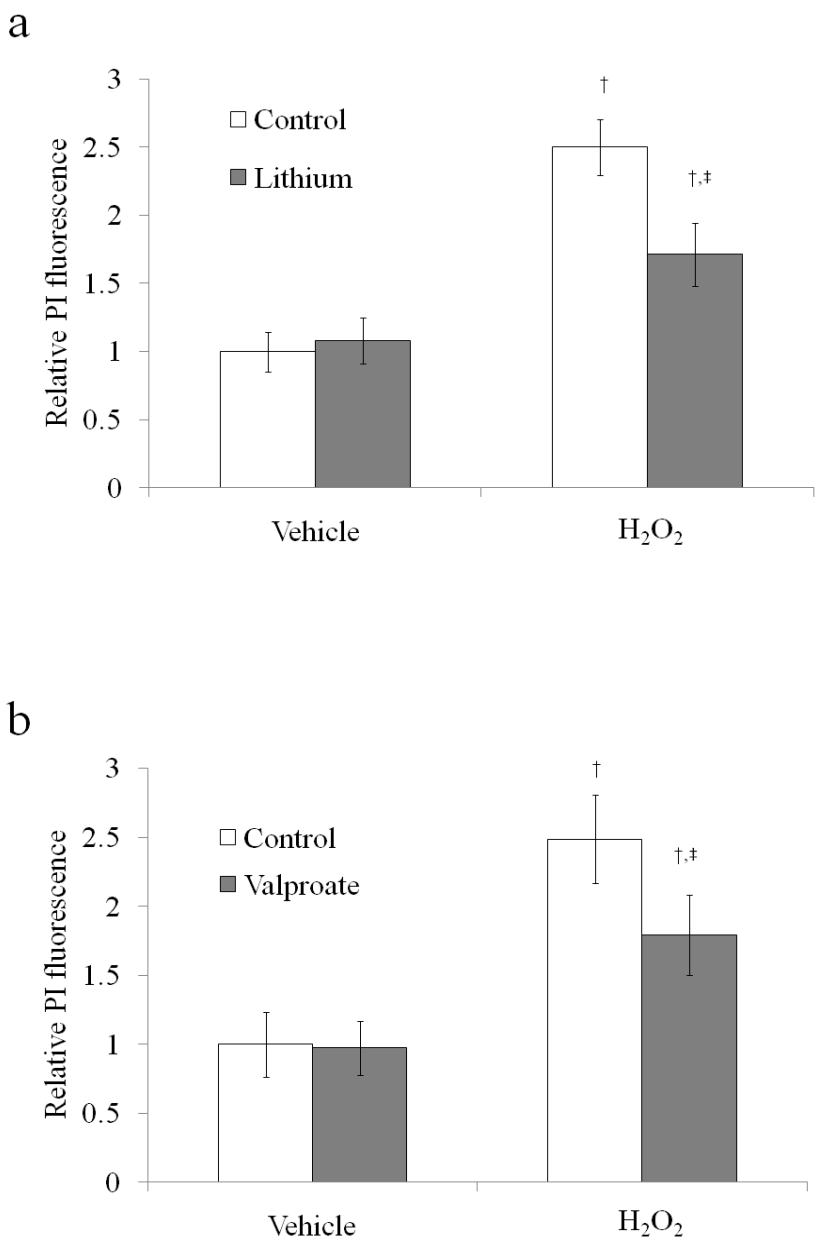

Figure 3.5 Lithium and valproate protect against H_2O_2-induced neuronal death. The levels of propidium iodide fluorescence in neuronal cultures pre-treated for 7 days with (a) 1mM lithium or (b) 0.60 mM valproate were normalized against vehicle-treated controls. The error bars represent SEM of at least 6 independent experiments. † represents $p < 0.05$ relative to vehicle-treated controls; ‡ represents $p < 0.05$ relative to untreated controls (Bonferroni).

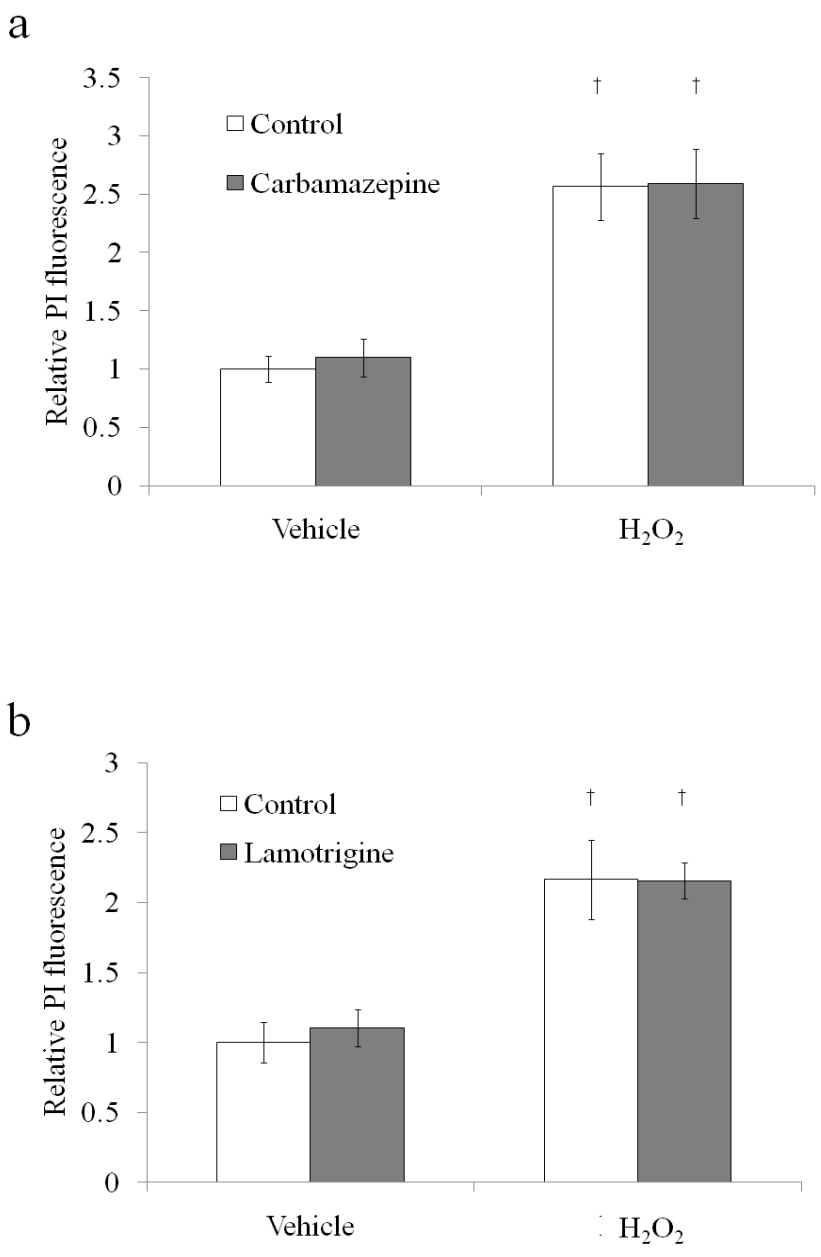

Figure 3.6 Carbamazepine and lamotrigine do not protect against H_2O_2-induced cell death. The levels of propidium iodide fluorescence in neuronal cultures pre-treated for 7 days with (a) 0.06 mM carbamazepine or (b) 0.10 mM lamotrigine were normalized against vehicle-treated controls. The error bars represent SEM of 8 independent experiments; † represents $p < 0.05$ relative to vehicle-treated controls (Bonferroni).

3.2.2 Effects of lithium and valproate against H_2O_2-induced neuronal death in the presence of BSO

Given the central role of GSH in the cellular defense against oxidative stress, and the suggestion that it may be involved in the neuroprotective effect of mood stabilizers (Cui et al., 2007), the next set of experiments examined whether depletion of GSH would counteract the neuroprotective effects of lithium and valproate against H_2O_2. Relative to vehicle, exposure of cultured neurons to 1 mM BSO, a γ-glutamylcysteine ligase inhibitor, for 24 hours did not have a significant effect on propidium iodide uptake. As well, incubation with BSO for 24 hours together with lithium or valproate did not significantly affect propidium iodide uptake of neurons that had been pre-treated with either lithium ($F_{3,12} = 2.19$, $p = 0.14$) or valproate ($F_{3,15} = 0.37$, $p = 0.77$) for 6 days, respectively.

As shown in Figure 3.7, a significant increase in propidium iodide uptake relative to vehicle was observed 24 hours after addition of 50 μM H_2O_2 to the culture media of untreated neurons (p's ≤ 0.001). This increase in fluorescence was unaffected by 24 hour incubation with 1 mM BSO (p's = 0.97- 1.00). In accordance with findings reported in the previous section (Figure 3.5a), pre-treatment with 1 mM lithium for 7 days significantly reduced H_2O_2 (50 μM)–induced neurotoxicity as indicated by a 16% decrease in propidium iodide uptake relative to controls ($p = 0.01$, Figure 3.7a). However, a significant neuroprotective effect of valproate against 50 μM H_2O_2 was not observed ($p = 1.00$, Figure 3.7b). Interestingly, incubation with 1 mM BSO for 24 hours abolished the protective effect of lithium pre-treatment against 50 μM H_2O_2 ($p = 0.38$, Figure 3.7a), suggesting that GSH may play a role in the neuroprotective effect of lithium treatment against H_2O_2-induced cell death. Similarly, there was no significant difference in 50 μM H_2O_2-induced cell death between control and valproate pre-treated neurons in the presence of 1 mM BSO ($p = 0.82$, Figure 3.7b).

