# THE NEUROPROTECTIVE PROPERTIES OF MOOD STABILIZERS: FROM GENE REGULATION TO ULTRASTRUCTURAL CHARACTERIZATION

By

CHRISTOPHER D. BOWN, B.SC.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

# NEUROPROTECTION AND MOOD STABILIZERS

DOCTOR OF PHILOSOPHY (2002)
(Medical Sciences)

McMaster University
Hamilton, Ontario

TITLE: The Neuroprotective Properties of Mood Stabilizers: from Gene Regulation to Ultrastructural Characterization

AUTHOR: Christopher D. Bown, B.Sc. (McMaster University)

SUPERVISOR: Professor L.T. Young

NUMBER OF PAGES: xii, 215

ABSTRACT

Bipolar disorder, or manic-depressive illness, is a common psychiatric illness affecting 1.5% of the population. It is a chronic illness that requires long-term (often lifetime) pharmacotherapy. The need for lifetime treatment with mood stabilizers (i.e. lithium, valproate and carbamazepine) and high relapse rates suggests that changes in gene expression may be involved in the mechanism of action of these drugs. Using differential display, we identified the 78-kilodalton glucose-regulated protein, GRP78, as being at valproate-regulated gene in both rat brain and cultured cells. GRP78 belongs to a family of resident endoplasmic reticulum proteins, referred to as the ER stress proteins, which includes GRP94 and calreticulin. These proteins act as both calcium binding proteins and molecular chaperones within the endoplasmic reticulum and when overexpressed protect the cell against cytotoxic insults. In addition to GRP78, we were able to show that GRP94 and calreticulin are also upregulated in a concentration- and time-dependent manner in response to valproate. This effect was shown to be either drug- or drug class-specific, as treatment with other psychotropic drugs did not elicit a similar response. In patient samples, all three ER stress proteins were increased in postmortem temporal cortex from depressed subjects who died by suicide. These results suggest a possible cytoprotective role for mood stabilizers, since studies have shown that valproate as well as lithium can regulate the expression of proteins with known cytoprotective properties. Indeed, when rat hippocampal neurons were treated for 7 days with the mood stabilizers, lithium, valproate and carbamazepine, a significant reduction in NMDA-mediated cytoplasmic vacuolization could be quantified using transmission electron microscopy. In conclusion, the results of this thesis contribute to a growing number of studies that propose neuroprotective properties for mood stabilizing drugs, and suggest that valproate might act in part by regulating ER stress proteins.

ACKNOWLEDGEMENTS

This thesis is dedicated to my wife, Tanya, who has provided unconditional support and understanding throughout the course of my studies. Without her love and encouragement many of my successes would have not been possible.

I would also like to extend a tremendous amount of gratitude to my supervisor, Dr. L. Trevor Young, for providing an environment for study where scientific inquiry was not bound by financial constraints. His work ethic and dedication to science are lessons that cannot be learned from textbooks.

I would also like to thank all those individuals that at sometime or another wandered into the laboratory, especially Dr. Jun-Feng Wang, Dr. Glenda MacQueen, Dr. Biao Chen, XiuJun Sun, Dar Dowlatshahi, Yarema Bezchlybnyk, and David Cadotte. I deeply appreciate the discussions and assistance that each provided over the course of my studies.

Finally, I would like to acknowledge the love and support of my parents who from an early age taught me to follow my dreams and that hard work will eventually turn those dreams into reality.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5-HIAA – 5-hydroxyinoleacetic acid
5-HT – serotonin
AC – adenylyl cyclase
ACTH – adrenocorticotropic hormone
AMI – Amitriptyline
ANOVA – analysis of variance
AP-1 – activator protein-1
Apaf-1 – apoptosis protease activating factor
APZ - Alprazolam
Bcl-2 – B cell lymphoma protein-2
BD – bipolar disorder
BDZ – Benzodiazepine
βAR – β-adrenergic receptors
BiP – immunoglobulin binding protein
bp – base pairs
BSA – bovine serum albumin
BTP – Benzotropine
BUP – Bupropion
BUS – Buspirone
CAD – caspase-activated deoxyribonulease
cAMP – cyclic adenosine-monophosphate
CBZ – Carbamazepine
CH - Chloral hydrate
CMI – Clomipramine
CNP – Clonazepam
CNS – central nervous system
CPT – Chlorprothixene
CPZ – Chlorpromazine
CRF – corticotropin-releasing factor
CSF – cerebral spinal fluid
CZP – Clozapine
DAG – diacylglycerol
DD-PCR – differential display polymerase chain reaction
DMI – Desipramine
DMSO – dimethylsulfoxide
DPH – Diphenhydramine
DSM – Diagnostic and Statistical Manual for Mental Disorders
ECL – enhanced chemiluminescence
ER – endoplasmic reticulum
ERSE – ER stress element
FLU – Fluoxetine
FZ – Flurazepam

GABA – γ-aminobutyric acid
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
GFAP – glial fibrillary acidic protein
GRP – glucose regulated protein
GSK3β – glycogen synthase kinase 3β
HAL – Haloperidol
HBSS – Hank's balanced salt solution
HSP70 – 70-kilodalton heat shock protein
HXZ – Hydroxyzine
IMI – Imipramine
$IP_3$ – inositol triphosphate
LZP – Lorazepam
MAOI – monoamine oxidase inhibitor
MAPK – mitogen-activated protein kinase
MARCKS – myristoylated alanine-rich C kinase substrate
MDD – major depressive disorder
MPT – Maprotiline
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NE – norepinephrine
NEF – Nefazadone
NMDA – N-methyl-D-aspartate
NMR – nuclear magnetic resonance
NOR – Nortriptyline
NSE – neuron-specific enolase
nt – nucleotides
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PDVF – polyvinylidene difluoride
PEBP2β – polyomavirus enhancer-binding protein 2β
PET – positron emission tomography
PHT – Phenytoin
PI – phosphoinositide
$PIP_2$ – phosphatidyl inositol
PKA – protein kinase A
PKC – protein kinase C
PLA2 – phospholipase A2
PLCβ - phospholipase Cβ
PTP – permeability transition pore
PXT – Paroxetine
RIS – Risperidone
RT-PCR – reverse transcriptase-polymerase chain reaction
SCZ – schizophrenia
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SER – Sertraline

SNRI – serotonin-norepinephrine reuptake inhibitor
SPECT – single photon emission computed tomography
SSPE – sodium chloride/sodium dihydrogen phosphate/EDTA
SSRIs – selective serotonin reuptake inhibitors
STS – staurosporine
TBHP – *tert*-butylhydroperoxideTCA – tricyclic antidepressant
TH – Thiothixene
THP – Trihexphenidyl
TRD – Thioridazine
TRI – Trimipramine
TZD – Trazadone
TZP – Temazepam
VEN – Venlafaxine
VPA – Valproate
VRG – Valpraote regulated gene
WHO – World Health Organization

# 1.0 INTRODUCTION

## 1.1 Mood disorders

Mood disorders include major depressive disorder and bipolar disorder, and together are the most common psychiatric illnesses (Goodwin and Jamison, 1990). The World Health Organization (WHO) recently identified five mental disorders, including major depression, to be among the ten leading causes of disability worldwide. In the 2001 World Health Report published by the WHO, it is estimated that 450 million people worldwide suffer from a mental disorder. Of that population it is estimated that 340 million people around the globe are affected by major depression. Great strides have been made in recent years to increase the quality of life of patients suffering from mood disorders by improving medications and therapies. Today, it is appreciated that mood disorders have a biological basis and may be the result of disturbances in neurochemical and neuroendocrine function leading to changes in gene expression and neuroanatomical abnormalities. However, the exact changes responsible for the disorders have yet to be elucidated.

### 1.1.1 Bipolar disorder

Bipolar affective disorder, or manic-depressive illness, is likely one of the oldest known mental illnesses and is characterized by episodes of mania and depression. The disorder was first noticed as far back as the second century. Some reports suggest that Aretaeus of Cappadocia, a city in ancient Turkey, first recognized some of the symptoms of mania and depression, and felt they could be linked to each other. Jules Falret was able to describe a distinction between moments of depression and heightened moods, and in 1875 his findings were termed Manic-Depressive Psychosis (Angst and Sellaro, 2000). However, it is the work of a German psychiatrist, Emil Kraeplin, in the early 1900's who is credited with defining bipolar disorder. Emil Kraeplin, in the textbook *Psychiatire Ein Lehrbuch fur Studieride und Arzte*, coined the term "manic-depressive" illness to explain how mania and depression affect the patient (Kraeplin, 1913). It was not until the release of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-III) in the 1980's that the diagnostic term bipolar disorder replaced manic-depressive illness, as proper terminology for the disorder.

Currently, bipolar disorder is one of the most commonly diagnosed psychiatric illnesses affecting approximately 1.5% of the population (Muller-Oerlinghausen et al., 2002). The natural course of bipolar disorder is one of alteration between states of illness and apparent health, even without treatment (Wolpert et al., 1990). Manic episodes are classified by the presence of three or more of the following: 1) inflated self-esteem or

grandiosity; 2) decreased need for sleep; 3) more talkative than usual or pressure to keep talking; 4) flight of ideas or subjective experience that thoughts are racing; 5) distractibility (i.e. attention too easily drawn to unimportant or irrelevant external stimuli); 6) increase in goal-directed activity (either socially, at work or school or sexually) or psychomotor agitation; and 7) excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g. engaging in unrestrained buying sprees, sexual indiscretions or poor business investments) (DSM-IV; Diagnostic and Statistical Manual for Mental Disorders-fourth edition). In contrast, depressive episodes are accompanied by those feelings described for major depressive disorder (described in section 1.1.2). Current DSM-IV criteria subdivides bipolar disorder into two distinct groups: bipolar I (having both depressive and manic episodes) or bipolar II (recurrent depression with hypomania, defined as manic behaviour falling short of full-blown mania) (MacQueen and Young, 2001). Patients with bipolar II disorder only show signs of hypomania, and not full blown mania, therefore misdiagnosis as unipolar depression is common.

The onset of bipolar disorder most often occurs in early adulthood and persists throughout life (Muller-Oerlinghausen et al., 2002). However, recent studies suggest the existence of an early onset childhood bipolar disorder. In fact, current theories suggest that the commonly diagnosed childhood disorder, attention-deficit hyperactivity disorder (ADHD) might be an early form of mania (Akin, 2001). Even from an early age, quality of life can be greatly impaired in bipolar disorder patients with higher than average rates of suicide and comorbidity. Approximately 25-50% of bipolar patients attempt suicide at

least once in their lifetime (reviewed by Jamison, 2000), which is five to ten times higher than the lifetime suicide attempt rates for the population as a whole provided by the World Health Organization (Welch, 2001). Comorbidity with other illnesses, particularly, substance abuse has been shown to be quite prevalent in patients with bipolar disorder. In a study by Regier et al. (Regier et al., 1990) the authors showed that individuals with bipolar I disorder had a 46% lifetime prevalence of alcohol-related disorders compared to only 14% of the population as a whole. Additional studies have also found that abuse of other drugs, including cocaine, marijuana and heroin, are more common in bipolar disorder than within the general population (Sherwood et al., 2001). Both comorbidity and higher than normal suicide rates in bipolar disorder patients presents a large economic burden as a result of the costs associated with treatment, loss of productive work and premature death (Wyatt and Henter, 1995). Therefore, identifying the mechanisms responsible for disease manifestation will provide opportunities to develop more effective treatments and improved prognosis for those affected with the disorder.

### 1.1.2 Major depressive disorder

Major depressive disorder, or unipolar depression, is the most common psychiatric illness with a lifetime prevalence of 16-20% (Doris et al., 1999). The World Health Organization recently reported that major depression would be one of the most important causes of ill health in this century. Although the symptoms of depression are frequently experienced by most individuals, clinical depression is different in that feelings of unhappiness and disappointment become qualitatively different, pervasive or interfere with normal function (Doris et al., 1999). DSM-IV describes an individual as having major depressive disorder when five or more of the following symptoms are present nearly everyday for a two week period: 1) depressed mood most of the day; 2) markedly diminished interest or pleasure in all, or almost all, activities most of the day; 3) significant weight loss when not dieting or weight gain, or decrease or increase in appetite; 4) insomnia or hypersomnia; 5) psychomotor agitation or retardation; 6) fatigue or loss of energy; 7) feelings of worthlessness or excessive or inappropriate guilt; 8) diminished ability to think or concentrate or indecisiveness; and 9) recurrent thoughts of death (DSM-IV, 1994).

As with bipolar disorder, major depression has an early age of onset (early 20's)(Doris et al., 1999). However, similarly to bipolar disorder, depression may be under diagnosed in children and adolescents. Recent reports suggest a prevalence of 2% in school-aged children and 5-8% in adolescence (reviewed by Son and Kirchner, 2000).

Interestingly, the gender ratio in prepubertal children is nearly 1:1, but dramatically shifts to 2:1 females to males following puberty, suggesting that endocrine factors (i.e. estrogens and progesterone) may have a role in the pathophysiology of the disorder (Doris et al., 1999; McEwen, 1998). Although depressive symptoms are often well managed by psycho- and pharmaco-therapy, a high rate of recurrence (~70%) (Post, 1992) and high mortality (10-15% suicide rate) and morbidity persists even during periods of euthymia (Mann, 1998).

## 1.2 Pharmacotherapies for mood disorders

Over the past fifty years, great advances have been made in the treatment options available for those suffering with mood disorders. Currently, drugs used to treat bipolar disorder are classified as mood stabilizers, and include lithium, valproate, and carbamazepine. Newer agents such as lamotrigine, clozapine and olanzapine have shown promise in certain patients (Compton and Nemeroff, 2000; Weizman and Weizman, 2001). Major depressive disorder is treated with drugs referred to as antidepressants and are divided into four main categories: tricyclic antidepressants (i.e. desipramine); serotonin selective reuptake inhibitors (i.e. fluoxetine); monoamine oxidase inhibitors (i.e. tranylcypromine); and atypical antidepressants (i.e. venlafaxine). Both classes of drugs, mood stabilizers and antidepressants, are long-term (often life-time) pharmacotherapies and most take several weeks to have an effect, suggesting their mechanism of action may reside in the regulation of gene expression.

### 1.2.2 Mood stabilizers

Lithium was one of the first drugs shown to have positive effects in the treatment of any psychiatric illness (Goodwin and Jamison, 1990; Weizman and Weizman, 2001). Today, lithium is still used as a first-line treatment for bipolar disorder and still may be the most effective anti-manic agent available (Poolsup et al., 2000). Poolsup and colleagues (2000) conducted meta-analysis examining the effectiveness of different mood stabilizers and other drugs in the treatment of mania and found that lithium may be the most effective agent for bipolar disorder, even when compared to other prescribed mood stabilizers such as valproate and carbamazepine. In addition to lithium's anti-manic and anti-depressive effects, this monovalent cation may also have anti-suicidal properties. A prospective study by Thies-Flechtner et al. (1996) studying the number of suicides in a group of subjects on different psychotropic drugs reported that none of the 188 subjects on lithium died by suicide, whereas, 4 of 143 died by suicide while on carbamazepine and 5 of 47 committed suicide while on the antidepressant, amitriptyline. Moreover, a meta-analysis of 22 studies concluded that subjects on lithium had an 82% lower chance of committing suicide while on the drug (Tondo et al., 2001). Although the remission rates achieved by lithium treatment in bipolar disorder have been shown to be double that of other mood stabilizers (Poolsup et al., 2000), the side-effects associated with the drug often become overwhelming for the patient and additional treatment strategies are required (Thase and Sachs, 2000). Common side effects of lithium treatment are: weight

gain, nausea, tremor, reduced sexual drive or performance, anxiety, hair loss, movement problems, or dry mouth. The side-effect profile for lithium illustrates the need for effective drugs to be developed that have far fewer side effects. Biochemical studies suggest that lithium treatment may have multiple physiological consequences within the cell and might in fact regulate ion transport, enzymatic activity, DNA binding, receptor sensitivity and gene expression (Borre and Kanner, 2001; Lenox et al., 1998).

Structurally unrelated to lithium, valproate or valproic acid is also regarded as a first-line treatment for bipolar disorder (Potter and Bowden, 1992). Originally developed as a drug for the treatment of epilepsy, the branched chained fatty acid (2-propylpentanoic acid) has been shown to have acute effects in treating mania in addition to preventing relapses when given chronically. Emilien et al. (1996) reported that in the published literature there was no significant difference between the response rates of bipolar disorder subjects to lithium or valproate, making valproate a safe alternative to lithium treatment. As with lithium, valproate has been shown to have numerous effects on neurons, including an overall depressant effect on synaptic transmission. The precise mechanism of action of valproate, however, has yet to be determined. Studies have shown that synaptosomal concentrations of γ-aminobutyric acid (GABA) are increased by valproate, possibly through activation of its major synthetic enzyme glutamic acid decarboxylase (Loscher, 1981). Valproate is also believed to downregulate excitatory amino acid (aspartate and glutamate) transmission (Cotariu et al., 1990). It may also possess a direct neuronal membrane depressant effect through enhancing $K^+$ channel function leading to hyperpolarization (Slater and Johnston, 1978). At the cellular level,

valproate has been shown to reduce protein kinase C (PKC) activity and decrease the expression of myristoylated alanine-rich C kinase substrate (MARCKS; Watson et al., 1998)). In addition, valproate has been reported to increase activator protein-1 (AP-1) binding (Asghari et al., 1998; Chen et al., 1997), suggesting that it may increase expression of those genes that contain such an element in their promoter region.

In addition to valproate, another anticonvulsant, carbamazepine, has been proven to also have mood stabilizing properties (Ballenger and Post, 1980; Post et al., 1987). However, compared to lithium and valproate, the evidence supporting carbamazepine as an effective mood stabilizing agent is far less convincing (Mitchell and Malhi, 2002). Meta analysis comparing lithium and carbamazepine reported that both drugs have similar response rates, however, carbamazepine was shown not to have any prophylactic benefits (Dardennes et al., 1995). Indeed, more double-blind randomized clinical trials will be required before carbamazepine is considered a first-line treatment for bipolar disorder. Currently, carbamazepine may only have prophylactic effects as an adjuvant or combination therapy.

Recent studies have been examining the effects of newer agents used to treat bipolar disorder, such as novel anticonvulsants (i.e. lamotrigine), or atypical antipsychotics, (i.e. clozapine or olanzapine). Data is rapidly accumulating on the effectiveness of these drugs in bipolar disorder (Mitchell and Malhi, 2002).

### 1.2.2 Antidepressants

Traditionally, development of pharmacological agents used to treat major depression has been driven by the monoamine hypothesis of depression, which essentially states that depressive symptoms are caused by decreased levels of norepinephrine and/or serotonin in vulnerable brain regions (discussed in detail in section 1.3.1). Most antidepressants are therefore developed to target various aspects of the synthesis and catabolism of these neurotransmitters and are classified as: tricyclic antidepressants, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors and atypical antidepressants. Tricyclic antidepressants include such compounds as; amitriptyline, clomipramine, desipramine, and imipramine. These agents are likely to influence catecholamine neurons by primarily inhibiting the presynaptic reuptake of norepinephrine; however, a secondary mechanism may result in elevated synaptic serotonin concentrations (Harms, 1983). In fact, early studies with imipramine were the first to show that tricyclic antidepressants did not affect the metabolism of norepinephrine but instead inhibited its reuptake into presynaptic neurons (Axelrod et al., 1961; Glowinski and Axelrod, 1964).

Targeting neurotransmitter reuptake led to the development of a whole new subclass of antidepressants, the selective serotonin reuptake inhibitors (SSRIs) (Fuller et al., 1974). SSRIs' primary mechanism of action is to inhibit serotonin reuptake sites. and these drugs include fluoxetine, sertraline, paroxetine, fluvoxamine and citalopram,

and have become the most widely prescribed first-line treatments for major depression (Sclar et al., 1998). SSRIs have been shown to have very similar efficacies to tricyclic antidepressants, but with far fewer side effects (Goldstein and Goodnick, 1998), resulting in high tolerability in patients. Fluoxetine (trade name Prozac®) is the most well-known SSRI, and has been estimated to have been prescribed to more than 35 million people world-wide, making it the most regularly prescribed antidepressant (Freemantle and Mason, 2000). SSRIs are believed to affect reuptake sites on neurons projecting from the brain stem region, in particular the raphe nuclei (Tork, 1990). Chronic inhibition of serotonin reuptake has been reported to result in increased levels of the available neurotransmitter at the synapse (Bel and Artigas, 1993; Rutter et al., 1994; Kreiss and Lucki, 1995; Arborelius et al., 1996). Long-term consequences of this shift in serotonin levels may in fact result in altered pre- and post-synaptic receptor densities and sensitivities. However, the effects are likely widespread and complex, since there are more than 14 serotonergic receptors with related functions affecting mood, anxiety, arousal, impulses, and aggression (Tork, 1990).

Monoamine oxidase inhibitors (MAOIs) have also been proven to have antidepressant properties (Freeman, 1993; Lecrubier and Guelfi, 1990). Several monoamine oxidases are located on the outer mitochondrial membrane in perikarya and noradrengeric neurons. The primary role of this enzyme is to convert monoamines, norepinephrine and serotonin, into their metabolites. By inhibiting the function of these enzymes, more neurotransmitter is sequestered by synaptic vesicles, in theory increasing the availability of the neurotransmitter for synaptic release. Although MAOIs are not

usually well tolerated because of their side-effect profile and their contraindications with other medications, several compounds have been approved for the treatment of depression, these include: phenelzine, tranylcypromine, and moclobemide.

Recently, newer atypical antidepressants have been developed that target additional components of the norepinephrine and serotonin metabolism and catabolism pathways. Venlafaxine is structurally unrelated to all other antidepressants, and has been described as a serotonin-norepinephrine reuptake inhibitor (SNRI). Studies have shown that *in vitro* venlafaxine blocks the synaptosomal uptake of norepinephrine, serotonin, and to a lesser extent dopamine (reviewed by Mendlewicz, 1995). Its effects on neurotransmitter reuptake may, in fact, be dose dependent, acting primarily as an SSRI at low doses and a norepinephrine reuptake inhibitor at higher doses (Andrews et al., 1996). Although initial studies suggest that there is no difference in efficacy between venlafaxine and SSRIs, venlafaxine treatment may, in fact, achieve more rapid response rates and greater remission rates compared to SSRIs (Entsuah et al., 2001). In addition, the favourable side-effect profile for venlafaxine may be better tolerated by patients (Mendels et al., 1993). Other atypical antidepressants include: bupropion, an antidepressant of the norepinephrine and dopamine systems; mirtazepine, which inhibits activity of presynaptic $\alpha_2$ inhibitory receptors; and reboxetine, a potent inhibitor of norepinephrine transport (Ascher et al., 1995; Schatzberg, 1998; Wheatley et al., 1998).

## 1.3 Theories regarding the pathophysiology of mood disorders

Although the clinical presentation of mood disorders is well established, an understanding of the pathophysiology responsible for the disorders is incomplete. One of the earliest hypotheses incorporated the idea that mood disorders were associated with alterations in neurotransmitter activity, in particular catecholamines and indoleamines. As advances were made in understanding the post-synaptic cellular and molecular changes associated with neurotransmitter release, signal transduction pathways were at the forefront of study into the mechanisms responsible for these disorders. Most recently, scientific advances in techniques available to study and identify changes in gene expression, as well as breakthroughs in the way the living brain can be imaged, have suggested that mood disorders may be the result of brain regional reductions in grey matter.

### 1.3.1 Catecholamine and indoleamine hypotheses

The hypothesis that mood disorders have a biological component has been studied since the 1960's. The catecholamine and indoleamine hypotheses of mood disorders were first proposed in the mid-1960's in two separate reviews (Coppen, 1967; Schildkraut, 1965). The catecholamine theory of mood disorders proposed that depression resulted from decreased norepinephrine and mania was the result of elevated norepinephrine levels at central adrenergic receptor sites (Schildkraut, 1965). The indoleamine hypothesis was derived from evidence suggesting that serotonin was responsible for the disorders, specifically, decreased serotonin levels caused depression (Coppen, 1967).

Interest in norepinephrine and serotonin as important neurotransmitters in mental illness arose when subjects given the tranquilizing substance reserpine, known to deplete brain amine levels, displayed profound behavioural depression during the course of the treatment (Coppen, 1967; Peterfy et al., 1976; Quetsch et al., 1959). A study by Lingjaerde (1963) reported similar findings when subjects were administered tetrabenazine, a compound with similar amine depleting effects. Alternatively, subjects given amphetamines, which are believed to lead to acute elevations in norepinephrine as well as dopamine showed euphoric or manic-type behaviours (Van Kammen and Murphy, 1975). Clinical studies measuring peripheral levels of serotonin and norepinephrine metabolites, 5-HIAA and MHPG respectively, have been conducted in an

attempt to support the catecholamine and indoleamine hypotheses. Peripheral measures in psychiatric illnesses are the only way to obtain information on mood state-dependent changes associated with the disorders. However, results from several studies suggest that peripheral measures of serotonin and norepinephrine metabolism from plasma, urine and cerebrospinal fluid (CSF) are inconsistent and fail to provide any evidence to support the catecholamine and indoleamine hypotheses (Geracioti, Jr. et al., 1997; Placidi et al., 2001 and references within). Confounding factors such as the time the sample was taken (Kirwin et al., 1997), diet (Williams et al., 1999) and even season of birth (Chotai and Asberg, 1999) have all been shown to have significant affects on CSF 5-H1AA and MHPG levels, thus masking any changes brought about by the disorder.

Strong evidence to support the catecholamine and indoleamine hypotheses has come from studies examining the mechanism of action of antidepressants and mood stabilizers. As described above (section 1.3.2), most antidepressants have been developed to target one or more of the elements involved in the synthesis and/or catabolism of norepinephrine or serotonin. The result of chronic treatment with any of these drugs is increased synaptic concentrations of serotonin or norepinephrine, suggesting that the pathophysiology of major depression involves decreased CNS levels of these neurotransmitters. It is less clear how mood stabilizers affect levels of these neurotransmitters. Interestingly, evidence from a limited number of studies suggests that mood stabilizers have similar effects on norepinephrine and serotonin. Lithium has been reported by several groups to increase serotonergic functioning, however, the mechanism of action of lithium on serotonin is yet to be fully understood (Price et al., 1990). In a

series of studies by Baf et al. (1994 a, b), both valproate and carbamazepine were shown to increase levels of both serotonin and norepinephrine. Valproate was shown to increase norepinephrine levels in the rat hippocampus and brain stem and serotonin levels in both the striatum- accumbens and brain stem (Baf et al., 1994a). Moreover, carbamazepine was shown to increase serotonin levels in an identical pattern as valproate, whereas, norepinephrine was increased only in the rat motor cortex and cerebellum (Baf et al., 1994b). Additional evidence to support the role of mood stabilizers in the regulation of serotonin and norepinephrine comes from separate studies showing that serotonin reuptake was blocked by lamotrigine and carbamazepine (Dailey et al., 1998; Southam et al., 1998).

Postmortem and brain imaging studies examining norepinephrine and serotonin in depression have had mixed results (reviewed by Ressler and Nemeroff, 1999; Staley et al., 1998). One of the confounding issues surrounding postmortem studies in depression is the assumption that individuals who commit suicide are depressed. Although, in theory, this assumption makes sense, there are a number of researchers and clinicians who suggest that suicidal tendency may in fact be an independent psychiatric illness with its own phenomenological and epidemiological profile (reviewed by Ahrens et al., 2000; Gross-Isseroff et al., 1998). In fact, suicide tendency may be associated with personality traits such as impulsivity and aggression, rather than depressed mood (Goodwin and Jamison, 1990). Moreover, case histories are often incomplete for these subjects and diagnosis is completed post-mortem. Furthermore, suicide rates may be higher in patients with bipolar disorder compared to major depressive disorder, suggesting generalization of

this group as depressed may be a misdiagnosis (Goodwin and Jamison, 1990). *In vivo* imaging studies may offer better insight into neurochemical changes associated with major depressive disorder. Only a limited number of positron emission tomography (PET) or single photon emission computed tomography (SPECT) studies have examined the role of serotonin in major depressive disorder, and these studies, too, have had mixed results (reviewed by Staley et al., 1998).

Although these hypotheses were developed nearly 40 years ago, very few studies have examined levels of norepinephrine or serotonin, including their metabolites or receptors, in post mortem samples from bipolar subjects. Young et al. (1994a) reported a significant increase in norepinephrine turnover in frontal, temporal and occipital cortices as well as the hypothalamus from bipolar disorder subjects compared to controls. In this particular study, the authors also reported a significant decrease in the levels of 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of serotonin, in frontal and parietal cortex of bipolar subjects. Since neurotransmitters and their metabolites are often difficult to measure in post-mortem brain, studies have focussed on identifying changes in receptor binding and densities. However, most studies have focused on identifying changes associated with major depressive disorder. The results of these studies support the notion that serotonin is likely dysregulated in major depressive disorder and suicide (reviewed in Dowlatshahi and Young, 2000; Mann, 1998; Stockmeier, 1997). The evidence to support the role of norepinephrine in major depression is less convincing (Klimek et al., 1997; Zhu et al., 1999). To date, only Young et al. (1994b) have measured norepinephrine receptor binding in bipolar disorder.

However, they were unable to show any differences in β-adrenergic binding in frontal, occipital or temporal cortex of bipolar disorder subjects (Young et al., 1994a).

### 1.3.2 Signal transduction

Relevant changes in second messengers and gene expression resulting from dysregulation of neurotransmitter systems may provide the biochemical link between behavioural and neurochemical alterations in mood disorders. Two important clinical findings suggest that changes downstream of neurotransmitter release may in fact be more relevant for understanding the biology of mood disorders. First, both mood stabilizers and antidepressants have been reported to have acute effects on neurotransmitter release, however, clinically there is delay of one to two weeks before the drugs appear to have efficacy (Goodwin and Jamison, 1990; Stassen et al., 1996). This would suggest that changes in gene expression are ultimately responsible for the effectiveness of these drugs, through correcting or normalizing changes in gene expression associated with the disease. Second, the need for long-term, often lifetime, treatment in mood disorders would suggest that these drugs do in fact regulate gene expression (Hyman and Nestler, 1996). Moreover, discontinuation of treatment does not immediately result in relapse but requires a few weeks before changes in mood are observed, suggesting that once treatment has ceased those genes affected by mood stabilizers or antidepressants gradually return to pretreatment levels (Post et al., 1992a).

Some of the first evidence to suggest that post-receptor mechanisms may indeed be associated with mood disorders showed that when β-adrenergic receptors (βAR) were stimulated, decreased adenylyl cyclase (AC) activity (see Figure 1.3.2.1 for

norepinephrine and serotonergic signal transduction) could be measured in platelets and lymphocytes from patients with mood disorders (Ebstein et al., 1987; Ebstein et al., 1988; Extein et al., 1979; Pandey et al., 1979). Activation of either the noradrenergic $\beta_1$AR or the serotonergic 5-$HT_{4,6,7}$ receptors results in cAMP generation through stimulation of AC. The results from these studies proposed that mood disorders may be the result of overexpression of βAR and that antidepressants and mood stabilizers may decrease the expression of these noradrenergic receptors. However, numerous studies have failed to validate this hypothesis, suggesting that post-receptor signalling molecules may be responsible for mediating these effects (reviewed by Vaidya and Duman, 2001). The study of G-proteins, as key molecules in the transduction of receptor activation to second messenger systems, have shown that elements immediately distal to the receptor have altered function in mood disorders. In fact, mostly consistent results have been found measuring G protein levels in peripheral samples from patients with mood disorders (Avissar et al., 1997a; Avissar et al., 1997b; Manji et al., 1995; Mitchell et al., 1997; Schreiber et al., 1991; Spleiss et al., 1998; Young et al., 1994b). Interestingly, increases in both level and activity of Gαs and Gαi appear to be correlated with the mood state of the patient (Avissar et al., 1997a; Manji et al., 1995; Mitchell et al., 1997; Schreiber et al., 1991). Evidence also suggests, at least in the rat brain, that lithium can attenuate the function of G-proteins, suggesting that G-protein function may be altered in bipolar disorder (Avissar et al., 1988).

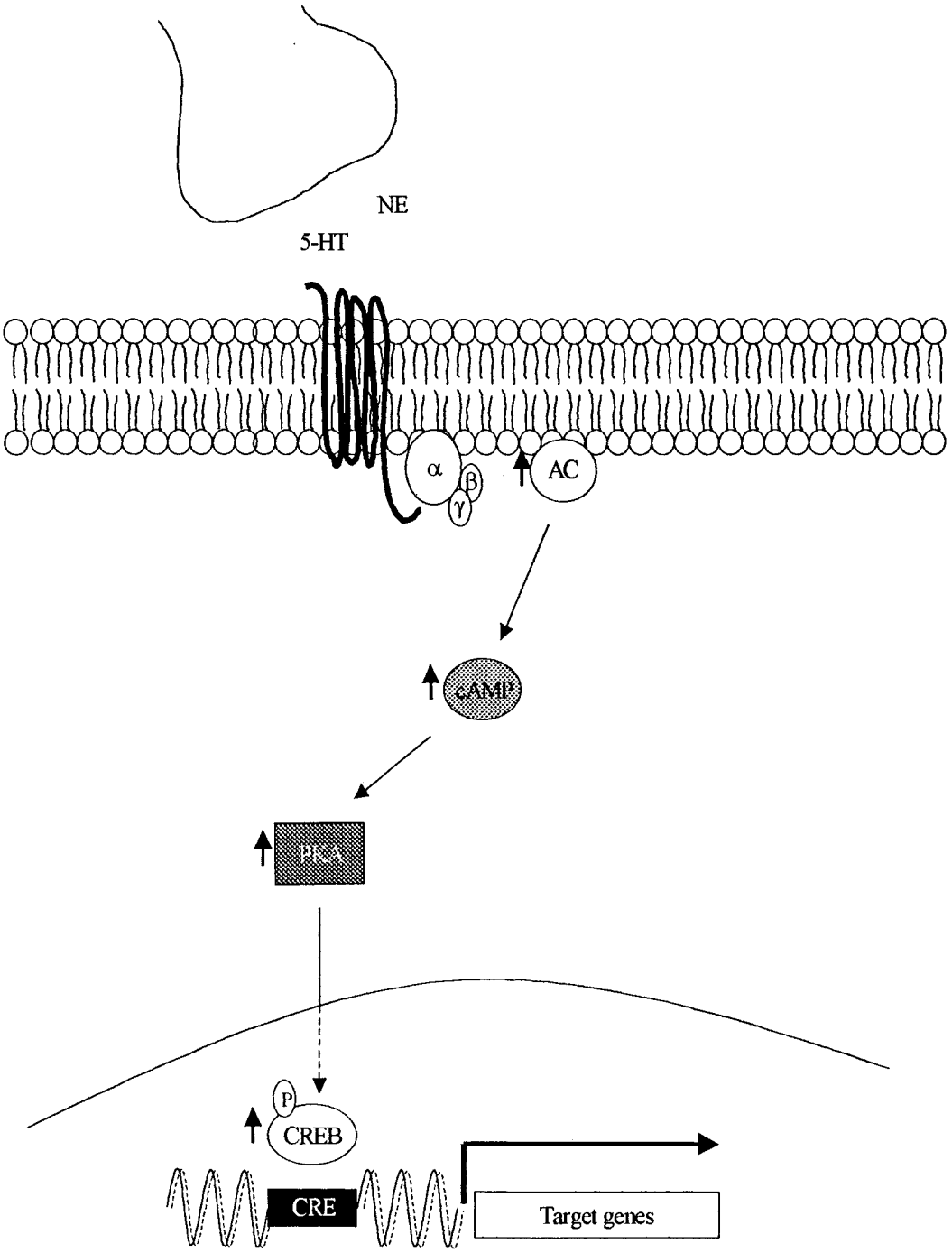

NE
5-HT
α
β
γ
AC
cAMP
PKA
P
CREB
CRE
Target genes

**Figure 1.3.2.1** *cAMP signal transduction.* Synaptic release of the neurotransmitters, norepinephrine (NE) and serotonin (5-HT), results in stimulation of G-protein coupled β-adrenergic receptor (βAR) and 5-$HT_{4,6,7}$ receptors, respectively. Post-synaptic signalling through these receptors results in activation of adenylyl cyclase (AC), which cause generation of cAMP. Generation of cAMP results in activation of protein kinase A (PKA), which translocates to the nucleus and phosphorylates the transcription factor CREB. Increased CREB phosphorylation causes the molecule to bind to the CRE consensus sequence and enhance the transcription of genes with CRE elements in their promoters.

Receptor-mediated stimulation of G-proteins can affect several different signalling pathways, including the cyclic adenosine-monophosphate (cAMP) pathway and the phosphoinositide (PI) pathway. As mentioned previously, activation of either the noradrenergic $\beta_1$AR or the serotonergic 5-$HT_{4,6,7}$ receptors results in cAMP generation through stimulation of AC. Studies measuring AC activity from mononucleocytes from depressed bipolar subjects have shown decreased activity compared to controls. In addition, lithium-treated euthymic bipolar patients also show decreased AC activity compared to controls (Avissar et al., 1997b; Avissar et al., 1996). Analogous processes may also occur in major depression (Wang et al., 1974). A study by Menkes et al. (1983) showed AC activity was increased in rat brain preparations following administration of several different classes of antidepressants. A similar effect was also reported for lithium treatment (Colin et al., 1991). Activation of AC catalyzes the production of cAMP from ATP, which is eventually degraded by phosphodiesterases. The importance of cAMP signalling in mood disorders is accentuated by recent reports that have shown rolipram, a selective phosphodiesterase inhibitor, has antidepressant effects (Zhu et al., 2001). Signalling by cAMP occurs via cAMP-dependent protein kinase A (PKA), which when activated regulates the activity of a number of substrates through phosphorylation of threonine residues (Popoli et al., 2001). Animal studies have shown that antidepressants can increase both PKA activity and nuclear translocation of the enzyme, suggesting these drugs do in fact regulate gene expression (Perez et al., 1989; Nestler et al., 1989). Studies on substrates of PKA phosphorylation have reported that the phosphorylation state of GTPase Rap1b in platelets from lithium-treated and euthymic bipolar patients is

increased (Perez et al., 1995; Perez et al., 2000a; Zanardi et al., 1997), suggesting that endogenous PKA levels or activity may be involved in mood disorders. However, the results of these studies have been questioned, since it has been shown that GTPase Rap1b levels are elevated in platelets from bipolar patients (Perez et al., 1999; Perez et al., 2000).

Changes in expression or activity of molecules of the cAMP signalling cascade (i.e. AC and PKA) have been shown to be associated with altered gene expression. The most likely candidate mediating these effects is the cAMP responsive transcription factor CREB. CREB enhances transcription of a number of genes (i.e. c-fos, BDNF, bcl-2 and others) through binding to the cAMP responsive element in the promoter region of these genes (Mayr and Montminy, 2001). Regulation of CREB by a variety of antidepressants in cultured cells and animal models of depression has been well characterized (Duman et al., 1997). Interestingly, the regulation of CREB by mood stabilizers in peripheral clinical samples from bipolar disorder patients have not revealed overwhelming changes in its expression.

Postmortem studies have confirmed many of the drug and blood studies, indicating that cAMP signalling may in fact be dysregulated in mood disorders. As mentioned previously, when Young et al. (1994b) measured βAR levels in post-mortem brain, no significant differences could be detected in frontal, occipital or temporal cortex of subjects with a previous history of mood disorders. The study of G protein abnormalities from postmortem subjects with mood disorders has been an area of intense interest. Further post-mortem studies by Young et al. (1991, 1993) showed that Gαs levels were

increased in frontal, temporal and occipital cortex in bipolar subjects, however, Gαi, Gαo and Gβ levels were unchanged. These changes in temporal and occipital cortex were later shown to be correlated to forskolin-stimulated AC activity. Friedman and Wang (1996) have also reported similar findings as the Young et al. (1994a) studies in frontal cortex of bipolar subjects. Fewer studies have focussed on G protein levels in postmortem samples from major depressive subjects. Two studies, with conflicting results, have shown changes in $G\alpha_{s \text{ and } i}$ levels in prefrontal cortex from depressed patients. Pacheco et al. (1996) reported increased Gαs and decreased Gαi levels in prefrontal cortex from depressed suicide subjects, whereas Garcia-Sevilla et al. (1999) reported increased Gαi levels in prefrontal cortex of depressed suicide subjects. Three other studies failed to find any measurable differences in G protein levels from postmortem brains of depressed subjects (Dowlatshahi et al., 1999; Cowburn et al., 1994; Ozawa et al., 1993). Downstream of the receptor G protein complex, changes in AC levels and cAMP binding have been measured in postmortem samples from depressed (Reiach et al., 1999) and bipolar subjects (Rahman et al., 1997). Although there is considerable evidence showing that CREB is modulated by antidepressants and has a role in behaviour, very few studies have been able to show the dysregulation of CREB in postmortem brain (Duman et al., 1997). CREB levels have been reported to be significantly increased in temporal cortex from a depressed subjects who had a history of antidepressant treatment (Dowlatshahi et al., 1998). However, no difference in CREB levels could be observed when the depressed group was compared to controls. This effect could be related to the region of the brain studied, since Odagaki et al. (2001) recently measured significant increases in

CREB levels (~121% compared to controls) in prefrontal cortex from depressed subjects. Whether mood stabilizers regulate CREB levels and function in cultured cells and animal models is less well studied (Wang et al., 1999a; Chen et al., 1999a). In post-mortem brain tissue, although no effect was found across all subjects with bipolar disorder, cerebral cortical CREB levels were lower in patients who were treated with mood stabilizers at the time of death compared to those who were not (Dowlatshahi et al., 1999).

Studies attempting to understand the molecular mechanisms that cause bipolar disorder have also focussed on the PI signalling pathway. Activation of Gq and Go coupled receptors initiates the PI signalling pathway, which eventually leads to release of $Ca^{2+}$ from intracellular stores and subsequent activation of $Ca^{2+}$ regulated enzymes. The steps and molecules involved in this pathway are shown in figure 1.3.2.2 The involvement of the PI pathway in bipolar disorder is well documented (Atack et al., 1995; Soares and Mallinger, 1996; Soares and Mallinger, 1997). Elements of the PI pathway have been shown to be common targets for both lithium and valproate. Using high resolution nuclear magnetic resonance (NMR) spectroscopy, O'Donnell et al. (2000) showed that in rat brain, levels of IP were increased and levels of myo-inositol were decreased following chronic treatment with lithium and valproate, suggesting that both mood stabilizers have an inhibitory effect on IMPase activity. In addition, studies on platelets from drug-free bipolar subjects show that PIP2 may be increased compared to controls (Soares et al., 2001). The only post-mortem study to examine the role of the

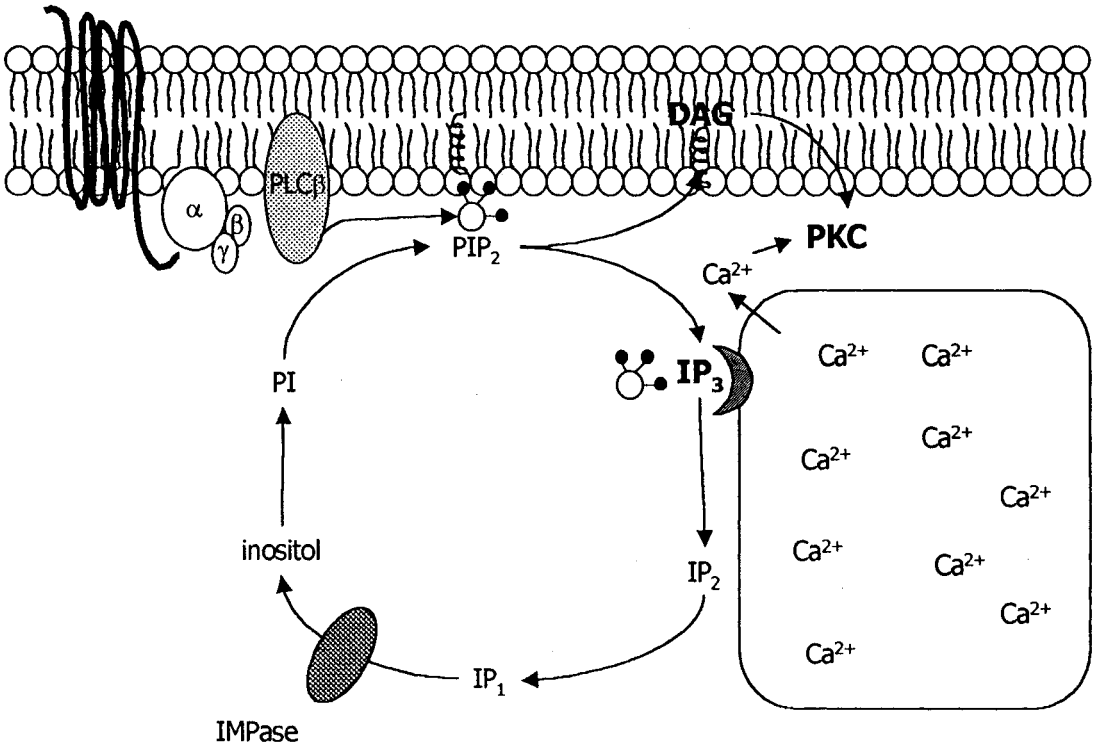

DAG
PLCβ
α
β
γ
PIP2
PKC
Ca2+
IP3
PI
inositol
IP2
IP1
IMPase

**Figure 1.3.2.2** *PI signal transduction.* Binding of a neurotransmitter to its receptor activates G proteins and the phospholipase C-β (PLCβ) and produces the second messengers diacylglycerol (DAG) and inositol triphosphate ($IP_3$) from phosphatidyl inositol ($PIP_2$). The $IP_3$ messenger diffuses through the cytoplasm to the ER, where it acts as the ligand for a ligand-gated $Ca^{2+}$ channel. $IP_3$ binding opens the channel and releases $Ca^{2+}$ from the ER in to cytoplasm. The released $Ca^{2+}$ and the DAG produced by PLCβ activate protein kinase C (PKC). $IP_3$ is recycled to the plasma membrane through a series of dephosphorylation reactions, eventually leading to the production of $PIP_2$ (modified from Kandel et al., 1991).

PI pathway in bipolar disorder measured a selective impairment in G protein-stimulated [3H]PI hydrolysis in occipital cortical membranes from bipolar subjects compared with controls (Jope et al., 1996).

The downstream consequence of altered PI signalling in bipolar disorder likely involve the release and possible depletion of $Ca^{2+}$ from intracellular stores, which has been shown to have deleterious effects on neuronal structure and function (Sapolsky, 2001). The involvement of the PI pathway in depression is less characterized, however, the limited number of studies investigating this pathway in depression have shown similar results. Mikuni et al. (1991) were the first to show that platelets from depressed patients, when stimulated with serotonin, had significant increases in IP levels compared to controls. This group went on to show that serotonin was also capable of increasing $Ca^{2+}$ mobilization in these same patient samples (Mikuni et al., 1992), which may be due to increased $IP_3$ receptor protein levels (Dwivedi et al., 1998). Similar results were obtained at approximately the same time by another laboratory (Atack et al., 1998), suggesting that depression may be in part to due to abnormalities in PI signalling. Karege et al. (1996) showed increased PI hydrolysis and $\alpha 2$ adrenergic receptor activation in platelets from depressed patients. Moreover, hyperactivity of the PI pathway has recently been demonstrated in platelets from depressed subjects (Pandey et al., 2001).

The evidence presented thus far overwhelmingly supports the theory that mood disorders are associated with altered signal transduction, which leads to changes in gene expression. Some of the early studies examining changes in gene expression in bipolar disorder were stimulated by studies showing that both lithium and valproate could

increase the binding of activator protein-1 (AP-1) to its consensus element (Asghari et al., 1998; Chen et al., 1997; Jope and Song, 1997; Ozaki and Chuang, 1997; Williams and Jope, 1995). This enhanced binding was accompanied by increased transcription of the AP-1 regulated gene, tyrosine hydroxylase (Chen et al., 1998). Interestingly, chronic lithium treatment significantly increased expression of tyrosine hydroxylase in rat hippocampus, frontal cortex and striatum (Chen et al., 1998). Another related finding has been the attenuation of glycogen synthase kinase 3β (GSK-3β) activity by both lithium and valproate (Chen et al., 1999b; Grimes and Jope, 2001a; Jope and Bijur, 2002). GSK-3β has been shown to have multiple substrates within the cell including c-jun, CREB and several cytoskeletal molecules (Grimes and Jope, 2001b). Although considerable evidence exists suggesting that mood stabilizers and antidepressants regulate signal transduction pathways and transcription factors, the actual genes affected have been elusive. The use of differential display PCR by a number of independent laboratories has identified several genes that may be regulated by mood stabilizers (reviewed by Wang et al., 2001). Among those genes identified as being regulated by mood stabilizers, the 78 kilodalton glucose regulated protein (GRP78) and B cell lymphoma protein-2 (bcl-2), have offered important clues as to the possible pathophysiological causes of bipolar disorder, and possibly to mood disorders as a whole. Our laboratory was able to show that rats treated for 21 days with valproate had a significant increase in GRP78 expression in cerebral brain regions compared to saline injected animals (Wang et al., 1999b). We also went on to show that this effect could be duplicated in cultured cells, with a nearly 50% increase in expression at therapeutically relevant concentrations of

valproate. At the same time, Chen and colleagues (1999c) showed that both lithium and valproate could upregulate the transcription factor polyomavirus enhancer-binding protein (PEBP) 2 β in rat frontal cortex. In this same study, they were also able to show that transcription of a PEBP2β regulated gene, B-cell lymphoma protein-2 (bcl-2), was enhanced following both lithium and valproate treatment. These two lines of evidence, combined with a growing number of reports on the neuroprotective properties of mood stabilizers and brain regional abnormalities in mood disorders, have prompted discussion about the possibility of mood disorders being associated with structural brain changes.

### 1.3.3 Cell loss or atrophy in patients with mood disorders: neuroprotection by mood stabilizers and antidepressants

Studies in our lab have shown that valproate can increase the expression of GRP78 and other members of the endoplasmic reticulum stress proteins family, GRP94 and calreticulin, in both rat brain and cultured cells (Bown et al., 2000; Chen et al., 2000c; Wang et al., 1999b). ER stress proteins have been shown in other studies to have two important functions within the cell: binding free $Ca^{2+}$ in the ER, and acting as molecular chaperones to assist in the proper folding and processing of newly synthesized proteins (Nigam et al., 1994). Overexpression of GRP78 and calreticulin have been shown to significantly increase the $Ca^{2+}$ binding capacity of ER stores and prevent the release of this bivalent cation into the cytoplasm during excitotoxic stress (Mery et al., 1996; Yu et al., 1999). The neuroprotective properties of valproate have been recently shown by Yuan et al. (2001), who reported that valproate could protect against age-associated neuronal death in cultured cells, and likely this effect is mediated by the mitogen-activated protein kinase (MAPK) pathway. Activation of the MAPK pathway has been shown to confer neuroprotection through regulation of survival factors such as bcl-2 and the growth associated protein, GAP43 (Yuan et al., 2001).

Lithium has also been reported to regulate expression of genes known to have neuroprotective properties. The anti-apoptotic factor bcl-2 has been shown to have increased expression following lithium treatment in both rat brain and cultured cells

(Chen et al., 1999c; Chen and Chuang, 1999). Although the most commonly described mechanism of action of bcl-2 is its interaction with pro-apoptotic factors (i.e. Bax), which prevents apoptosis, Bcl-2 might also have a role in regulating $Ca^{2+}$ flux across the ER membrane. Lam et al. (1994) showed that in mouse lymphoma cells, overexpression of Bcl-2 reduced the depletion of ER $Ca^{2+}$ stores by the irreversible ER $Ca^{2+}$-ATPase inhibitor, thapsigargin. The regulation of ER stress proteins and Bcl-2 by mood stabilizing agents suggests that these two drugs could increase the $Ca^{2+}$-binding ability of the neuron and maintain $Ca^{2+}$ homeostasis, providing added protection against sudden $Ca^{2+}$ influxes (reviewed in Bown et al., 2002). Dysregulation of $Ca^{2+}$ homeostasis in bipolar disorder has long been thought to play a significant role in the disorder (Dubovsky et al., 1992). Clinical studies have shown elevated levels of intracellular $Ca^{2+}$ in platelets and lymphocytes from bipolar disorder compared to the control group (Dubovsky et al., 1992; Emamghoreishi et al., 1997). More recently, Hough et al. (1999) reported that lymphocytes and platelets from bipolar disorder patients had greater concentrations of intracellular $Ca^{2+}$ compared to controls following exposure to thapsigargin, suggesting that ER $Ca^{2+}$ stores in these patients may be more sensitive to the neurochemical changes associated with bipolar disorder.

The neuroprotective role of lithium was first shown in neurons by protecting against glutamate-mediated excitotoxicity (Dixon and Hokin, 1998). They showed that chronic treatment with lithium upregulated the synaptosomal uptake of glutamate. These results were further advanced by Nonaka et al. (1998), who showed that lithium could robustly protect rat cerebellar granule cells from glutamate-induced excitotoxicity by

inhibiting N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx. Nonaka and colleagues (1998) propose that lithium may regulate NMDA receptor function by inhibiting GSK-3β, which could in turn decrease the phosphorylation state of NMDA receptor subunits. This particular mechanism of action on NMDA receptor subunits may be shared between both lithium and valproate, since studies have shown that valproate can also inhibit the activity of GSK-3β (Chen et al., 1999b). Carbamazepine may have a direct effect on the NMDA receptor, thereby preventing influx of $Ca^{2+}$ during activation (Ambrosio et al., 1999; Hough et al., 1999). In an electrophysiology study, Hough et al. (1996) showed that rat cerebellar granule cells simultaneously exposed to carbamazepine and NMDA had a decrease in $Ca^{2+}$ influx compared to when NMDA was administered alone. These results further accentuate the potential role of mood stabilizers, in particular lithium, in regulating $Ca^{2+}$ homeostasis.

Neuronal survival may also be enhanced following treatment with antidepressants. The regulation of the cAMP pathway by antidepressants and the enhanced transcriptional activity of CREB on BDNF expression have been well characterized in cultured cells and rat brain (Morinobu et al., 1999; Nibuya et al., 1995; Nibuya et al., 1996; Thome et al., 2000). Recently, Chen et al. (2001) showed that overexpression of CREB in rat hippocampus produced antidepressant effects in these animals in the learned helplessness model of depression as measured by forced swim tests. This group has gone on to show that upregulation of BDNF by antidepressants may lead to hippocampal neurogenesis, as well as improved performance in behavioural paradigms of depression (Malberg et al., 2000). The antidepressant effect of BDNF has

been further studied by Shirayama et al. (2002), who showed in rats that BDNF injected directly into the dentate gyrus and CA3 regions of the hippocampus produced antidepressant-type behaviours in the learned helplessness paradigm of depression.

The neuroprotective properties of mood stabilizers and antidepressants, *in vitro*, are quite interesting considering the recent postmortem and brain imaging studies that have shown structural abnormalities in mood disorders (reviewed by Manji et al., 2001; Vawter et al., 2000). Advances in brain imaging techniques and the availability of postmortem brains from subjects with extensive medical histories, such as those from the Stanley Neuropathology Consortium, have allowed for more careful studies to be conducted examining the structural abnormalities associated with mood disorders. Volumetric studies have shown that patients with mood disorders have reduced grey matter volumes in the areas of the hippocampus (Bremner et al., 2000; Sheline et al., 1996; Sheline et al., 1999), orbital and medial prefrontal cortex (Drevets et al., 1997) compared to healthy controls. Postmortem studies have produced similar results as the imaging studies, showing reduced neuronal densities in the hippocampus (Benes et al., 1998), anterior cingulate cortex (Benes et al., 2001; Cotter et al., 2001), dorsolateral prefrontal cortex (Rajkowska et al., 2001) and orbitofrontal cortex (Drevets, 2000). Reduced glial densities may also be an important factor in the neurobiology of mood disorders, and in fact have been shown to be decreased in dorsolateral prefrontal cortex caudal orbitiofrontal cortex (Cotter et al., 2002; Rajkowska et al., 2001), and subgenual prefrontal cortex (Ongur et al., 1998; Rajkowska et al., 1999). The findings from these and other studies (Drevets, 2000) suggest that mood disorders are associated with

decreased regional brain volume, which may be in part due to loss of neurons, glia or both, and one mechanism of action of mood stabilizers might be to prevent or reverse these losses. Indeed, a recent volumetric study on bipolar disorder subjects showed that 4 weeks of treatment with lithium could significantly increase grey matter volume in these subjects (Moore et al., 2000).

## 1.4 Goals and Objectives

The clinical features, neurochemistry and neuropathology of mood disorders strongly indicate a biological basis for the illnesses. A number of studies have provided insights into potential mechanisms and systems that may be responsible for the illnesses, however, the precise defect has yet to be elucidated. Since mood disorders patients show response to long-term (lifetime) pharmacological dependence (antidepressants and mood stabilizers), it has been suggested that adaptational changes in gene expression are critical to their prophylactic effects. Therefore, the goals of the research described in this thesis are:

**1) determine if valproate can regulate gene expression, and further characterize any genes that appear to be regulated by the mood stabilizer;**

**2) determine if those changes in gene expression identified in animal models and cultured cells can be quantified in post-mortem brain samples from subjects with a history of psychiatric illness and healthy controls;**

**3) determine if other psychotropic drugs can regulate the expression of valproate-regulated genes, and**

**4) determine if mood stabilizers can protect against excitotoxic insults in rat hippocampal cultures.**

The specific objectives of this thesis are:

1) *To demonstrate that the 78 kilodalton glucose-regulated protein (GRP78) is regulated by chronic valproate treatment in both rat cerebral cortex and cultured cells;*

2) *To determine whether other members of the ER stress protein family, GRP94 and calreticulin, are regulated by valproate in cultured cells;*

3) *To determine whether the expression of ER stress proteins have altered expression in post-mortem temporal cortex from subjects with a history of major depressive disorder, bipolar disorder, and schizophrenia compared to non-psychiatric, non-neurologic controls;*

4) *To identify whether other psychotropic drugs have a similar effect on the expression of ER stress proteins in cultured cells;*

5) *To test whether, rat hippocampal neurons, pretreatment with mood stabilizers in primary cell cultures can protect against the ultrastructural changes induced by NMDA exposure; and*

6) *To determine whether NMDA-mediated cytoplasmic vacuolization is associated with loss of cell viability and the cell death pathways involved.*

## 2.0 Materials and Methods

### 2.1 Cell culture

Rat C6 glioma cells (American Type Tissue Collection; Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Cultures were maintained at $37^0$C in a 5.0% $CO_2$ environment. Culture flasks and plates were purchased from Fisher Scientific (Nepean, ON Canada). Media was replaced with fresh media every 48 hours. Cell viability was confirmed by trypan blue exclusion and was consistently greater than 98% and shown not to be different across different treatment groups.

## 2.2 Drug treatment

Cells were treated with mood stabilizers or antidepressants by supplementing the normal culture media with the desired concentration of the drug. The mood stabilizers, lithium chloride (BDH Laboratory Supplies, Poole, Dorset, England) and sodium valproate (ICN Biochemicals Inc, Aurora, OH, USA) were dissolved in cell culture grade distilled water, filtered through a 0.2 μm filter and added to the culture media. Carbamazepine (ICN Biochemicals Inc, Aurora, OH, USA) was dissolved in 100% dimethylsulfoxide (DMSO) and filtered before being added to the culture media. In experiments where carbamazepine was used, a group treated with DMSO was included as a control. The antidepressants, desipramine (ICN Biochemicals Inc, Aurora, OH, USA), venlafaxine HCl (WY-45030-W; WYETH, Montreal, QC, Canada) and tranylcypromine (Sigma, St.Louis MO, USA) were all dissolved in cell culture grade distilled water, filtered through a 0.2 μm filter and subsequently added to the culture media. N-methyl-D-aspartate (NMDA; Sigma, St.Louis MO, USA) was dissolved in cell culture grade distilled water. Culture media was supplemented with each agent for the desired time period.

## 2.3 RNA isolation and quantification

Isolation of total RNA from cells was carried out using Gibco™ TRIzol® reagent and the procedure described by the manufacturer (Invitrogen Canada Inc, Burlington ON, Canada). Cells were washed 3 times in phosphate buffered saline (PBS), followed by 5 minutes of incubation with TRIzol® reagent. Isolated cells suspended in TRIzol® were transferred to 15 ml Falcon tubes (BD Biosciences, Bedford MA, USA). Chloroform (0.2ml per 1ml TRIzol®) was mixed in with the TRIzol® and incubated at room temperature for 3 minutes. Samples were centrifuged (12,000 x *g*) for 15 minutes at $4^0$C. The aqueous layer was removed and isopropyl alcohol (1 ml per 1 ml TRIzol® reagent) was added, and incubated overnight at $-20^0$C. Samples were then centrifuged (12,000 x *g*) for 10 minutes at $4^0$C. Pellets were then washed with cold 75% ethanol (1 ml ethanol/1 ml TRIzol®) and further centrifuged (12,000 x *g*) for 2 minutes at $4^0$C. Pellets were then air dried on ice for 15 minutes and dissolved in DEPC-treated distilled water.

RNA concentrations were determined by measuring the absorbance at 260 nm and the purity determined by the 260:280 ratio. Only samples with purity ratios greater than 1.6 were used for further study. Three micrograms of total RNA was subjected to electrophoresis on a 0.8% agarose gel to check the integrity of the RNA (18S and 28S rRNA bands).

## 2.4 Protein isolation

Isolation of protein was carried using a whole cell extraction protocol (Korner et al., 1989). Cells were washed three times with PBS and cell lysis buffer (20 mM HEPES pH 7.0, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol) was added to each well. Repeated freeze-thaw cycles in a dry ice-ethanol bath, detached cells from the substratum. Cells were then gently agitated in the extraction buffer for 90 minutes at $4^0$C. Cell lysates were then centrifuged (14,000 x *g*) for 25 minutes at $4^0$C. The supernatant was then removed and stored at – $80^0$C. Protein concentrations were determined according to the method of Bradford (1976). Samples were diluted 1:50 in distilled water, then 2.5 volumes Bio-Rad Protein Assay dye reagent (BioRad Laboratories, Mississauga ON Canada) was added to each sample and the absorbance at 595 nm was measured using a Multiskan Plus version 1.43 microplate reader (Labsystems Inc, Franklin MA, USA). A standard curve of known bovine serum albumin concentrations was included on each plate to determine the concentration of each sample.

## 2.5 Complementary DNA probes

Complementary DNA (cDNA) fragments for grp78 and calreticulin were synthesized from rat C6 glioma cell mRNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) procedures, as described by the manufacturer (Perkin Elmer Life Sciences, Montreal QC, Canada), were followed using specific primer sequences for rat grp78 and calreticulin (Genbank Accession numbers S63521, X53363). RT-PCR was carried out in a master mix solution containing 5 mM $MgCl_2$, 1 X PCR buffer II, 1 mM each of dGTP, dATP, dTTP, dCTP, 1 U/μl RNase inhibitor, 2.5 U/ml MuLV reverse transcriptase, 2.5 μM oligo $d(T)_{16}$, and 0.9 μg of template RNA. RT-PCR proceeded in a GeneAmp PCR System 9600 (Perkin Elmer Life Sciences, Montreal QC, Canada) using the following program: $42^0$C for 15 minutes, $99^0$C for 5 minutes, and $5^0$C for 5 minutes. PCR amplification was carried out in a master mix containing 1.75 mM $MgCl_2$, 1X PCR buffer II, 2.5U/100 μl AmpliTaq® DNA polymerase and 100 pmole of each primer. The specific primers were as follows: grp78 $^{5'}$ CCACCGTAACAATCAAGGTC $^{3'}$ (upstream) and $^{5'}$CACGTGAGCAACTGCTAATG $^{3'}$ (downstream), and for calreticulin $^{5'}$ GTTCACCGTGAAGCATGAGC $^{3'}$ (upstream) and $^{5'}$ CAGTCCTCAGGCTTCTAAGC $^{3'}$ (downstream). PCR was performed using the following program: $95^0$C for 105 seconds; 45 cycles of $95^0$C for 15 seconds followed by $56^0$C for 30 seconds; and a final extension at $72^0$C for 7 minutes. A grp94 cDNA fragment was synthesized from a p4A3 plasmid containing a hamster cDNA fragment (a generous gift from Dr. Amy S. Lee,

University of Southern California) using the PCR protocol described above. PCR amplification of the hamster grp94 cDNA was conducted using the following primers: $^{5'}$ GCCTGTTGGATGAATACTGC $^{3'}$ (upstream) and $^{5'}$ TCTGATCAGCGGATGTCTG $^{3'}$ (downstream).

The amplified 767 nucleotide (nt) rat grp78, 375 nt grp 94 and 476 nt calreticulin cDNA fragments were isolated using the Qiaex II gel extraction kit (Qiagen Inc, Mississauga ON, Canada). Bands were excised from the gel and dissolved at 37$^0$C in 3 volumes of Buffer QX1. A volume of 10 µl QIAEX II was added to the sample and incubated at 50$^0$C for 10 minutes, vortexing the sample every two minutes. Samples were then centrifuged at 14,000 x *g* for 30 seconds, and supernatant removed. The pellet was then resuspended in 500 µl of Buffer QX1 and centrifuged at 14,000 x *g* for 30 seconds. The pellet was then washed twice with 500 µl of Buffer PE and centrifuged at 14,000 x *g* for 30 seconds. The pellet was then air dried for 15 minutes at room temperature. Dissolving the pellet in distilled water and incubating at room temperature for 5 minutes eluted DNA. Following centrifugation at 14,000 x *g* for 30 seconds the supernatant was removed and stored at –80$^0$C. To improve yield, an additional elution step was performed and the supernatant from that step was added to the original purified cDNA. Specificity of each cDNA fragment was confirmed by sequencing performed by the staff at the Institute for Molecular Biology (MOBIX; McMaster University) using the dideoxy chain-termination method (Sanger et al., 1977).

## 2.6 Northern blot hybridization

Northern blot hybridization was carried out following the method described by Wang and Young (1996). Twenty micrograms of total RNA was separated according to size on a 1% agarose gel with 2.2 M formaldehyde. RNA was capillary transferred to a nitrocellulose membrane via a capillary reaction overnight. RNA was cross-linked to the membrane by ultra-violet light (Stratagene Corporation, LaJolla CA, USA). Membranes were then prehybridized for 3 hours at $42^0$C in a buffer containing 10% dextran sulfate, 50% deionized formamide, 5 M SSPE, 5X Denhardt's reagent and 1% SDS. Hybridization proceeded overnight at $42^0$C in fresh hybridization buffer containing 6 x $10^6$ cpm of $\alpha$-$^{32}$P dCTP labelled probe. Following hybridization, membranes were rinsed three times with 2X SSC, 0.1% SDS followed by two washes at room temperature with 2X SSC, 0.1% SDS for 15 minutes. Membranes were exposed to a Molecular Dynamics Phosphor Screen (Amersham Biosciences Inc, Piscataway NJ, USA) and quantified using the Molecular Dynamics PhosphorImager and ImageQuant software version 5.60 (Amersham Biosciences Inc, Piscataway NJ, USA).

## 2.7 Slot blot hybridization

Slot blot hybridization was used to measure differences in mRNA levels following drug treatment. Five micrograms of total RNA from each sample was applied to a Zeta-probe membrane (BioRad Laboratories, Mississauga ON Canada) using a slot blot apparatus (BioRad Laboratories, Mississauga ON Canada) under low vacuum. The membrane was then washed twice under a low vacuum with 10X SSC. Finally, membranes were UV crosslinked for 30 seconds with a UV crosslinker (Stratagene Corporation, LaJolla CA, USA). Membranes were prehybridized in 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, 1 mM EDTA at $65^0$C for 30 minutes. Following prehybridization, a total of 6 x $10^6$ cpm of a-$^{32}$P dCTP labelled probe was placed in the prehybridization solution for overnight hybridization. Unbound probe was washed off the membrane in a series of 20 minute stringency washes using 2X SSC, 0.1% SDS at various incubation temperatures. Membranes were exposed to the Molecular Dynamics Phosphor Screen (Amersham Biosciences Inc, Piscataway NJ, USA) and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software version 5.60 (Amersham Biosciences Inc, Piscataway NJ, USA).

## 2.8 Immunoblotting

### 2.8.1 Rat C6 glioma cells

Ten micrograms of whole cell extract was subjected to SDS-PAGE with a 10% acrylamide gel for 90 minutes at 120 V. A standard curve of control C6 glioma cell protein was run on every gel (2.5-20 μg) to ensure assays are performed within the linear range and allowing for comparisons across blots. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences Inc, Piscataway NJ, USA ) at 100 V for one hour, equal loading and transfer was detected using Ponseau S staining. Membranes were blocked in 5% milk-PBS (30 minutes at room temperature) and incubated overnight at $4^0$C with monoclonal antisera for GRP78 (Stressgen Biotechnologies, Victoria, BC Canada) at a 1:500 dilution in blocking buffer. Polyclonal antisera for GRP94 (Stressgen Biotechnologies, Victoria, BC Canada) at a dilution of 1:2,500 and polyclonal antisera for calreticulin (Stressgen Biotechnologies, Victoria, BC Canada) at a dilution of 1:20,000 were all used to detect those specific proteins. Blots were incubated with an appropriate secondary antibody, goat anti-mouse IgG for GRP78 (Santa Cruz Biotechnology Inc, Santa Cruz CA, USA) at a dilution of 1:2000, goat anti-rat IgG for GRP94 (Santa Cruz Biotechnology Inc, Santa Cruz CA, USA) at a dilution of 1:4,000 and goat anti-rabbit IgG for calreticulin (Upstate Biotechnologies; Lake Placid, NY USA) at a dilution of 1:20,000 conjugated to horseradish peroxidase diluted in 5% milk-PBS for 1 hour at room temperature. Specificity of the secondary antibodies was

determined by incubating the membrane with only the secondary antibody. The secondary antibodies alone detected no bands. Immunoreactive bands were detected with an enhanced chemiluminescence (ECL) system (Amersham Biosciences Inc, Piscataway NJ, USA). The blots were subsequently exposed to Kodak X-OMAT film (Eastman Kodak Company, Toronto ON, Canada) and immunoreactivity determined by densitometry using the SCID image analysis system (ImageExperts, Oakville, ON Canada).

2.8.2 Postmortem brain

Immunoblotting was carried out following the same method as described above (2.8.1), using 30 μg of protein per lane. Tissue samples from one healthy comparison subject were run on every gel (10-40 μg) to ensure assays were performed within the linear range and to allow comparisons across blots. The optical density for 30 μg of protein, as determined from the standard curve, was used to determine the relative immunoreactivity for each of the samples. Samples were run in duplicate and blind to diagnosis. If greater than 20% variability was found between the two relative immunoreactivity values for each subject, the sample was repeated. Primary monoclonal antisera for both GRP78, which immunocross reacts with GRP94 (1:2,500 dilution in 5% milk-PBS) and polyclonal antisera for calreticulin (1:25,000 dilution) were obtained from Stressgen Biotechnologies (Victoria, BC Canada). Secondary antibodies, goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:2,500 (Santa Cruz Biotechnologies, address) for GRP78/94 and goat anti-rabbit IgG diluted 1:25,000 for calreticulin (Upstate Biotechnologies, Lake Placid, NY USA) were used, followed by detection with ECL (Amersham Biosciences Inc, Piscataway NJ, USA). Immunoreactive bands were quantified by densitometry using the SCID image analysis system (ImageExperts, Oakville, ON Canada).