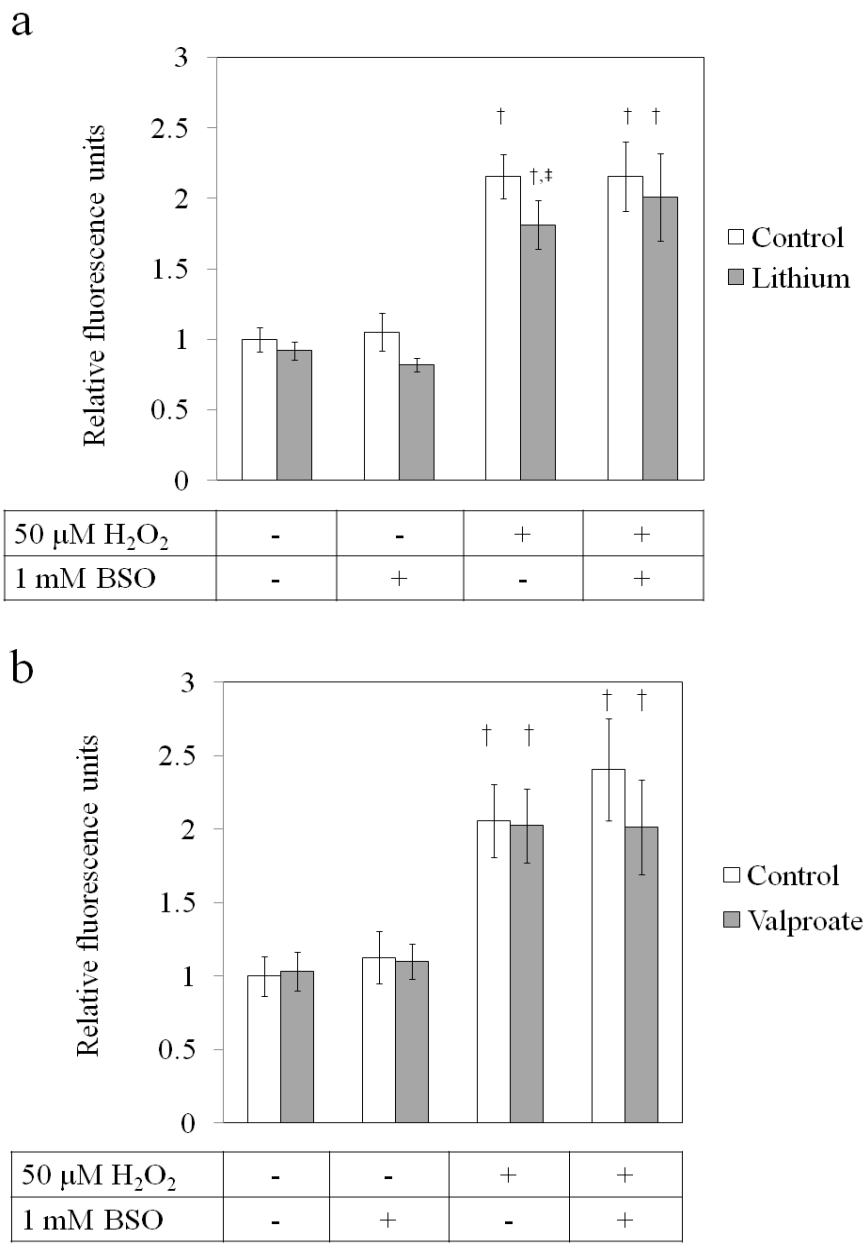

Figure 3.7 BSO attenuates the neuroprotective effect of lithium against H_2O_2 (50 μM)–induced cytotoxicity. The levels of propidium iodide fluorescence in neuronal cultures treated with (a) lithium or (b) valproate were normalized against vehicle-treated controls. The error bars represent SEM of at least 7 independent experiments. † represents $p < 0.05$ relative to vehicle-treated controls; ‡ represents $p < 0.05$ relative to untreated controls (Bonferroni).

3.2.3 Putative effects of lithium and valproate on intracellular GSH and GSSG levels

The results presented in Section 3.2.2 suggest that changes in GSH metabolism may be involved in the neuroprotective effect of lithium. This possibility was further tested by examining whether alterations in GSH and GSSG levels occur in neurons treated chronically (7-day) with 1mM lithium. With regards to valproate, the role of GSH in the neuroprotective effect of this mood stabilizer was not clearly established in the previous experiment. However, pre-treatment with 0.60 mM valproate for 7 days protected against the neurotoxicity of 100 μM H_2O_2 (Section 3.2.1) and has also been shown to increase the levels of GSH-T in primary rat cortical neurons (Cui et al., 2007). Thus, the effect of 7 day pre-treatment with 0.60 mM valproate on the levels of GSH and GSSG levels was also measured.

In untreated primary rat cortical neurons, the average concentrations of GSH and GSSG were 13.36 ± 1.40 nmol/ mg protein (n = 3) and 1.44 ± 0.25 nmol/ mg protein (n = 3), respectively. Chronic treatment with 1 mM lithium (GSH 15.29 ± 1.63 nmol/ mg protein and GSSG 1.99 ± 0.41 nmol/ mg protein, n = 3) or 0.60 mM valproate (GSH 12.92 ± 1.58 nmol/ mg protein and GSSG 2.13 ± nmol/ mg protein, n=3) did not significantly alter the levels of GSH-T ($F_{2,4}$ = 0.68, p = 0.56; Figure 3.8a), GSH ($F_{2,4}$ = 0.52, p = 0.63; Figure 3.8b) and GSSG ($F_{2,4}$ = 3.72, p = 0.12; Figure 3.8c) in primary rat cortical neurons.

It has been suggested that the ratio between GSH and GSSG provides an estimate of the cellular oxidative status (Schafer and Buettner, 2001). In addition, this ratio can also reflect the changes in GSH and GSSG levels due to alterations in GSH metabolism including *de novo* GSH synthesis, GSH conjugation, and GSSG recycling (Dringen, 2000b). Consistent with the findings presented above, no significant differences in GSH:GSSG ratios were detected in lithium-treated neurons (8.61 ± 2.22) relative to untreated controls (10.47 ± 3.57) ($F_{2,4}$ = 1.08, p = 0.42, Figure 3.8d). Interestingly, valproate pre-treatment resulted in a 40% decrease in GSH:GSSG ratio (6.04 ± 0.70); however, this difference did not reach statistical significance (p = 1.00) likely due to the high variability in the data. Overall, the above findings suggest that 7-day pre-treatment with either lithium or valproate is unlikely to alter either the cellular reductive potential or the mechanisms involved in GSH metabolism.

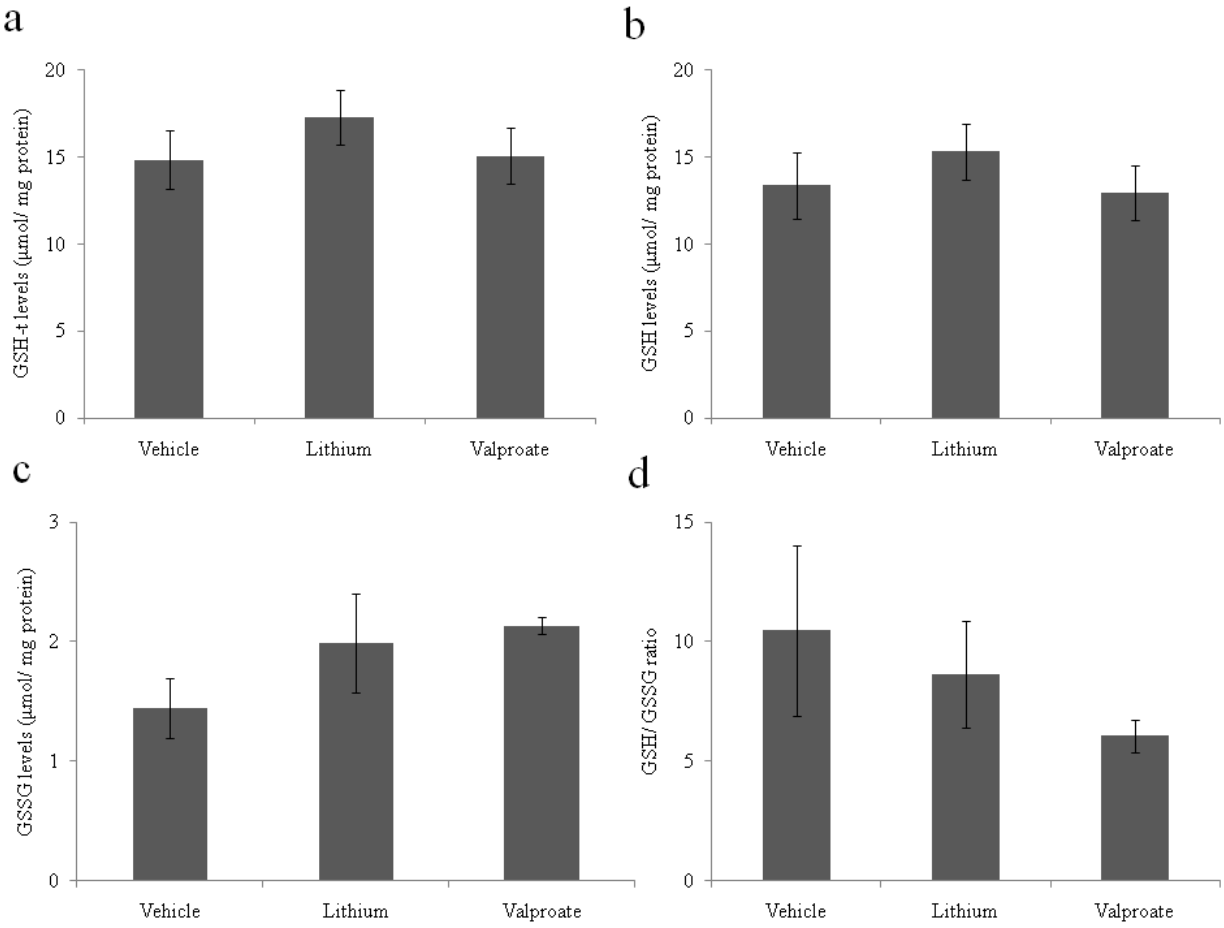

Figure 3.8 Lithium and valproate do not alter GSH metabolism in primary rat cortical neurons. The levels of GSH and GSSG in primary rat cortical neurons were determined spectrophotometrically and expressed as (a) GSH-T, (b) GSH, (c) GSSG, and (d) ratio of GSH:GSSG. Differences among treatment groups were analyzed using repeated-measures ANOVA. Error bars represent SEM of 3 independent experiments.