## 2.9 Post-mortem brain

Postmortem brain tissue was obtained from the Stanley Foundation Neuropathology Consortium (4 groups of n=15 age- and sex-matched subjects, i.e. subjects with BD, subjects with major depressive disorder (MDD), subjects with schizophrenia (SCZ) and non-psychiatric, non-neurologic comparison subjects) (Torrey et al., 2000)(Table 1). Temporal cortex was dissected according to the gyral and sulcal landmarks as defined by Brodmann (1909). Two neuropathologists made microscopic examinations on all cases with toxicological examinations conducted on the majority. Extensive medical records were available for all subjects and were reviewed by two psychiatrists. Interviews were conducted with family members, and for selected cases, with the treating mental health professional by one psychiatrist to clarify clinical information. Based on data gathered from medical records and interviews, diagnoses were established by two psychiatrists using DSM-IV criteria. Control subjects were confirmed to be free of both psychiatric illness and substance abuse by the same methods. Cause of death, substance abuse history, medications at time of death and lifetime intake of antipsychotics were available for all subjects (Table 2).

## 2.10 Primary rat hippocampal culture

Rat hippocampi were isolated from 16-18 day old rat fetuses, as originally described by Banker and Cowan (1977). Hippocampal neurons were dispersed and cultured following the procedure detailed by Brewer et al. (1993). Briefly, two hippocampi were isolated from each fetal rat brain, defined by the clearly visible hippocampal fissure and the developing fimbria. Cells were mechanically dispersed in $Ca^{2+}$- and $Mg^{2+}$-free Hank's Balanced Salt Solution (HBSS: Sigma, St. Louis, MO USA) supplemented with 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). An equal volume of HBSS containing $Ca^{2+}$ and $Mg^{2+}$ and the described supplements was added to the cell suspension. Cells were collected by centrifugation at 1,000 x *g* for 4 minutes. Cells were resuspended in Gibco Neurobasal™ media (Invitrogen Canada Inc, Burlington ON, Canada) containing B27 supplements (Invitrogen Canada Inc, Burlington ON, Canada), 0.5 mM L-glutamine, 1% penicillin/streptomycin and 25 μM glutamate. An equal concentration of cells were plated on poly-D-lysine (MW 30,000 – 70,000) coated vessels and maintained at $37^0$C in a 5.0% $CO_2$ humid environment. Cells were plated at a density of 50,000 cells/$cm^2$. Following four days of culture an equal volume of fresh Neurobasal™ media without glutamate was added to each plate and culture maintained for two more days. On the sixth day, media was removed and replaced with fresh glutamate-free Neurobasal™ media and further cultured for 7 days. All drug treatments were started on the sixth day.

## 2.11 Immunocytochemistry

The relative distribution of neurons to glia in the culture was determined by immunocytochemistry (Brewer et al., 1993). Cells were grown on poly-D-lysine coated Lab-Tek 2-Chamber Slides (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were fixed in 4% paraformaldehyde for 1 hour at $37^0$C, then blocked for 5 minutes in 1% bovine serum albumin (BSA), 5% normal goat serum and 0.05% Triton X-100. Rabbit anti-rat neuron-specific enolase (NSE, Polysciences Inc., Warrington PA, USA) diluted 1:1000 in blocking buffer and monoclonal antisera for glial fibrillary acidic protein (GFAP, Sigma Inc., St. Louis MO, USA) at a dilution of 1:400 were used to detect the specific cell types. Slides were incubated with an appropriate fluorescently tagged secondary antibody, rhodamine anti-rabbit IgG for NSE (Molecular Probes Inc., Eugene OR, USA)(1:100 dilution in 1% BSA, 1% normal goat serum, 0.05% Triton X-100) and fluorescein anti-mouse IgG for GFAP (Molecular Probes Inc., Eugene OR, USA)(1:50 dilution). Neurons and glia were imaged using a Carl Zeiss 510 confocal laser scanning microscope (Carl Zeiss Inc., Oberkochen, Germany) with excitation and emission spectra set to 570/590 nm for visualization of neurons and 494/518 nm for glia.

## 2.12 Electron microscopy

Neurons were grown for 13 days on 2-chamber slides coated with poly-D-lysine (MW 30,000-70,000). The Electron Microscopy staff at McMaster University assisted in the preparation of neurons for electron microscopy. Neurons were fixed in 2.0% glutaraldehyde buffered in 0.1M sodium cacodylate, pH 7.4 for 30 minutes at room temperature. Slides were rinsed in 0.2M sodium cacodylate, pH 7.4 and post-fixed with 1% osmium tetroxide in sodium cacodylate buffer for 1 hour at room temperature. Slides were dehydrated by a graded series of ethanol and further dehydrated in 100% propylamide. Neurons were embedded in Beem #3 capsules with Epon resin and polymerized overnight at $60^0$C. Capsules were then snapped off the slides, transferring neurons on the slide to the plastic resin capsule. Ultra-thin sections (~70 nm) were cut from each capsule and floated onto 200 mesh copper/palladium grids and stained for contrast with uranyl acetate and lead citrate. Grids were observed using a transmission electron microscope (JEOL 1200EX; JEOL USA, Inc., Peabody MA, USA) at 80 kV.

## 2.13 Quantification of cytoplasmic vacuolization

An investigator blind to the treatment group quantified cytoplasmic vacuolization. For some sections, blinding occurred by masking the treatment with a random code. The investigator recorded the number of cytoplasmic vacuoles present in fifty randomly chosen neurons. In addition, the location of the neuron was also noted by the investigator to avoid multiple counting of a single neuron. A neuron was characterized by the size of the cell (> 7.5 μm), the densely packed cytoplasm and the large circular nucleus (Fawcett, 1994). A cytoplasmic vacuole was defined as a circular, electron translucent space between the nucleus and plasma membrane of a neuron (Cameron, 1952). These vacuoles could be clearly differentiated between holes that occasionally occur in the ultrathin sections using transmission electron microscopy by the intensity of the electron beam on the phosphorous screen. Neurons sectioned in a way that no nuclear material could be seen were not included in this evaluation.

## 2.14 Fluorescent determination of cell viability

The percentage of dead neurons compared to the total number of neurons was determined using the LIVE/DEAD® viability/cytotoxicity kit and the procedure described by the manufacturer (Molecular Probes, Eugene OR, USA). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeable calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells. Ethdium homodimer (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

Neurons were grown on poly-D-lysine coated Lab-Tek 2-Chamber slides (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were washed in PBS, then incubated at $37^0$C in PBS containing 2 μM calcein AM and 4 μM EthD-1 for 45 minutes. Following incubation, cells were washed with PBS and fixed for 15 minutes with a 4% paraformaldehyde solution. Following fixation cells were washed with PBS and coverslips were mounted using Aquamount (BDH Laboratory Supplies, Poole, Dorset, England) and imaged using a Carl Zeiss 510 confocal laser scanning microscope (Carl Zeiss Inc., Oberkochen, Germany) with excitation and emission spectra set to 485/530 nm for visualization of live cells and 485/590 nm for dead cells. Images were

taken using a 20X objective lens and the numbers of live and dead cells were manually counted from the image. Two randomly chosen fields were focused on and the image captured from 4 replications for each group.

## 2.15 MTT assay

Cell viability was determined by measuring the cleavage of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis MO, USA) into blue-coloured formazan by the mitochondrial enzyme succinate-dehydrogenase, with modifications from the originally described procedure (Manji et al., 2000a; Mosmann, 1983). Rat hippocampal neurons were cultured on 12-well poly-D-lysine (MW 30,000-70,000) coated plates (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Culture media was removed and cells were rinsed with PBS. Cells were then cultured for one hour in media supplemented with 125 μg/ml MTT. Following incubation, media was aspirated and cells were dissolved in 200 μl DMSO and the plates were vigorously shaken for 5 minutes. Three aliquots of 50 μl DMSO were removed from each well and transferred to a 96-well plate (Nalge Nunc International, Rochester NY, USA). The optical density was measured using an autonomic Multiskan Plus Version 1.43 plate reader (Labsystems Inc, Franklin MA, USA) with absorbance set at 540 nm. The mean absorbance for the three aliquots constituted the total absorbance of the sample. Controls cells that were not exposed to any cytotoxic agents were used as a negative control and STS treated cells were positive controls for the MTT assay.

## 2.16 Caspase 3 activity

Caspase-3 activity was quantified using the EnzChek® Caspase-3 activity kit and the protocol described by the manufacturer (Molecular Probes Inc, Eugene OR, USA). Rat hippocampal neurons were cultured on 12-well poly-D-lysine (MW 30,000-70,000) coated plates (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were lysed in the supplied cell lysis buffer, using repeated freeze-thaw cycles. Cells were then centrifuged (5,000 rpm for 5 minutes) and the supernatant transferred to a 96-well microplate (Nalge Nunc International, Rochester NY, USA). An equal volume of reaction buffer (20 PIPES, pH 7.4, 4 mM EDTA, 0.2% CHAPS) containing 0.2 mM Z-DEVD-AMC substrate was added to each well and incubated at room temperature and protected from light for 30 minutes. Fluorescence was measured in a CytoFluor II Fluorescence microplate reader (Applied Biosystems, Foster City CA, USA) with the excitation and emission spectra set to 350/450 nm.

## 2.17 TUNEL-staining

The ApopTag® peroxidase *in situ* apoptosis detection kit (Intergen Company, Purchase, NY USA) was used to detect the presence of fragmented deoxyribonucleic acid, independent of the pathway or enzyme responsible for the cleavage, following the protocol described by the manufacturer. Cells were grown on poly-D-lysine coated Lab-Tek 2-Chamber slides (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were fixed in 1% paraformaldehyde in PBS, pH 7.4 for 1 hour at $37^0$C. Slides were washed twice in PBS, then incubated at $-20^0$C in 2:1 ethanol:acetic acid solution for 5 minutes. Following incubation, cells were further washed in PBS. Endogenous peroxidases were quenched by incubating cells at room temperature in 3.0% hydrogen peroxide in PBS for 5 minutes. Cells were then incubated at room temperature for 10 seconds with the supplied equilibration buffer. Terminal deoxynucleotidyl transferase (TdT) was then added to each slide and incubated in a humidified chamber at $37^0$C for one hour. The reaction was then stopped by incubating slides at room temperature for 10 minutes in the stop/wash buffer supplied by the manufacturer. Samples were then washed three times in PBS, and then incubated in a humidified chamber at room temperature for 30 minutes with anti-digoxigenin peroxidase conjugate. Following incubation cells were washed in PBS, then exposed to peroxidase substrate for 5 minutes at room temperature. Samples were then washed in distilled water, and then incubated in distilled water at room temperature for 5 minutes. Slides were counter

stained in 0.5% methyl green, which selectively stains the cytoplasm for 10 minutes at room temperature, and then further washed in distilled water. A final wash in 100% N-butanol for 30 seconds completed the procedure. Coverslips were mounted on the slides using Aquamount (BDH Laboratory Supplies, Poole, Dorset, England). Images were digitized using a Bioquant Pure Color Windows 98 imaging system (R&M Biometrics, Nashville, TN, USA) attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany).

## 2.18 Data and statistical analysis

All data analysis was conducted using Minitab version 12 (Minitab Inc., State College PA, USA) on an International Business Machines personal computer using a Windows based operating system. Statistical analysis was conducted on raw data and converted to percentages only for graphical display.

Table 2.9.1: Group characteristics of postmortem temporal cortex from the Stanley Foundation Neuropathology Consortium

| | Schizophrenia | Bipolar disorder | Major depressive disorder | Controls |
|---|---|---|---|---|
| Age | 44.2 (25-62) | 42.3 (25-61) | 46.5 (30-65) | 48.1 (29-68) |
| Sex | 9 M, 6 F | 9 M, 6 F | 9 M, 6 F | 9 M, 6 F |
| Race | 13 W, 2 As | 14 W, 1 B | 15 W | 14 W, 1B |
| Postmortem interval (hrs) | 33.7 (12-61) | 32.5 (13-62) | 27.5 (7-47) | 23.7 (8-42) |

Table 2.9.2: Subject information provided by the Stanley Foundation Neuropathology Consortium

a) Schizophrenia

| Age/Sex/Race | PMI (hrs) | Medications at time of death | Cause of death | Substance abuse | Family history |
|---|---|---|---|---|---|
| 30/F/W | 60 | DMI, TH | suicide | yes | $2^0$ |
| 52/M/W | 61 | none | cardiac | no | $1^0$ |
| 30/M/W | 32 | RIS, TRD | pneumonia | no | $2^0$ |
| 62/F/As | 26 | none | MVA | no | none |
| 60/F/W | 40 | none | cardiac | no | none |
| 60/M/W | 31 | TRD, AMI | drowning | no | none |
| 32/M/W | 19 | CZP | Acute alcohol intoxication | yes | none |
| 31/M/W | 14 | CZP | suicide | no | $2^0$ |
| 58/F/W | 26 | HAL, DPH | cardiac | no | $1^0$ |
| 25/M/W | 32 | RIS, PXT | suicide | no | $2^0$ |
| 44/M/W | 50 | HAL, CBZ, FLU, CNP, BTP | cardiac | no | $1^0$ |
| 44/M/W | 29 | CZP, CPZ, Li | pulmonary | yes | $2^0$ |
| 56/F/As | 12 | HAL Li, DPH, CH | suicide | no | $1^0$ |
| 35/M/W | 35 | CZP, CPZ, BTP, DPH, MPT | cardiac | no | $2^0$ |
| 49/F/W | 38 | HAL, CZP, CNP | cardiac | no | $1^0$ |

b) Bipolar disorder

| Age/Sex/Race | PMI (hrs) | Medications at time of death | Cause of death | Substance abuse | Family history |
|---|---|---|---|---|---|
| 25/F/W | 24 | TH, CBZ, Li, TZD | suicide | yes | $2^0$ |
| 48/F/W | 22 | VPA, CBZ, CPT, SER | pneumonia | yes | none |
| 37/F/W | 29 | Li, BUP, CNP, LZP | suicide | no | $1^0$ |
| 54/M/W | 39 | Li, CBZ | subdural hematoma | no | $1^0$ |
| 30/M/W | 31 | Li, CZP | pneumonia | no | none |
| 30/M/W | 56 | None | suicide | no | none |
| 57/M/W | 19 | HAL, DPH | cardiac | no | N/A |
| 34/M/W | 23 | RIS, VPA, VEN | suicide | no | $1^0$ |
| 48/M/W | 13 | None | suicide | no | $2^0$ |
| 31/M/W | 28 | HAL,TZD, THP | suicide | yes | $2^0$ |
| 30/M/W | 45 | VPA, BUP | suicide | no | $1^0$ and $2^0$ |
| 50/F/B | 18 | None | malnutrition | yes | $1^0$ |
| 61/F/W | 60 | FLU, VPA | suicide | no | $1^0$ |
| 50/M/W | 19 | VPA, CZP, FZ, BTP | suicide | no | none |
| 50/F/W | 62 | VPA, CMI | pulmonary | no | $2^0$ |

c) Major depressive disorder

| Age/Sex/Race | PMI (hrs) | Medications at time of death | Cause of death | Substance abuse | Family history |
|---|---|---|---|---|---|
| 32/F/W | 47 | IMI, AMI, NOR, CNP | suicide | no | N/A |
| 53/F/W | 40 | Li, TZD | acute alcohol intoxication | yes | $1^0$ |
| 44/F/W | 32 | FLU, IMI, LZP | suicide | no | $1^0$ and $2^0$ |
| 65/M/W | 19 | PHT | cardiac | no | $1^0$ |
| 52/M.W | 12 | None | cardiac | no | none |
| 46/M/W | 26 | DPH, CNP | suicide | no | $1^0$ |
| 42/F/W | 25 | FLU, Li | cardiac | no | $1^0$ |
| 51/M/W | 26 | NEF, HXZ | suicide | no | $1^0$ |
| 39/M/W | 23 | None | suicide | yes | $1^0$ |
| 42/M/W | 7 | TZP | suicide | no | $1^0$ |
| 56/M/W | 23 | SER | cardiac | no | $2^0$ |
| 56/F/W | 28 | VEN, BUS, APZ | pulmonary | no | none |
| 30/F/W | 33 | NOR, APZ, CMI | suicide | no | $1^0$ |
| 43/M/W | 43 | TRI | cardiac | yes | N/A |
| 47/M/W | 28 | FLU, NEF | cardiac | no | $1^0$ |

d) Control

| Age/Sex/Race | PMI (hrs) | Medications at time of death | Cause of death | Substance abuse | Family history |
|---|---|---|---|---|---|
| 52/M/W | 28 | None | cardiac | no | none |
| 44/F/W | 25 | None | cardiac | no | none |
| 59/M/W | 26 | None | cardiac | no | none |
| 52/M/W | 8 | None | cardiac | no | none |
| 52/M/W | 22 | None | cardiac | no | none |
| 53/M/W | 28 | None | cardiac | no | none |
| 44/M/W | 10 | None | cardiac | no | none |
| 35/F/W | 23 | None | cardiac | no | none |
| 41/M/B | 11 | None | pulmonary | no | none |
| 42/M/W | 27 | None | cardiac | no | none |
| 35/F/W | 40 | None | pulmonary | no | none |
| 68/F/W | 13 | None | pulmonary | no | none |
| 58/M/W | 27 | None | cardiac | no | none |
| 29/F/W | 42 | None | MVA | no | $2^0$ |
| 57/F/W | 26 | None | MVA | no | none |

*Abbr.* APZ, Alprazolam; AMI, Amitriptyline, BDZ, Benzodiazepine; BTP, Benzotropine, BUP, Bupropion; BUS, Buspirone; CBZ, carbamazepine, CH, Chloral hydrate; CPZ, Chlorpromazine; CPT, Chlorprothixene; CMI, Clomipramine; CNP, Clonazepam; CZP, Clozapine; DMI, desipramine; DPH, Diphenhydramine, FLU, Fluoxetine; FZ, Flurazepam; HAL, Haloperidol; HXZ, Hydroxyzine; IMI, Imipramine; LZP, Lorazepam; MPT, Maprotiline; NEF, Nefazadone; NOR, Nortriptyline, PXT, Paroxetine, PHT, Phenytoin, RIS, Risperidone; SER, Sertraline; TZP, Temazepam; TRI, Trimipramine; TRD, Thioridazine, TH, Thiothixene, TZD, Trazadone; THP, Trihexphenidyl; VEN, Venlafaxine

## 3.1 Differential Display PCR Reveals Novel Targets for the Mood-stabilizing Drug Valproate Including the Molecular Chaperone GRP78

Jun-Feng Wang, Christopher Bown, L. Trevor Young

*Published in* Molecular Pharmacology 55: 521-527 (1999)



***Rationale***: The clinical features, genetics, and neurochemistry of bipolar disorder strongly indicate a biological basis for the illness, although the precise defect has yet to be discovered. Since bipolar disorder patients have shown response to long-term (lifetime) pharmacology (lithium, valproate, carbamazepine), it has been suggested that adaptational changes in gene expression are critical to their prophylactic effects. Using differential display PCR to identify valproate-regulated genes in rat brain, we identified six differentially regulated genes. One of these genes had 100% homology to the rat 78 kilodalton glucose-regulated gene (GRP78). The research described in this paper involved identification and characterization of GRP78 as a valproate regulated gene. Optimistically, by identifying genes regulated by mood stabilizers, we will understand the biochemical defects associated with the disorder, which will hopefully lead to better treatment options for patients.

***Involvement:*** Differential display PCR as well as the initial confirmation experiments were carried out by JFW. Characterization of GRP78 protein expression and further confirmation of GRP78 in cultured cells was carried out by CDB. All work and manuscript editing was supervised by LTY.

0026-895X/99/030521-07$3.00/0

MOLECULAR PHARMACOLOGY, 55:521–527 (1999).

# Differential Display PCR Reveals Novel Targets for the Mood-Stabilizing Drug Valproate Including the Molecular Chaperone GRP78

JUN-FENG WANG, CHRISTOPHER BOWN, and L. TREVOR YOUNG

*Department of Psychiatry and Behavioral Neurosciences, McMaster University, Hamilton, Ontario, Canada*

Received June 15, 1998; accepted November 25, 1998

This paper is available online at http://www.molpharm.org

## ABSTRACT

Differential display polymerase chain reaction was used to identify genes regulated by the mood-stabilizing drug valproate (VPA). Four differentially displayed valproate-regulated gene fragments were isolated in rat cerebral cortex after i.p. injection of sodium VPA (300 mg/kg) for 3 weeks, and their expression was confirmed by Northern and slot blot analysis in rat cerebral cortex and C6 glioma cells. Sequencing analysis revealed three previously unidentified cDNA fragments in addition to a sequence with 100% homology with a molecular chaperone, 78-kDa glucose-regulated protein (GRP78). VPA treatment did not increase mRNA expression of 70-kDa heat shock protein, which is a related stress-induced molecular chaperone protein. All four candidate genes, including *GRP78*, showed similar VPA concentration-dependent increases in mRNA abundance. Another commonly prescribed mood-stabilizing anticonvulsant, carbamazepine, also increased *GRP78* mRNA expression in C6 glioma cells, whereas lithium had no effect at doses up to 2 mM. Immunoblotting revealed that GRP78 protein levels were also increased in C6 glioma cells treated with VPA under the same conditions. Nuclear runoff analysis showed that VPA increased *GRP78* gene transcription. Because GRP78 possesses molecular chaperone activity, binds $Ca^{2+}$ in the endoplasmic reticulum, and protects cells from the deleterious effects of damaged proteins, the present findings suggest that VPA (and possibly carbamazepine) treatment may target one or more of these processes.

The mood-stabilizing drug sodium valproate (VPA) is a branched-chain saturated fatty acid (2-propylpentanoic acid) that possesses anticonvulsant effects in the treatment of epilepsy (Penry and Dean, 1989). Recent evidence has established that VPA has a mood-stabilizing effect; this drug is quickly becoming a first line treatment for bipolar disorder (BD) because it has a broad spectrum of efficacy in patients with this illness (McElroy et al., 1992; Bowden, 1996). Long-term prophylactic treatment with mood-stabilizing drugs is currently recommended for BD. It is believed that chronic treatment with these drugs regulates gene expression (Post, 1992; Hyman and Nestler, 1996). The specific targets of these drugs are, however, poorly understood. Increased $Na^+$ channel subunit mRNA levels have been reported after VPA treatment (Yamamoto et al., 1997). VPA has also been shown to decrease myristoylated alanine-rich C kinase substrate expression (Lenox et al., 1996). VPA increases activator protein-1 binding in cultured cells (Chen et al., 1997; Asghari et al., 1998), suggesting that genes containing this consensus sequence in their promoter may be targets of this drug. The available evidence is far from explaining the mechanism of this drug. Therefore, isolation of specific valproate-regulated genes (VRGs) is important, particularly if the products of these genes are found to be relevant to the pathophysiology of BD.

Differential display polymerase chain reaction (PCR) was developed to identify differentially expressed genes and to detect individual mRNA differences in various sets of mammalian cells (Liang et al., 1992). Side by side comparison of RNA samples from different cells allows the identification of differentially expressed genes. Amplified cDNAs can then be used as probes to isolate genes from genomic and cDNA libraries for further molecular characterization. To date, these methods have been successfully applied to isolate differentially expressed genes in cancer (Liang et al., 1992), heart disease (Utans et al., 1994), and diabetes (Nishio et al., 1994). We used this method to isolate differentially expressed genes in rat cerebral cortex after administering VPA for 3 weeks. Cerebral cortex was chosen because this is the region in which abnormalities have been reported in BD patients

This work was supported by a Stanley Foundation Research Grant (L.T.Y). J.F.W. is a Canadian Psychiatric Research Foundation fellow. L.T.Y. is a career scientist of the Ontario Ministry of Health.

**ABBREVIATIONS:** CNS, central nervous system; VRG, valproate-regulated gene; GRP78, 78-kDa glucose-regulated protein; HSP70, 70-kDa heat shock protein; VPA, valproate; BD, bipolar disorder; PCR, polymerase chain reaction; SSPE, sodium chloride/sodium dihydrogen phosphate/EDTA; bp, base pairs; CBM, carbamazepine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and where mood stabilizers have been shown to have numerous effects (Song and Jope, 1992; Li et al., 1993). One of these genes is the 78-kDa glucose-regulated protein (GRP78), a molecular chaperone that participates in protein trafficking and endoplasmic reticulum (ER) calcium homeostasis. We have confirmed the specificity and dose-response relationship of *GRP78* regulation by VPA. Additionally, VPA treatment resulted in increased GRP78 protein levels.

## Materials and Methods

**Animal Treatment and Cell Culture.** Male Sprague-Dawley rats (200–250 g) were injected i.p. with VPA (300 mg/kg) or 0.9% saline (control) once daily for 21 days. Rats were weighed weekly to control for nutritional status, sacrificed by decapitation, and their brains were dissected rapidly in 0.32 M sucrose at 4°C. All areas of cerebral cortex were dissected, pooled for each animal, and used immediately for RNA extraction. Rat C6 glioma cells were grown in a medium containing Dulbecco's modified Eagle's medium and 10% fetal calf serum. Cells were treated at various times in the presence or absence of different concentrations of VPA. Cell viability was greater than 98% as confirmed by tryptan blue exclusion and was not different across drug treatments (data not shown).

**Differential Display PCR.** Total RNA from tissues and cell lines was isolated using Trizol reagent (Gibco Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Chromosomal DNA was digested with RNase-free DNase I (Boehringer Mannheim, Laval, Quebec) for 30 min at 37°C. The concentration of RNA was determined by measuring the absorbance at 260 nm and the purity determined by the 260:280 ratio. Three micrograms of total RNA was subjected to electrophoresis on a denaturing 1% agarose gel to check the integrity of the RNA (18S and 28S rRNA bands).

All reagents used in differential display PCR were obtained from GenHunter Corporation (Nashville, TN) except *Taq* polymerase, which was obtained from Perkin-Elmer Corp. (Branchburg, NJ). DNA-free RNA was transcribed to cDNA with reverse transcriptase using three different anchored primers: oligo(dTG), oligo(dTA), and oligo(dTC), respectively. Primers for PCR reaction were one of eight arbitrary primers and one of oligo(dTG), oligo(dTA), or oligo(dTC). The PCR reaction mixture (20 $\mu$l) contained 2 $\mu$M of each primer, 25 $\mu$M dNTP, 0.5 $\mu$M [$\alpha$-$^{35}$S]dATP (1200 Ci/mmol) and 1 U of *Taq* polymerase. The PCR reaction (40 cycles) consisted of sequential incubations for 30 s at 94°C, for 2 min at 40°C, and for 30 s at 72°C, with a final extension for 5 min at 72°C. The amplified cDNAs were then separated on 6% denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-Omat film. Differentially displayed cDNAs were then recovered from the gel and reamplified by PCR using the same primers. The reamplified cDNAs were extracted from a 1.5% agarose gel using a QIAEX Gel Extraction Kit (Qiagen Inc., Chatsworth, CA); the protocol was provided by the manufacturer.

**DNA Cloning and Sequence Analysis.** Differentially displayed PCR products were ligated into a pNoTA/T7 vector at 25°C for 30 min with T4 DNA ligase (5 Prime-3 Prime, Inc., Boulder, CO). Competent bacterial cells were transformed with the ligated construct. Plasmid DNA from subclones containing inserts were then purified using a Wizard Miniprep DNA purification system (Promega Biotec, Madison, WI). Inserts were sequenced by the dideoxy chain-termination method (Sanger et al., 1977). The sequencing products were run by electrophoresis on a 6% polyacrylamide-urea gel and identified by autoradiography. Sequences thus derived were compared for homology to the sequences present in the current GenBank database through the National Center for Biotechnology Information with the BLASTN program (Altschul et al., 1990).

**Northern Hybridization and Slot Blot Analysis.** Total RNA from rat cerebral cortex or C6 glioma cells was applied to nylon membranes using either a slot blot apparatus or by capillary transfer after electrophoresis in 1% agarose/formaldehyde gel, and ultraviolet-cross-linked. Membranes were then prehybridized at 42°C for 3 h in 5× sodium chloride/sodium dihydrogen phosphate/EDTA (SSPE), 50% formamide, 5× Denhardt's, 1.0% SDS, 5% dextran sulfate, and salmon sperm DNA (100 $\mu$g/ml) and then hybridized at 42°C overnight in this buffer with differentially displayed cDNA probes derived as described above. cDNA probes were labeled with [$\alpha$-$^{32}$P]dCTP by using a random prime labeling method (Feinberg and Vogelstein, 1983). Membranes were then washed at room temperature for 45 min in 2× SSPE and 0.1% SDS twice and at 58°C for 15 min in 0.1× SSPE and 0.1% SDS twice. Membranes were exposed overnight at −80°C to Kodak X-Omat film with an intensifying screen.

**Nuclear Runoff.** Nuclear runoff assays were performed as previously described (Lofquist et al., 1995). Briefly, C6 cells were treated or untreated with VPA at 1 mM for 1 week. The cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40). Several microliters of cell lysate were examined with a hemacytometer on a phase-contrast microscope to determine whether the cells were free of membrane components. Nuclei were collected by centrifugation. The nuclei were resuspended in 100 $\mu$l of glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). Runoff transcription was initiated by adding 100 $\mu$l of reaction buffer [(10 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM dithiothreitol, 40 units/ml RNasin, 1 mM ATP, 1 mM GTP, 1 mM CTP, and 25 $\mu$l of [$\alpha$-$^{32}$P]UTP (3000 Ci/mmol)] at 30°C for 30 min. DNA was digested by adding 1 $\mu$l of 20,000 U/ml RNase-free DNase, whereas protein was digested by adding 10 $\mu$l of proteinase K (20 mg/ml). Newly transcribed RNA was purified by 25:24:1 buffered phenol/chloroform/isoamyl alcohol, followed by precipitation with isopropyl alcohol. The membrane with cDNAs applied by slot blotting was then prehybridized at 42°C for 3 h in 5× SSPE, 50% formamide, 5× Denhardt's, 1.0% SDS, and salmon sperm DNA (100 $\mu$g/ml), and was hybridized at 42°C overnight in this buffer with the $^{32}$P-labeled newly transcribed RNA. After hybridization, membranes were then washed at room temperature for 45 min in 2× SSPE and 0.1% SDS twice and at 58°C for 15 min in 0.1× SSPE and 0.1% SDS twice. Membranes were exposed overnight at −80°C to Kodak X-Omat film with an intensifying screen.

**Immunoblotting Analysis.** Cells were washed in PBS (100 mM NaCl, 100 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.5) and then suspended in cell lysis buffer (150 mM NaCl, 15 mM $Mg_2Cl$, 1 mM EGTA, 50 mM HEPES-KOH, 10% glycerol, 1% Triton X-100, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and 2 $\mu$M benzamidine. After gentle agitation for 30 min at 4°C, cell lysates were centrifuged for 10 min at 12,000*g*. Supernatants were used for immunoblotting analysis. The protein concentration was determined according to the method of Bradford (1976).

Five micrograms of protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 12% acrylamide gel for 1 h at 120 V. Proteins were transferred to polyvinylidene difluoride membranes at 100 V for 1 h, blocked in 3% milk-PBS (30 min, 22°C), and incubated overnight at 22°C with a monoclonal antisera for GRP78 (StressGen Biotechnologies Corp., Victoria, BC) at a 1:5000 dilution. Blots were then incubated with secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:2000 in blocking buffer for 1 h at 22°C. Immunoreactive bands were detected with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The blots were subsequently exposed to Kodak X-Omat.

**Analysis and Interpretation of Data.** All sequence data were aligned with sequences available in GenBank. Data from slot blot, nuclear runoff, and immunoblotting studies were obtained by densitometric analysis of autoradiograms using the Northern Exposure program from ImagExperts, Inc. (Oakville, Ontario, Canada). Results were expressed as a percentage of control. Changes in gene expression after drug treatment were expressed as the mean ± S.E.M. from three separate experiments. Statistical significance of

differences between means was determined by Student's *t* tests or one-way ANOVA.

## Results

**Identification of Candidate VRGs by Differential Display PCR.** With differential display PCR, six cDNA PCR products were differentially expressed in the rat cerebral cortex between controls and rats treated for 3 weeks with VPA at 300 mg/kg i.p.. The six differentially expressed cDNA PCR products were then excised from the 6% denaturing polyacrylamide gel and reamplified with the same primers used in differential display PCR. These six candidate VRGs were purified and ligated into a pNo-TA/T7 cloning vector. The differentially expressed candidate VRG cDNA inserts were sequenced and named: VRG1 [112 base pairs (bp)], VRG2 (106 bp), VRG3 (146 bp), VRG4 (105 bp), VRG5 (62 bp), and VRG6 (386 bp). VRG1 and VRG3–6 are previously unidentified cDNA fragments and showed no significant homology (<85%) to any entry in existing nucleic acid databases available through the National Center for Biotechnology Information. VRG2, however, displayed 100% homology to the *GRP78*, which is a stress-induced molecular chaperone. Figure 1 shows an autoradiograph of VRG2 demonstrating increased expression after VPA treatment. The cDNA fragments of candidate VRGs were radioactively labeled for Northern blot hybridization. Total RNA from cerebral cortex of rats treated for 3 weeks with VPA at 300 mg/kg i.p. and from control animals were used for Northern blot analysis. VRG1, VRG2, VRG4, and VRG6 were verified to be differentially expressed by Northern blot analysis (Fig. 2), whereas no expression of VRG3 or VRG5 was detected in rat brain.

**Dose-Response Relationship of VRG Expression.** To further investigate the effect of VPA on the differentially expressed candidate VRGs (VRG1, VRG2, VRG4, and VRG6), we determined VPA dose-response curves for these genes. First, VRG1, VRG2, VRG4, and VRG6 were confirmed to be differentially expressed by Northern blot analysis in C6 glioma cells treated with 1 mM VPA for 1 week. We used C6 cells to study the dose-response effect on *GRP78* because 1) C6 cells are commonly used to study the mechanism of mood stabilizers including VPA, and 2) *GRP78* is known to be expressed in this cell line (Brostrom et al., 1991; Chen et al., 1996, 1997). As shown in Fig. 2, all four genes were increased by VPA in C6 glioma cells as in rat cerebral cortex. Next, C6 glioma cells were exposed to VPA at different concentrations of 0, 0.25, 0.5, and 1 mM for 1 week and VRG1, VRG2, VRG4, and VRG6 mRNAs were quantitated by slot blot hybridization. These concentrations represent serum levels commonly used to treat patients (Bowden et al., 1996). The results indicated that VPA increased VRGs' mRNA expressions in a dose-dependent manner and that VPA dose-response curves for these genes showed similar patterns (Fig. 3).

**Effects of VPA on *GRP78* and Heat Shock Protein 70 (*HSP70*) mRNA Levels.** HSP70 is a related stress-induced molecular chaperone. To study the specificity of VPA on *GRP78* mRNA expression and to further verify the effect of VPA on *GRP78*, *HSP70* and *GRP78* mRNA levels were measured with Northern blot analysis using *HSP70* and *GRP78* coding region sequences as probes in rat cerebral cortex and C6 glioma cells. Seven hundred sixty-seven-bp *GRP78* and 495-bp *HSP70* cDNA probes were generated by reverse transcription-PCR. The *GRP78* upstream primer (5'-CCACCG-TAACAATCAAGGTC-3') and downstream primer (5'-CACGTGAGCAACTGCTAATG-3'), which are located in regions +276 to +295 and +1024 to +1043 of the *GRP78* gene, and *HSP70* upstream primer (5'-GGTGCTGACCAA-GATGAAG-3') and downstream primer (5'-CAGAGAGTC-GATCTCCAGG-3'), which are located in regions +543 to +561 and +1020 to +1038 of *HSP70* gene were used, respectively, for PCR amplification. *GRP78* and *HSP70* cDNA probes from PCR were confirmed by sequencing analysis. As shown in Fig. 4, VPA increased *GRP78*, but not *HSP70*, mRNA in both rat cerebral cortex and C6 glioma cells. VPA at 1 mM also showed time-dependent increases of *GRP78* mRNA after treatment for 1 and 5 h, and 1, 3, 5, and 7 days. After 7 days of treatment with 1 mM VPA, the drug was subsequently withdrawn from cells to determine the reversibility of *GRP78* induction and rule out cell death as a result

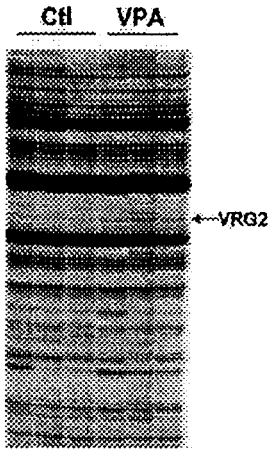



Fig. 1. Differential display of mRNA in control (Ctl) and sodium VPA (300 mg/kg i.p. for 3 weeks)-treated rats. Each lane represents tissue from one animal. A sample of 0.2 $\mu$g of total RNA from each animal was used for differential display PCR. Arrows point to the differentially displayed product VRG2.

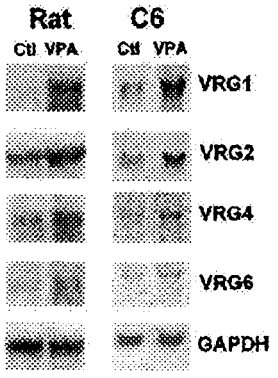



Fig. 2. Northern blot analysis of candidate VRGs in rat cerebral cortex and C6 glioma cells. Total RNA was isolated from rat cerebral cortex after 3 weeks of sodium VPA (300 mg/kg i.p.) treatment or from rat C6 glioma cells after 1 week of 1-mM VPA compared with untreated control (Ctl) tissues. Twenty-five micrograms of RNA was used for Northern blot analysis using probes prepared from products of differential display (VRG1, VRG2, VRG4, and VRG6) or the housekeeping gene *GAPDH*.

of VPA treatment. As shown in Fig. 5, the *GRP78* mRNA level was decreased from 65 ± 9% to 16 ± 8% at 6 h, and to 6 ± 8% at 24 h after VPA withdrawal.

**Effect of Other Mood Stabilizers such as Lithium and Carbamazepine on *GRP78* mRNA Abundance.** To further investigate possible similarities between VPA and the two other mood stabilizers commonly used to treat BD, we determined the effect of therapeutically relevant concentrations of lithium chloride (LiCl) and carbamazepine (CBM) on *GRP78* gene expression by slot blot analysis. As shown in Fig. 6, 0.5 to 2 mM LiCl and 0.025 and 0.05 mM CBM had no effect on *GRP78* gene expression. VPA at 0.5 mM and 1.0 mM significantly increased *GRP78* gene expression by 31.6 ± 6.2% ($p < .05$) and 50.5 ± 8.9% ($p < .05$,) respectively. CBM at 0.1 mM also increased *GRP78* gene expression by 25.2 ± 5.3% ($p < .05$). There was no difference in the level of hybridization of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) compared to control cells after any of these treatments.

**Effect of VPA on *GRP78* Gene Transcription.** To determine whether the changes in the *GRP78* mRNA level is contributed to by the induction of *GRP78* transcription, we used nuclear runoff analysis to measure the effect of VPA on transcription of the *GRP78* gene. Five micrograms of empty plasmid DNA or plasmid DNA with *GRP78* cDNA insert was blotted to nylon membranes. C6 glioma cells were treated with and without VPA at 1 mM for 1 week. Nuclei were isolated from control and VPA-treated cells. Newly transcribed RNAs from nuclei of control and VPA-treated cells were hybridized with *GRP78* cDNA and empty plasmid DNA blots. As shown in Fig. 7, VPA treatment induced a significant increase of *GRP78* gene transcription in C6 glioma cells.

**Effect of VPA on GRP78 Protein Level.** To determine whether the changes in the *GRP78* mRNA level contribute to GRP78 translation, we measured the effect of VPA on the GRP78 protein level using immunoblotting analysis. Figure 8 shows that VPA treatment of C6 glioma cells for 1 week at therapeutically relevant concentrations (0.25–1.0 mM) (Bowden et al., 1996; Chen et al., 1996, 1997; Asghari et al., 1998) increased the GRP78 protein level in a dose-dependent manner.

## Discussion

Differential display PCR revealed four differentially expressed gene products in rat cerebral cortex after i.p. injection with VPA (300 mg/kg) for 3 weeks. Although the identity of three of these gene products is presently unknown, the expression of a cDNA with 100% homology to the gene coding for the molecular chaperone GRP78 was markedly increased after VPA treatment. The expression of this cDNA in rat cerebral cortex was confirmed by Northern blot hybridization. Quantitative slot blot analysis demonstrated that VPA increased the expression of these candidate genes in a dose-dependent manner at therapeutically relevant concentrations in rat C6 glioma cells. These results strongly suggest that specific genes, including the molecular chaperone *GRP78*, are regulated by VPA treatment. Furthermore, *GRP78* mRNA expression was also increased by treatment with the closely related mood-stabilizing anticonvulsant carbamazepine, but not lithium chloride. These results are consistent with the current notion that psychotropic drug treatment may act through long-term adaptational changes in the central nervous system (CNS) to bring about their therapeutic effects (Post, 1992; Hyman and Nestler, 1996).

GRP78 is induced as part of the stress response in eukaryotic cells (Brostrom and Brostrom, 1998). This gene product functions in endoplasmic reticulum (ER) glycoprotein trafficking, possesses molecular chaperone activity, and protects cells from the deleterious effects of damaged proteins. GRP78 is constitutively expressed in the ER, and transcription of the

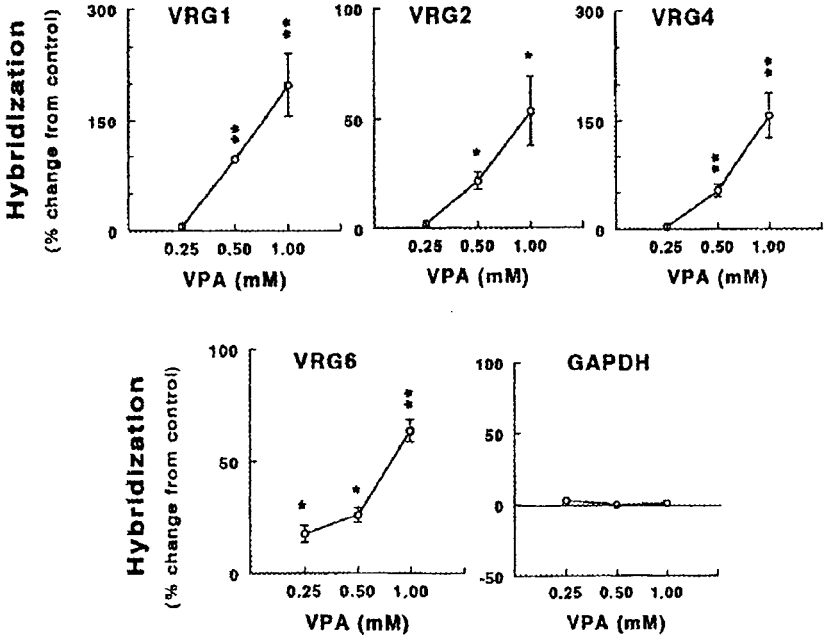



Fig. 3. The effects of different doses of VPA on candidate VRG expression. Rat C6 glioma cells were grown for 1 week with 0.25, 0.5, and 1 mM VPA. Control cells (Ctl) were treated identically but without addition of drug. Total RNA was analyzed by slot blot hybridization using [$\alpha$-$^{32}$P]dCTP-labeled probes prepared from products of differential display (VRG1, VRG2, VRG4, and VRG6) or the housekeeping gene *GAPDH*. Autoradiograms were quantitated by densitometry. Results are the mean ± S.E.M. of triplicate determinations. $^{*}p < .05$ and $^{**}p < .01$ significantly different from controls by ANOVA.

*GRP78* gene is elevated in response to malfolded proteins and treatments that interfere with protein glycosylation, protein trafficking, and ER storage of calcium (Kim et al., 1987; Wooden et al., 1991; Little et al., 1994). The molecular chaperone activities of GRP78 make it critical to ER functioning. GRP78 assists in the folding of the newly synthesized proteins and the acquisition of their correct tertiary and quaternary structure (Bole et al., 1986; Lee, 1987). Malfolded protein binds to GRP78 ultimately to be disposed of by nonlysosomal proteolytic process (Bonifacino and Lippincott-Schwartz, 1991; Werner et al., 1996).

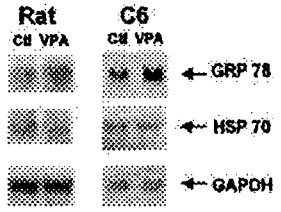



**Fig. 4.** Northern blot analysis of *GRP78* and *HSP70* in the rat cerebral cortex and C6 glioma cells. Total RNA was isolated from rat cerebral cortex after 3 weeks of VPA (300 mg/kg i.p.) and from C6 glioma cells grown for 1 week with 1 mM VPA and untreated control (Ctl) tissues. Total RNA was used by Northern blot analysis using probes prepared from *GRP78* cDNA, *HSP70* cDNA, or the housekeeping gene *GAPDH*.

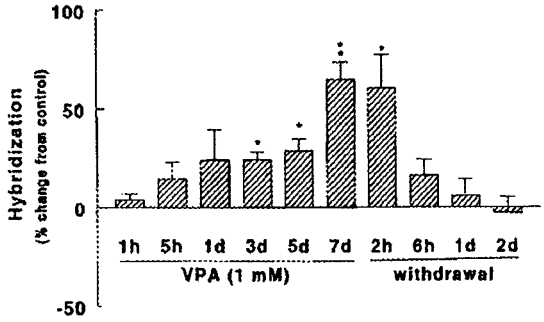



**Fig. 5.** Time course and withdrawal of VPA on *GRP78* gene expression. C6 glioma cells were treated with 1 mM VPA for 1 and 5 h (h), and 1, 3, 5, and 7 days (d). VPA was withdrawn at 2 h, 6 h, 1 day, and 2 days after a 1-week treatment with VPA at 1 mM. Control cells were treated identically but without addition of drug. Autoradiograms were quantitated by densitometry. Results are the mean ± S.E.M. of triplicate determinations. $^{*}p < .05$ and $^{**}p < .01$ significantly different from control by ANOVA.

Our results indicate that chronic VPA treatment increases both GRP78 mRNA and protein levels at therapeutically relevant concentrations in rat C6 glioma cells. Although the functional relevance of these changes is not yet known, the fact that VPA increased GRP78 protein levels suggests that these changes may be relevant to the pharmacological action of VPA. It has been previously reported that classical inducers of stress proteins such as thapsigargin lead to 6-fold or higher induction of *GRP78* in various cell lines (Miles et al., 1994; Hsieh et al., 1996). In the present study, VPA induced *GRP78* more modestly than thapsigargin, suggesting that the effect of VPA on *GRP78* may be due to a different mechanism. Thapsigargin depletes ER $Ca^{2+}$, which in turn inhibits ER protein processing and cellular protein synthesis. These changes after thapsigargin treatment secondarily lead to *GRP78* induction, which may protect the cell against damage or cell death that would ultimately occur after ER $Ca^{2+}$ depletion. Our results suggest that *GRP78* induction by VPA is not the result of ER damage or insult. Indeed, withdrawal of VPA after 1 week of treatment resulted in a return to baseline of *GRP78* mRNA levels, which occurs as early as 6 h.

There is increasing interest in the role of GRP78 in CNS disease and injury. GRP78 expression is induced after CNS injuries such as seizures, global ischemia, and acute trauma (Lowenstein et al., 1994), and in neurodegenerative diseases like Alzheimer's (Hamos et al., 1991). *GRP78* induction by chronic VPA treatment may play a neuroprotective role by clearing malfolded proteins. Recently, chronic lithium treatment at therapeutically relevant concentrations was found to protect cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity (Nonaka et al., 1998). In our study, chronic lithium treatment did not alter *GRP78* gene expression, suggesting that these drugs may both have neuroprotective effects, but these effects occur through different pathways.

It has recently been shown that GRP78 not only participates in protein trafficking but is an important $Ca^{2+}$-binding

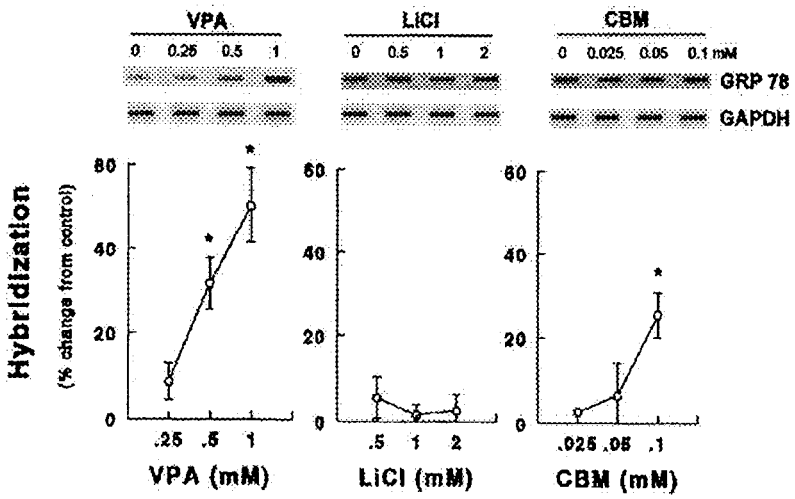



**Fig. 6.** Effects of sodium VPA, LiCl, and CBM on *GRP78* gene expression. C6 glioma cells were grown for 1 week with VPA at concentrations of 0.25, 0.5, and 1 mM; LiCl at 0.5, 1, and 2 mM; CBM at 0.025, 0.05, and 0.1 mM. Control cells were treated identically but without addition of drug. Total RNA was analyzed by slot blot hybridization using [$\alpha$-$^{32}$P]dCTP-labeled probe of *GRP78* or the housekeeping gene *GAPDH*. Autoradiograms were quantitated by densitometry. Results are the mean ± S.E.M. of triplicate determinations. $^{*}p < .05$ significantly different from control by ANOVA.

protein in the ER (Lièvremont et al., 1997). Overexpression of exogenous hamster GRP78 in HeLa cells induced increases of the ER $Ca^{2+}$ storage capacity (Lièvremont et al., 1997). Therefore, GRP78 may play an important role in the control of the ER luminal $Ca^{2+}$ homeostasis. Increased expression of the molecular chaperone GRP78 after VPA suggests that this drug may regulate $Ca^{2+}$ homeostasis. Consistent with this hypothesis is the fact that VPA has been shown to inhibit 5-hydroxytryptamine-induced increase of intracellular free calcium in C6 glioma cells (Yamaji et al., 1996). Lithium was also found to inhibit *N*-methyl-D-aspartate receptor-mediated calcium influx (Nonaka et al., 1998). Because increased intracellular calcium levels have consistently been reported in blood cells obtained from patients with BD (Dubovsky et al., 1989; Emamghoreishi et al., 1997), a novel target for VPA that could regulate intracellular $Ca^{2+}$ levels is of potential importance.

HSP70 is another molecular chaperone that is induced by stress. *HSP70* mRNA levels were not regulated by VPA treatment, suggesting specific regulation of *GRP78* by VPA. It has been shown that the stress response is associated with the independent induction of two groups of evolutionarily conserved proteins, HSPs and glucose-regulated proteins (GRPs). HSP70 induction is fostered by damaged or aberrant cytosolic, nuclear, and mitochondrial proteins caused by thermal or chemical insult, whereas the accumulation of abnormally or poorly processed proteins within the lumen of the ER causes GRP78 to be synthesized (Brostrom and Brostrom, 1998). Increased *GRP78*, but not *HSP70*, expression after VPA treatment suggests that this drug may specifically regulate protein processing linked to *GRP78* induction.

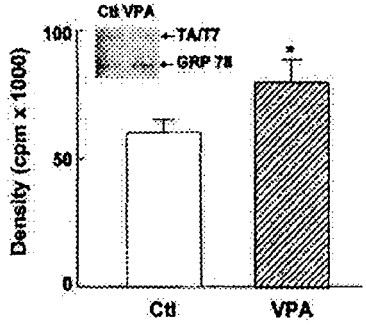



**Fig. 7.** Effect of sodium VPA on *GRP78* transcription. Nuclei were isolated from C6 glioma cells after treatment without (Ctl) or with 1 mM VPA. Equal amounts (cpm) of $^{32}P$-labeled nuclear runoff RNA were hybridized to slot blots containing 5 μg of *GRP78* plasmid cDNA and the empty plasmid vector pNoTA/T7 DNA (TA/T7). Results are the mean ± S.E.M. of triplicate determinations. $^*p < .05$ significantly different from control by ANOVA.

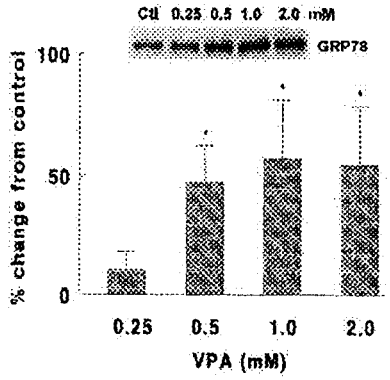



**Fig. 8.** Effects of VPA on GRP78 protein levels. C6 glioma cells were treated with VPA at 1 mM for 1 week. Control cells (Ctl) were treated identically but without addition of drugs. Five micrograms of protein was analyzed by immunoblotting analysis using a GRP78 antisera. Autoradiograms were quantitated by densitometry. Results are the mean ± S.E.M.. $^*p < .05$ significantly different from control by ANOVA.

The increase of *GRP78* mRNA expression by VPA appears to occur in part at the level of transcription based on the results from the nuclear runoff assay. The dose-response curves for *GRP78* and the other candidate VRGs are highly similar, suggesting that VPA could regulate these genes through a common mechanism. Indeed, it is possible that there is a VPA-responsive sequence in the promoter of these genes similar to that found for ethanol (Hsieh et al., 1996). Although VPA has been found to increase AP-1 DNA binding activity (Chen et al., 1997; Asghari et al., 1998), there is no known AP-1 site in the *GRP78* promoter, suggesting the potential importance of other elements (Resendez et al., 1988). The identity of a VPA responsive *cis*-acting element and isolation of *trans*-acting factors need to be further investigated. The role of the other unidentified candidate VRGs induced by VPA remains to be determined. Although all of the VRGs isolated here show induction at therapeutically relevant VPA concentrations, further studies are needed to characterize the therapeutic importance of these changes in gene expression.

In conclusion, chronic VPA treatment resulted in the increased expression of several mRNAs in rat cerebral cortex and C6 glioma cells, one of which has been identified as the molecular chaperone GRP78. The results of the present study demonstrate the potential utility of differential display PCR to identify the targets of psychotropic drugs and suggest potentially important and previously unidentified targets for VPA.

**Acknowledgments**

We thank Dr. M. Fahnestock and Dr. R. Mishra for invaluable discussions.

**References**

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410.

Asghari V, Wang JF, Reiach JS and Young LT (1998) Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 binding activity in human neuroblastoma SH-SY5Y cells. *Mol Brain Res* 58:95–102.

Bole DG, Hendershot LM and Kearney JF (1986) Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in non-secreting and secreting hybridomas. *J Cell Biol* 102:1558–1566.

Bonifacino JS and Lippincott-Schwartz J (1991) Degradation of proteins within the endoplasmic reticulum. *Curr Opin Cell Biol* 3:592–600.

Bowden CL (1996) Role of newer medications for bipolar disorder. *J Clin Psychopharmacol* 16(Suppl):48S–55S.

Bowden CL, Janicak PG, Orsulak P, Swann AC, Davis JM, Calabrese JR, Goodnick P, Small JG, Rush AJ, Kimmel SE, Risch SC and Morris DD (1996) Relation of serum valproate concentration to response in mania. *Am J Psychiatry* 153:765–770.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–252.

Brostrom CO and Brostrom MA (1998) Regulation of translational initiation during cellular responses to stress, in *Progress in Nucleic Acid Research and Molecular Biology*, vol 58, pp 79–125. Academic Press, San Diego.

Brostrom MA, Prostko CR, Gmitter-Yellen D, Grandison LJ, Kuznetsov G, Wong WL and Brostrom CO (1991) Inhibition of translational initiation by metalloendoprotease antagonists. Evidence for involvement of sequestered $Ca^{2+}$ stores. *J Biol Chem* 266:7037–7043.

Chen G, Manji HK, Wright CB, Hawver DB and Potter WZ (1996) Effects of valproic

acid on beta-adrenergic receptors, G-proteins, and adenylyl cyclase in rat C6 glioma cells. *Neuropsychopharmacology* **15**:271–280.

Chen G, Yuan P, Hawver DB, Potter WZ and Manji HK (1997) Increase in AP-1 transcription factor DNA binding activity by valproic acid. *Neuropsychopharmacology* **16**:238–45.

Dubovsky SL, Christiano J, Daniell LC, Franks RD, Murphy J, Adler L, Baker N and Harris RA (1989) Increased platelet intracellular calcium concentration in patients with bipolar affective disorders. *Arch Gen Psychiatry* **46**:632–638.

Emamghoreishi M, Schlichter L, Li PP, Parikh S, Sen J, Kamble A and Warsh JJ (1997) High intracellular calcium concentrations in transformed lymphoblasts from subjects with bipolar I disorder. *Am J Psychiatry* **154**:976–982.

Feinberg AP and Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**:6–13.

Hamos JE, Oblas B, Pulaski-Salo D, Welch WJ, Bole DG and Drachman DA (1991) Expression of heat shock proteins in Alzheimer's disease. *Neurology* **41**:345–350.

Hsieh K-P, Wilke N, Harris A and Miles M (1996) Interaction of ethanol with inducers of glucose-regulated stress proteins: Ethanol potentiates inducers of grp78 transcription. *J Biol Chem* **271**:2709–2716.

Hyman SE and Nestler EJ (1996) Initiation and adaptation: A paradigm for understanding psychotropic drug action. *Am J Psychiatry* **153**:151–162.

Kim YK, Kim KS and Lee AS (1987) Regulation of the glucose-regulated protein genes by beta-mercaptoethanol requires de novo protein synthesis and correlates with inhibition of protein glycosylation. *J Cell Physiol* **133**:533–539.

Lee AS (1987) Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. *Trends Biochem Sci* **12**:20–23.

Lenox RH, McNamara RK, Watterson JM and Watson DG (1996) Myristoylated alanine-rich C kinase substrate (MARCKS): A molecular target for the therapeutic action of mood stabilizers in the brain? *J Clin Psychiatry* **57(Suppl 13)**:23–31.

Li PP, Young LT, Tam YK, Sibony D and Warsh JJ (1993) Effects of chronic lithium and carbamazepine treatment on G protein subunit expression in rat cerebral cortex. *Biol Psychol* **34**:167–170.

Liang P, Averboukh L, Keyomarsi K, Sager R and Pardee AB (1992) Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. *Cancer Res* **52**:6966–6968.

Lièvremont J-P, Rizzuto R, Hendershot L and Meldolesi J (1997) BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of $Ca^{2+}$. *J Biol Chem* **49**:30873–30879.

Little E, Ramakrishnan M, Roy B, Gazit G and Lee AS (1994) The glucose-regulated proteins (GRP78 and GRP94): Functions, gene regulation, and applications. *Crit Rev Eukaryotic Gene Expr* **4**:1–18.

Lofquist AK, Mondal K, Morris JS and Haskill JS (1995) Transcription-independent turnover of I kappa B alpha during monocyte adherence: Implications for a translational component regulation I kappa B alpha/MAD-3 mRNA levels. *Mol Cell Biol* **15**:1737–1746.

Lowenstein DH, Gwinn RP, Seren MS, Simon RP and McIntosh TK (1994) Increased expression of mRNA encoding calbindin-Dw8K, the glucose-regulated proteins, or the 72kDA heat-shock protein in three models of acute CNS injury. *Mol Brain Res* **22**:299–308.

McElroy SL, Keck PE Jr, Pope HG Jr and Hudson JI (1992) Valproate in the treatment of bipolar disorder: Literature review and clinical guidelines. *J Clin Psychopharmacol* **12(Suppl)**:42S–52S.

Miles MF, Wilke N, Elliot M, Tanner W and Shah S (1994) Ethanol-responsive genes in neural cells include the 78-kilodalton glucose-regulated protein (GRP78) and 94-kilodalton glucose-regulated protein (GRP94) molecular chaperones. *Mol Pharmacol* **46**:878–879.

Nishio Y, Aiello LP and King GL (1994) Glucose induced genes in bovine aortic smooth muscle cells identified by mRNA differential display. *FASEB J* **8**:103–106.

Nonaka S, Hough CJ and Chuang DM (1998) Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting *N*-methyl-D-aspartate receptor-mediated calcium influx. *Proc Natl Acad Sci USA* **95**:2642–2647.

Penry HK and Dean JC (1989) The scope and use of valproate with epilepsy. *J Clin Psychiatry* **49(Suppl)**:17–22.

Post RM (1992) Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. *Am J Psychiatry* **149**:999–1010.

Resendez E, Wooden SK and Lee A (1988) Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. *Mol Cell Biol* **8**:4579–4584.

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**:5463–5467.

Song L and Jope RS (1992) Chronic lithium treatment impairs phosphatidylinositol hydrolysis in membranes from rat brain regions. *J Neurochem* **58**:2200–2206.

Utans U, Liang P, Wyner LR, Karnovsky MJ and Russell M (1994) Chronic cardiac rejection: Identification of five upregulated genes in transplanted hearts by differential mRNA display. *Proc Natl Acad Sci USA* **91**:6463–6467.

Werner ED, Brodsky JL and McCracken AA (1996) Proteasome-dependent endoplasmic reticulum-associated protein degradation: An unconventional route to a familiar fate. *Proc Natl Acad Sci USA* **93**:13797–13801.

Wooden SK, Li-Jing L, Navarro D, Qadri I, Pereira L and Lee AS (1991) Transactivation of the grp78 promoter by malfolded proteins, glycosylation block, and calcium ionophore is mediated through a proximal region containing a CCAAT motif which interacts with CTF/NF-1. *Mol Cell Biol* **11**:5612–5622.

Yamaji K, Kagaya A, Uchitomi Y, Yokata N and Yamawaki S (1996) Effects of carbamazepine and sodium valproate on 5-HT-induced calcium increase in individual C6 rat glioma cells. *Neuropsychobiology* **34**:22–25.

Yamamoto R, Toshihiko Y, Kobayashi H, Yokoo H and Wada A (1997) Up-regulation of sodium channel subunit mRNAs and their cell surface expression by antiepileptic valproic acid: Activation of calcium channel and catecholamine secretion in adrenal chromaffin cells. *J Neurochem* **68**:1655.

**Send reprint requests to:** Dr. L. Trevor Young, Department of Psychiatry, McMaster University, 1200 Main St. W. HSC Room 3G57, Hamilton, Ontario, Canada, L8N 3Z5. E-mail: youngt@fhs.mcmaster.ca

## 3.2 Increased Expression of Endoplasmic Reticulum Stress Proteins Following Chronic Valproate Treatment of Rat C6 Glioma Cells

Christopher D. Bown, Jun-Feng Wang, L. Trevor Young

Reprinted from Neuropharmacology 39: 2162-2169., Copyright (2000), with permission from Elsevier Science

***Rationale:*** Previous studies in our laboratory had identified GRP78 as a valproate regulated gene in both rat cerebral cortex and rat C6 glioma cells. As it turns out, GRP78 belongs to a larger family of proteins, termed the ER stress proteins, which include GRP94 and calreticulin. These proteins are referred as ER stress proteins, since their expression is increased in response to various stressful stimuli, such as disruptions in intracellular $Ca^{2+}$ homeostasis, malfolded or damaged proteins and oxidative stress. In response to stress, the expression of all three proteins is increased, which has been shown to protect the cell from cytotoxic damage or death. Since upregulation of this family of proteins may have important neuroprotective properties, we questioned whether valproate could also increase the expression of GRP94 and calreticulin. The research in this paper shows the characterization of the ER stress proteins as a valproate-regulated family of proteins. The results of this study also contribute to the growing number of studies showing that mood stabilizers can regulate neuroprotective genes.

***Involvement:*** The research described in this paper as well as preparation of the manuscript was conducted by CDB. Both JFW and LTY provided technical advice and manuscript editing.

Pergamon

Neuropharmacology 39 (2000) 2162–2169

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

# Increased expression of endoplasmic reticulum stress proteins following chronic valproate treatment of rat C6 glioma cells

Christopher D. Bown, Jun-Feng Wang, L. Trevor Young *

*Mood Disorders Program, Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, Ontario, Canada, L8N 3Z5*

Accepted 11 January 2000

**Abstract**

The anticonvulsant sodium valproate has been shown to be an effective treatment for bipolar disorder, however, its precise mechanism of action has yet to be determined. It has been suggested that adaptational changes in gene expression are critical for valproate's prophylactic effects. Previous studies in our lab have shown that one gene that may be regulated by valproate is the 78-kilodalton glucose-regulated protein (GRP78). We report that treatment of rat C6 glioma cells with valproate can also increase the expression of additional endoplasmic reticulum stress proteins, GRP94 and calreticulin. All three proteins showed similar concentration-dependent increases in messenger RNA abundance. Chronic (seven days) treatment significantly increased GRP78 and GRP94 messenger RNA expression, whereas calreticulin expression increased after both acute and chronic treatment. Increases in mRNA expression corresponded to a similar increase in protein expression. The roles of GRP78, GRP94 and calreticulin as molecular chaperones and calcium binding proteins, suggest that these results might have functional relevance to the therapeutic action of valproate. 

*Keywords:* GRP78; GRP94; Calreticulin; Mood stabilizers; Valproate; Anticonvulsants

## 1. Introduction

Bipolar disorder (BD) is a well-characterized mood disorder with clinical features and genetics that strongly indicate a biological basis for the illness. While 60% of BD patients respond well to long-term pharmacotherapy with mood stabilizing drugs, the precise defect of the disorder has yet to be elucidated. It has been proposed that adaptational changes in gene expression are critical to the prophylactic effects of these drugs (Post, 1992).

Sodium valproate (VPA) is a branched chain fatty acid (2-propylpentanoic acid), that was originally prescribed as an anticonvulsant, but has more recently become a first line treatment for bipolar disorder (Pope et al., 1991). In addition to its role in the treatment of epilepsy, VPA has been shown to effectively treat mania when administered acutely as well as helping to prevent relapses when given chronically (Bowden et al., 1994). Like other mood stabilizing drugs, the precise mechanism of action of VPA has yet to be determined.

Our lab has used differential display polymerase chain reaction (PCR) to study the regulation of gene expression by the mood stabilizers, VPA and lithium (Wang and Young, 1996; Wang et al., 1999). One gene shown to be regulated by VPA was identified as the molecular chaperone, 78-kilodalton glucose-regulated protein (GRP78). GRP78, along with GRP94 and calreticulin, comprise the resident endoplasmic reticulum (ER) stress proteins (Gething, 1997). These proteins are all constitutively expressed and act as molecular chaperones capable of binding $Ca^{2+}$ (Nigam et al., 1994). In addition to its ER lumenal functions, calreticulin has also been shown to have additional cellular functions, including

*Abbreviations:* ANOVA=analysis of variance; BD=bipolar disorder; ECL=enhanced chemiluminescence; ER=endoplasmic reticulum; ERSE=ER stress response element; GAPDH=glyceraldehyde 3-phosphate dehydrogenase; GRP78=78-kDa glucose-regulated protein; nt=nucleotides; PBS=phosphate-buffered saline; PCR=polymerase chain reaction; RT=reverse transcriptase; SDS-PAGE=sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSPE=sodium chloride/sodium dihydrogen phosphate/EDTA; VPA=sodium valproate.

* Corresponding author. Tel.: +1-905-525-9140 ext. 22501; fax: +1-905-522-8804.

*E-mail address:* youngt@mcmaster.ca (L.T. Young).

0028-3908/00/$ - see front matter 
PII: S0028-3908(00)00029-0

cell adhesion and gene expression (reviewed by Burns et al., 1994). ER stress proteins have been shown to be up-regulated in response to various cellular insults (Gething, 1997). In turn, up-regulation of ER stress proteins has been shown to prevent ER $Ca^{2+}$ depletion and inhibit unfolded protein aggregation, thus protecting the cell from damage or death (Gething and Sambrook, 1992; Liu et al., 1997; Yu et al., 1999).

As GRP78 was identified as a VPA regulated gene (Wang et al., 1999), an interesting question arises concerning the effects of VPA on the expression of closely related ER stress proteins, GRP94 and calreticulin. This study investigates further the effects of VPA on the regulation of GRP78 in addition to that of GRP94 and calreticulin. The identification of cellular targets that are regulated by mood stabilizers will allow for the future development of novel drugs that target specific abnormalities of mood disorders and have superior efficacy.

## 2. Methods

### 2.1. Cell culture and treatment

Rat C6 glioma cells (American Type Tissue Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine at 37°C in a 5.0% $CO_2$ atmosphere. Drug treatment was carried out by supplementing the culture media with sodium valproate (ICN Biomedicals Inc). Cell viability was greater than 98% as confirmed by Tryptan Blue exclusion and was not different across drug treatments.

### 2.2. Northern and slot blot hybridization

Isolation of total RNA from rat C6 glioma cells was carried out using Trizol reagent, and the procedure described by the manufacturer (Gibco BRL, Life Technologies). Standard Northern hybridization procedures were followed (Wang and Young, 1996). Briefly, 20 μg of total RNA was separated according to size on a 1% agarose gel with 2.2 M formaldehyde. RNA was capillary transferred to a Zeta-probe membrane (BioRad) overnight. Membranes were then prehybridized for 3 h at 42°C in a buffer containing 10% dextran sulfate, 50% deionized formamide, 5M SSPE, 5× Denhardt's reagent and 1% sodium dodecyl sulfate (SDS). Radioactive labeling of each cDNA fragment was conducted using a random priming labeling kit and the protocol described by the manufacturer (Boehringer Mannheim). Hybridization proceeded overnight at 42°C in fresh hybridization buffer containing $\alpha$-$^{32}$P dCTP-labeled probe. Slot blot hybridization was used to determine the expression levels of grp78, grp94 and calreticulin mRNA after VPA treatment, as described previously (Wang et al., 1999).

### 2.3. Complementary DNA probes

cDNA fragments for grp78 and calreticulin were synthesized from rat C6 glioma cell mRNA. Reverse transcriptase–PCR (RT-PCR) procedures, as described by the manufacturer (Perkin Elmer), were followed using specific primer sequences for rat grp78 and calreticulin. The 767 nucleotide (nt) rat grp78 cDNA fragment was synthesized using the following oligonucleotides as primers: 5′ CCACCGTAACAATCAAGGTC 3′ (upstream) and 5′ CACGTGAGCAACTGCTAATG 3′ (downstream). A 476 nt cDNA fragment specific to rat calreticulin was synthesized using 5′ GTTCACCGTGAAGCATGAGC 3′ (upstream) and 5′ CAGTCCTCAGGCTTCTAAGC 3′ (downstream) oligonucleotides as primers. Amplified cDNA fragments were run on a 1.2% agarose gel containing ethidium bromide and then isolated using the Qiaex II gel extraction kit (Qiagen). Specificity of cDNA fragments was confirmed by sequencing using the dideoxy chain-termination method (Sanger et al., 1977). The 375 nt cDNA fragment for grp94 was synthesized from the p4A3 plasmid which contains a hamster grp94 cDNA fragment (a generous gift from Dr A.S. Lee, University of Southern California) using standard PCR techniques. The hamster grp94 cDNA plasmid has been previously shown to recognize rat grp94 mRNA (Lee et al., 1983). PCR amplification of the hamster grp94 cDNA was conducted using the following primers: 5′ GCCTGTGGATGAATACTGC 3′ (upstream) and 5′ TCTGATCAGCGGATGTCTG 3′ (downstream). The amplified product was isolated as described above.