3.2.4 Putative effects of lithium and valproate on neuronal G*clc* and G*clm* expression

The results of the previous study suggest that pre-treatment with either lithium or valproate had no apparent effect on the levels of GSH and the GSH:GSSG ratio. However, data regarding the synthesis and/or function of enzymes that catalyze the production and consumption of GSH are needed to further establish whether GSH metabolism is involved in the neuroprotective effects of lithium and valproate. An earlier study demonstrated that in primary rat cortical neurons, chronic treatment with lithium or valproate increased the levels of both GSH and GCLC protein (Cui et al., 2007). Given that the catalytic and modifier sub-units of GCL regulate the synthesis of GSH (Section 1.5), this study examined whether pre-treatment with 1mM lithium or 0.60 mM valproate for 7 days would increase the mRNA expression of *Gclc* and *Gclm*.

To rule out the possibility that therapeutic levels of lithium and valproate might indirectly regulate the mRNA expression of *Gclc* and *Gclm* through the production of ROS, the mRNA levels of *Hmox-1*, an index of oxidative stress (Bauer and Bauer, 2002; Ryter and Choi, 2002), were also measured using quantitative RT-PCR.

The results of repeated measures ANOVA indicate that the mRNA levels of *Gclc* ($F_{2,6} = 2.48$, $p = 0.16$) and *Gclm* ($F_{2,6} = 3.44$, $p = 0.10$) did not differ significantly between controls and lithium- or valproate-treated neurons (Figure 3.9). A 30% increase in mRNA levels of *Gclm* was observed in valproate-treated neurons relative to vehicle controls; this difference, however, did not reach statistical significance ($p = 0.45$; Figure 3.9). While lithium had no significant effect on the mRNA level of *Hmox-1* ($p = 1.00$), valproate treatment decreased *Hmox-1* mRNA in primary rat cortical neurons by 60%. This difference, however, was not statistically significant ($p = 0.07$).

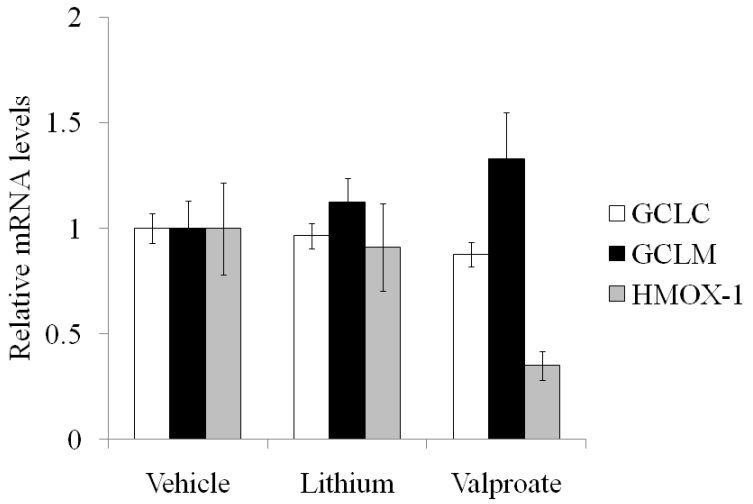

Figure 3.9 Lithium and valproate do not alter *Gclc, Gclm,* and *Hmox-1* expression in primary rat cortical neurons. mRNA levels were determined by quantitative RT-PCR. The mRNA levels of *Gclc*, *Gclm,* and *Hmox-1* were normalized against the mRNA levels of *Gapdh*. Differences in $2^{-\Delta Ct}$ values among treatment groups (Schmittgen and Livak, 2008) were analyzed using repeated-measures ANOVA followed by Bonferroni post-hoc analysis. Data are presented as mRNA levels relative to vehicle. Error bars represent SEM of 4 independent experiments.

3.3 In vivo experiments

3.3.1 Animal treatment

In vitro, neurons are devoid of the three-dimensional intercellular interactions that influence cellular development and function within the tissue matrix. Hence, animal studies were performed to determine the effects of chronic treatment of lithium and valproate on brain regional GSH metabolism and mRNA expression of Gclc and Gclm.

Weight gain and drug serum levels

Rats fed with regular, lithium-containing, and valproate-containing chow gained weight continuously throughout the treatment period. The average weights of animals during the 30-day period are shown in Figure 3.10. At the start of treatment, there was no significant difference in the weights of animals across treatment groups (control 314.24 ± 2.77 g, n= 20; lithium 312.10 ± 2.70 g, n= 20; valproate 312.02 ± 2.35 g, n=20; $F_{2,57} = 0.224$, $p = 0.80$). Weight gain was significantly slower in rats treated chronically with lithium ($p < 0.01$) and valproate ($p < 0.01$) relative to controls. On the 29th day following the start of treatment, there was a significant effect of treatment on weight ($F_{2,57} = 54.71$, $p < 0.001$) with lithium-treated (380.37 ± 6.93 g, $p < 0.01$) and valproate-treated (422.2 ± 5.45 g, $p < 0.01$) animals weighing significantly less than control animals (472.97 ± 6.00 g).

Serum lithium concentrations at the time of sacrifice ranged from 0.37 to 0.97 mM with a mean of 0.71 ± 0.04 mM (n = 20) while the average serum valproate level was 245.45 ± 19.78 μM (n = 20) ranging from 132 μM to 452 μM.

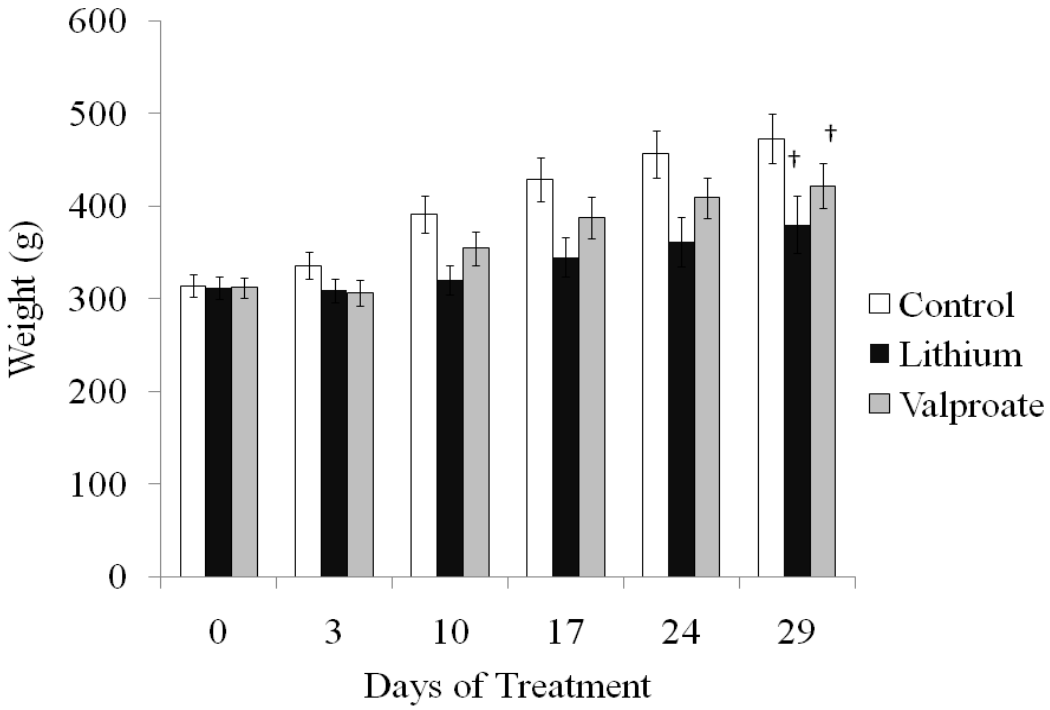

Figure 3.10. Weights of animals fed with control, lithium, or valproate chow over the course of treatment. There was no significant difference in the weights of animals in the control, lithium, and valproate groups at the beginning of treatment. All the animals gained weight during the 30-day treatment period. At the end of treatement, control animals weighed significantly more than lithium- and valproate-treated animals. † represents $p < 0.05$ compared to control. Error bars represent SEM of 20 animals.

3.3.2 Assessment of the effects of lithium and valproate on GSH metabolism within the brain

Extending the in vitro studies, the effect of mood stabilizers on glutathione metabolism was also assessed by measuring the levels of GSH and GSSG in vivo within the hippocampus. Although the pre-frontal cortex was also an area of interest, these parameters were not examined in this brain region due to lack of material after processing the harvested tissues for immunoblotting and RT-PCR. In animals fed with regular chow, GSH-T was 24.42 ± 1.46 μmol/ mg protein (n=5). Chronic treatment with either lithium (GSH-T 22.93 ± 1.11 μmol/ mg protein, n=5) or valproate (20.39 ± 1.33 μmol/ mg protein, n=5) did not significantly alter the GSH-T within the hippocampus ($F_{2,12}$ = 0.52, p = 0.61; Figure 3.11a). In control animals, the concentrations of GSH and GSSG were 22.61 ± 1.54 μmol/ mg protein (n = 5) and 1.81 ± 0.24 μmol/mg protein (n = 5), respectively. Chronic lithium treatment had no significant effect on the levels of GSH (21.14 ± 1.09 μmol/ mg protein, $F_{2,12}$ =2.27, p = 0.15, Figure 3.11b) and GSSG (1.78 ± 0.05 μmol/ protein, n=5, $F_{2,12}$ = 1.20, p = 0.34, Figure 3.11c). Meanwhile, valproate caused a decrease in the levels of GSH (18.25 ± 1.28 μmol/ mg protein, n=5, Figure 3.11b) and an increase in the amount of GSSG (2.13 ± 0.13 μmol/ mg protein, n=5, Figure 3.11c) in the hippocampus; these differences, however, did not reach statistical significance. Interestingly, a significant effect of treatment on GSH:GSSG ratio was detected ($F_{2,12}$ = 4.15, p = 0.04, Figure 3.11d), with valproate-treated animals having a lower hippocampal GSH:GSSG ratio (8.62 ± 0.66) relative to controls (13.54 ± 1.68) (p = 0.03). The hippocampal GSH:GSSG ratio of lithium-treated animals (11.84 ± 0.61), however, did not differ significantly from controls (p = 0.54).