### 2.4. Immunoblotting

Protein was isolated following a whole-cell extraction protocol (Lievremont et al., 1997; Wang et al., 1999). Ten micrograms of whole cell extract was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 10% acrylamide gel, and immunoblotted following the protocol of Wang et al. (1999). All antibodies used for immunoblotting were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Monoclonal antisera for GRP78 at a 1:500 dilution in blocking buffer, polyclonal antisera for GRP94 at a dilution of 1:2,500 and polyclonal antisera for calreticulin at a dilution of 1:20,000 were also used to detect the specific proteins. Blots were incubated with an appropriate secondary antibody, goat anti-mouse IgG for GRP78 (Santa Cruz Biotechnologies)(1:2,000 dilution), goat anti-rat IgG for GRP94 (Santa Cruz Biotechnologies)(1:4,000 dilution) and goat anti-rabbit IgG for calreticulin (Upstate Biotechnologies)(1:20,000 dilution) conjugated to hor-

seradish peroxidase diluted in 5% milk–phosphate-buffered saline (PBS). Immunoreactive bands were detected with an enhanced chemiluminescence (ECL) system (Amersham). The blots were subsequently exposed to Kodak XAR-5 film, and immunoreactivity determined by densitometry using the Northern Exposure program from ImagExperts (Oakville, ON, Canada).

### 2.5. Data and statistical analysis

Equal loading of Northern blots was determined using a 28S ribosomal RNA oligonucleotide. All slot blot hybridization measures were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data from immunoblotting was normalized to a standard from one control group run on all gels. Differences between VPA treated and untreated cells were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

## 3. Results

To determine whether chronic treatment with VPA could increase the expression of grp78, grp94 and calreticulin, rat C6 glioma cells were treated for seven days at 1 mM concentration. This cell line was used since both mood stabilizing drugs and cellular insults can increase ER stress proteins in these cells (Brostrom et al., 1991; Chen et al. 1996, 1997; Chen et al., 1999a; Wang et al., 1999). Following seven days of treatment, total RNA was isolated and expression of grp78, grp94 and calreticulin was measured using Northern blot analysis. All three ER stress proteins were up-regulated following VPA treatment (Fig. 1).

The effect of varying doses of VPA on the expression of GRP78, GRP94 and calreticulin was investigated. Dose–response curves were determined for rat C6 glioma cells treated for one week with 0, 0.25, 0.5, 0.75, 1, 1.5 or 2 mM VPA (Fig. 2). Concentrations between 0.5 and 1 mM are commonly used for in vitro experiments examining the mood stabilizing effects of VPA (Chen et al. 1994, 1997; Chen et al., 1999b; Lenox et al., 1996; Wang et al., 1999; Watson et al., 1998). Clinically, total VPA serum concentrations between 0.5 and 1 mM are observed to be effective in the treatment of BD (Bowden et al., 1996). Both grp78 and grp94 mRNA levels were increased in a dose-dependent manner, with significant increases resulting from concentrations between 1 mM and 2 mM ($P<0.05$). Calreticulin mRNA expression was also increased in a dose-dependent manner with significance ($P<0.01$) resulting from VPA concentrations of 1 mM to 2 mM. The results indicate that VPA can increase the expression levels of grp78, grp94 and calreticulin mRNA in a dose-dependent manner and that VPA dose–response curves for these genes show similar patterns.

Time-course studies examining the effects of VPA on grp78, grp94, and calreticulin mRNA expression were also conducted (Fig. 3). Rat C6 glioma cells were treated with 1 mM VPA for 2 h, 6 h, one day or seven days. Total RNA from these cells was examined for the expression of each ER stress protein using slot blot hybridization. Both grp78 and grp94 mRNA were significantly ($P<0.05$) increased after seven days, with an approximate increase of 60% over control in both cases. Expression of calreticulin mRNA was significantly increased ($P<0.05$) following 6 h of VPA treatment, with expression reaching a plateau at one day and remaining at this level following seven days of treatment.

To determine the reversibility of grp78, grp94 and calreticulin induction, drug withdrawal studies were conducted on C6 glioma cells treated for seven days with 1 mM VPA (Fig. 4). Following the pretreatment period, the drug was removed from the media for 2, 6, 24 or 48 h and grp78, grp94 and calreticulin mRNA levels were subsequently quantified using slot blot hybridization. Six hours following the removal of the VPA, grp78, grp94 and calreticulin mRNA levels returned to those determined for cells that did not receive treatment. All three ER stress proteins showed similar patterns of expression following withdrawal of VPA treatment.

To determine whether increased mRNA levels are associated with increased protein levels, the effects of VPA treatment on ER stress protein levels was also examined (Fig. 5). Rat C6 glioma cells were grown for seven days in media containing 0, 0.25, 0.5, 1, or 2 mM of VPA and protein from their whole-cell lysate was analyzed using immunoblotting techniques. As shown in Fig. 5, both GRP78 and calreticulin protein levels were increased in a dose-dependent fashion after chronic VPA treatment ($P<0.05$). Although not statistically significant, there was an apparent trend towards an increase in GRP94 protein expression following 1 mM VPA treatment.

## 4. Discussion

The goal of this study was to examine the effect of VPA on the expression of the ER stress proteins. Previously, our lab used differential display PCR on rat cerebral cortex to identify genes regulated by VPA treatment and found a resulting increase in GRP78 expression (Wang et al., 1999). This finding illustrated the importance of studying the effects of VPA on ER stress proteins, and here we report these effects for GRP78, GRP94 and calreticulin. A dose-dependent relationship between grp78, grp94 and calreticulin mRNA with VPA concentration was observed following

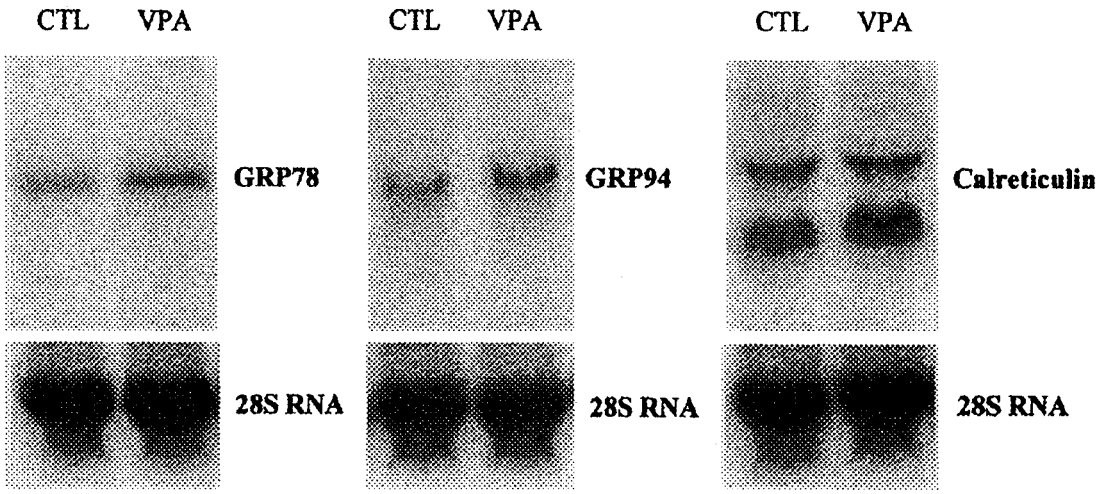



Fig. 1. Northern blot analysis of grp78, grp94 and calreticulin mRNA following VPA treatment. Rat C6 glioma cells were grown for one week with 1 mM VPA. Control cells (CTL) were treated identically but without addition of the drug. Twenty micrograms of total RNA was analyzed by Northern blot hybridization using [α-$^{32}$P]dCTP-labeled probes prepared from cDNA fragments specific to grp78, grp94 and calreticulin mRNA. Equal loading was determined by the expression of 28S ribosomal RNA.

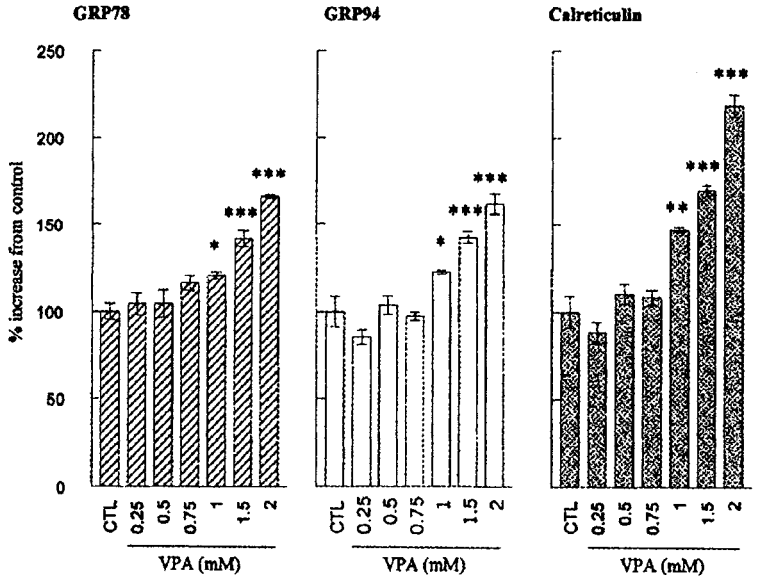



Fig. 2. The effect of different doses of VPA on grp78, grp94 and calreticulin mRNA levels. Rat C6 glioma cells were grown for one week with 0.25, 0.5, 0.75, 1, 1.5, and 2 mM VPA. Control cells (CTL) were treated identically but without addition of the drug. Total RNA was analyzed by slot blot hybridization using [α-$^{32}$P]dCTP-labeled probes prepared from cDNA fragments specific to grp78, grp94 and calreticulin mRNA or the housekeeping gene GAPDH. All values obtained were normalized to GAPDH levels. Quantification of mRNA levels was done using a PhosphorImager. All results are the mean±S.E.M. of triplicate determinations. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ significantly different from control by one-way ANOVA followed by Dunnett's multiple comparison test.

seven days of treatment. Results from the time-course experiments indicate that calreticulin shows an acute response (<one day) to VPA treatment, whereas the increase in expression of grp78 and grp94 occurs after a more chronic (approx. seven days) treatment with the drug. Protein levels of ER stress proteins were also measured to determine whether affects on protein levels are complementary to the resulting increases in grp78, grp94 and calreticulin mRNA. We found that GRP78 and calreticulin protein levels are also increased by VPA in a dose-dependent manner with a trend towards increased GRP94 protein levels. It is unknown whether these find-

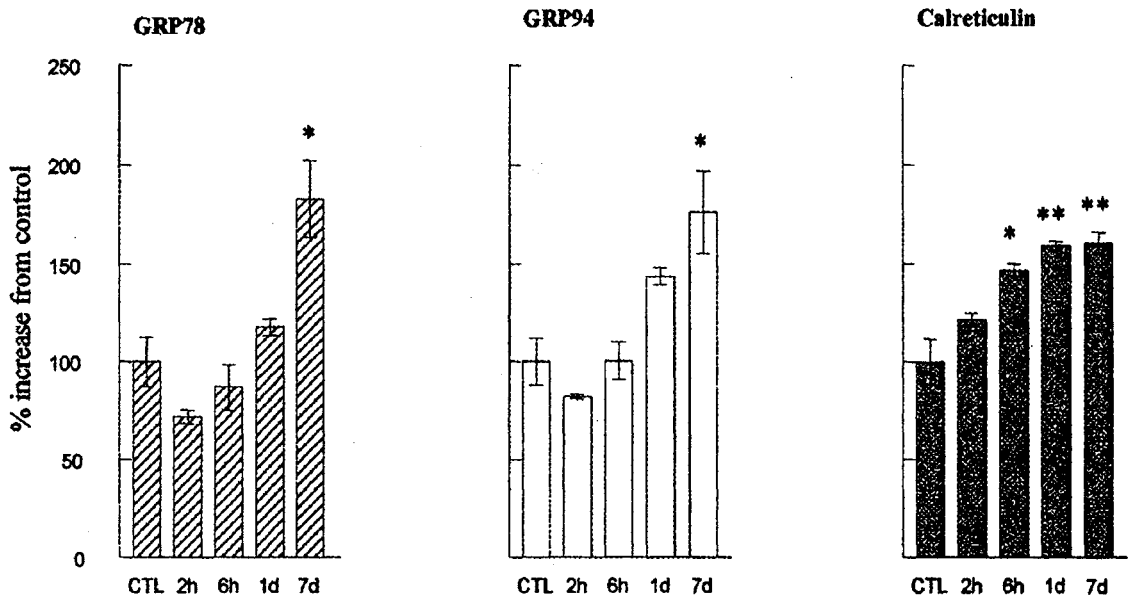



Fig. 3. Time-course of VPA on grp78, grp94 and calreticulin gene expression. Rat C6 glioma cells were grown for 2 h, 6 h, one day or seven days with 1 mM VPA. Control cells (CTL) were treated identically but without addition of the drug. Total RNA was analyzed by slot blot hybridization using [α-$^{32}$P]dCTP-labeled probes prepared from cDNA fragments specific to grp78, grp94 and calreticulin mRNA or the housekeeping gene GAPDH. All values obtained were normalized to GAPDH levels. Quantification of mRNA levels was done using a PhosphorImager. All results are the mean±S.E.M. of triplicate determinations. *$P<0.05$ and **$P<0.01$ significantly different from control by one-way ANOVA followed by Dunnett's multiple comparison test.

ings relate to either VPA's mood stabilizing or anticonvulsant clinical effect.

Northern blot analysis shows that seven days of treatment with 1 mM VPA increases expression of grp78, grp94 and calreticulin mRNA with the calreticulin probe recognizing two bands. Other studies have detected the presence of another calreticulin mRNA species, but the precise identity of this mRNA is unclear (Fliegel et al., 1989; Johnson et al., 1992). Both bands showed nearly identical increases over control when treated with 1 mM VPA.

The similar pattern of expression resulting for all three ER stress proteins in response to VPA indicates that a common signaling pathway might be responsible for their regulation. The mRNA levels of all three ER stress proteins show an approximate 50% increase in expression at the therapeutic dose of 1 mM with a more robust increase observed at 2 mM. Regulatory elements common to all three genes have been shown to exist. Yoshida et al. (1998) describe one such element, the ER stress response element (ERSE), which is responsible for co-transcriptional activation of the human GRP78, GRP94 and calreticulin genes in response to ER stress. Although the upregulation of ER stress protein genes by VPA was much more modest than the increase described by activation of ERSE, the likelihood exists that this, or a similar signaling pathway, might be responsible for the increase observed following VPA treatment. VPA has also been found to increase AP-1 DNA binding activity (Asghari et al., 1998; Chen et al., 1997). However, calreticulin is the only gene in this family known to contain an AP-1 site (McCauliffe et al., 1992; Waser et al., 1997). This lends thought to the possibility that an unidentified common regulatory element activated by VPA is present in the promoter region of these three genes.

As various lethal cellular insults result in the induction of ER stress proteins (Gething, 1997) and VPA becomes cytotoxic at concentrations greater than 10 mM, (Martin and Regan, 1991) we examined the reversibility of this induction following VPA treatment to confirm that the observed increase in ER stress protein expression did not result from cellular damage. When VPA was removed from the media following seven days of treatment, all three ER stress protein levels returned to basal expression. The observed pattern of expression suggests that the up-regulation of the ER stress proteins are in fact due to the presence of VPA and not damage done by VPA to the cell. An increase due to ER damage should have resulted in mRNA levels either remaining elevated or decreasing significantly below control levels following removal of VPA. Electron micrograph studies show that changes in ER structure following exposure to toxic agents exist for up to 12 h (Regan et al., 1995). This suggests that if VPA did damage the structure of the ER following seven days of treatment then the same

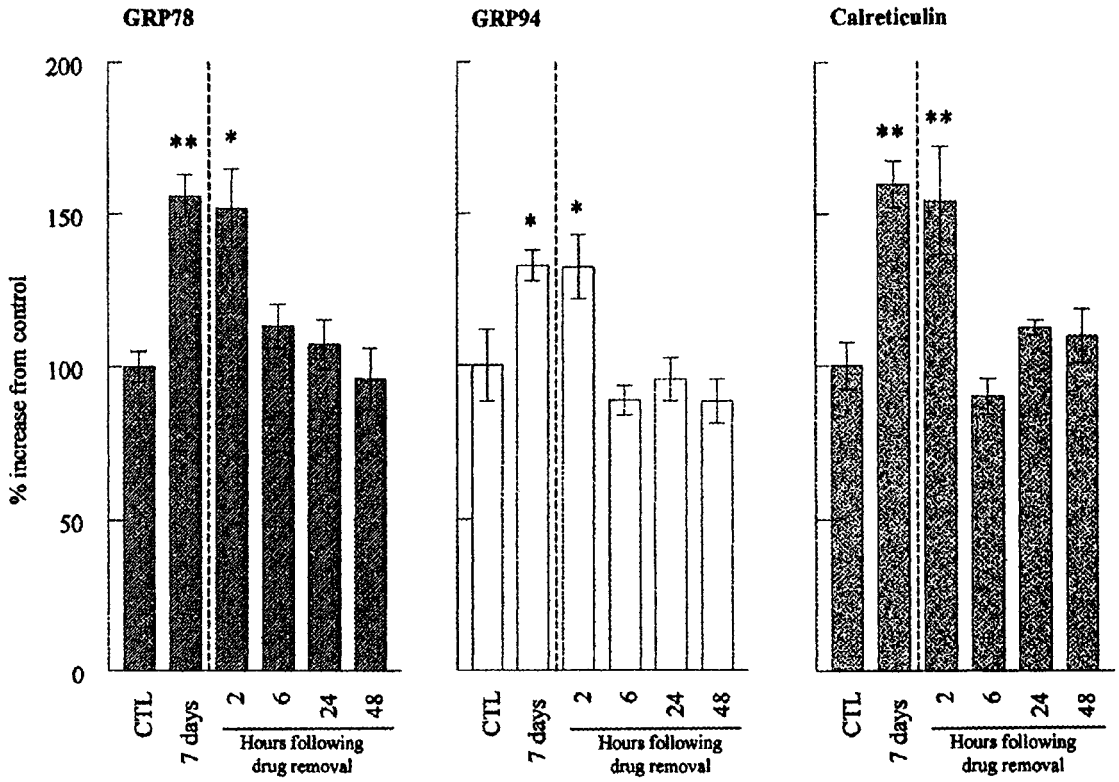



Fig. 4. Effect of VPA withdrawal on grp78, grp94 and calreticulin gene expression. Rat C6 glioma cells were grown for seven days with 1 mM VPA. Following seven days of treatment the drug was removed from the media and grp78, grp94 and calreticulin mRNA levels determined at 2, 6, 24 and 48 h. Control cells (CTL) were treated identically but without addition of the drug. Total RNA was analyzed by slot blot hybridization using [$\alpha$-$^{32}$P]dCTP-labeled probes prepared from cDNA fragments specific to grp78, grp94 and calreticulin mRNA or the housekeeping gene GAPDH. All values obtained were normalized to GAPDH levels. Quantification of mRNA levels was done using a PhosphorImager. All results are the mean±S.E.M. of triplicate determinations. *$P<0.05$, and **$P<0.01$ significantly different from control by one-way ANOVA followed by Dunnett's multiple comparison test.

pattern of gene expression would exist for longer than 12 h. In addition, the disaggregation of polyribosomes that occurs following thapsigargin treatment exists up to 4 h following treatment and reaggregation occurs in a irregular shape (Doutheil et al., 1997). Since grp78, grp94 and calreticulin mRNA levels returned to basal levels and remained constant, it can be postulated that VPA alone is responsible for the up-regulation of these ER stress proteins. However, further studies well be needed to prove this result unequivocally.

The observed increases in both transcription and translation of ER stress proteins in response to VPA renders it probable that the increased protein levels have functional roles within the cell. Although the functional relevance of these changes is not yet known, it is possible that these changes are of significant importance to the pharmacological action of VPA. The up-regulation of ER stress proteins by VPA may indicate a potential mechanism by which the drug elicits its therapeutic effect.

## 5. Conclusion

Valproate, a commonly prescribed anticonvulsant and mood stabilizing drug, is capable of up-regulating the expression of GRP78, GRP94 and calreticulin, collectively referred to as the ER stress proteins. The roles of these ER stress proteins as molecular chaperones as well as calcium binding proteins, suggest that these results might have functional relevance to the therapeutic action of VPA. The identification of genes that are regulated by anticonvulsant and mood stabilizing drugs may allow for the future development of novel drugs with superior efficacy and which target specific abnormalities in epilepsy and mood disorders.

## Acknowledgements

This work was supported by a Stanley Foundation Research Grant (L.T.Y.). J.F.W. is a Canadian Psychi-

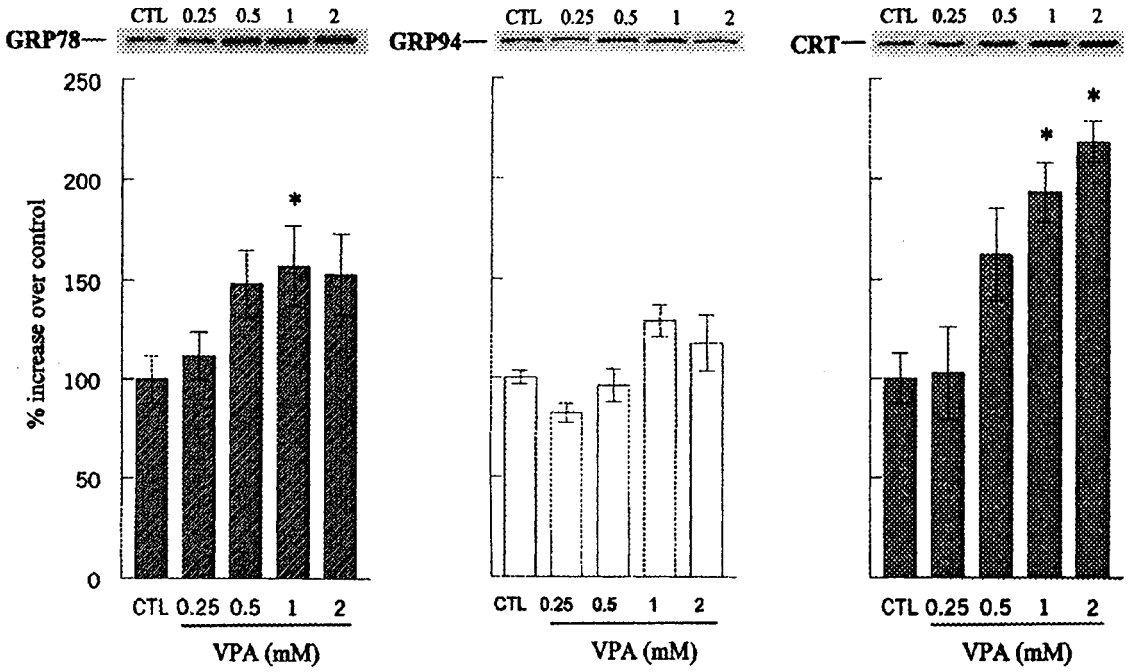



Fig. 5. Effect of VPA on GRP78, GRP94 and calreticulin protein levels. Rat C6 glioma cells were treated with VPA at 0.25, 0.5, 1, and 2 mM for one week. Control cells (CTL) were treated identically by without addition of VPA. Ten micrograms of protein was analyzed by immunoblotting analysis using GRP78, GRP94 and calreticulin antisera. Autoradiograms were quantified by densitometry. Results are the mean±S.E.M. of triplicate determinations. *$P<0.05$ significantly different from controls by one-way ANOVA followed by Dunnett's multiple comparison test.

atric Research Foundation fellow. L.T.Y. is a career scientist of the Ontario Ministry of Health.

## References

Asghari, V., Wang, J.F., Reiach, J.S., Young, L.T., 1998. Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. Molecular Brain Research 58, 95–102.

Bowden, C.L., Brugger, A.M., Swann, A.C., Calabrese, J.R., Janicak, P.G., Petty, F., Dilsaver, S.C., Davis, J.M., Rush, A.J., Small, J.G., Garza-Trevino, E.S., Risch, S.C., Goodnick, P.J., Morris, D.D., 1994. Efficacy of dialproex vs lithium and placebo in the treatment of mania. Journal of the American Medical Association 271, 918–924.

Bowden, C.L., Janicak, P.G., Orsulak, P., Swann, A.C., Davis, J.M., Calabrese, J.R., Goodnick, P., Small, J.G., Rush, A.J., Kimmel, S.E., Risch, S.C., Morris, D.D., 1996. Relation of serum valproate concentration to response in mania. American Journal of Psychiatry 153, 765–770.

Brostrom, M.A., Prostko, C.R., Gmitter-Yellen, D., Grandison, L.J., Kuznetsov, G., Wong, W.L., Brostrom, C.O., 1991. Inhibition of translational initiation by metalloendoprotease antagonists. Evidence for involvement of sequestered $Ca^{2+}$ stores. Journal of Biological Chemistry 266, 7037–7043.

Burns, K., Atkinson, E.A., Bleackley, R.C., Michalak, M., 1994. Calreticulin: from $Ca^{2+}$ binding to control of gene expression. Trends in Cell Biology 4, 152–154.

Chen, G., Manji, H.K., Hawver, D.B., Wright, C.B., Potter, W.Z., 1994. Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon in vitro. Journal of Neurochemistry 63, 2361–2364.

Chen, G., Manji, H.K., Wright, C.B., Hawver, D., Potter, W.Z., 1996. Effects of valproic acid on β-adrenergic receptors, G proteins and adenylyl-cyclase in rat C6 glioma cells. Neuropsychopharmacology 15, 271–280.

Chen, G., Yuan, P., Hawver, D.B., Potter, W.Z., Manji, H.K., 1997. Increase in AP-1 transcription factor DNA binding activity by valproic acid. Neuropsychopharmacology 16, 238–245.

Chen, G., Zeng, W.Z., Yuan, P.X., Huang, L.D., Jiang, Y.M., Zhao, Z.H., Manji, H.K., 1999a. The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. Journal of Neurochemistry 72, 879–882.

Chen, G., Yuan, P.X., Jiang, Y.M., Huang, L.D., Manji, H.K., 1999b. Valproate robustly enhances AP-1 mediated gene expression. Molecular Brain Research 64, 52–58.

Doutheil, J., Gissel, C., Oschlies, U., Hossmann, K.A., Paschen, W., 1997. Relation of neuronal endoplasmic reticulum calcium homeostasis to ribosomal aggregation and protein synthesis: implications for stress-induced suppression of protein synthesis. Brain Research 775, 43–51.

Fliegel, L., Burns, K., MacLennan, D.H., Reithmeier, R.A., Michalak, M., 1989. Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. Journal of Biological Chemistry 264, 21522–21528.

Gething, M.-J., 1997. Guidebook to Molecular Chaperones and Protein-Folding Catalysts. Oxford University Press, Oxford.

Gething, M.J., Sambrook, J., 1992. Protein folding in the cell. Nature 355, 33–45.

Johnson, R.J., Liu, N.G., Fishman, J.B., Dixon, J.D., Fine, R.E., 1992.

Isolation of a calreticulin-like calcium binding protein from bovine brain. Molecular Brain Research 12, 69–76.

Lee, A.S., Delegeane, A.M., Baker, V., Chow, P.C., 1983. Transcriptional regulation of two genes specifically induced by glucose starvation in a hamster mutant fibroblast cell line. Journal of Biological Chemistry 258, 597–603.

Lenox, R.H., McNamara, R.K., Watterson, J.M., Watson, D.G., 1996. Myristoylated alanine-rich C kinase substrate (MARCKS): a molecular target for the therapeutic action of mood stabilizers in the brain? Journal of Clinical Psychiatry 57 (Suppl. 13), 23–31.

Lievremont, J.-P., Rizutto, R., Hendershot, L., Meldolesi, J., 1997. BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of $Ca^{2+}$. Journal of Biological Chemistry 272, 12003–12009.

Liu, H., Bowes III, R.C., van De Water, B., Sillence, C., Nagelkerke, J.F., Stevens, J.L., 1997. Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, $Ca^{2+}$ disturbances, and cell death in renal epithelial cells. Journal of Biological Chemistry 272, 21751–21759.

Martin, M.L., Regan, C.M., 1991. The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. Brain Research 554, 223–228.

McCauliffe, D.P., Yang, Y.S., Wilson, J., Sontheimer, R.D., Capra, J.D., 1992. The 5′-flanking region of the human calreticulin gene shares homology with the human GRP78, GRP94, and protein disulfide isomerase promoters. Journal of Biological Chemistry 267, 2557–2562.

Nigam, S.K., Goldberg, A.L., Ho, S., Rhode, M.F., Bush, K.T., Sherman, M.Y.U., 1994. A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes $Ca^{2+}$ binding proteins and member of the thioredoxin superfamily. Journal of Biological Chemistry 269, 1744–1749.

Pope, H.G., McElroy, S.L., Keck, P.E., Hudson, J.I., 1991. Valproate in the treatment of acute mania. Archives of General Psychiatry 48, 62–68.

Post, R.M., 1992. Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. American Journal of Psychiatry 149, 999–1010.

Regan, R.F., Panter, S.S., Witz, A., Tilly, J.L., Giffard, R.G., 1995. Ultrastructure of excitotoxic neuronal death in murine cortical culture. Brain Research 705, 188–198.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA 74, 5463–5467.

Wang, J.F., Young, L.T., 1996. Differential display PCR reveals increased expression of 2′,3′ cyclic nucleotide 3′-phosphodiesterase by lithium. Federation of the European Biochemical Societies Letters 386, 225–229.

Wang, J.F., Bown, C., Young, L.T., 1999. Differential display PCR reveals novels targets for the mood stabilizing drug valproate including the molecular chaperone, glucose-regulated protein 78. Molecular Pharmacology 55, 521–527.

Waser, M., Mesaeli, N., Spencer, C., Michalak, M., 1997. Regulation of calreticulin gene expression by calcium. Journal of Cell Biology 138, 547–557.

Watson, D.G., Watterson, J.M., Lenox, R.H., 1998. Sodium valproate down-regulates the myristoylated alanine-rich C kinase substrate (MARCKS) in immortalized hippocampal cells: a property of protein kinase C-mediated mood stabilizers. Journal of Pharmacology and Experimental Therapeutics 285, 307–316.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., Mori, K., 1998. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Journal of Biological Chemistry 273, 33741–33749.

Yu, Z., Luo, H., Fu, W., Mattson, M.P., 1999. The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Experimental Neurology 155, 302–314.

## 3.3 Increased Temporal Cortex ER Stress Proteins in Depressed Subjects Who Died By Suicide

Christopher Bown, Jun-Feng Wang, Glenda MacQueen, L. Trevor Young

Reprinted from Neuropsychopharmacology 22: 327-332., Copyright (2000), with permission from Elsevier Science

***Rationale:*** Our previous studies have shown that chronic valproate treatment can increase the expression of the ER stress proteins, GRP78, GRP94 and calreticulin. Since ER stress proteins have been shown to have both $Ca^{2+}$-binding and molecular chaperone properties, one or both of these processes might be important for the mechanism of action of valproate. Interestingly, it has been shown by a number of independent laboratories that $Ca^{2+}$ homeostasis may be disrupted in bipolar disorder. Therefore, upregulation of ER stress proteins by valproate may contribute to the $Ca^{2+}$ binding capacity of the cell and restore intracellular $Ca^{2+}$ concentrations. Increased expression of ER stress proteins may enhance the resistance of neurons to cytotoxic insults, thereby protecting vulnerable brain regions in bipolar disorder. The research described in this paper measures the expression of GRP78, GRP94 and calreticulin in post-mortem temporal cortex of age- and sex-matched subjects with a history of major depression, bipolar disorder, schizophrenia, as well as healthy controls.

***Involvement:*** The research described in this paper as well as preparation of the manuscript was conducted by CDB. GM aided in statistical analysis. Both JFW and LTY provided technical advice and manuscript editing.

ELSEVIER

BRIEF REPORT

# Increased Temporal Cortex ER Stress Proteins in Depressed Subjects Who Died by Suicide

*Christopher Bown, B.Sc., Jun-Feng Wang, M.D., Ph.D., Glenda MacQueen, M.D., Ph.D., and L. Trevor Young, M.D., Ph.D.*

*Regulation of ER stress proteins, such as the 78-kilodalton glucose regulated protein (GRP78) by chronic treatment with mood stabilizing drugs suggests that this family of proteins may be involved in the pathophysiology of mood disorders. Indeed, increased levels of GRP78, GRP94, and calreticulin, a third member of the ER stress protein family, were found in temporal cortex of subjects with major depressive disorder who died by suicide compared with controls and subjects who died by other means. No such differences were found in subjects with other psychiatric disorders such as bipolar disorder or schizophrenia. These data suggest a potential role for ER stress proteins in severe depression that merits further study.*
*[Neuropsychopharmacology 22:327–332, 2000]* 

KEY WORDS: *GRP78; GRP94; Calreticulin; Depression; Suicide; Antidepressants; Anticonvulsants*

Current theories of the pathophysiology of mood disorders suggest that prolonged increases in glucocorticoid levels, likely the result of prolonged environmental stress, lead to biochemical changes in cortical and limbic neurons that leave individuals vulnerable to depression (Duman et al. 1997). A number of studies have found evidence of neuronal atrophy and loss of hippocampal neurons in response to stress (Magarinos et al. 1996; Uno et al. 1989; Sapolsky et al. 1985), and a report of reduced hippocampal volumes in elderly patients with histories of depression suggests that analogous processes may occur in individuals with depression (Sheline et al. 1996).

From the Mood Disorders Program, McMaster University, Hamilton, Ontario, Canada.

Address correspondence to: L. Trevor Young, M.D., Ph.D., McMaster University, Department of Psychiatry and Behavioural Neurosciences, HSC-4N77A, 1200 Main Street West, Hamilton, Ontario, Canada, L8N 3Z5.

Received April 29, 1999; revised July 12, 1999; accepted July 16, 1999.

Some models of mood disorders (Hyman and Nestler 1996; Post 1992) have attempted to incorporate the notion of compensatory processes into an understanding of the pathophysiologic changes that occur in patients with mood disorders, but few studies have tested these notions empirically (Post 1992). Part of the difficulty has been identifying neuroprotective mechanisms that may be of relevance for mood disorders.

Recently, we found that treatment with the mood stabilizing drugs, valproate and carbamazepine, increased expression of the 78-kilodalton glucose-regulated protein (GRP78) (Wang et al. 1999). GRP78 is a member of the ER stress protein family that includes GRP94 and calreticulin, all of which function as $Ca^{2+}$ binding proteins and molecular chaperones to assist in the regulation of protein folding (Gething 1997). These proteins have neuroprotective properties, are induced by seizures and ischemic damage, and may be involved in Alzheimer's disease pathology (Yu et al. 1999; Lowenstein et al. 1994; Hamos et al. 1991).

Increased expression of ER stress proteins by mood stabilizing drugs suggest that these proteins may be in-



Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/00/$–see front matter
PII S0893-133X(99)00091-3

volved in the pathophysiology of mood disorders, perhaps as compensatory factors induced in response to stress-related neuronal damage. Further evidence for a role of neuroprotective proteins in mood disorders comes from recent reports of increased Bcl-2 levels, another neuroprotective anti-apoptotic factor, after treatment with mood stabilizers (Chen et al. 1999; Chen and Chuang 1999). To test the hypothesis that ER stress proteins may be important in the pathophysiology of mood disorders, we measured GRP78, GRP94 and calreticulin in postmortem samples of patients with mood disorders, schizophrenia and nonpsychiatric controls.

## METHODS

Postmortem brain tissue was obtained from the Stanley Foundation Neuropathology Consortium (4 groups of $n$ = 15 age- and sex-matched subjects, i.e., subjects with bipolar disorder (BD), subjects with Major Depressive Disorder (MDD), subjects with schizophrenia (SCZ), and non-psychiatric, non-neurologic comparison subjects) (Johnston et al. 1997). Details on dissection and clinical characteristics have been previously published (Dowlatshahi et al. 1998, 1999).

Briefly, all medical records for psychiatric cases were reviewed by two psychiatrists. Diagnosis was determined according to DSM-IV criteria. If there was disagreement about the diagnosis, a third psychiatrist read the records. If a diagnosis could not be established, a family member was then interviewed. All interviewed relatives were first degree except for two (one aunt and one father-in-law). Similarly, a clinical interview was performed with a family member of each normal control. Cause of death, substance abuse history, medications at time of death and lifetime intake of antipsychotics was available for all subjects (details for MDD subjects shown in Table 1).

Sections were cut from coronal blocks. All sections are cut perpendicular to the anterior-posterior axis of the brain. All cortical layers were included as was the underlying white matter. The thickness of underlying white matter in the block was not predetermined and therefore could have varied between subjects. The sections obtained were approximately one gram, and further dissected into 50 mg sections to include a full thickness section.

Total protein was prepared using a whole cell extraction protocol of 50 mg of temporal cortex tissue (Brodmann Areas 20 and 21) (Dowlatshahi et al. 1998; Wang et al. 1999). Briefly, cells were sonicated in 3 v. of 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 50 mM Hepes-KOH, 10% glycerol, 1% Triton X-100 supplemented with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml pepstatin. Following sonication cells were centrifuged at 14,000g for 20 min at 4°C and the supernatants used. Immunoblotting was carried out following the method of Wang et al. (1999) using 30 μg of protein per lane. Equal loading and transfer was detected

**Table 1.** Cause of Death and Treatment Information of Subjects with MDD

| Age/Sex | Postmortem Interval (hours) | Antidepresant/ Mood Stabilizer Medications at Time of Death | Other Medications | Cause of Death | Alcohol/ Substance Abuse |
|---|---|---|---|---|---|
| 32/F | 47 | TCA[b,c] | BDZ | Suicide | None |
| 44/F | 32 | TCA[b,c], SSRI[c] | BDZ | Suicide | None |
| 46/M | 26 | None[a,b] | BDZ, diphenhydramine | Suicide | None |
| 51/M | 26 | Nefazadone[a] | Hydroxyzine | Suicide | Past |
| 39/M | 23 | None[a,b] | None | Suicide | Current |
| 42/M | 7 | None[a,b] | None | Suicide | None |
| 30/F | 33 | TCA[b,c], SSRI[c] | BDZ | Suicide | None |
| 53/F | 40 | Li, trazadone | None | Acute alcohol intoxication | Current |
| 65/M | 19 | None[b,c] | Phenytoin | Cardiac | None |
| 52/M | 12 | None | None | Cardiac | None |
| 42/F | 25 | SSRI, Li | None | Cardiac | None |
| 56/M | 23 | SSRI | None | Cardiac | None |
| 56/F | 28 | SSRI | BDZ, buspirone | Pulmonary | None |
| 43/M | 43 | TCA | None | Cardiac | Current |
| 47/M | 28 | Nefazadone, SSRI | None | Cardiac | None |

*Abbreviations*: TCA, tricyclic antidepressant; SSRI, selective serotonin reuptake inhibitor; Li, lithium; BDZ, benzodiazepine.
[a]Antidepressant/mood stabilizer treatment was confirmed with toxicological data obtained from urine.
[b]Antidepressant/mood stabilizer treatment was confirmed with toxicological data obtained from blood.
[c]Antidepressant/mood stabilizer treatment was confirmed with toxicological data obtained from brain.

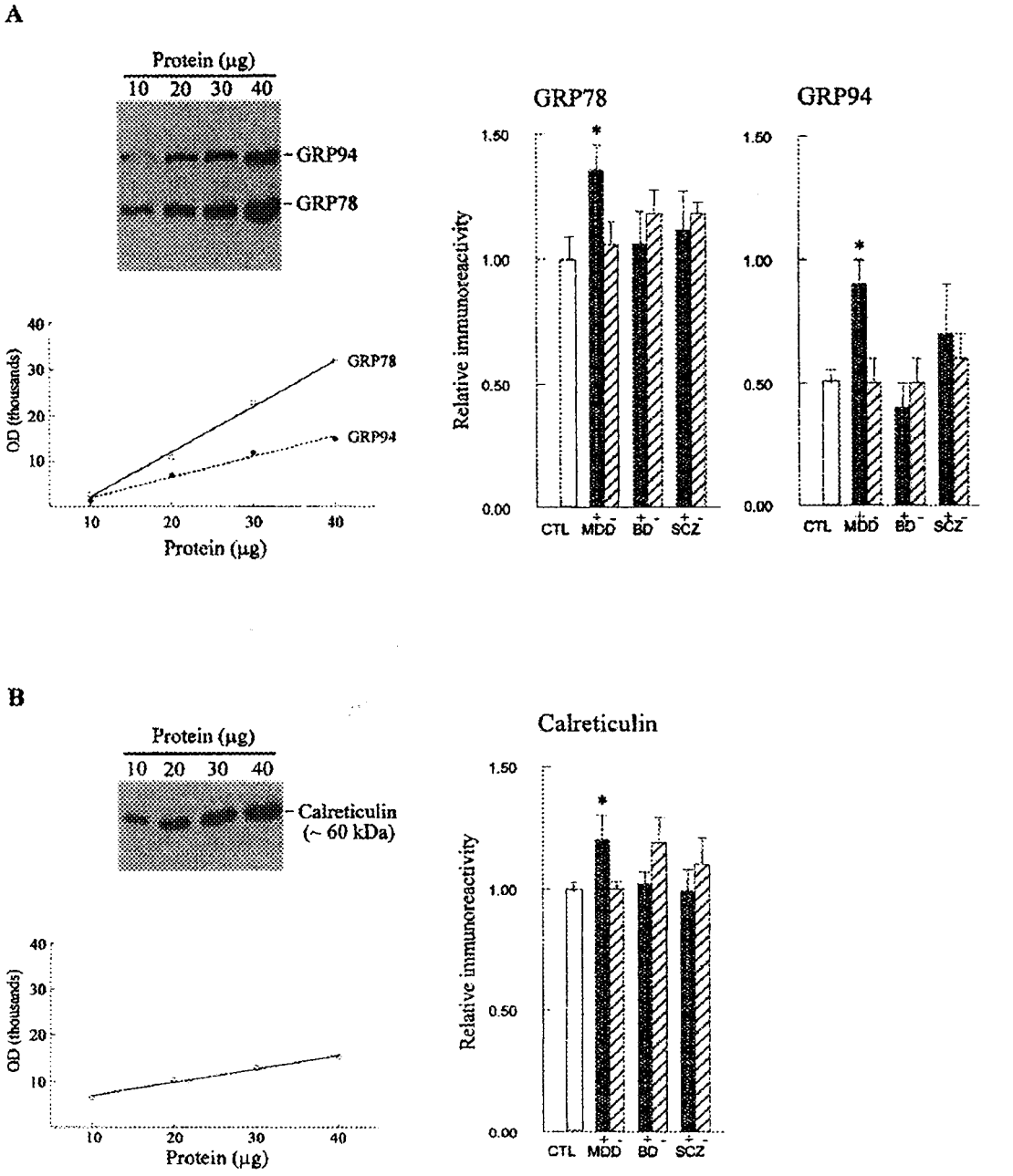



**Figure 1.** Effect of diagnosis and suicide on GRP78, GRP94, and calreticulin immunoreactivity in postmortem temporal cortex. Immunoblots represent linear ranges of protein used for standardization. Ten to 40 μg of whole cell extract from temporal cortex from one healthy comparison subject was run on every gel. A standard curve relating optical density to protein concentration from one healthy comparison subject was constructed for each blot and used to determine the relative immunoreactivity for each subject. MDD suicide (+) subjects ($n = 7$) had significantly higher GRP78 (A), GRP94 **(A)**, and calreticulin **(B)** immunoreactivities compared to controls (CTL, $n = 15$) and MDD non-suicide (−) subjects ($n = 8$). Both BD ($n = 9$ for suicide) and SCZ ($n = 4$ for suicide) subjects showed no increase in immunoreactivity in any of the proteins compared to the CTL group or non-suicide subjects (BD, $n = 6$; SCZ, $n = 11$). Autoradiograms were quantified by densitometry. Results are mean ± S.E.M. $^*p < .05$ significantly different from controls and non-suicide MDD subjects by ANOVA.

using Ponseau S staining. Tissue samples from one healthy comparison subject were run on every gel (10–40 μg) to ensure assays were performed within the linear range and to allow comparisons across blots (Figure 1).

A standard curve relating optical density to protein concentration from the one comparison subject was constructed for each blot. The optical density for 30 μg of protein, as determined from the standard curve, was used to determine the relative immunoreactivity for each of the samples. All blots were repeated and tested blind to diagnosis. If greater than 20% variability was found between the two relative immunoreactivity values for each subject, the sample was repeated. Primary monoclonal antisera for GRP78, which cross-reacts with GRP94 (1:2500 dilution) and polyclonal antisera for calreticulin (1:25,000 dilution) were obtained from Stressgen Biotechnologies (Victoria, B.C.). Secondary antibodies, goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:2500 (Santa Cruz Biotechnology, Santa Cruz, CA) for GRP78/94 and goat anti-rabbit IgG diluted 1:25,000 for calreticulin (Upstate Biotechnologies, Lake Placid, NY) were used, followed by detection with enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL). Immunoreactive bands were quantified by densitometry using the SCID image analysis system (ImageExperts, Oakville, ON). Statistical analysis was conducted using one-way analysis of variance (ANOVA).

## RESULTS

Subject characteristics for the MDD group are shown in Table 1. Figure 1 shows that immunoblotting with GRP78/94 or calreticulin antisera yielded immunoreactive bands at 78, 94, and 60 kDa, previously identified as GRP78, GRP94, and calreticulin, respectively. To determine if age, sex, and postmortem delay affected GRP78, GRP94 and calreticulin protein expression, we examined protein levels across these variables in the entire sample. There were no significant effects of age, sex or postmortem delay on GRP78, GRP94, and calreticulin immunoreactivity in the temporal cortex ($p > .05$ in all cases). No significant diagnostic differences ($p > .05$) were found in levels of GRP78, GRP94, and calreticulin when comparing BD, MDD, and SCZ subjects with control subjects. However, we found significantly increased levels of GRP78 ($F = 3.50$, $df = 2, 27$, $p = .045$), GRP94 ($F = 4.41$, $df = 2, 27$, $p = .022$), and calreticulin ($F = 4.52$, $df = 2, 27$, $p = .020$) in the MDD group when comparing subjects who suicided with controls or with those who died by other means (Figure 1). GRP78, GRP94, and calreticulin levels were increased by 35%, 64%, and 20%, respectively, over control subjects and 28%, 69%, and 20% over non-suicide MDD subjects.

Although it cannot be known for certain in all subjects whether the treatment history reflects the actual medication taken by the patient at the time of death, we found no differences comparing subjects who were treated with either antidepressants, anticonvulsants, or lithium at the time of death compared to those who were not on any of these pharmacological agents ($p > .05$ in all cases).

## DISCUSSION

In the present study we found significantly increased levels of GRP78, GRP94, and calreticulin in temporal cortex of subjects with MDD who died by suicide compared to those who did not. It is unlikely that these differences are the result of suicide itself because no differences were found in patients with BD or SCZ who died by suicide. Furthermore, it does not appear that subjects who died by suicide had different patterns of pharmacologic treatment or greater rates of substance abuse to account for the increased levels in the ER stress proteins (see Table 1), and given the consistency of the pattern across the three proteins, it is very unlikely that this is a spurious finding.

Induction of ER stress proteins in MDD may be an attempt to compensate for the toxic effect of prolonged stress and glucocorticoid release on vulnerable brain regions such as the temporal cortex. In support of this hypothesis is the fact that dysregulation of the HPA axis, which controls glucocorticoid levels is particularly common in severe forms of depression and in those patients with suicidal ideation or attempts (Lopez et al. 1997).

Given the nature of the postmortem tissue used in this study it is not possible to know whether MDD patients who died by suicide were more severely ill at the time of death, or had differences in response or exposure to antidepressants. Since we controlled for other risk factors for suicide such as age, gender and substance abuse (Mann et al. 1999), differences in illness severity or response to treatment between the two groups seems to be likely explanations. Whatever the reason for these differences, upregulation of ER stress proteins in temporal cortex of MDD subjects who commit suicide may be important for several reasons.

Since calreticulin modulates glucocorticoid responsive gene expression (Michalak et al. 1996), it is interesting to speculate that this may have been an attempt to compensate for high levels of circulating glucocorticoids on these vulnerable brain regions. Moreover, since studies have shown that increased expression of ER stress proteins prevents $Ca^{2+}$ depletion from the ER and protects against cellular damage and death (Lievremont et al. 1997; Liu et al. 1997, 1998; Yu et al. 1999), it is possible that an elevation of these proteins in this subgroup of patients may have been an attempt to compen-

sate for these neuropathologic changes in this patient group. Indeed, several recent studies are suggestive of cellular loss and glial changes in cortical regions of patients with MDD, which may be correlated with severity of illness (Ongur et al. 1998; Rajkowska et al. 1999).

These results need to be interpreted with caution as the functional significance of these differences needs to be established as well as measurement of these proteins in other brain regions. Although clinical information on these subjects appears to be comprehensive and detailed, we cannot rule out the possibility that an undetermined clinical variable contributed to the pattern of results. Nonetheless, the present findings, even with a relatively small sample size suggest that further study of the role of ER stress proteins in MDD and its treatment are worthwhile.

## ACKNOWLEDGMENTS

This work was supported by the Theodore and Vada Stanley Foundation (L.T.Y.) and the Medical Research Council of Canada (LTY). Dr. L. T. Young is a career scientist of the Ontario Ministry of Health. Dr. G. MacQueen is a fellow of the Canadian Psychiatric Research Foundation. Postmortem brains were donated by the Stanley Foundation Brain Consortium courtesy of Drs. Llewellyn B. Bigelow, Juraj Cervenak, Mary M. Herman, Thomas M. Hyde, Joel E. Kleinman, Jose D. Paltan, Robert M. Post, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken.

## REFERENCES

Chen G, Zeng WZ, Yuan PX, Huang LD, Jiang YM, Zhao ZH, Manji HK (1999): The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. J Neurochem 72:879–882

Chen RW, Chuang DM (1999): Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. J Biol Chem 5:6039–6042

Dowlatshahi D, MacQueen GM, Wang JF, Young LT (1998): Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. Lancet 352:1754–1755

Dowlatshahi D, MacQueen GM, Wang JF, Reiach JS, Young LT (1999): G-protein coupled cAMP signalling in postmortem brain of subjects with mood disorders: effects of diagnosis, suicide and treatment at the time of death. J Neurochem 73:1121–1126

Duman RS, Heninger GR, Nestler EJ (1997): A molecular and cellular theory of depression. Arch Gen Psychiatry 54:597–606

Gething M-J (1997): Guidebook to Molecular Chaperones and Protein-folding Catalysts. Oxford, Oxford University Press

Hamos JE, Oblas B, Pulaski-Salo D, Welch WJ, Bole DG, Drachman DA (1991): Expression of heat shock proteins in Alzheimer's disease. Neurology 41:345–350

Hyman SE, Nestler EJ (1996): Initiation and adaptation: a paradigm for understanding psychotropic drug action. Am J Psychiatry 153:151–162

Johnston NL, Cerevnak J, Shore AD, Torrey EF, Yolken RH (1997): Multivariate analysis of RNA levels from postmortem human brains as measured by three different methods of RT-PCR: Stanley Neuropathology Consortium. J Neurosci Meth 77:83–92

Lievremont J-P, Rizutto R, Hendershot L, Meldolesi J (1997): BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of $Ca^{2+}$. J Biol Chem 272:12003–12009

Liu H, Bowes RC 3rd, van de Water B, Sillence C, Nagelkerke JF, Stevens JL (1997): Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, $Ca^{2+}$ disturbances, and cell death in renal epithelial cells. J Biol Chem 272:21751–21759

Liu H, Miller E, van de Water B, Stevens JL (1998): Endoplasmic reticulum stress proteins block oxidant-induced $Ca^{2+}$ increases and cell death. J Biol Chem 273:12858–12862

Lopez JF, Vazquez DM, Chalmers DT, Watson SJ (1997): Regulation of 5-HT receptors and the hypothalamic-pituitary-adrenal axis. Implications for the neurobiology of suicide. Ann NY Acad Sci 836:106–134

Lowenstein DH, Gwinn RP, Seren MS, Simon RP, McIntosh TK (1994): Increased expression of mRNA encoding calbindin-D28K, the glucose-regulated proteins, or the 72 kDA heat-shock protein in three models of acute CNS injury. Mol Brain Res 22:299–308

Magarinos AM, McEwan BS, Flugge G, Fuchs E (1996): Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. J Neurosci 16:3534–3540

Mann JJ, Waternaux C, Haas GL, Malone KM (1999): Toward a clinical model of suicidal behavior in psychiatric patients. Am J Psychiatry 156:181–189

Michalak M, Burns K, Andrin C, Mesaeli N, Jass GH, Busaan JL, Opas M (1996): Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression. J Biol Chem 271:29436–29445

Ongur D, Drevets WC, Price JL (1998): Glial reduction in the subgenual prefrontal cortex in mood disorders. Proc Natl Acad Sci USA 95:13290–13295

Post RM (1992): Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. Am J Psychiatry 149:999–1010

Rajkowska G, Miguel-Hidalgo JJ, Wei J, Dilley G, Pittman SD, Meltzer HY, Overholser JC, Roth BL, Stockmeier CA (1999): Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. Biol Psychiatry 45:1085–1098

Sapolsky RM, Krey LC, McEwen BS (1985): Prolonged glucocorticoid exposure reduces hippocampal neuron number: Implications for aging. J Neurosci 5:1222–1227

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier

MW (1996): Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci USA 93:3908–3913

Uno H, Tarara R, Else JG, Suleman MA, Sapolsky RM (1989): Hippocampal damage associated with prolonged and fatal stress in primates. J Neurosci 9:1705–1711

Wang JF, Bown C, Young LT (1999): Differential display PCR reveals novels targets for the mood stabilizing drug valproate including the molecular chaperone, glucose-regulated protein 78. Mol Pharmacol 55:521–527

Yu Z, Luo H, Fu W, Mattson MP (1999): The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Exp Neurol 155:302–314

## **3.4** The Regulation of GRP78 and Calreticulin by Desipramine, Venlafaxine and Tranylcypromine in Rat C6 Glioma Cells

Christopher D. Bown, L. Trevor Young

***Rationale:*** Previously we have shown that the mood stabilizer, valproate, could significantly increase the expression of GRP78, GRP94 and calreticulin in both a concentration- and time-dependent manner. We went on to show that in post-mortem temporal cortex from subjects with a history of major depressive disorder who died by suicide, a significant increase in all three proteins, GRP78, GRP94 and calreticulin was present compared to depressed subjects who died by other means and to healthy controls. This result suggests that dysregulation of ER stress proteins in major depression may be a marker of vulnerability of certain brain regions to the prolonged effects of stress and increased glucocorticoid release. Since we had this finding in post-mortem brain from subjects with a history of major depression, and we had previously shown that at least one mood stabilizer could regulate the expression of the ER stress proteins, we examined whether other psychotropic drugs, in particular antidepressants, could also regulate their expression. The research described in this section examines the concentration- and time-dependent regulation of GRP78 and calreticulin by a tricyclic antidepressant (desipramine), a monoamine oxidase inhibitor (tranylcypromine) and a serotonin-norepinephrine reuptake inhibitor (venlafaxine).

***Involvement:*** All research described in this section was conducted by CDB. Supervision was provided by LTY.

## THE REGULATION OF GRP78 AND CALRETICULIN BY DESIPRAMINE, VENLAFAXINE AND TRANYLCYPROMINE IN RAT C6 GLIOMA CELLS

### 3.4.3 ABSTRACT

Recent studies have shown brain matter volume reductions in certain vulnerable brain regions in subjects diagnosed with major depressive disorder. The exact mechanism(s) responsible for these reductions in brain matter have yet to be elucidated. However, chronic stress and hyperactivity of the hypothalamic-pituitary-adrenal axis likely contribute to these neuropathological defects through increased glucocorticoid release. Previously we have measured increased temporal cortex ER stress proteins in depressed subjects who died by suicide. Since one member of the ER stress proteins family, calreticulin has been shown to regulate glucocorticoid-mediated gene expression, and since we have previously shown that other psychotropic drugs could upregulate the expression of calreticulin, we were interested in studying the regulation of ER stress proteins by antidepressants. We treated rat C6 glioma cells with different doses of the tricyclic antidepressant, desipramine, the serotonin-norepinephrine reuptake inhibitor, venlafaxine, and the monoamine oxidase inhibitor, tranylcypromine for either 24 hours or 7 days and then measured the mRNA levels of GRP78 and calreticulin. Desipramine or venlafaxine treatment did not significantly influence the expression of GRP78 or calreticulin, whereas 7 days of 5 μM tranylcypromine treatment reduced the expression of both GRP78 and calreticulin by ~23%. The results of this study suggest that our

previous finding showing valproate could regulate the expression of ER stress proteins is likely either drug- or drug class-specific, since no one particular antidepressant we tested significantly affected ER stress protein expression.

### 3.4.4 INTRODUCTION

Major depressive disorder (MDD) is the most commonly diagnosed psychiatric illness, affecting 16-20% of the population (Doris et al., 1999). Early clinical observations and recent systematic studies overwhelmingly document a greater role for psychosocial stressors in association with the first episode of MDD than with subsequent episodes. Hypotheses suggest that sensitization both to stressors and episodes occur and become encoded at the level of gene expression (Post, 1992b). Thus, both stressors and episodes may leave residual traces and vulnerabilities that result in further occurrences of the illness. The role of stress in MDD is further exemplified in the fact that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis in patients with MDD is one of the most consistent findings in biological psychiatry (Lopez et al., 1997).

The HPA axis is the classic neuroendocrine system that responds to stress. Perception of stress by an organism triggers a series of events whose final result is the secretion of glucocorticoids (cortisol in humans, corticosteroids in rats) from the adrenal cortex (Lopez et al., 1991). Glucocorticoids bind to glucocorticoid receptors in hippocampal neurons, which then mediates an inhibitory influence on corticotrophin-releasing factor (CRF) secretion from the hypothalamus. Decreased CRF consequently inhibits pituitary adrenocorticotropic hormone (ACTH) secretion, thus inhibiting

glucocorticoid release from the adrenal cortex. This negative feedback of the glucocorticoid stress response is critical to the adaptation and survival of the organism.

Current theories of the pathophysiology of MDD suggest that chronic increases in glucocorticoid levels, likely the result of prolonged environmental stress, lead to biochemical and physiological changes in temporal lobe neurons that leave individuals vulnerable to depression (Duman et al., 1997). Recently, studies have actually shown that hippocampal volumes are decreased in elderly patients with histories of depression (Sheline et al., 1996; Bremner et al., 2000), which is likely a result of duration of the illness and not age-related atrophy (Sheline et al., 1999).

Following the identification of ER stress proteins as being regulated by the mood stabilizer, valproate Bown et al., 2000a, Wang et al., 1999) we were interested in studying the levels of ER stress proteins from postmortem temporal cortex brain tissue from control and psychiatric subjects. The results of this postmortem brain study showed that ER stress proteins levels are significantly elevated in MDD subjects who commit suicide when compared to controls and to those MDD subjects who died by some other fate (Bown et al., 2000b). Although the significance of the increase in this select subgroup has yet to be determined, the fact that it was an increase only seen in the MDD suicide group suggests that the increase might reflect severity of illness (Josepho and Plutchik, 1994). Induction of ER stress proteins in MDD may be an attempt to compensate for the effect of prolonged stress and glucocorticoid release of vulnerable brain regions. Since one of the ER stress proteins, calreticulin, is known to modulate glucocorticoid-sensitive gene expression (Burns et al., 1994), and antidepressants have

been shown to effect glucocorticoid function, studying the role of antidepressants on the expression of ER stress proteins might help explain the clinical findings, as well as provide a potential mechanism of action of antidepressants.

### 3.4.5 METHODS

*Cell Culture and treatment*

Rat C6 glioma cells (American Type Tissue Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine at $37^0$C in a 5.0% $CO_2$ atmosphere. Drug treatment was carried out by supplementing the culture media with desipramine (ICN Biomedicals), venlafaxine HCl (WY-45030-W; WYETH, Montreal, QC, Canada) or tranylcypromine (Sigma, St. Louis MO, USA). Culture media was changed every 24 hours for the duration of the treatment.

*cDNA probes for ER stress proteins*

cDNA fragments for rat grp78 and calreticulin were synthesized from rat C6 glioma mRNA. Reverse transcriptase PCR (RT-PCR) procedures, as described by the manufacturer (Perkin Elmer Life Sciences, Montreal QC, Canada), were followed using specific primer sequences designed from the cDNA sequences for rat grp78 and

calreticulin (Genbank Accession numbers S63521, X53363). The 767 nucleotide (nt) rat grp78 cDNA fragment was synthesized using the following oligonucleotides as primers: 5' CCACCGTAACAATCAAGGTC 3' (upstream) and 5' CACGTGAGCAACTGCTAATG 3' (downstream). A 476 nt cDNA fragment specific to rat calreticulin was synthesized using: 5' GTTCACCGTGAAGCATGAGC 3' (upstream) and 5' CAGTCCTCAGGCTTCTAAGC 3' (downstream) oligonucleotides as primers. Amplified cDNA fragments were run on a 1.2% agarose gel containing ethidium bromide and then isolated using the Qiaex II gel extraction kit (Qiagen, Inc, Mississauga ON, Canada). Specificity of cDNA fragments was confirmed by sequencing using the dideoxy chain termination method (Sanger et al., 1977). Both of these cDNA probes have been shown to be specific for GRP78 and calreticulin, as previously shown by Northern blot hybridization (Bown et al., 2000b).

Radioactive labelling of each cDNA fragment was conducted using a random priming labelling kit and the protocol described by the manufacturer (Roche Diagnostic Canada, Laval, QC Canada). Fifty μCi of $\alpha$-$^{32}$P was used to label approximately 40 ng of cDNA template. One μl of $\alpha$-$^{32}$P dCTP labelled probe was placed in a vial containing liquid scintillation mixture (Amersham Biosciences Inc, Piscataway NJ, USA) and radioactivity of the probe sequence measured using a Beckman scintillation counter.

*Slot blot hybridization*

Isolation of total RNA from rat C6 glioma cells was carried out using Trizol reagent and the procedure described by the manufacturer (Invitrogen Canada Inc, Burlington ON, Canada). Slot blot hybridization was used to determine expression levels of grp78, and calreticulin mRNA after antidepressant treatment, as described previously (Wang et al., 1999b). Briefly, 5 μg of total RNA from each sample was transferred to a nitrocellulose membrane using a slot blot apparatus (BioRad Laboratories, Mississauga ON Canada) under low vacuum. The membrane was then washed twice under a low vacuum with 10X SSC. Finally, membranes were UV crosslinked for 30 seconds in a UV crosslinker (Stratagene Corporation, LaJolla CA, USA). Membranes were prehybridized in 0.5M $NaHPO_4$ (pH 7.2), 7% SDS, 1 mM EDTA at $65^0$C for 30 minutes. Following prehybridization, a total of 6 x $10^6$ cpm of $\alpha$-$^{32}$P dCTP labelled probe was placed in the prehybridization solution overnight. Unbound probe was washed off the membrane in a series of 20 minute washes using 2X SSC, 0.1% SDS at graded incubation temperatures. Membranes obtained from slot blot hybridizations were exposed to a Phosphor Screen (Amersham Biosciences Inc, Piscataway NJ, USA) and quantified using a PhosphorImager and ImageQuant software version 5.60 (Amersham Biosciences Inc, Piscataway NJ, USA).

*Data and statistical analysis*

Differences between antidepressant treated and untreated cells were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

All RNA measures were normalized to the expression of 28S ribosomal RNA. Statistical analysis was performed on raw data and converted to percentages only for graphical display.

### 3.4.6 RESULTS

To determine the effect of the antidepressants, desipramine (DMI), venlafaxine (VEN) and tranylcypromine (TCP) treatment on the expression of the ER stress proteins, GRP78 and calreticulin, we treated rat C6 glioma cells with 0.1, 1 or 10 μM of DMI, 0.1, 1 or 10 μM VEN or 0.2, 1, or 1 μM of TCP for 24 hours or 7 days. Our laboratory and others have shown that rat C6 glioma express GRP78 and calreticulin, as well as regulate gene expression in response to mood stabilizer and antidepressant treatment (Bown et al., 2000b; Wang et al., 1999b; Chen and Rasenick, 1995). Following the treatment period, total RNA was isolated and mRNA levels of GRP78 and calreticulin were measured using slot blot hybridization.

Rat C6 glioma cells treated with DMI for 24 hours or 7 days failed to increase the expression of either GRP78 or calreticulin at any of the doses of the drug examined (Figure 1). Cells treated with VEN for 24 hours or 7 days also failed to significantly increase the expression of either GRP78 or calreticulin (Figure 2). Rat C6 glioma treated with TCP for 7 days did have a significant reduction in GRP78 mRNA levels ($p<0.05$) (Figure 3a). Calreticulin expression was also significantly reduced following 7 days of 1

and 5 μM TCP treatment (Figure 3b). However, both GRP78 and calreticulin levels only reached a maximal reduction of ~ 23% following 7 days of 5 μM TCP treatment.

### 3.4.7 DISCUSSION

The goal of this pilot study was to determine whether acute or chronic treatment of rat C6 glioma cells with antidepressants could affect the expression of the ER stress proteins, GRP78 and calreticulin. Previously, we have shown that the mood stabilizer valproate could significantly increase the expression of the ER stress proteins, GRP78, GRP94 and calreticulin (Bown et al., 2000b; Wang et al., 1999b). We also measured increased temporal cortex ER stress proteins in depressed subjects who died by suicide (Bown et al., 2000a). Induction of ER stress proteins in MDD may be an attempt to compensate for the toxic effect of prolonged stress and glucocorticoid release on vulnerable regions such as the temporal cortex, a brain region containing important limbic structures, including the hippocampus and amygdala.

In the present study we were unable to detect any significant changes in either GRP78 or calreticulin mRNA levels following acute or chronic treatment with the norepinephrine reuptake inhibitor, DMI or the serotonin-norepinephrine reuptake inhibitor, VEN. Although a sizable increase in GRP78 mRNA levels were measured following 7 days of VEN treatment, and an increase in calreticulin mRNA was measured following 24 hours of treatment, the variability within each group prevented any statistical difference from being detected. An increased number of samples in each group

would likely decrease the variability within each group and provide a better understanding of the effect VEN has on ER stress proteins. We were able to measure a statistically significant decrease in GRP78 and calreticulin mRNA following 7 days of treatment with 5 μM TCP, a known monoamine oxidase inhibitor. A dose-dependent decrease in calreticulin mRNA was measured following 7 days of TCP treatment. However, even at the maximum dose tested we only measured a 23% decrease in both GRP78 and calreticulin mRNA levels following 7 days of TCP treatment.

We were unable to measure large differences in expression of GRP78 or calreticulin following treatment with antidepressants, the results suggest that our previous finding with valproate is likely drug specific (Bown et al., 2000b). Pretreatment with valproate caused C6 glioma cells to produce a greater than 50% increase in the expression of GRP78, GRP94 and calreticulin. We also showed that lithium, a commonly prescribed mood stabilizer, was unable to upregulate the expression of GRP78 (Wang et al., 1999b). Alternatively, carbamazepine, a commonly prescribed mood stabilizer belonging to the same drug class as valproate, mood stabilizing anticonvulsants, did significantly increase the expression of GRP78 at supratherapeutic doses. Therefore, the regulation of ER stress proteins may be either drug- or drug class-specific. Additional studies using other mood stabilizing anticonvulsants (i.e. lamotrigine, gabapentin) will have to be conducted to delineate the effect of these drugs on the ER stress proteins.

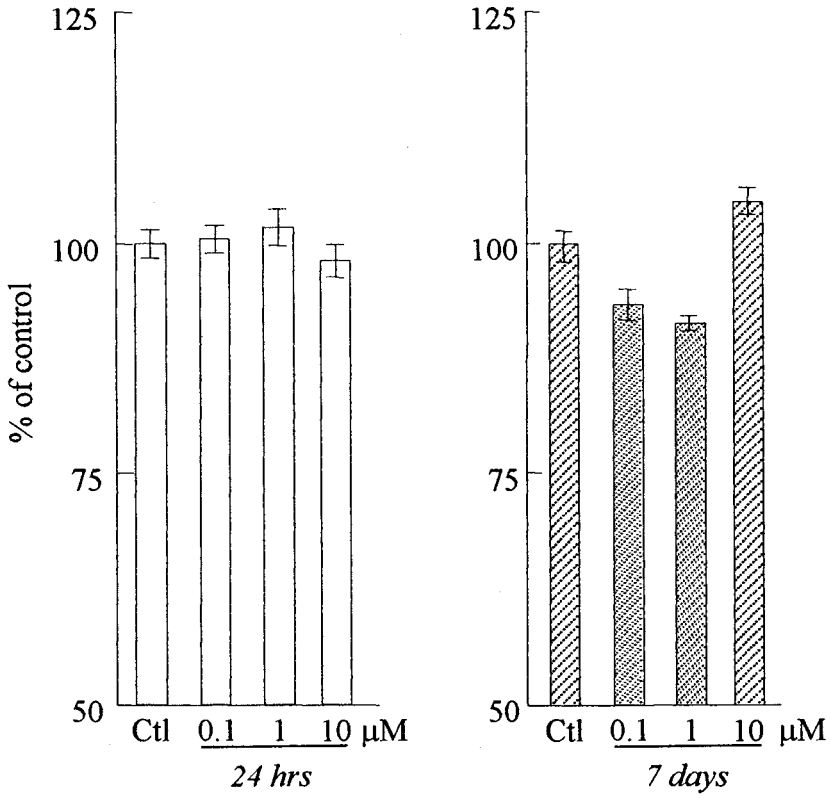
In the current study, we measured levels of only GRP78 and calreticulin following antidepressant treatment. In a previous study examining the effect of valproate

on ER stress proteins, we measured levels of all three ER stress proteins, GRP78, GRP94 and calreticulin (Bown et al., 2000b). We identified increased mRNA levels for all three ER stress proteins, but only measured significant changes in protein levels with GRP78 and calreticulin. Therefore, in this pilot study we first looked at the expression of GRP78 and calreticulin, not GRP94, since they appear to be more susceptible to pharmacological treatment. We chose drugs from three of the four major classes of drugs used to treat MDD patients, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and atypical antidepressants and treated C6 glioma cells with doses that included the therapeutically relevant concentration for each drug. SSRIs were not used in this study since C6 glioma cells appear not to express serotonin transporters or the 5-HT1a receptor, which are thought to be cell surface targets of SSRIs (Storring et al., 1999; Johnson RA et al., 1998). A more relevant cell line to study the effect of SSRIs on the expression of ER stress proteins would be the rat serotonergic raphe cell line RN46A. Since we had previously shown that rat C6 glioma cells do in fact regulate gene expression in response to pharmacological treatment, we did not feel that changing cell lines would be advantageous for this pilot study. In addition, our previous results using lithium, valproate and carbamazepine were obtained in rat C6 glioma cells, so changing cell lines would not allow us to make meaningful comparisons across the different drug classes. Future studies would likely include the use of different cell lines and primary culture, such as the rat raphe RN46A cell line and primary rat hippocampal neurons, which have both been shown to express constitutively high levels of the 5-HT1A receptor and have been shown to respond to serotonin (Eaton et al., 1995; Welner et al., 1989).