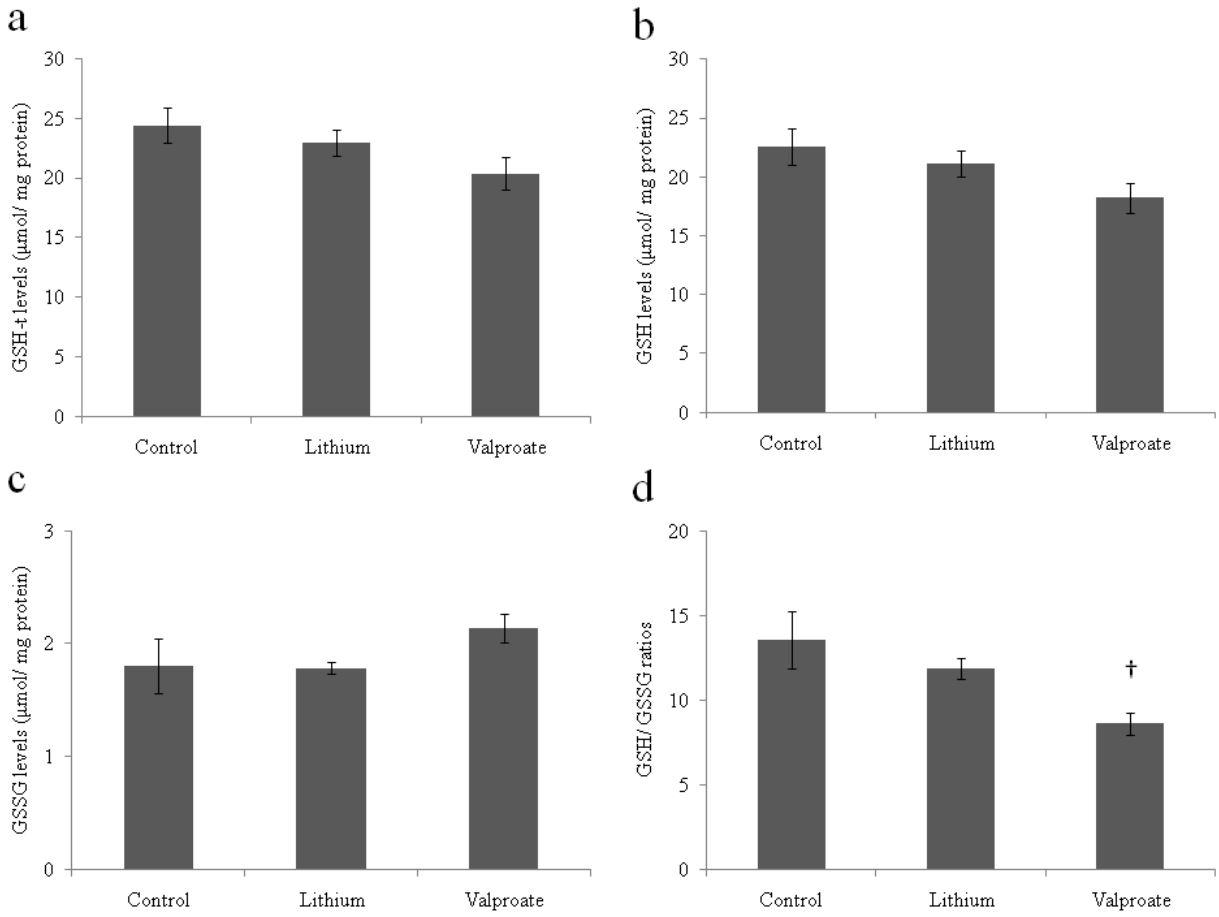

Figure 3.11 Lithium and valproate do not increase the levels of GSH within the brain. The levels of GSH and GSSG within the hippocampus were determined spectrophotometrically and expressed as (a) GSH-T, (b) GSH, (c) GSSG, and (d) ratio of GSH:GSSG. Error bars represent SEM of 5 independent samples. † represents $p < 0.05$ compared to control (Dunnett's test).

3.3.3 Putative effects of lithium and valproate on *Gclc* and *Gclm* expression within the brain

In conjunction with measures of hippocampal GSH and GSSG, the mRNA levels of *Gclc*, *Gclm* and *Hmox-1*, the prototypical ARE-regulated gene, were examined in the hippocampus and pre-frontal cortex following chronic treatment with lithium and valproate. Analysis of data obtained using quantitative RT-PCR revealed that there were no significant effects of treatment on the mRNA levels of hippocampal *Gclc* ($F_{2,27} = 2.14$, $p = 0.14$, Figure 3.12a); *Gclm* ($F_{2,27} = 3.12$, $p = 0.06$, Figure 3.12a); and *Hmox-1* ($F_{2,12} = 0.68$, $p = 0.53$, Figure 3.12a). Similarly, chronic treatment with lithium and valproate did not significantly alter the expression of *Gclc* ($F_{2,27} = 2.14$, $p = 0.14$, Figure 3.12b), *Gclm* ($F_{2,26} = 0.45$, $p = 0.64$, Figure 3.12b), and *Hmox-1* ($F_{2,12} = 1.91$, $p = 0.19$, Figure 3.12b) within the pre-frontal cortex. Consistent with the results of quantitative RT-PCR, analysis of immunoblotting results (Figure 3.13a) also revealed that protein levels of GCLC in the hippocampus ($F_{2,27} = 2.21$, $p = 0.13$; Figure 3.13b) and pre-frontal cortex ($F_{2,27} = 1.49$, $p = 0.24$; Figure 3.13b) did not differ significantly across treatment conditions.

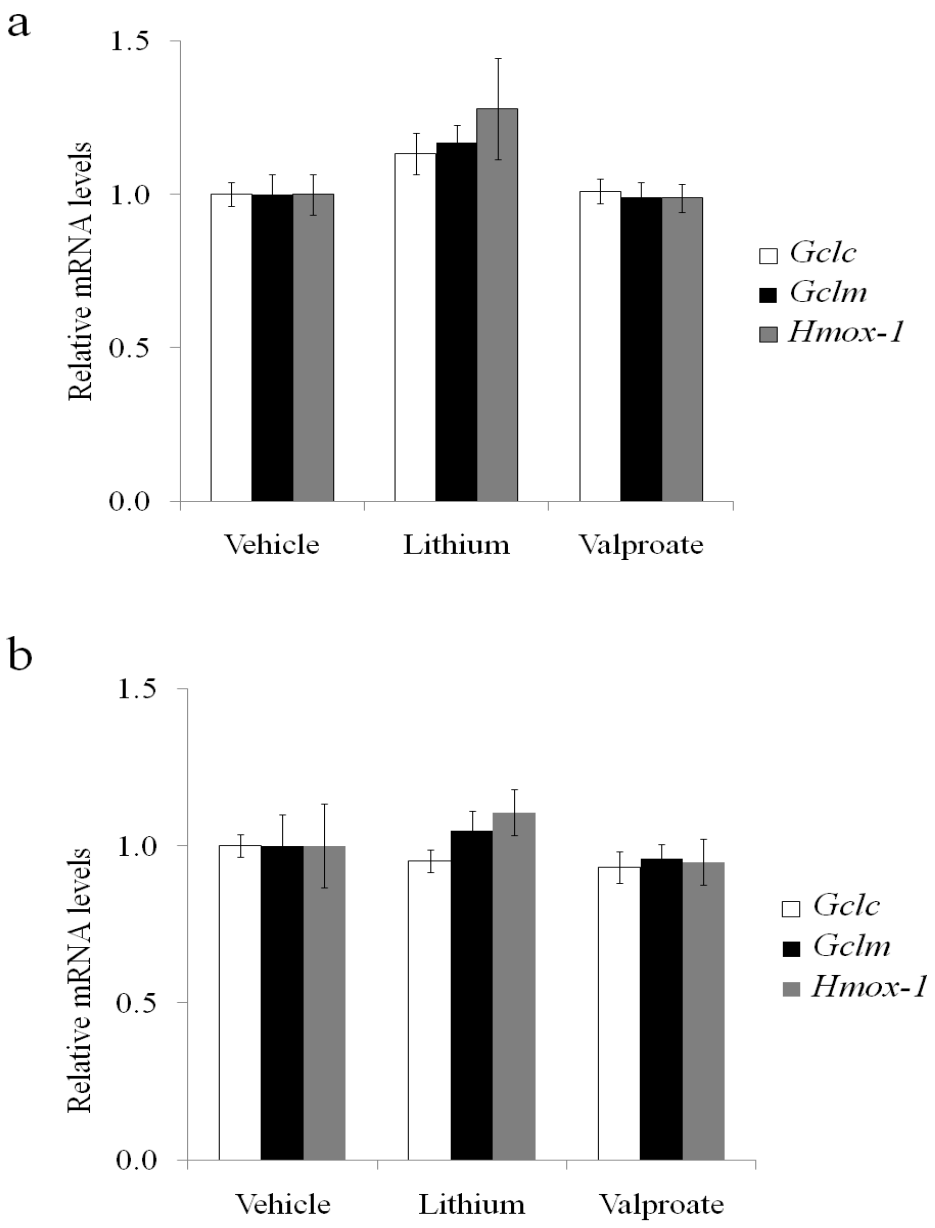

Figure 3.12 Lithium and valproate do not alter *Gclc*, *Gclm*, and *Hmox-1* expression. Gene expression was measured within the (a) hippocampus and the (b) pre-frontal cortex. The levels of mRNA were determined by quantitative RT-PCR. The mRNA levels of *Gclc*, *Gclm* and *Hmox-1*were normalized against levels of *Gapdh* mRNA. Differences in $2^{-\Delta Ct}$ values among treatment groups were analyzed using one-way ANOVA. Data are presented as mRNA levels relative to untreated controls. Error bars represent SEM of 5 to 10 samples.

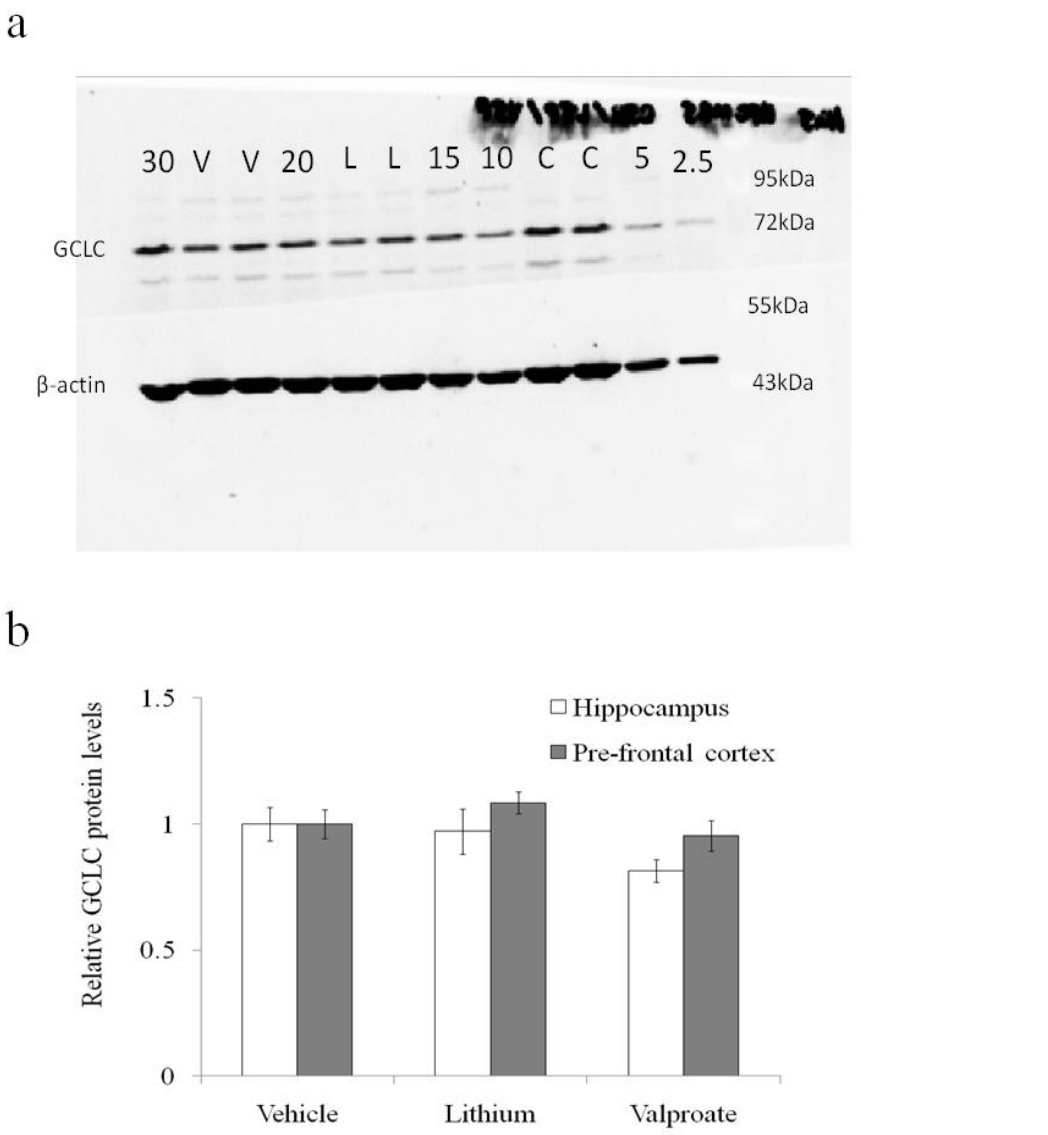

Figure 3.13 Neither lithium nor valproate alters GCLC protein levels within the rat hippocampus and pre-frontal cortex. (a) Representative immunoblot: Purified hippocampal proteins were resolved through gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was probed using antibodies for rat GCLC (72 kDa) and rat β-actin (42 kDa), which was used as a loading control. Control (C), lithium (L), and valproate (V) samples were loaded at 15 µg/ well. A standard curve was generated by loading the indicated amounts (in µg) of protein obtained from pooled rat cortex. (b) No significant effect of treatment on GCLC protein levels was detected in the hippocampus and the pre-frontal cortex. Error bars represent SEM of 10 independent samples.

4 Discussion

This study sought to examine whether the putative neuroprotective effects of mood stabilizers are mediated by changes in GSH metabolism that enhance the cellular reductive potential. Experiments were conducted to test the hypothesis that the neuroprotective effect against oxidative stress-induced cell death, previously associated with chronic lithium or valproate treatment, is a common property shared by other mood stabilizers namely carbamazepine and lamotrigine. Further investigation was conducted to determine whether this neuroprotective effect is due to an increase in the levels of GSH and/or GSH:GSSG ratio—an effect that would enhance the reducing capacity of primary rat cortical neurons and select brain regions. In addition, this study also examined whether chronic mood stabilizer treatment enhances GSH synthesis through induction of *Gclc* and *Gclm*. The results of in vitro studies using primary rat cortical neurons revealed that the neuroprotection against the oxidative stress inducing agent, H_2O_2, is limited to lithium and valproate. Moreover, treatment with BSO was shown to abrogate the neuroprotective effect of lithium, raising the possibility that the neuroprotective effect of this mood stabilizer is dependent on GCL-mediated GSH synthesis. However, subsequent experiments demonstrated that neither lithium nor valproate pre-treatment altered the levels of GSH and the GSH:GSSG ratios. Also, chronic mood stabilizer treatment did not affect the expression of *Gclc* and *Gclm* in primary rat cortical neurons. More importantly, these findings were corroborated by the results of the experiments conducted in vivo. In the rat hippocampus, chronic lithium and valproate treatment did not affect GSH levels. Meanwhile, chronic valproate treatment was even associated with a significant reduction in the GSH:GSSG ratio. Furthermore, the expression of *Gclc* and *Gclm*, as well as the protein levels of GCLC in the hippocampi and pre-frontal cortices, did not significantly differ between controls and lithium- or valproate-treated animals. Taken together, these findings do not support the hypothesis that GSH metabolism is involved in the neuroprotective effect of lithium and valproate against H_2O_2-induced cytoxicity.

4.1 Lithium and valproate, but not carbamazepine and lamotrigine, protect against H_2O_2-induced cell death

In this study, cell death was assessed by measuring the fluorescence intensity of propidium iodide, a membrane-impermeant dye which intercalates with the exposed DNA of non-viable cells (Dengler et al., 1995; Rudolph et al., 1997). This assay has previously been used to examine the toxicity of glutamate in primary cortical neurons and has been shown to produce results that correlated with the measure of cell death obtained using the lactate dehydrogenase (LDH) assay (Rudolph et al., 1997; Sattler et al., 1997). In designing this study, the two other commonly used methods of measuring end-stage cell death, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and LDH assays, were also considered. The MTT assay is based on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide into formazan by mitochondrial succinic dehydrogenase. Thus, the results of the MTT assay may be confounded by factors that alter mitochondrial function but do not necessarily affect cellular viability. Similar to the MTT assay, the LDH assay relies on the detection of tetrazolium formed by the action of lactate dehydrogenase which is released upon cell lysis. Due to its dependence on enzymatic function, the results of the LDH assay are subject to changes in temperature and pH. In addition, the activity of the released LDH is subject to degradation, with a half-life of approximately 9 hours (Promega technical pamphet). In primary rat cortical neurons, necrosis, as indicated by membrane blebbing, has been observed as early as 3 hours after H_2O_2 addition (Whittemore et al., 1995). Thus, the use of LDH activity as a measure of cell death could potentially underestimate the toxicity of H_2O_2. Since the propidium iodide assay depends on the exclusion of the dye by viable cells, this method allows for measurement of necrosis as well as apoptosis while circumventing the issues that are inherent in the other cell death assays. However, the nature of this assay precludes differentiation between apoptosis and necrosis.

The results of this study confirmed that chronic treatment with lithium or valproate at clinically-relevant concentrations prevents oxidative damage and cell death in primary rat cortical neurons, in accordance with results of previous studies examining the protective effect of these drugs in primary rat cortical neurons and human SH-S5Y5 neuroblastoma cells (Cui et al., 2007; Lai et al., 2006). While lithium and valproate protected against the neurotoxicity of 100 μM

H_2O_2, only lithium attenuated cell death induced by a lower concentration (50 μM) of the same stressor. Given the similar extent of neuroprotection exerted by lithium and valproate against 100 μM H_2O_2 and the simultaneous culturing and treatment of neurons, the lack of effect of valproate in these set of experiments is likely due to other extraneous factors. These issues may include inconsistencies in the preparation of the valproate solution and variations in the batches of neurons rather than a reflection of differences in overall culture conditions or in the efficacy of lithium and valproate.

In comparison to lithium and valproate, this study, which measured cell death in primary cortical neurons following incubation with H_2O_2, did not detect a significant neuroprotective effect associated with chronic treatment with carbamazepine or lamotrigine. Indeed, there is a paucity of data supporting the neuroprotective effect of these mood stabilizers against oxidative stress. Recently, however, a number of studies have presented indirect evidence suggesting that chronic treatment with either carbamazepine or lamotrigine at therapeutically relevant concentrations may alter intracellular signaling mechanisms that influence neuronal viability. For example, the mRNA and protein levels of the neuroprotective factors BDNF and bcl-2 were increased in the frontal cortex of rats treated chronically with either carbamazepine or lamotrigine (Chang et al., 2009). Furthermore, it has been reported that treatment with carbamazepine or lamotrigine for 7 days increased the protein levels of GCLC as well as the levels of GSH in human SH-SY5Y neuroblastoma cells (Cui et al., 2007) and primary rat cortical neurons (Bakare et al., 2009). In addition, chronic treatment with lamotrigine in vivo has also been shown to reverse the decrease in GSH-Px activity and the levels of vitamin C, vitamin A, and β-carotene and attenuate the increase in lipid peroxides induced by chronic mild stress (Eren et al., 2007). Although these findings allude to changes that conceivably enhance cellular resilience, there have been no prior studies directly demonstrating the neuroprotective effects of either carbamazepine or lamotrigine against oxidative stress-induced cell death. In fact, Gao and Chuang (1992) have shown that carbamazepine, at levels within the therapeutic range (20 – 50 μM) (McNamara, 2006), reduced [^{3}H] ouabain binding to Na^+-K^+ ATPase, an index of neuronal survival in mixed neuron-astrocyte cultures (Markwell et al., 1991). This detrimental effect on neuronal survival, as assessed by morphological analysis, DNA fragmentation, and MTT reduction, was more pronounced at supra-therapeutic concentrations (100 μM) (Gao et al., 1995; Nonaka et al., 1998).