Although we were not able to detect any significant changes in GRP78 and calreticulin expression following treatment with DMI in rat C6 glioma cells, others have shown that DMI can regulate gene expression in C6 glioma cells. One of the most consistent findings of DMI on C6 glioma cells is the significant reduction in expression of β-adrenergic receptors (βARs) following more than 3 days of 10 μM DMI treatment (Fishman and Finberg, 1987; Fowler and Brannstrom, 1990; Manji et al., 1991a; Manji et al., 1991b). Manji et al. (1991b) showed that coincubation of C6 glioma cells with the phospholipase A2 (PLA2) inhibitor mecacrine or the protein kinase C (PKC) inhibitor H7, during chronic treatment with 10 μM DMI attenuated the downregulation of βARs, suggesting that DMI mediates its effect through the PKC pathway. These effects on gene expression by DMI are likely downstream or a result of desensitization of the norepinephrine reuptake receptor, the primary mechanism of action for DMI (reviewed by Frazer, 2001)). The primary mechanism of action of VEN is inhibition of serotonin-norepinephrine reuptake (Dawson et al., 1999). In fact, microdialysis studies in rat brain have shown that norepinephrine levels are increased following treatment with VEN (Dawson et al., 1999). Studies on gene expression following VEN treatment have shown the glucocorticoid receptor mRNA levels are significantly decreased (~45 %) in rat hippocampus (Yau et al., 2001), and levels of neuroprotective superoxide dismutase mRNA are increased in PC12 cells acutely treated with the drug (Li et al., 2000). A recent study using isolated synaptosomes from rat brain showed that high concentrations of VEN could block ATP-dependent $Ca^{2+}$-uptake by the ER (Couture et al., 2001).

The effect of TCP on rat C6 glioma cell gene expression is not as well understood. In fact, only two studies have treated C6 glioma cells with TCP and both were at concentrations high enough to induce DNA fragmentation (Slamon et al., 2001; Slamon and Pentreath, 2000). TCP is a known MAOI, which attenuates the action of MAO by preventing the breakdown of norepinephrine and dopamine, resulting in more availability of these neurotransmitters in the presynaptic terminal. TCP has been shown to increase gene expression in rats. Ainsworth et al. (1998) measured significant differences in central dopamine D2-like but not D1-like receptor function in rats following chronic treatment with TCP. The results from the present study were not conclusive enough to speculate on the role of ER stress proteins following antidepressant treatment. However, additional cell lines, animal models and a wider spectrum of antidepressants should be used before the regulation of ER stress proteins by antidepressants can be elucidated.

*A) GRP78*




*B) Calreticulin*

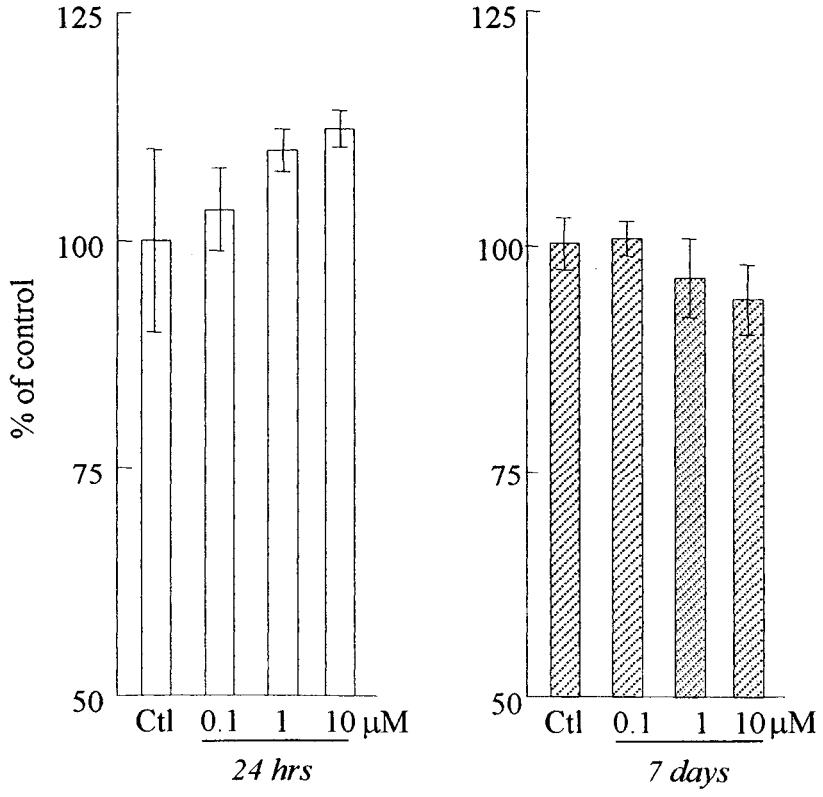
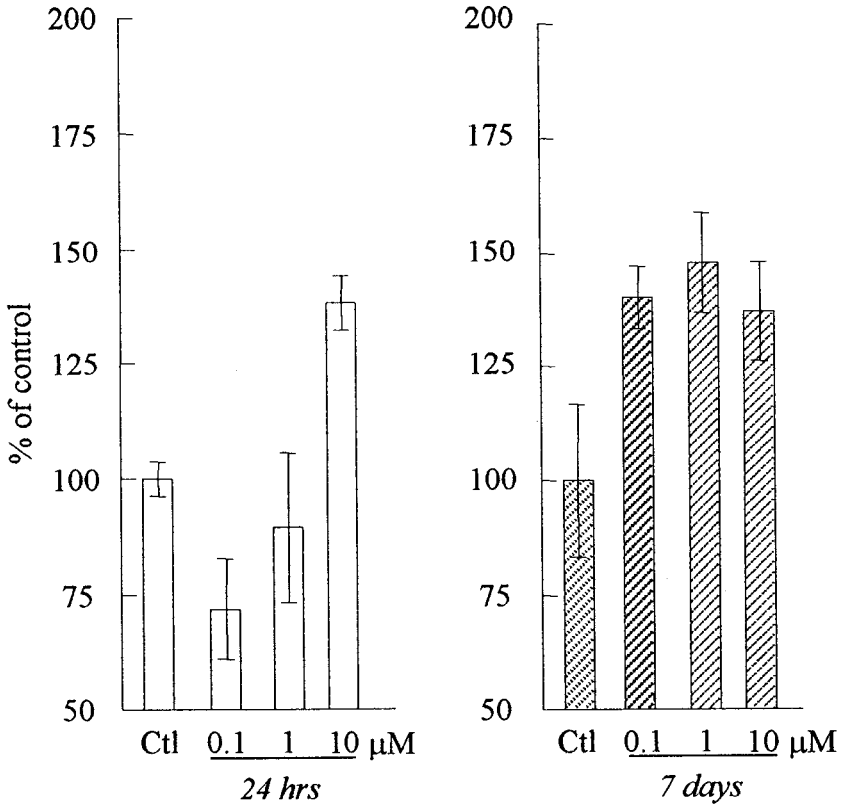


**Figure 3.4.6.1.** The effect of desipramine treatment on the expression of A) GRP78 and B) calreticulin mRNA levels in rat C6 glioma cells. C6 glioma cells were treated for 24 hours or 7 days with 0.1, 1 or 10 μM of desipramine. All values have been normalized to the expression of 28S ribosomal RNA. N=4 for all treatment groups.

*A) GRP78*




*B) Calreticulin*

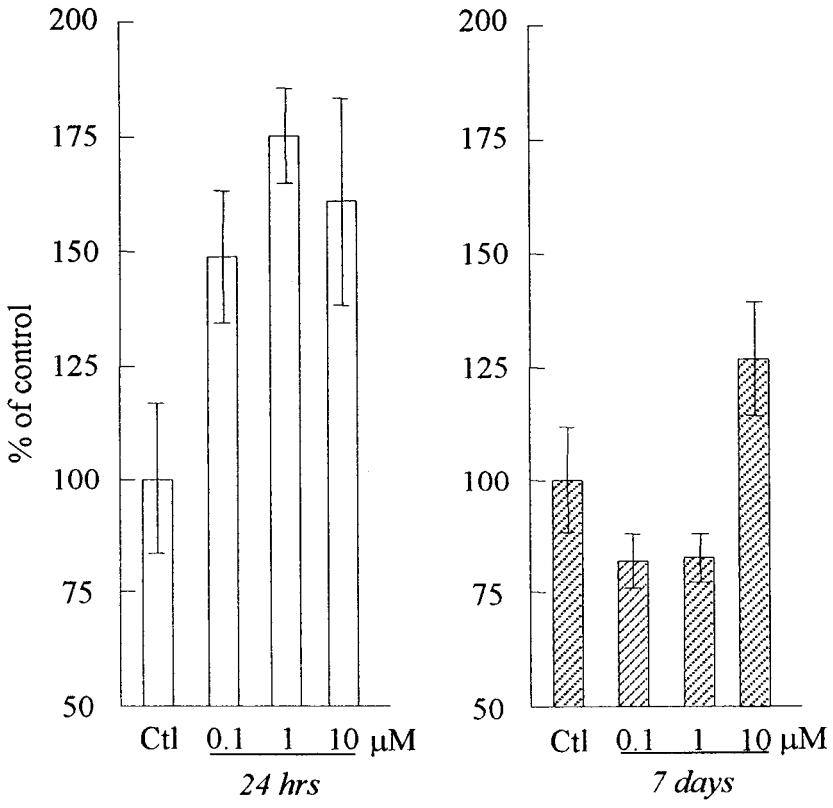
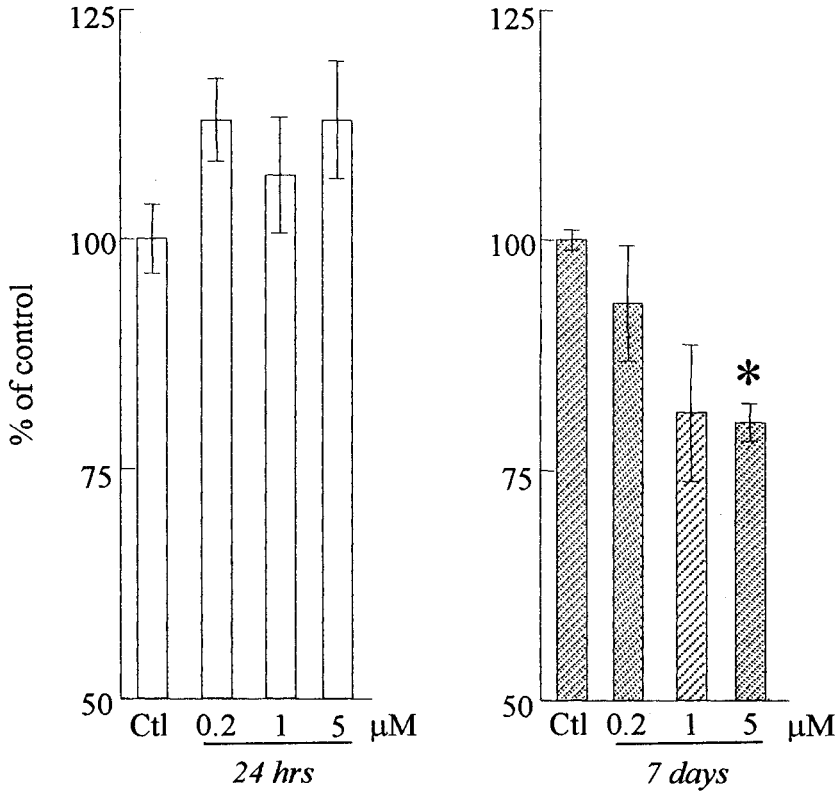


**Figure 3.4.6.2.** The effect of venlafaxine treatment on the expression of A) GRP78 and B) calreticulin mRNA levels in rat C6 glioma cells. C6 glioma cells were treated for 24 hours or 7 days with 0.1, 1 or 10 μM of venlafaxine. All values have been normalized to the expression of 28S ribosomal RNA. N=4 for all treatment groups.

*A) GRP78*




*B) Calreticulin*

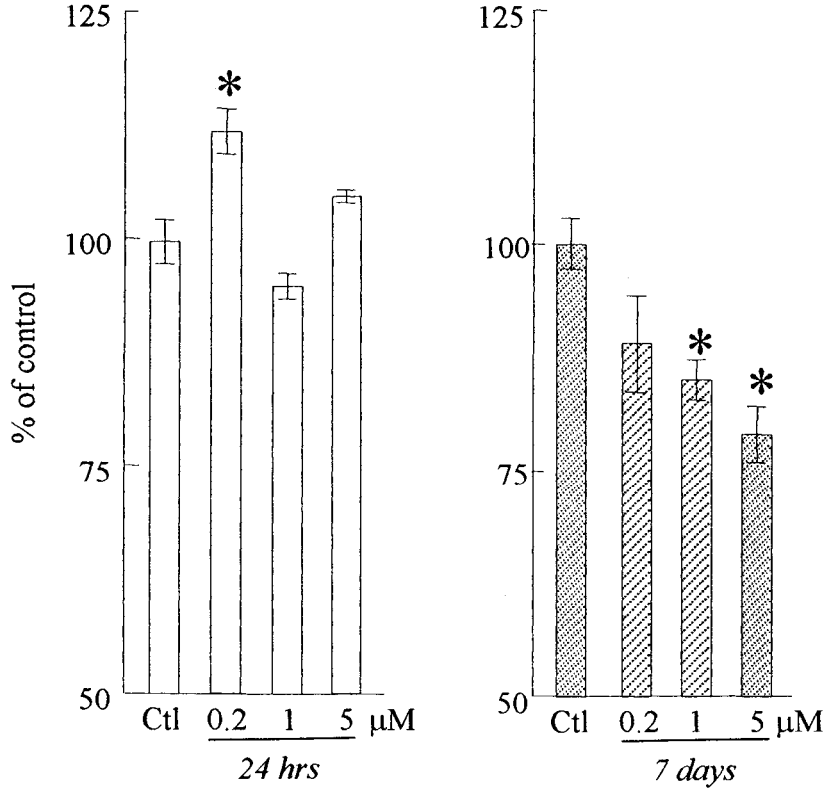


**Figure 3.4.6.3.** The effect of tranylcypromine treatment on the expression of A) GRP78 and B) calreticulin mRNA levels in rat C6 glioma cells. C6 glioma cells were treated for 24 hours or 7 days with 0.2, 1 or 5 μM of tranylcypromine. All values have been normalized to the expression of 28S ribosomal RNA. N=4 for CTL, 1 and 5 μM and N=3 for 0.2. * $p < 0.05$ significantly different by one way ANOVA followed by Dunnett's multiple comparisons test.

**3.5** Attenuation of NMDA-Mediated Cytoplasmic Vacuolization in Primary Rat Hippocampal Neurons by Mood Stabilizers

Christopher D. Bown, Jun-Feng Wang, L. Trevor Young

*Submitted to:* Neuroscience on February 22, 2002

***Rationale:*** Previous results have shown that ER stress proteins are regulated by valproate in rat brain and cultured cells. Other studies have shown in various neuronal cell lines that if ER stress proteins are overexpressed prior to a cytotoxic insult, then the cell displays resistance to the insult. Therefore, valproate treatment may protect cells against damaging insults by increasing the expression of members of the ER stress protein family. Increasing evidence suggests that mood stabilizers may have a common mechanism of action through regulation of neuroprotective proteins. Moreover, post-mortem and brain imaging studies suggest that mood disorders may be associated with cell loss and/or atrophy in vulnerable brain regions, such as the hippocampus. Since we have shown that valproate can regulate the expression of a set of resident endoplasmic reticulum proteins known to have neuroprotective properties, we were interested in examining the ultrastructural appearance of rat hippocampal neurons pretreated with lithium, valproate and carbamazepine, proceeded by exposure to an excitotoxic insult, NMDA.

***Involvement:*** The research described in this paper was conducted by CDB. Both JFW and LTY provided technical advice and manuscript editing.

# ATTENUATION OF NMDA-MEDIATED CYTOPLASMIC VACUOLIZATION IN PRIMARY RAT HIPPOCAMPAL NEURONS BY MOOD STABILIZERS

## ABSTRACT

Recent post-mortem and brain imaging studies suggest that decreased neuronal and glial densities may account for cell loss in vulnerable brain regions such as the hippocampus and the frontal cortex in patients with bipolar disorder. Investigations into the mechanisms of action of mood stabilizers suggest that these drugs may regulate the expression of neuroprotective genes and protect against excitotoxicity. In this study, we characterized the ultrastructural appearance of rat hippocampal neurons pretreated with mood stabilizers and then exposed to the glutamate receptor agonist N-methyl-D-aspartate. Using transmission electron microscopy we found that rat hippocampal neurons exposed to 0.5 mM N-methyl-D-aspartate for 10 minutes produced more cytoplasmic vacuolization than in control neurons. Chronic treatment with mood stabilizers, lithium, valproate or carbamazepine for 7 days at therapeutically relevant concentrations fully attenuated N-methyl-D-aspartate-mediated cytoplasmic vacuolization. These results suggest that inhibition of neurotoxicity may be involved in the action of mood stabilizers.

KEYWORDS: vacuolization, lithium, valproate, carbamazepine, neuroprotection, transmission electron microscopy

## INTRODUCTION

The most widely prescribed drugs for bipolar disorder are commonly referred to as mood stabilizers and include lithium, valproate and carbamazepine. Studies from a number of independent laboratories examining their mechanisms of action suggest that treatment with these drugs is likely to produce neuroprotective effects (reviewed by (Manji et al., 2000a). The role of lithium in protecting against glutamate-mediated excitotoxicity was first studied by Dixon and Hokin (Dixon and Hokin, 1998) in research showing that chronic treatment with lithium up-regulated the synaptosomal uptake of glutamate. These results were further advanced by Nonaka et al. (Nonaka et al., 1998) who found that lithium robustly protects rat cerebellar granule cells from glutamate-induced excitoxicity by inhibiting N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx. In our laboratory, we found that valproate at therapeutically relevant concentrations provides a neuroprotective effect by reducing oxidant TBHP induced LDH release in rat C6 glioma cells and also by reducing DNA fragmentation caused by ER Ca2+-ATPase inhibitor thapsigargin in primary cultured rat cerebral cortex (Bown et al., 2001). Recently, a number of studies have demonstrated that lithium and valproate increase the expression of the protooncogene bcl-2 and that valproate increases expression of ER stress proteins GRP78, GRP94 and calreticulin (Bown et al., 2000; Chen et al., 2000a; Chen et al., 1999a; Chen and Chuang, 1999; Wang et al., 1999). Bcl-2 is an antiapoptotic factor and inhibits cytochrome c release from mitochondria (Adams and Cory, 1998; Bruckheimer et al., 1998). ER stress proteins have dual functions within

the cell, by acting as molecular chaperones and $Ca^{2+}$ binding proteins. Both bcl-2 and ER stress proteins have been shown to produce cytoprotective effects (Liu et al., 1997; Liu et al., 1998; van de Water et al., 1999; Yu et al., 1999; Manji et al., 2000a). All of these studies indicate that a neuroprotective mechanism may play an important role in the action of mood stabilizers.

We report here our research suggesting that chronic treatment with lithium, valproate and carbamazepine at therapeutically relevant concentrations also produces a neuroprotective effect by inhibiting glutamate receptor agonist NMDA-mediated cytoplasmic vacuolization in cultured rat hippocampal neurons. Neuronal vacuolization has been consistently reported to be involved in neurotoxicity (Meldrum, 1986; Olney et al., 1989, 1991; Phillips et al., 2001). Hippocampus was studied because it is a region in which abnormalities have been reported in bipolar disorder and where mood stabilizers have previously been shown to have a variety of effects (Dowlatshahi et al., 2000; Chen et al., 2000b; Chen et al., 1999b; Fatemi et al., 2001; Benes et al., 2001; Law and Deakin, 2001; Noga et al., 2001; Fatemi et al., 2000; Eastwood and Harrison, 2000; Vawter et al., 1999; Strakowski et al., 1999; Lenox et al., 1992; Watson et al., 1998) .

# MATERIALS AND METHODS

## *Isolation and culture of primary rat hippocampal neurons*

Rat hippocampi were isolated from 16-18 day old rat fetuses, as originally described by Banker and Cowan (Banker and Cowan, 1977). Hippocampal neurons were dissociated and cultured following the procedure detailed by Brewer et al. (1993). Briefly, two hippocampi were isolated from each fetus and mechanically dissociated in $Ca^{2+}$- and $Mg^{2+}$-free Hank's Balanced Salt Solution (HBSS) supplemented with 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). An equal volume of HBSS containing $Ca^{2+}$ and $Mg^{2+}$ was added to the cell suspension. Cells were collected by centrifugation at 1,000 x *g* for 4 minutes. Cells were resuspended in Neurobasal™ media (GIBCO BRL Inc) containing B27 supplements (GIBCO BRL Inc), 0.5 mM L-glutamine, 1% penicillin/streptomycin and 25 μM glutamate. An equal concentration of cells was ($2.7 \times 10^6$ cells/well) plated on poly-D-lysine (MW 30,000 – 70,000) coated vessels and maintained at $37^0$C in a 5.0% $CO_2$ humid environment. After four days, an equal volume of fresh Neurobasal™ media without glutamate was added to the existing media and cells cultured for another two days. On the sixth day, media was removed and replaced with fresh glutamate-free Neurobasal™ media and cultured for a further 7 days. Lithium chloride (BDH Chemicals) at a concentration of 1 mM, 0.6 mM valproate (ICN Biochemical), 50 mM carbamazepine (ICN Biochemicals) dissolved in 0.05%

dimethylsulfoxide (DMSO) or 0.05% DMSO (vehicle) were added to the media on day six in the appropriate experiments.

*Immunocytochemistry*

The relative distribution of neurons to glia in the culture was determined by immunocytochemistry (Brewer et al., 1993). Cells were grown on poly-D-lysine coated Lab-Tek 2-Chamber Slides (Nalge Nunc International) for a total of 13 days. Cells were fixed in 4% paraformaldehyde for 1 hour at $37^0$C, then blocked for 5 minutes in 1% bovine serum albumin (BSA), 5% normal goat serum and 0.05% Triton X-100. Rabbit anti-rat neuron-specific enolase (NSE, Polysciences Inc.) diluted 1:1000 in blocking buffer and monoclonal antisera for glial fibrillary acidic protein (GFAP, Sigma Inc) at a dilution of 1:400 were used to detect the specific cell types. Slides were incubated with an appropriate fluorescence tagged secondary antibody, rhodamine anti-rabbit IgG for NSE (Molecular Probes ; 1:100 dilution in 1% BSA, 1% normal goat serum, 0.05% Triton X-100) and fluorescein anti-mouse IgG for GFAP (Molecular Probes; 1:50 dilution). Neurons and glia were imaged using a Carl Zeiss 510 confocal laser scanning microscope (Jena, Germany) with excitation and emission spectra set to 570/590 nm for visualization of neurons and 494/518 nm for glia.

*Transmission electron microscopy*

Rat hippocampal neurons were cultured in Neurobasal media supplemented with B27 supplements for 13 days on 2-chamber microscope slides (Nalge-Nunc). Neurons were fixed in 2.0% glutaraldehyde buffered in 0.1M sodium cacodylate, pH 7.4 for 30 minutes at room temperature. Slides were rinsed in 0.2M sodium cacodylate, pH 7.4 and post-fixed with 1% osmium tetroxide in sodium cacodylate buffer for 1 hour at room temperature. Slides were dehydrated by a graded series of ethanol and further dehydrated in 100% propylamide. Neurons were embedded in Beem #3 capsules with Epon resin and incubated overnight at 60°C. Capsules were then snapped off the slides, transferring neurons on the slide to the plastic resin capsule. Ultra-thin sections (~70 nm) were cut from each capsule and floated onto 200 mesh copper/palladium grids and stained for contrast with uranyl acetate and lead citrate. Grids were observed using a transmission electron microscope (JEOL 1200EX) at 80 kV.

*Quantification of cytoplasmic vacuolization*

To quantify ultrastructural changes in cultured cells, we counted the number of cytoplasmic vacuoles in individual cells. Cytoplasmic vacuoles were quantified since they were easily identifiable following NMDA exposure and provided a morphological difference associated with treatment. The number of cytoplasmic vacuoles present in fifty

randomly chosen neurons was recorded blindly. All of the experiments were repeated twice for each condition. Cells were identified as neurons based on the size of the cell (> 7.5 μm), the densely packed cytoplasm and the large circular nucleus (Fawcett, 1994). A cytoplasmic vacuole was defined as a circular, electron translucent space between the nucleus and plasma membrane of a neuron (Cameron, 1952). These vacuoles could be clearly differentiated from small tears that occasionally occur in the ultrathin sections using transmission electron microscopy by the intensity of the electron beam on the phosphorous screen. Sections in which nuclei could not be visualized were excluded from analysis.

*Cell viability*

The percentage of dead to live neurons was determined using the LIVE/DEAD® viability/cytotoxicity kit and the procedure described by the manufacturer (Molecular Probes, Eugene OR, USA). Cells were grown on poly-D-lysine coated Lab-Tek 2-Chamber Slides (Nalge Nunc International) for a total of 13 days. Cells were washed in PBS and incubated at 37$^0$C in PBS containing 2 μM calcein AM and 4 μM EthD-1 for 45 minutes. Following incubation, cells were washed with PBS and fixed for 15 minutes with a 4% paraformaldehyde solution. Following fixation, cells were washed with PBS, and coverslips were mounted using Aquamount (BDH Laboratory Supplies, Poole, Dorset, England) and imaged using a Carl Zeiss 510 confocal laser scanning microscope (Jena, Germany) with excitation and emission spectra set to 485/530 nm for visualization

of live cells and 485/590 nm for dead cells. Images were taken using a 20X objective lens and the number of live and dead cells were manually counted from the image. Two randomly chosen fields were focused on and the image captured from 4 replications for each group.

Cell viability was determined by measuring the cleavage of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis MO, USA), into blue-coloured formazan by the mitochondrial enzyme succinate-dehydrogenase, with modifications from the originally described procedure (Manji et al., 2000b; Mosmann, 1983). Rat hippocampal neurons were cultured on 12-well poly-D-lysine (MW 30,000-70,000) coated plates (Nalge Nunc International) for a total of 13 days. Culture media was removed and cells were rinsed with PBS. Cells were then cultured for one hour in media supplemented with 125 μg/ml MTT. Following incubation, media was aspirated and cells were dissolved in 200 μl DMSO, and the plates were vigourously shaken for 5 minutes. Three aliquots of 50 μl DMSO were removed from each well and transferred to a 96-well plate (Nalge Nunc International). The optical density was measured using a microplate reader (Titertek Multiscan) with absorbance set at 540 nm. The mean absorbance for the three aliquots constituted the total absorbance of the sample.

*Statistical analysis*

All statistical analysis was conducted using Minitab version 12.0. Statistical differences between treatment groups were evaluated using the unpaired Student's t-test. All values are expressed as the mean cytoplasmic vacuolization per section of 50 individual neurons.

## RESULTS

First, we measured the neuron to glial cell ratio in cultured hippocampal cells using immunocytochemistry with primary antibodies to both neurons (anti-neuron specific enolase) and glia (anti-glial fibrillary acidic protein) and fluorescent secondary antibodies. Our results (Figure 1A) showed that glial cells in our cultured cells are less than 0.02%, which is consistent with other reports in primary serum-free cultured hippocampal neurons (Brewer et al., 1993; Brewer, 1999; Bartlett and Banker, 1984). Cultured hippocampal neurons are pyramidal, based upon their shape as described by the previous work of Banker and Cowan (Banker and Cowan, 1977), which characterized primary rat hippocampal neurons in culture. The ultrastructural appearance of primary rat hippocampal neurons as determined by transmission electron microscopy showed

characteristic neuronal features such as a large circular nucleus, densely packed cytoplasm and easily definable ER and mitochondria (Figure 1B).

Next, we measured the effect of lithium, valproate and carbamazepine on changes of ultrastructural appearance after NMDA treatment in cultured hippocampal neurons using transmission electron microscopy. Typical ultrastructural characteristics such as shape and composition of the nucleus, plasma membrane integrity, shape and translucency of mitochondria, aggregation pattern of polyribosomes and the amount of cytoplasmic vacuolization, were all noted in 50 randomly chosen neurons from each condition. As a quantifiable marker of ultrastructural changes in cells, we counted the number of cytoplasmic vacuoles from these neurons. All of the experiments were repeated twice. Treatment with NMDA at 0.5 mM for 10 minutes showed a significant amount of cytoplasmic vacuolization. NMDA treated neurons had a two-fold increase in the amount of vacuoles present ($p<0.05$) in their cytoplasm when compared with control neurons (Figures 2 and 3). Although chronic treatment with lithium (1 mM), valproate (0.6 mM) and carbamazepine (0.05 mM) at their therapeutically relevant concentrations for 7 days did not alter the ultrastructural appearance of hippocampal neurons, chronic treatment with all of these drugs prevented any statistically significant increase in cytoplasmic vacuolization caused by NMDA (Figures 2 and 3). Neurons pretreated with DMSO, the vehicle for carbamazepine, also showed a significant amount of cytoplasmic vacuolization.

To determine whether NMDA-mediated cytoplasmic vacuolization was associated with reduced cell viability, we calculated the number of live and dead cells.

The number of EthD-1 positive cells were manually counted and a percentage calculated from the total number of cells visible (Figure 4a). NMDA-mediated cytoplasmic vacuolization was not associated with reduced cell viability (96% v.s. 95% live cells, controls v.s. NMDA).Cell viability was also determined by measuring the absorbance of formazan, the cleavage product of MTT by functioning mitochondrial enzyme succinate-dehydrogenase (Figure 4b). Using this marker of cell viability NMDA was not shown to significantly reduce cell viability compared to control groups. Exposure of rat hippocampal neurons to NMDA only decreased cell viability by 9% compared to control cells.

## DISCUSSION

In the present study, we found that chronic treatment with any of the three commonly prescribed mood stabilizers, lithium, valproate or carbamazepine, at therapeutically relevant concentrations for 7 days, significantly blocked glutamate receptor agonist NMDA-induced cytoplasmic vacuolization in primary cultured rat hippocampal neurons. None of these treatments alone had an effect on the ultrastructural appearance of these cells. These results add to the growing body of evidence suggesting that neuroprotection may be an important factor in the action of mood stabilizers as long-term prophylactic treatment for bipolar disorder. Administration of NMDA at 0.5 mM for 10 minutes produces cytoplasmic vacuolization in primary cultured rat hippocampal neurons but the same treatment does not change cell viability, which indicates that

cytoplasmic vacuolization may be an early stage of cell toxicity when induced by NMDA.

Cytoplasmic vacuolization is a phenomenon commonly described in the literature, yet the mechanism responsible for this morphological characteristic has been elusive (reviewed by (Henics and Wheatley, 1999)). Previous studies have shown that activation of the NMDA receptor can mediate cytoplasmic vacuolization (Freeman and Goldberg, 1994; Goldberg and Bateman, 1993). Vacuolization has been shown to occur as the result of organelle swelling, in particular the endoplasmic reticulum and mitochondria (Puka-Sundvall et al., 2000; Isaev et al., 1996). Sudden elevations in intracellular $Ca^{2+}$ levels have been shown to be quickly sequestered by both the mitochondria and the endoplasmic reticulum (Puka-Sundvall et al., 2000; Wang and Thayer, 1996). If the rate of $Ca^{2+}$ influx into the organelle exceeds the processing capacity of the organelle, then swelling will likely occur (Brustovetsky and Dubinsky, 2000). Therefore, a disruption of intracellular $Ca^{2+}$ homeostasis, as a result of influx by voltage-gated $Ca^{2+}$ channels, is a possible cause of mitochondria and endoplasmic reticulum swelling which maybe a possible explanation for the results we observed.

Although the morphological appearance of the rat hippocampal neurons following NMDA exposure suggests compromised cell viability, we did not find decreased cell viability following NMDA exposure under these experimental conditions. Interestingly, other studies have reported similar findings in various cell lines (Freeman and Goldberg, 1994; Henics et al., 1993; Henics and Wheatley, 1997). Moreover, in many instances, vacuolization can be fully reversible with removal of the toxic agent from the culture

media (Pollanen et al., 1990). This would suggest that cytoplasmic vacuolization, is not a measure of cell death but instead is likely an indication that the neuron is under cytotoxic stress. In fact, Meldrum (1986) showed that pyramidal neurons of the CA1 and CA3 region of the rat hippocampus developed $Ca^{2+}$ containing vacuoles following status epilepticus. These vacuoles preceeded cell death and were suggested to be a morphological characteristic of cytotoxicity. If the stress, in this case NMDA, is not removed from the culture media cytoplasmic vacuolization will continue to a point where cell viability is compromised. In fact when we exposed neurons to 0.5 mM NMDA for 6 hours, significant (~ 40%) loss of cell viability could be measured (data not shown).

Although the significance of prevention of cytoplasmic vacuolization in hippocampal cells is not fully understood, these observations are likely an indication that these mood stabilizers are capable of preventing or decreasing the cytotoxic effects of NMDA. Dixon and Hokin (Dixon and Hokin, 1998) reported that chronic lithium treatment up-regulated mouse synaptosomal uptake of glutamate. Since accumulation of extracellular glutamate contributes to excitotoxicity, this suggests that lithium may have a protective role through increase of glutamate uptake. In addition, Nonaka et al. (Nonaka et al., 1998) found that lithium protects cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity by inhibiting calcium influx. Carbamazepine was also reported to inhibit $Ca^{2+}$ influx by a direct effect on the NMDA receptor in rat cerebellar granule cells (Hough et al., 1996; Puka-Sundvall et al., 2000; Ambrosio et al., 1999).

Valproate has also been shown to have neuroprotective effects. For example, valproate was found to protect cultured cerebellar granule cells against apoptosis induced by low K+ (Mora et al., 1999). Chronic treatment with valproate was also found to reduce oxidant TBHP induced LDH release in rat C6 glioma cells and to reduce thapsigargin induced DNA fragmentation in rat cerebral cortex (Bown et al., 2001). Chronic treatment with valproate also inhibited age associated DNA fragmentation in cerebral cortex (Bown et al., 2001). Several molecular mechanisms have been identified for valproate which may be relevant to neuroprotection and more specifically to the findings of cytoplasmic vacuolization reported in the present study. Previously, we reported that valproate significantly increased the expression of the endoplasmic reticulum (ER) stress proteins, including GRP78, GRP94 and calreticulin, in both cultured cells and rat brain (Bown et al., 2000; Chen et al., 2000a; Wang et al., 1999). This family of proteins has several important functions including binding free $Ca^{2+}$ in the ER (Nigam et al., 1994). For example, overexpression of GRP78 and calreticulin increased $Ca^{2+}$ binding capacity of ER stores, and thus prevented its release into cytoplasm during excitotoxic stress (Mery et al., 1996; Yu et al., 1999). Moreover, other targets of valproate may also account for these neuroprotective effects including Bcl2 (Manji et al., 2001) or glycogen synthase kinase 3β (Chen et al., 1999c).

In summary, in this study we show that three commonly prescribed mood stabilizing drugs, lithium, valproate and carbamazepine, are able to protect rat hippocampal neurons against NMDA-mediated cytoplasmic vacuolization. The ability of mood stabilizers to regulate expression of neuroprotective genes and prevent

ultrastructural changes caused by excitotoxic insults may prove to be important in understanding bipolar disorder. Recent studies have suggested that the pathophysiology of bipolar disorder may involve structural abnormalities such as cell loss and atrophy in vulnerable brain regions, however the mechanism responsible for these changes is currently unknown, and excitotoxic damage is just one possibility. Future studies to identify the biochemical agents responsible for causing neuronal loss in bipolar disorder subjects may allow for more efficient pharmacotherapies to be developed that target specific molecules and have fewer side effects.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Theodore and Vada Stanley Foundation (LTY), Ontario Mental Health Foundation (JFW, LTY) and the Natural Sciences and Engineering Research Council of Canada (CDB). The authors wish to extend their appreciation to Larry Arsenault, Ernie Spitzer, Michael Moore and the rest of the staff at the McMaster University Electron Microscopy Facility for their assistance in sample preparation and Ben Doxtator for assistance in examining the sections.