4.2 Chronic mood stabilizer treatment and GSH metabolism

This study demonstrated that incubation with L-BSO—a widely-used, non-competitive inhibitor of GCL—abolished the neuroprotective effects of lithium against H_2O_2 in vitro, suggesting that the action of this mood stabilizer is likely dependent on the maintenance of GSH levels attributed to constitutive de novo synthesis. As previously mentioned (Section 1.5), GSH is important in the regulation of ROS levels and limits oxidative stress-induced cellular damage. Hence, increasing GSH, and subsequently raising the GSH:GSSG ratio, is an attractive mechanism by which lithium and valproate could enhance the reducing capacity of neurons and brain tissue against oxidative stress. Based on the results showing that lithium and valproate increased GSH-T and the protein levels of GCLC in primary rat cortical neurons, Cui and colleagues (Cui et al., 2007) suggested that mood stabilizers increase GSH by up-regulating its de novo synthesis. Contrary to this hypothesis, the present study did not detect a significant effect of lithium or valproate treatment on neuronal and hippocampal GSH levels—a finding that argues against the involvement of GSH synthesis in the mechanism of action of these mood stabilizers. In addition to de novo synthesis, treatment with lithium or valproate could also potentially boost the levels of GSH by increasing the turnover of GSSG—a notion supported by clinical studies showing that mood stabilizer treatment can increase the activities of GPx (Andreazza et al., 2007a) and GRed (Andreazza et al., 2009). However, the present study revealed that the ratio of GSH:GSSG in primary rat cortical neurons was unaffected by chronic lithium and valproate treatment. The lack of chronic lithium effect on the GSH:GSSG ratio was also observed in the rat hippocampus where valproate caused a decrease. The unexpected reduction in GSH:GSSG ratio, in accordance with trends of lower GSH and higher GSSG relative to controls, may be attributed to the valproate-induced inhibition of GRed, the enzyme that reduces GSSG back to GSH (Cotariu et al., 1992; Gupta et al., 2004). Collectively, this set of findings suggests that treatment with lithium or valproate is unlikely to induce changes to the GSH metabolism under normal, stress-free conditions. Moreover, since there is no increase in the GSH:GSSG couple, the major redox buffer within cells (Schafer and Buettner, 2001), these results also suggest that treatment with either lithium or valproate is unlikely to enhance, and may even decrease, the reducing capacity of neurons and the intact brain.

The discrepancy between the in vitro results presented here and the findings reported by others (Cui et al., 2007) may lie in the difference in culture conditions. In the study by Cui and

colleagues, the primary cortical neurons were maintained for 14 days in Neurobasal media supplemented with B-27. The standard formulation of this media includes antioxidants namely GSH, vitamin E, catalase, and superoxide dismutase. In my study, the amount of antioxidants present in the culture was gradually decreased starting on 7 DIV, 6 days prior to treatment with H_2O_2. By comparison, the number of days the antioxidants were present in the culture media were not specified (Cui et al., 2007). Although it has been shown that neuronal viability is unaffected by the presence or absence of antioxidants after 1 DIV (Perry et al., 2004), it is not clear if different levels of antioxidants during neuronal culture may modulate the intracellular levels of GSH and GSSG.

In light of results indicating that chronic treatment with lithium as well as valproate did not regulate neuronal and hippocampal GSH levels and GSH:GSSG ratios, it is possible that the loss of lithium's neuroprotective effect in the presence of BSO may be due to the additive effects of GCL inhibition and H_2O_2. While BSO was not sufficient to induce cell death (Figure 3.4), the combination of BSO and H_2O_2 may have presented a greater degree of stress that would antagonize the neuroprotective effect of lithium. However, there was no difference in the extent of neurotoxicity induced by 50 μM H_2O_2 in the absence or presence of BSO, suggesting that BSO did not potentiate the toxicity of H_2O_2. Alternatively, the possibility that BSO may inhibit an as yet identified enzyme(s) that plays a crucial role in the neuroprotective mechanism of lithium cannot be excluded. Since its development, the analysis of the binding specificity of BSO has been limited to glutamine synthetase and GCL (Griffith and Meister, 1979). That BSO could directly interact with and inhibit other enzymes that may be involved in regulating neuronal viability has not been well-characterized. In addition, BSO could also indirectly impede enzyme function through reduction of intracellular GSH content (White and Cappai, 2003). In addition to its central role in the defense against oxidative stress, increasing evidence suggests that GSH may also modulate several cellular processes including kinase-dependent signalling mechanisms (Filomeni et al., 2002) and the apoptotic cascade (Franco and Cidlowski, 2009). By depleting GSH levels, BSO could potentially interfere with adaptive cellular mechanisms and nullify the neuroprotective effect of mood stabilizers against oxidative stress-induced cell death. This hypothesis could be further tested by measuring the levels of GSH and GSSG under such conditions and examining the correlation between these parameters and the activity of enzymes such as MAP kinases and caspases.

4.3 Chronic mood stabilizer treatment and *Gclc*, *Gclm* expression

As discussed earlier (Section 1.5), GCL, comprised of GCLC and GCLM, is the rate-limiting enzyme in the de novo synthesis of GSH. It has previously been shown that increased expression and protein levels of the catalytic and modifier subunits of this enzyme protect against various pro-oxidant insults in vitro. In SH-SY5Y neuroblastoma cells, the induction of *Gclc* and *Gclm* and the concomitant increase in levels of GSH have been shown to protect against oxidative stress induced by prolonged exposure to iron loads (Aguirre et al., 2007) and acrolein exposure (Jia et al., 2009). Similarly, increased expression of *Gclc* and *Gclm* and elevated GSH levels have also been shown to enhance the resilience of primary cortical cells against oxidative stress (Ogawa et al., 2008; Saito et al., 2007). In a study examining the role of GSH in the neuroprotective effect of mood stabilizers, Cui and coworkers (Cui et al., 2007) reported that chronic treatment of primary rat cortical neurons with lithium or valproate increased GCLC protein levels. In striking contrast, the present study did not detect a significant effect of chronic lithium or valproate treatment on the expression of *Gclc* and *Gclm* mRNA levels in primary rat cortical neurons as well as in the rat hippocampus and the prefrontal cortex. These findings suggest that the previously reported increase in the levels of GCLC protein following chronic mood stabilizer treatment (Cui et al., 2007) is not likely due to transcriptional induction of *Gclc*. Moreover, within the two brain regions examined, chronic treatment with lithium or valproate did not affect the protein levels of GCLC. While the present results are in contrast with the data presented by Cui and colleagues (Cui et al., 2007), the lack of treatment effect on *Gclc* and *Gclm* mRNA expression as well as GCLC protein levels are consistent with my findings of comparable GSH levels following chronic lithium and valproate treatment in vitro and in vivo.

4.4 Putative mechanisms mediating the neuroprotective effects of lithium and valproate

The results of the present study argue against the involvement of GSH metabolism in the neuroprotective effect of lithium and valproate. However, these two mood stabilizers have been shown to target several proteins (Section 1.4) and could potentially regulate multiple signaling cascades that interact with the biochemical pathways activated by oxidative stress. As illustrated in Figure 4.1, ROS readily react with biological macromolecules resulting in oxidative damage to DNA (Whittemore et al., 1995), proteins (Stadtman, 1992; Stadtman and Berlett, 1997), and lipids (Sewerynek et al., 1995), facilitate mitochondrial permeability transition (Takeyama et al., 2002), inhibit protein phosphatases (Chen et al., 2009), and stimulate cyclo-oxygenase 2 (COX-2) activity (Fatokun et al., 2007). These immediate actions of ROS subsequently result in downstream effects that eventually lead to cell demise. DNA damage could activate PARP, which has been shown to precipitate cell death (Cole and Perez-Polo, 2002) either through energy loss (Ha and Snyder, 1999) or activation of the apoptosis inducing factor (Yu et al., 2002). Lipid peroxidation could lead to loss of membrane integrity and contribute towards formation of protein aggregates that would impede proteasome activity (Friguet and Szweda, 1997; Shringarpure et al., 2000). The inhibition of protein degradation could induce ER stress and apoptosis, especially under conditions where calcium buffering is lost due to mitochondrial permeability transition. Besides elevating intracellular calcium levels, increased mitochondrial permeability would also result in the uncoupling of oxidative phosphorylation cascades, and the release of ROS (Kannan and Jain, 2000). More recently, Chen and colleagues (Chen et al., 2009) suggested that inhibition of protein phosphatase 2A and 5 may explain the previously observed activation of JNK and ERK 1/ 2 MAPK, an event that facilitated cell death following exposure to H_2O_2 (Crossthwaite et al., 2002; Ishikawa and Kitamura, 2000; Ruffels et al., 2004). While the effectors of COX-2 have not been identified, inhibition of this enzyme has been shown to mitigate cell death induced by H_2O_2 (Fatokun et al., 2007).

Although GSH metabolism is not involved, the observed neuroprotective effects of lithium and valproate against H_2O_2 could be mediated by the action of these mood stabilizers on one or more of the pathways linking oxidative stress and cell death, as shown in Figure 4.1. These mood stabilizers could reduce the ROS levels and directly prevent oxidative damage by up-regulating the activity of antioxidant enzymes. This idea is supported by the results from clinical studies

indicating that chronic mood stabilizer treatment increases in the activities of SOD and CAT, which catalyzes the dismutation of superoxide to O_2 and H_2O_2, and the decomposition of H_2O_2 to O_2 and H_2O, respectively. In addition, lithium and valproate could also potentially antagonize the deleterious effect of H_2O_2 on mitochondrial function. These mood stabilizers could decrease the probability of mitochondrial permeability transition as chronic treatment with these drugs has been shown to increase membrane potential (Bachmann et al., 2009). Also, lithium and valproate have also been shown to block the initiation of the apoptotic cascade likely due to the induction of the anti-apoptotic protein Bcl-2 (Bachmann et al., 2009; Chen et al., 1999b; Chen and Chuang, 1999; Corson et al., 2004; Creson et al., 2009; Hao et al., 2004; Hiroi et al., 2005; Laeng et al., 2004; Lai et al., 2006; Li et al., 2008; Michaelis et al., 2006; Pan et al., 2005; Senatorov et al., 2004; Tsai et al., 2008; Wei et al., 2001; Wlodarczyk et al., 1996; Yazlovitskaya et al., 2006; Yuan et al., 2001) as well as decrease the expression of proapoptotic factors, such as p53 and Bax (Chen and Chuang, 1999). Furthermore, lithium and valproate have also been shown to induce HSP70 (Bijur and Jope, 2000; Kim et al., 2005; Marinova et al., 2009; Pan et al., 2005; Ren et al., 2004; Ren et al., 2003) and ER chaperone proteins including GRP78, GRP 94, and calreticulin (Bown et al., 2000b; Chen et al., 2000; Hiroi et al., 2005; Kim et al., 2005; Shao et al., 2006; Shi et al., 2007; Wang et al., 1999b)—effects that could facilitate protein degradation and prevent the accumulation of protein aggregates.

With regards to signaling cascades, lithium has been shown to inhibit JNK and p38 MAPK (Chen et al., 2003; Hongisto et al., 2003), activation of which are typically associated with neuronal cell death. Interestingly, activation of ERK MAPK, which has been implicated in oxidative stress-induced cell death, has also been associated with the neuroprotective effects of lithium (Di Daniel et al., 2005; Einat et al., 2003; Mai et al., 2002; Michaelis et al., 2006; Nielsen et al., 2008; Straiko et al., 2009; Xia et al., 2008; Yan et al., 2007; Young et al., 2008) and valproate (Biermann et al., 2010; Creson et al., 2009; Einat et al., 2003; Hao et al., 2004; Pan et al., 2005; Yuan et al., 2001). This pro-survival activity may be due to the activation of ERK MAPK in the absence of p38 induction which accompanies oxidative stress. More recently, Bosetti and colleagues (2002) demonstrated that chronic lithium treatment downregulates the cytotoxic activity of COX-2, providing yet another avenue by which lithium could inhibit oxidative stress-induced cell death.

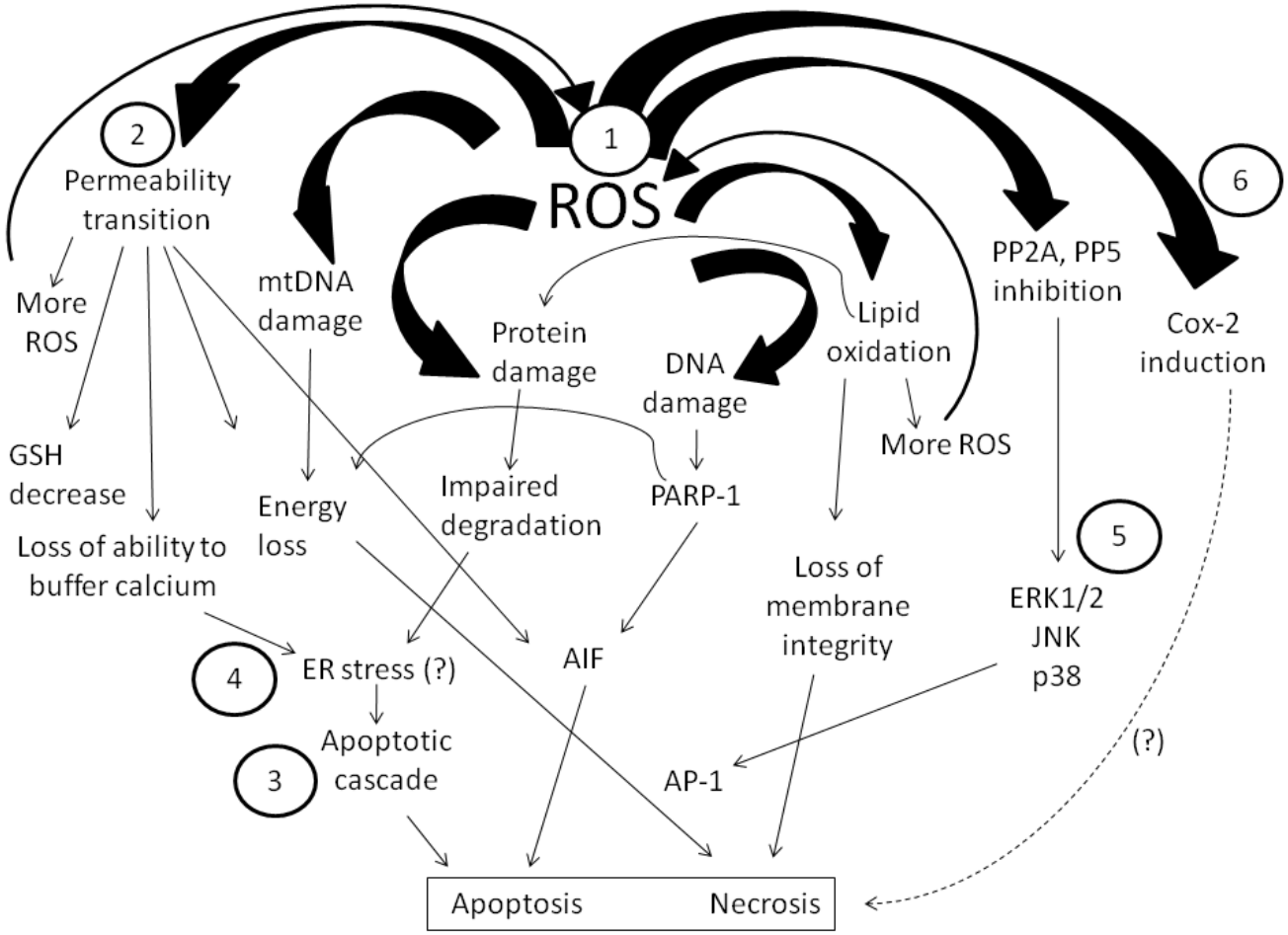

Figure 4.1 Effects of lithium and valproate on pathways linking oxidative stress and cell death. The immediate effects (→) of ROS trigger downstream events (→) that lead to cell death. Lithium and/ or valproate can inhibit cell death by (1) neutralizing ROS through induction of SOD and CAT, (2) blocking mitochondrial permeability transition, (3) antagonizing the apoptotic cascade through Bcl-2 induction, (4) up-regulating chaperone proteins, (5) activating ERK and inhibiting of JNK and p38 MAPK, and (6) inhibiting COX-2 activation.

4.5 Potential limitations

Overall, the results of this study are not consistent with the results of earlier studies that implicate the involvement of GSH metabolism in the neuroprotective effect of lithium and valproate. Although corroborated by data obtained from experiments conducted in vitro and in vivo, these findings should be interpreted with caution owing to several limitations in the present study. First, the levels of GSH and GSSG were measured in only 4 separate sets of cultured neurons, raising the possibility that the observed changes in GSSG levels and GSH/ GSSG following treatment may reach statistical significance if the sample size was increased. Power analysis estimates that a sample size of at least n=8 would be required to achieve a power of 80% with a one-sided test and having a Type I error (α) of 0.05, a Type II error (β) of 0.20. Thus a larger sample size would be necessary to clarify whether chronic treatment with lithium or valproate would decrease GSH/ GSSG, as suggested by the observed trend in Figure 3.8d.

Second, propidium iodide fluorescence was the only index used to measure cell death. As mentioned earlier (Section 4.1), this assay, which relies on the incorporation of the membrane-impermeant dye into exposed DNA, cannot distinguish between apoptosis and necrosis. The ability to discriminate between these modes of cell death can aid in identifying pathways that mediate the neuroprotective effects lithium or valproate. Also, this fluorescence-based assay is highly dependent on the even distribution of neurons growing in a monolayer. The clumping of cells and the presence of sparse regions result in altered fluorescence readings, which increase inter-well variability but do not reflect changes in cellular viability. Besides this end-stage assay, the measurement of apoptotic indices such as caspase 3 activation and cytochrome c release through immunolabeling and DNA fragmentation through terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) would address these limitations and enhance the evaluation of cell death.

Third, the effects of chronic mood stabilizer treatment on cellular viability and GSH metabolism were examined using primary rat cortical neurons. While this in vitro approach offers several advantages over experimental designs involving immortalized neuronal cell lines, it is unlikely that these cultured primary rat neurons would exhibit disease-specific phenotypes. As mentioned earlier, platelets, lymphocytes, lymphoblasts and differentiated olfactory neurons derived from BPD patients exhibit changes in cellular function including gene expression (Section 1.2.2),

second messenger signaling (Section 1.2.3.2), calcium regulation (Section 1.2.3.3), and UPR (Section 1.2.3.4). Moreover, Andreopoulus and colleagues (Andreopoulus 2004) demonstrated that chronic lithium treatment reduces the levels of TRPC3 in B lymphoblasts derived from BPD patients but not in healthy controls, suggesting that the response to mood stabilizers may be differentially altered in cells established from BPD patients. Thus, these current results which are obtained from unaffected neurons may underestimate the involvement of cellular mechanisms in the therapeutic action of mood stabilizers. In the same vein, the mRNA and protein levels of GCLC and GCLM as well as GSH and GSSG were measured in brain regions of healthy animals that were chronically treated with lithium or valproate. Hence, this paradigm may not effectively mirror the effect of these mood stabilizers in the context of disease.

Fourth, exogenous H_2O_2 was used exclusively to induce oxidative stress. A number of reports suggest that the mechanisms activated by this mode of oxidative stress differ from signalling pathways triggered by H_2O_2 produced from intracellular sources (Boutahar et al., 2008; Choi et al., 2005; King and Jope, 2005; Veal et al., 2007). For instance, in human neuroblastoma SH-SY5Y cells, GSK3β inhibitors attenuated mitochondrial inhibitor, rotenone- but not H_2O_2-induced cell death (King and Jope, 2005; Lai et al., 2006), suggesting GSK3β-mediated pathway is involved in cytotoxicity induced by intrinsic, but not extrinsic, oxidative stress. Although the cytoprotective effects of lithium and valproate against oxidative stress induced by H_2O_2 and rotenone have previously been demonstrated (Lai et al., 2006), the possibility that carbamazepine and lamotrigine may regulate other pathways and exert differential effects against an intrinsic- versus exogenous-derived oxidative stress cannot be discounted.

Fifth, the protein levels of GCLC were not measured in primary rat cortical neurons. Rather, this study assessed the effect of lithium and valproate treatment on *Gclc* and G*clm* mRNA levels. An analysis of the relationship between mRNA and protein levels revealed moderate and varied correlations suggesting the potential limitation of using mRNA levels to predict protein levels (Guo et al., 2008). Hence, it is not possible to conclude whether chronic mood stabilizer treatment induced changes at the protein level in vitro in spite of quantitative RT-PCR results indicating that lithium and valproate do not affect *Gclc* expression in primary rat cortical neurons. However, given that lithium and valproate did not alter *Gclc* mRNA expression and protein levels of GCLC in rat hippocampus and pre-frontal cortex, the current in vitro results also imply that any differences in the protein levels of GCLC in primary rat cortical neurons

following mood stabilizer treatment are not likely due to changes in the transcriptional regulation of *Gclc*.

4.6 Future directions

In light of increasing interest in the involvement of oxidative stress in the pathophysiology of BPD, the results of this study warrants further investigation into the role of antioxidant mechanisms in the action of mood stabilizers. Future studies could address the following questions.

Could mood stabilizers influence GSH metabolism under chronic mild oxidative stress conditions?

The up-regulation of GSH synthesis in response to oxidative stress (Chen et al., 2006b; Kondo et al., 1999; Liu et al., 2001; Saito et al., 2007; Schuliga et al., 2002; Shi et al., 1994) raises the possibility that the presence of ROS could influence the putative effect of mood stabilizers on GSH metabolism. Efforts to explore the possibility that mood stabilizers act in concert with intracellular defense mechanisms would be greatly aided by a comprehensive approach combining cellular and pre-clinical animal models. Through in vitro studies, the effect of chronic mood stabilizer treatment on cell viability, ROS generation, GSH metabolism, and gene expression can be evaluated in primary neurons subjected to chronic mild oxidative stress induced by mitochondrial inhibitors such as malonate, 6-hydroxydopamine, rotenone and MPP^+. The use of this chronic regimen is particularly relevant to BPD as mitochondrial dysfunction has been implicated in the pathophysiology of the disorder. Alternatively, the same measures can be determined in patient-derived neurons by taking advantage of developments in directed differentiation of induced pluripotent stem cells (iPSC) and neural progenitor derived from the olfactory neuroepithelium. Studies have shown that human iPSCs are able to differentiate into functional neurons characterized by the capacity to fire action potentials and by the presence of neuronal markers including MAP2 and choline acetyltransferase (ChAT) (Hu et al., 2010; Karumbayaram et al., 2009). As well, functional neurons have also been derived from human olfactory neuroepithelial-derived progenitors following application of growth promoters including retinoic acid, forskolin, and sonic hedgehog (Zhang et al., 2006). Ideally, these differentiated neurons would provide a disease-relevant in vitro model for examining the effect of mood stabilizers. However, it is currently not known whether the phenotype of these

differentiated neurons is identical to that displayed by mature neurons in the brain of BPD patients.

Complementary to studies using cellular models, the effect of chronic mood stabilizer treatment on brain GSH metabolism, redox status, and behaviour should also be examined in animal models of BPD. Although the antioxidative properties of mood stabilizers have been demonstrated in the more traditional models of mood dysregulation including amphetamine–induced mania (Frey et al., 2006b) and chronic variate stress (de Vasconcellos et al., 2006), the effect of mood stabilizers on redox status have yet to be examined in more relevant models of mania and depression. Animal models, which carry mutations in genes shown to be affected in patients, were developed based on increased understanding of molecular abnormalities associated with BPD (Malkesman et al., 2009). Models wherein lithium and valproate have been shown to attenuate manic and/or depressive-like behaviours include transgenic mice expressing mutant mitochondria DNA polymerase (POLG) in a neuron specific manner; knockout mice in which genes encoding glutamate receptor 6, *Erk*, or *bcl-2* have been inactivated; and mice with a mutated *Clock* gene (Malkesman et al., 2009). Mice over-expressing GSK3β have also been shown to exhibit hyperactivity and increased sensorimotor reactivity (Prickaerts et al., 2006). However, there are currently no publications characterizing the effects of lithium and valproate in this model (Kato et al., 2007; Malkesman et al., 2009). It would be of interest to analyze the effects of mood stabilizers on GSH levels, antioxidant enzyme activities, and oxidative stress markers in these animal models.

What other mechanisms could underlie the antioxidant effects of lithium and valproate?

The lack of changes in GSH metabolism following chronic treatment with either lithium or valproate underscores the contribution of other mechanisms in mediating the neuroprotective effect of these agents against H_2O_2. As discussed earlier (Section 4.4), lithium and valproate have been shown to target mitochondria and ER-related proteins that could regulate the pathways linking oxidative stress and cell death. Given these findings, it is likely that mitochondrial stabilization and an enhanced ER stress response are involved in the neuroprotective effects of lithium and valproate against oxidative stress. Preliminary experiments to test this hypothesis could involve measurement of mitochondrial function and ER stress response in lithium or valproate-treated neurons that exhibit tolerance to oxidative

stress insults. Changes in mitochondrial stability can be assessed by monitoring mitochondrial membrane potential ($\Delta\Psi_m$) using fluorescent dyes including JC-1 and rhodamine 123. Meanwhile, markers of the unfolded protein response including phosphorylated protein kinase-like ER-resident kinase (phospho-PERK), CCAAt/enhancer binding protein (C/EBP) homologous protein (CHOP), IRE1, an ER transmembrane kinase/ ribonuclease, the ER chaperone BiP, and the transcription factors XBP1 and ATF4 can be measured using immunodetection techniques. Studies that would further explore the involvement of the mitochondria and ER could aid in the development of new interventions for BPD that are geared towards improved organelle function.

Are neuroprotective effects against oxidative stress crucial for mood stabilizer action?

Along with lithium and valproate, carbamazepine and lamotrigine are also indicated for treatment of BPD (Section 1.1). Unlike the first two mood stabilizers, however, these anticonvulsants did not protect neurons against H_2O_2-induced cell death. Moreover there are limited data supporting the antioxidant properties of carbamazepine and lamotrigine (Section 1.4), suggesting that neuroprotective effects against oxidative stress may not be involved in the action of mood stabilizers. The efficacy of supplementation with NAC, a thiol antioxidant and GSH precursor, in alleviating the symptoms of BPD has previously been demonstrated in a double-blind placebo-controlled randomized trial (Berk et al., 2008). However, in addition to neutralization of ROS, NAC has also been shown to inhibit nitric oxide production (Bergamini et al., 2001; Cuzzocrea et al., 2000) and potentially affect cellular signaling mechanisms. Thus further work is needed to delineate the role of antioxidant effects in the treatment of BPD. Future studies could examine whether supplementation with other antioxidants that are transported across the blood-brain barrier such as vitamin E, vitamin A, melatonin, and alpha lipoic acid (Gilgun-Sherki et al., 2001) could decrease the severity of BPD symptoms.

Are abnormalities in GSH metabolism relevant to the pathophysiology of BPD?

As GSH is a major component of the endogenous antioxidant system, additional studies evaluating the relationship between GSH metabolism and BPD would enhance understanding of the involvement of oxidative stress in the pathophysiology of the disorder. Data from clinical (Andreazza et al., 2007a; Andreazza et al., 2009) and post-mortem (Benes et al., 2006) studies

have already suggested that disturbances in GSH metabolism may accompany BPD. Indeed, an association between GSH deficiency and behavioural and cognitive dysfunction has been demonstrated in animal models (Dean et al., 2009; Steullet et al.). However, characterization of GSH synthesis and consumption in the context of BPD is still limited. Studies comparing the frequencies of GCLC, GCLM, and GS polymorphisms among patients, family members, and healthy controls would be valuable in determining whether genetic variants can predispose individuals to BPD. A similar analysis in patients with schizophrenia revealed that oxidative stress, which has also been implicated in the pathophysiology of this disorder, may be due to a mutation in GCLC resulting in impaired GSH synthesis (Gysin et al., 2007; Gysin et al., 2009). Furthermore, assessment of the activities of GSH-related enzymes in peripheral tissue along with assays of oxidative stress indicators, including GSH and GSSG levels, during the early and late stages of the disorder could reveal whether progression of BPD is associated with alterations in GSH metabolism that may increase cellular vulnerability to oxidative stress.

4.7 Conclusion

Overall, the results of this study indicate that GSH metabolism is unlikely to be involved in the neuroprotective effect of lithium and valproate. By comparing the putative effects of lithium, valproate, carbamazepine, and lamotrigine against H_2O_2-induced cell death in primary rat cortical neurons, this study demonstrated that neuroprotection against oxidative stress is not a property shared by these four mood stabilizers. While these results further establish the antioxidative properties of lithium and valproate, these findings do not support neuroprotection against H_2O_2-induced oxidative stress in the mechanism of action of carbamazepine and lamotrigine.

Although BSO treatment attenuated the neuroprotective effect of lithium, it is unlikely that an increase in cellular reductive capacity due to elevated levels of GSH is involved in the mechanism of action of lithium. As demonstrated in primary rat cortical neurons and in the hippocampus, chronic treatment with lithium and valproate, at therapeutically relevant concentrations, did not increase GSH and GSH:GSSG ratio. These findings suggest that previously identified molecular targets which include (1) SOD and CAT induction, (2) increased mitochondrial stabilization, (3) enhancement of ER stress response, (4) activation of pro-

survival (5) MAPK signaling, and (6) inhibition of pro-apoptotic mechanisms are more likely to play a role in mediating the neuroprotective effects of lithium and valproate.

That the neuroprotective effect of lithium and valproate is mediated by the up-regulation of *Gclc* and *Gclm* is an attractive hypothesis as earlier studies have reported an association between oxidative stress tolerance and increased protein levels of GCLC and GCLM (Aguirre et al., 2007; Jia et al., 2009; Ogawa et al., 2008; Saito et al., 2007). However, the results of present *in vitro* and *in vivo* experiments indicate that under basal conditions, chronic treatment with either lithium or valproate does not affect *Gclc* and *Gclm* expression. It is possible that under chronic oxidative stress conditions, mood stabilizers may enhance the induction of antioxidant response genes which might include *Gclc* and *Gclm*. This hypothesis remains to be tested.

Future studies, using a combination of disease-relevant in vitro and in vivo models, could examine the contribution of previously identified targets to the antioxidant effects of lithium and valproate. In addition, clinical studies could also evaluate the efficacy of antioxidant supplements in mood stabilization and analyze potential alterations in GSH metabolism in BPD patients. Together, the results of these in vitro and in vivo studies can guide the development of new interventions for BPD.

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