REFERENCES

Adams,J.M. and Cory,S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Ambrosio,A.F., Silva,A.P., Malva,J.O., Soares-da-Silva,P., Carvalho,A.P., and Carvalho,C.M. (1999). Carbamazepine inhibits L-type Ca2+ channels in cultured rat hippocampal neurons stimulated with glutamate receptor agonists. Neuropharmacology *38*, 1349-1359.

Banker,G.A. and Cowan,W.M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res. *126*, 397-42.

Bartlett,W.P. and Banker,G.A. (1984). An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. J. Neurosci. *4*, 1944-1953.

Benes,F.M., Vincent,S.L., and Todtenkopf,M. (2001). The density of pyramidal and nonpyramidal neurons in anterior cingulate cortex of schizophrenic and bipolar subjects. Biol. Psychiatry *50*, 395-406.

Bown,C.D., Wang,J., and Young,L.T. (2000). Increased expression of endoplasmic reticulum stress proteins following chronic valproate treatment of rat C6 glioma cells. Neuropharmacology *39*, 2162-2169.

Bown,C.D., Wang,J.F., Azzam,J.E., Sun,X.J., and Young,L.T. (2001). Protective effect of the mood stabilizers lithium and valproate. Soc. Neurosci. Abstr. *27*, 872.9.

Brewer,G.J. (1999). Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture. Exp. Neurol. *159*, 237-247.

Brewer,G.J., Torricelli,J.R., Evege,E.K., and Price,P.J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. *35*, 567-576.

Bruckheimer,E.M., Cho,S.H., Sarkiss,M., Herrmann,J., and McDonnell,T.J. (1998). The Bcl-2 gene family and apoptosis. Adv. Biochem. Eng Biotechnol. *62*, 75-105.

Brustovetsky,N. and Dubinsky,J.M. (2000). Dual responses of CNS mitochondria to elevated calcium. J. Neurosci. *20*, 103-113.

Cameron,G.R. (1952). Pathology of the cell. (London: Oliver & Boyd).

Chen,B., Wang,J.F., Hill,B.C., and Young,L.T. (1999b). Lithium and valproate differentially regulate brain regional expression of phosphorylated CREB and c-Fos. Brain Res. Mol. Brain Res. *70*, 45-53.

Chen,B., Wang,J.F., and Young,L.T. (2000b). Chronic valproate treatment increases expression of endoplasmic reticulum stress proteins in the rat cerebral cortex and hippocampus. Biol. Psychiatry *48*, 658-664.

Chen,B., Wang,J.F., and Young,L.T. (2000a). Chronic valproate treatment increases expression of endoplasmic reticulum stress proteins in the rat cerebral cortex and hippocampus [In Process Citation]. Biol. Psychiatry *48*, 658-664.

Chen,G., Huang,L.D., Jiang,Y.M., and Manji,H.K. (1999c). The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. J. Neurochem. *72*, 1327-1330.

Chen,G., Zeng,W.Z., Yuan,P.X., Huang,L.D., Jiang,Y.M., Zhao,Z.H., and Manji,H.K. (1999a). The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. J. Neurochem. *72*, 879-882.

Chen,R.W. and Chuang,D.M. (1999). Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. J. Biol. Chem. *274*, 6039-6042.

Dixon,J.F. and Hokin,L.E. (1998). Lithium acutely inhibits and chronically up-regulates and stabilizes glutamate uptake by presynaptic nerve endings in mouse cerebral cortex. Proc. Natl. Acad. Sci U. S. A *95* , 8363-8368.

Dowlatshahi,D., MacQueen,G., Wang,J.F., Chen,B., and Young,L.T. (2000). Increased hippocampal supragranular Timm staining in subjects with bipolar disorder. Neuroreport *11*, 3775-3778.

Eastwood,S.L. and Harrison,P.J. (2000). Hippocampal synaptic pathology in schizophrenia, bipolar disorder and major depression: a study of complexin mRNAs. Mol. Psychiatry *5*, 425-432.

Fatemi,S.H., Earle,J.A., and McMenomy,T. (2000). Reduction in Reelin immunoreactivity in hippocampus of subjects with schizophrenia, bipolar disorder and major depression. Mol. Psychiatry *5*, 654-63, 571.

Fatemi,S.H., Earle,J.A., Stary,J.M., Lee,S., and Sedgewick,J. (2001). Altered levels of the synaptosomal associated protein SNAP-25 in hippocampus of subjects with mood disorders and schizophrenia. Neuroreport *12*, 3257-3262.

Fawcett,D.M. (1994). Bloom and Fawcett, a textbook of histology. (New York: Chapman & Hall).

Freeman,J.K. and Goldberg,M.P. (1994). Confocal microscopic visualization of MK-801-induced cytoplasmic vacuoles in vitro. Psychopharmacol. Bull. *30*, 541-547.

Goldberg,M.P. and Bateman,M.C. (1993). Taxol attenuates glutamate receptor-mediated neurite swelling in vitro. Soc. Neurosci. Abstr. *19*, 26.

Henics,T., Koszegi,T., Szucs,G., Kellermayer,M.S., Jr., and Kellermayer,M. (1993). Transient cytoplasmic vacuolization in cultured normal and neoplastic cells treated with low molecular weight human serum ultrafiltrate: is out inside? Physiol Chem. Phys. Med. NMR *25*, 227-236.

Henics,T. and Wheatley,D.N. (1997). Vacuolar cytoplasmic phase separation in cultured mammalian cells involves the microfilament network and reduces motional properties of intracellular water. Int. J. Exp. Pathol. *78*, 343-354.

Henics,T. and Wheatley,D.N. (1999). Cytoplasmic vacuolation, adaptation and cell death: a view on new perspectives and features. Biol. Cell *91*, 485-498.

Hough,C.J., Irwin,R.P., Gao,X.M., Rogawski,M.A., and Chuang,D.M. (1996). Carbamazepine inhibition of N-methyl-D-aspartate-evoked calcium influx in rat cerebellar granule cells. J. Pharmacol. Exp. Ther. *276*, 143-149.

Isaev,N.K., Zorov,D.B., Stelmashook,E.V., Uzbekov,R.E., Kozhemyakin,M.B., and Victorov,I.V. (1996). Neurotoxic glutamate treatment of cultured cerebellar granule cells induces Ca2+ -dependent collapse of mitochondrial membrane potential and ultrastructural alterations of mitochondria. FEBS Lett. *392*, 143-147.

Law,A.J. and Deakin,J.F. (2001). Asymmetrical reductions of hippocampal NMDAR1 glutamate receptor mRNA in the psychoses. Neuroreport *12*, 2971-2974.

Lenox,R.H., Watson,D.G., Patel,J., and Ellis,J. (1992). Chronic lithium administration alters a prominent PKC substrate in rat hippocampus. Brain Res. *570*, 333-340.

Liu,H., Bowes,R.C., III, van de,W.B., Sillence,C., Nagelkerke,J.F., and Stevens,J.L. (1997). Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca2+ disturbances, and cell death in renal epithelial cells. J. Biol. Chem. *272*, 21751-21759.

Liu,H., Miller,E., van de,W.B., and Stevens,J.L. (1998). Endoplasmic reticulum stress proteins block oxidant-induced Ca2+ increases and cell death. J. Biol. Chem. *273*, 12858-12862.

Manji,H.K., Moore,G.J., and Chen,G. (2000b). Clinical and preclinical evidence for the neurotrophic effects of mood stabilizers: implications for the pathophysiology and treatment of manic-depressive illness. Biol. Psychiatry *48*, 740-754.

Manji,H.K., Moore,G.J., and Chen,G. (2000a). Lithium up-regulates the cytoprotective protein Bcl-2 in the CNS in vivo: a role for neurotrophic and neuroprotective effects in manic depressive illness. J. Clin. Psychiatry *61 Suppl 9*, 82-96.

Manji,H.K., Moore,G.J., and Chen,G. (2001). Bipolar disorder: leads from the molecular and cellular mechanisms of action of mood stabilizers. Br. J. Psychiatry Suppl *41*, s107-s119.

Meldrum B.S. (1986) Cell damage in epilepsy and the role of calcium in cytotoxicity. Adv. Neurology *44*, 849-55.

Mery,L., Mesaeli,N., Michalak,M., Opas,M., Lew,D.P., and Krause,K.H. (1996). Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx. J. Biol. Chem. *271*, 9332-9339.

Mora,A., Gonzalez-Polo,R.A., Fuentes,J.M., Fiedorowicz,A., Figiel,I., Kaminska,B., Zaremba,M., Wilk,S., and Oderfeld-Nowak,B. (1999). Different mechanisms of protection against apoptosis by valproate and Li+ Dentate granule neuron apoptosis and glia activation in murine hippocampus induced by trimethyltin exposure. Brain Res. *266*, 886-891.

Mosmann,T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods *65*, 55-63.

Nigam,S.K., Goldberg,A.L., Ho,S., Rohde,M.F., Bush,K.T., and Sherman,M.Y. (1994). A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily. J. Biol. Chem. *269*, 1744-1749.

Noga,J.T., Vladar,K., and Torrey,E.F. (2001). A volumetric magnetic resonance imaging study of monozygotic twins discordant for bipolar disorder. Psychiatry Res. *106*, 25-34.

Nonaka,S., Hough,C.J., and Chuang,D.M. (1998). Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting N-methyl-D-aspartate receptor-mediated calcium influx. Proc. Natl. Acad. Sci U. S. A *95*, 2642-2647.

Olney, J.W., Labruyere, J. and Price, M.T. (1989). Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. Science 244, 1360-1362.

Olney, J.W., Labruyere, J., Wang, G., Wozniak, D.F., Price, M.T. and Sesma, M.A. (1991). NMDA antagonist neurotoxicity: Mechanism and prevention. Science 254, 1515-1518.

Phillips M, Wang C, Johnson KM. (2001). Pharmacological characterization of locomotor sensitization induced by chronic phencyclidine administration. J Pharmacol Exp Ther 296, 905-13

Pollanen,M.S., Quinn,B.A., Wollenberg,G.K., and Hayes,M.A. (1990). Reversible mitochondrial swelling in cultured rat hepatocytes exposed to 1,2-dimethylhydrazine. Exp. Mol. Pathol. *52*, 170-178.

Puka-Sundvall,M., Hallin,U., Zhu,C., Wang,X., Karlsson,J.O., Blomgren,K., and Hagberg,H. (2000). NMDA blockade attenuates caspase-3 activation and DNA fragmentation after neonatal hypoxia-ischemia. Neuroreport *11*, 2833-2836.

Strakowski,S.M., DelBello,M.P., Sax,K.W., Zimmerman,M.E., Shear,P.K., Hawkins,J.M., and Larson,E.R. (1999). Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch. Gen. Psychiatry *56*, 254-260.

van deWater,B., Wang,Y., Asmellash,S., Liu,H., Zhan,Y., Miller,E., and Stevens,J.L. (1999). Distinct endoplasmic reticulum signaling pathways regulate apoptotic and necrotic cell death following iodoacetamide treatment. Chem. Res. Toxicol. *12*, 943-951.

Vawter,M.P., Howard,A.L., Hyde,T.M., Kleinman,J.E., and Freed,W.J. (1999). Alterations of hippocampal secreted N-CAM in bipolar disorder and synaptophysin in schizophrenia. Mol. Psychiatry *4*, 467-475.

Wang,G.J. and Thayer,S.A. (1996). Sequestration of glutamate-induced Ca2+ loads by mitochondria in cultured rat hippocampal neurons. J. Neurophysiol. *7*, 1611-1621.

Wang,J.F., Bown,C., and Young,L.T. (1999). Differential display PCR reveals novel targets for the mood-stabilizing drug valproate including the molecular chaperone GRP78. Mol. Pharmacol. *55*, 521-527.

Watson,D.G., Watterson,J.M., and Lenox,R.H. (1998). Sodium valproate down-regulates the myristoylated alanine-rich C kinase substrate (MARCKS) in immortalized hippocampal cells: a property of protein kinase C-mediated mood stabilizers. J. Pharmacol. Exp. Ther. *285*, 307-316.

Yu,Z., Luo,H., Fu,W., and Mattson,M.P. (1999). The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Exp. Neurol. *155*, 302-314.

A

B

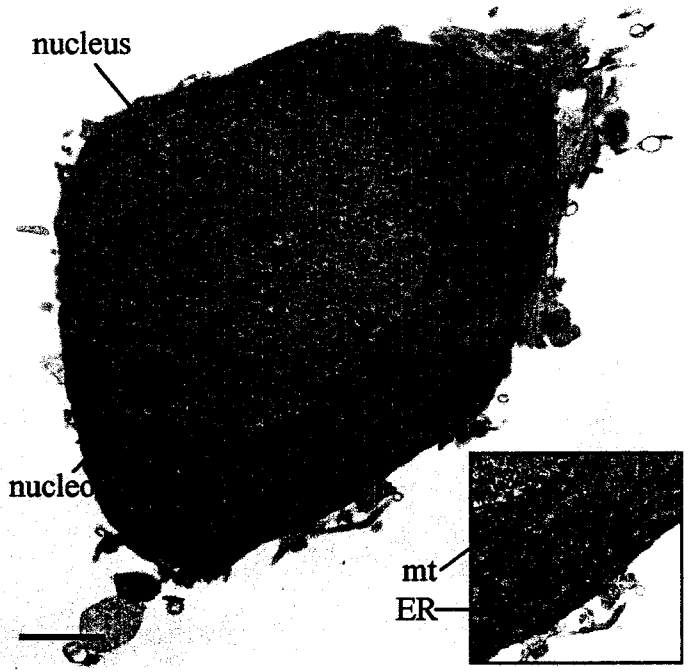


Figure 1. *Microscopic and ultrastructural appearance of rat hippocampal neurons.* Rat hippocampal neurons were grown on poly-D-lysine coated chamber slides in Neurobasal™ media for 13 days. A) Neurons were detected using an NSE primary antibody. Cultures contained <0.2% glia. B) Electron micrograph of a hippocampal neuron. Major subcellular structures are identified; nucleus, nucleolus, mitochondria (mt) and endoplasmic reticulum (ER). Bar = 2 μm.

control
Li
VPA
CBZ
2 μM
control
NMDA

Figure 2. *Pretreatment of rat hippocampal neurons with mood stabilizers prevents NMDA-mediated cytoplasmic vacuolization.* Representative electron micrographs of ultrathin sections of hippocampal neurons. Rat hippocampal neurons were grown in culture for 13 days in media supplemented with no mood stabilizers (control), 1 mM lithium (Li), 0.6 mM valproate (VPA), or 50 μM carbamazepine (CBZ) for the final 7 days. Following pre-treatment, neurons were exposed to 0.5 mM NMDA for 10 minutes followed by 90 minutes of culture in NMDA-free media. Vacuoles are identified by arrows. Bar = 2 μm.

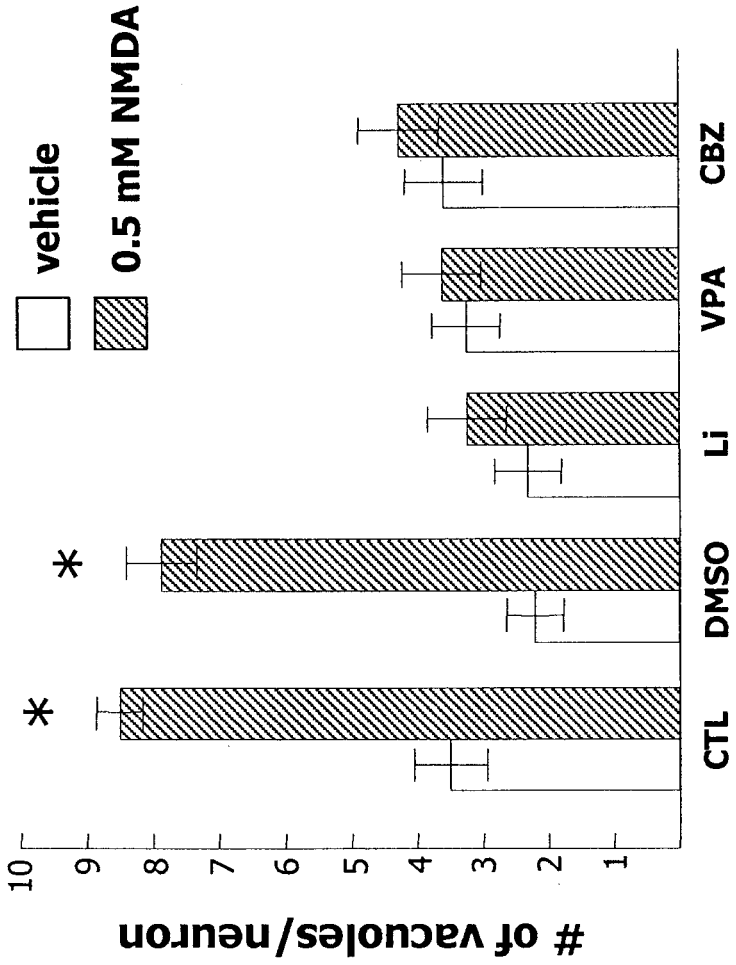

vehicle
0.5 mM NMDA
# of vacuoles/neuron
10
9
8
7
6
5
4
3
2
1
*
*
CTL
DMSO
Li
VPA
CBZ

Figure 3. *NMDA significantly increases the amount of cytoplasmic vacuolization in rat hippocampal neurons, which is protected against by pre-treatment with mood stabilizers.* Rat hippocampal neurons were grown in culture for 13 days in media supplemented with no mood stabilizers (CTL), 0.05% DMSO, 1 mM lithium (Li), 0.6 mM valproate (VPA) or 50 μM carbamazepine (CBZ) for the final 7 days. Following pre-treatment, neurons were exposed to 0.5 mM NMDA for 10 minutes followed by 90 minutes of culture in NMDA-free media. Number of cytoplasmic vacuoles per cell was determined through counting the number of cytoplasmic vacuoles present in 50 randomly chosen neurons by an investigator blind to the sample treatment. The experiments were repeated twice for each condition. Values are expressed as the average ± S.E.M. * $p < 0.05$ significantly different from vehicle treated neurons, determined by unpaired Student's t-test.

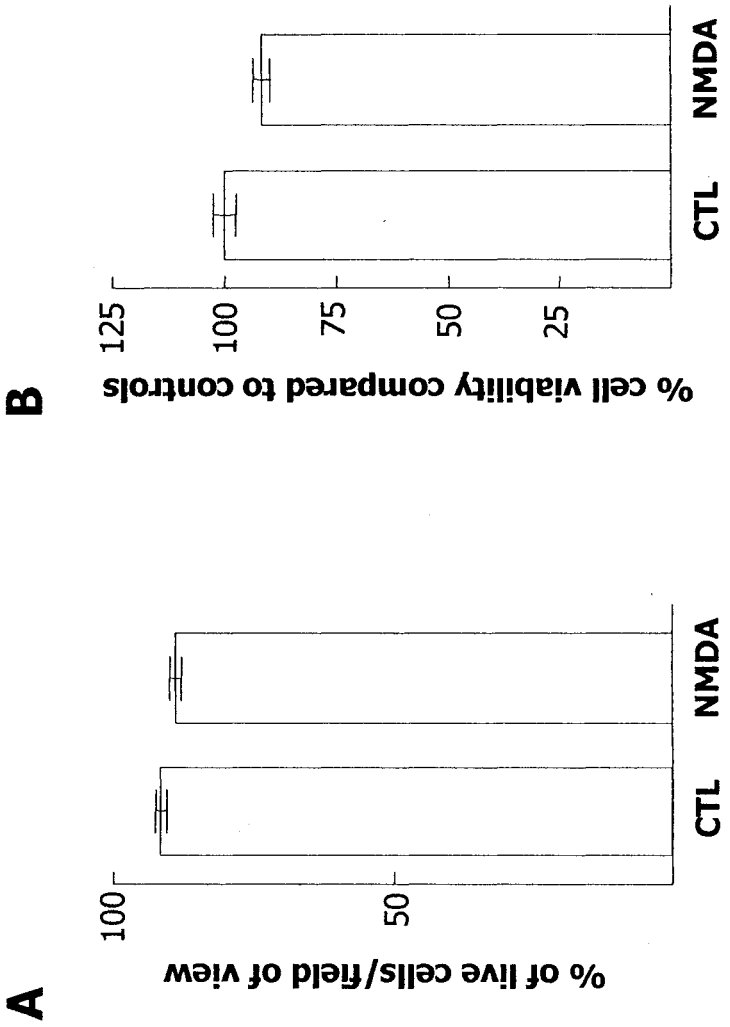

A
% of live cells/field of view
100
50
CTL
NMDA
B
% cell viability compared to controls
125
100
75
50
25
CTL
NMDA

Figure 4. *NMDA-mediated cytoplasmic vacuolization does not significantly reduce cell viability in rat hippocampal neurons.* Rat hippocampal neurons were grown in culture for 13 days in Neurobasal media. NMDA was introduced to the media for 10 minutes at a concentration of 0.5 mM and then the culture was continued for another 90 minutes in NMDA-free media. A) The percentage of live cells was calculated in two randomly chosen fields of view from 4 replications of each group using the LIVE/DEAD cytotoxicity/cell viability kit. Values represent the mean percentage of live cells per field of view ± S.E.M. B) Cell viability was determined by measuring absorbance of formazan at 540 nm produced by the cleavage of MTT by mitochondrial enzyme succinate-dehydrogenase. Values represent the mean percentage of cell viability with controls representing 100% cell viability ± S.E.M.

**3.6** Caspase 3 Activation and DNA Fragmentation are not Associated with NMDA-Mediated Cell Death in Primary Rat Hippocampal Neurons

Christopher D. Bown, L. Trevor Young

***Rationale:*** Our previous studies showed that NMDA could induce cytoplasmic vacuolization in primary rat hippocampal neurons, which could be completely blocked when neurons were treated for 7 days prior to the excitotoxic insult with lithium, valproate or carbamazepine. Interestingly, rat hippocampal neurons exposed to 10 minutes of NMDA were highly vacuolated, however, no quantifiable decrease in cell viability could be measured. In this study we questioned whether prolonged exposure (6 hours) to NMDA could produce a loss in cell viability. Moreover, since it has been suggested that cytoplasmic vacuolization is associated with apoptosis, we were interested in measuring classic markers of apoptosis, capsase 3 activity and DNA fragmentation, following NMDA exposure.

***Involvement:*** All research described in this section was conducted by CDB. Supervision was provided by LTY.

# CASPASE 3 ACTIVATION AND DNA FRAGMENTATION ARE NOT ASSOCIATED WITH NMDA-MEDIATED CELL DEATH IN PRIMARY RT HIPPOCAMPAL NEURONS

## ABSTRACT

Recent imaging and post-mortem studies have shown that mood disorders, bipolar disorder and major depressive disorder, are associated with cell loss in vulnerable brain regions. The ability of mood stabilizers, lithium, valproate and carbamazepine to regulate genes that have known cytoprotective properties accentuates the importance of studying how mood stabilizers can regulate cell death pathways and protect neurons against cell damage and death. In a recent study, we showed that rat hippocampal neurons pretreated with mood stabilizers could completely prevent NMDA-mediated cytoplasmic vacuolization. Since cytoplasmic vacuolization has been shown to be a morphological feature of apoptosis, we were interested in determining whether NMDA could activate classic apoptotic pathways in rat hippocampal neurons. Although rat hippocampal neurons exposed to 1 mM NMDA for 6 hours did have a significant reduction in cell viability, markers of apoptotic cell death, such as caspase 3 activity and DNA fragmentation could not be detected. These results would suggest that, in primary rat hippocampal neurons, NMDA causes cell death via some other mechanism than those described in classic apoptotic models.

# INTRODUCTION

The need for long-term pharmacotherapy in bipolar disorder and the high relapse rate associated with non-compliance suggest that lasting changes in gene expression may account for the mechanism of action of mood stabilizers (Post, 1992a). Identifying those genes regulated by the commonly prescribed mood stabilizers, lithium, valproate (VPA) and carbamazepine (CBZ), has been an area of extensive investigation in the field of bipolar disorder research. Results from a number of independent laboratories have shown that mood stabilizers regulate the expression of various neuroprotective or apoptotic factors i.e. GRP78, Bcl-2, Bax, p53, GSK-3β (Bown et al., 2000b; Chen et al., 1999c; Chen and Chuang, 1999; Wang et al., 1999b). Moreover, mood stabilizers have been shown to protect various neuronal cell lines against cytotoxic cell death (Mora et al., 1999; Chen and Chuang, 1999). We have recently shown that cultured rat hippocampal neurons exposed to NMDA had a two-fold increase in the amount of cytoplasmic vacuolization (Bown et al., 2002). Pretreatment of rat hippocampal neurons with lithium, VPA or CBZ for 7 days protected neurons against NMDA-mediated cytoplasmic vacuolization. Interestingly, NMDA-mediated cytoplasmic vacuolization was not shown to be associated with any reduction in cell viability.

Cytoplasmic vacuolization is a phenomenon commonly described in the literature, yet the mechanism responsible for this morphological characteristic has been elusive

(reviewed by Henics and Wheatley, 1999). Previous studies have shown that activation and blockade of the NMDA receptor can mediate cytoplasmic vacuolization (Goldberg and Bateman, 1993); Freeman and Goldberg, 1994). In neurons exposed to NMDA, a significant increase in cytoplasmic vacuolization was visualized by confocal microscopy staining for microtubule networks (Goldberg and Bateman, 1993). In a follow-up study this group also showed that treatment with neurotoxic concentrations of the NMDA receptor antagonist MK-801 also caused significant vacuolization (Freeman and Goldberg, 1994). These results suggest that severe disruption of normal $Ca^{2+}$ homeostasis, mediated by the NMDA receptor, can result in cytoplasmic vacuolization and possible neurotoxicity.

Cytoplasmic vacuolization has been shown to be a morphological characteristic of apoptosis (Clarke, 1990). Apoptosis, or programmed cell death, has been implicated in a number of human diseases, including: Huntington's, Alzheimer's, Parkinson's and dementia (Davies and Ramsden, 2001; Engidawork et al., 2001; Hartmann and Hirsch, 2001). The term apoptosis was first used by Kerr and colleagues (Kerr et al., 1972) to described a basic cellular phenomena where cells shrink, present cell surface molecules that are usually not present, and undergo nuclear changes such as DNA fragmentation. Since the early morphological characterization of apoptotic cells, a great deal has been learned about the biochemical changes that occur during apoptosis. The most commonly studied pathways of apoptotic cell death are the death receptor- and mitochondrial-mediated pathways. However, these might not be the only apoptotic pathways, since recent studies have shown that the endoplasmic reticulum may in fact be able to initiate

the apoptotic cascade (Rao et al., 2001). Death receptor mediated apoptosis is activated when ligands, such as CD95 and TNF bind to death receptors, causing the eventual activation of caspase 8, which acts as a link between the death receptor and mitochondrial-mediated apoptotic pathways (Budihardjo et al., 1999). Caspase 8 cleaves the pro-apoptotic factor, Bid, allowing it to translocate to the surface of the mitochondrial outer membrane. The biochemical events associated with mitochondrial mediated apoptosis are likely triggered by cytotoxic insults, such as activation of NMDA gated $Ca^{2+}$ influx (Sugawara et al., 2002). Much of the $Ca^{2+}$ that enters the cell following NMDA-receptor activation is sequestered by the mitochondria, causing a depolarizing effect on the mitochondrial membrane (Duchen, 2000). Members of the bcl-2 pro-apoptotic family (i.e. Bax, Bak, Bad, Bid and Bim) that are inactive in normal cells become activated to form the membrane permeability transition pore (PTP) connecting the cytosol with the lumen of the mitochondria. The PTP provides a channel for the exchange of ions and molecules between the cytosol and the luminal space of the mitochondria. The PTP also allows for factors sequestered within the lumen of the mitochondria, such as cytochrome c, to be released into the cytosol (Budd et al., 2000). Cytochrome c, when released into the cytoplasm, forms a heterodimer with apoptosis protease activating factor-1 (Apaf-1) causing the cleavage of pro-caspase 9 into active caspase 9 (Budihardjo et al., 1999). Activation of caspase 9 initiates the mitochondrial-mediated caspase cascade. Caspase 9 then cleaves pro-caspase 3 into active caspase 3, which is thought to be the so-called "point of no return" for apoptosis, subsequently leading to cell death. The endpoint for the biochemical events associated with apoptosis

occurs when caspase 3 activates caspase-activated deoxyribonuclease (CAD), which translocates to the nucleus to degrade chromosomal DNA (Cao et al., 2001).

In this study we wanted to further examine the results of our previous study that showed primary rat hippocampal neurons exposed to NMDA had a significantly higher amount of cytoplasmic vacuolization compared to controls and those neurons pretreated with mood stabilizers. Cytoplasmic vacuolization has been shown to be associated with activation of the apoptotic pathway (Clarke, 1990), therefore we questioned whether the morphology we observed using electron microscopy was the beginning of apoptosis. Since activation of caspase 3 has been shown to be a common downstream target of different apoptotic pathways (Brecht et al., 2001), we studied its activity in primary rat hippocampal neurons following 6 hours of NMDA exposure. We also studied the endpoint of apoptosis, DNA fragmentation, by visualizing the presence of TUNEL-positive cells following NMDA exposure. Neither measure of apoptosis provided any evidence to suggest that NMDA is causing activation of a caspase 3-mediated apoptotic pathway in rat hippocampal neurons.

## MATERIALS AND METHODS

### *Primary rat hippocampal culture*

Rat hippocampi were isolated from 16-18 day old rat fetuses, as originally described by Banker and Cowan (Banker and Cowan, 1977). Hippocampal neurons were

dispersed and cultured following the procedure detailed by Brewer and colleagues (Brewer et al., 1993). Briefly, two hippocampi were isolated from each fetus, located by using the hippocampal fissure and the developing fimbria as landmarks. Cells were mechanically dispersed in $Ca^{2+}$- and $Mg^{2+}$-free Hank's Balanced Salt Solution (HBSS: Sigma, St. Louis, MO USA) supplemented with 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). An equal volume of supplemented HBSS containing $Ca^{2+}$ and $Mg^{2+}$ was added to the cell suspension. Cells were collected by centrifugation at 1,000 x *g* for 4 minutes. Cells were resuspended in Gibco Neurobasal™ media (Invitrogen Canada Inc, Burlington ON, Canada) containing B27 supplements (Invitrogen Canada Inc, Burlington ON, Canada), 0.5 mM L-glutamine, 1% penicillin/streptomycin and 25 μM glutamate. An equal concentration of cells were plated on poly-D-lysine (MW 30,000 – 70,000) coated vessels and maintained at $37^0$C in a 5.0% $CO_2$ humid environment. Cells were plated at a density of 50,000 cells/$cm^2$. Following four days of culture an equal volume of fresh Neurobasal™ media without glutamate was added to each plate and culture maintained for two more days. On the sixth day, media was removed and replaced with fresh glutamate-free Neurobasal™ media and further cultured for 7 days.

*MTT assay*

Cell viability was determined by measuring the cleavage of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis MO, USA), into blue-coloured formazan by the mitochondrial enzyme succinate-

dehydrogenase, with slight modifications from the originally described procedure (Manji et al., 2000a; Mosmann, 1983). Rat hippocampal neurons were cultured on 12-well poly-D-lysine (MW 30,000-70,000) coated plates (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Culture media was removed and cells were rinsed with PBS. Cells were then cultured for one hour in media supplemented with 125 μg/ml MTT. Following incubation, media was aspirated and cells were dissolved in 200 μl DMSO and the plates were vigorously shaken for 5 minutes. Three aliquots of 50 μl DMSO were removed from each well and transferred to a 96-well plate (Nalge Nunc International, Rochester NY, USA). The optical density was measured using an automatic Multiskan Plus Version 1.43 plate reader (Labsystems Inc, Franklin MA, USA) with absorbance set at 540 nm. The mean absorbance for the three aliquots constituted the total absorbance of the sample.

*Caspase 3 activity*

Caspase-3 activity was quantified using the EnzChek® Caspase-3 activity kit and the protocol described by the manufacturer (Molecular Probes Inc, Eugene OR, USA). Rat hippocampal neurons were cultured on 12-well poly-D-lysine (MW 30,000-70,000) coated plates (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were lysed in the supplied cell lysis buffer, using repeated freeze-thaw cycles. Cells were then centrifuged (5,000 rpm for 5 minutes) and the supernatant transferred to a 96-well microplate (Nalge Nunc International, Rochester NY, USA). An equal volume

of reaction buffer (20 PIPES, pH 7.4, 4 mM EDTA, 0.2% CHAPS) containing 0.2 mM Z-DEVD-AMC substrate was added to each well and incubated at room temperature and protected from light for 30 minutes. Fluorescence was measured in a CytoFluor II Fluorescence microplate reader (Applied Biosystems, Foster City CA, USA) with the excitation and emission spectra set to 350/450 nm.

*TUNEL-staining*

The ApopTag® peroxidase *in situ* apoptosis detection kit (Intergen Company, Purchase, NY USA) was used to detect the presence of fragmented deoxyribonucleic acid, following the protocol described by the manufacturer. Cells were grown on poly-D-lysine coated Lab-Tek 2-Chamber Slides (Nalge Nunc International, Rochester NY, USA) for a total of 13 days. Cells were fixed in 1% paraformaldehyde in PBS, pH 7.4 for 1 hour at $37^0$C. Following incubation, cells were further washed in PBS. Endogenous peroxidases were quenched by incubating cells at room temperature in 3.0% hydrogen peroxide in PBS for 5 minutes. Cells were then incubated at room temperature for 10 seconds with the supplied equilibration buffer. TdT enzyme was then added to each slide and incubated in a humidified chamber at $37^0$C for one hour. The reaction was then stopped by incubating the slides at room temperature for 10 minutes in the stop/wash buffer supplied by the manufacturer. Samples were then washed three times in PBS, and then incubated in a humidified chamber at room temperature for 30 minutes with anti-digoxigenin-peroxidase conjugate. Following incubation, cells were washed in PBS, and

then exposed to peroxidase substrate for 5 minutes at room temperature. Samples were then washed in $dH_2O$, and then incubated in $H_2O$ at room temperature for 5 minutes. Slides were countered stained in 0.5% methyl green for 10 minutes at room temperature, and then further washed in $dH_2O$. A final wash in 100% N-butanol for 30 seconds completed the procedure. Coverslips were mounted to the slides using Aquamount (BDH Laboratory Supplies, Poole, Dorset, England). Images were digitized using a Bioquant Pure Color Windows 98 imaging system (R&M Biometrics, Nashville, TN, USA) attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany).

*Data and statistical analysis*

All data analysis was conducted using Minitab version 12 (Minitab Inc., State College PA, USA) on an International Business Machines personal computer running a Windows based operating system. Statistical analysis was conducted on raw data and converted to percentages only for graphical display. Statistical differences were detected using Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparisons test.

RESULTS

Previous studies have shown that rat hippocampal neurons exposed to NMDA for 10 minutes produced a two-fold increase in cytoplasmic vacuolization. Interestingly, this

morphology was not associated with reduced cell viability (Bown et al., 2002). To determine whether prolonged NMDA exposure would eventually reduce cell viability, we exposed rat hippocampal neurons to 0.25, 0.5 and 1 mM NMDA for 6 hours. Another group of neurons were exposed to 0.3 μM staurosporine (STS), a known activator of neuronal apoptosis, for the same period of time. Prolonged exposure of hippocampal neurons to both NMDA and STS significantly reduced the amount of cell viability compared to controls, as determined by measuring the cleavage of MTT into blue-coloured formazan by the mitochondrial enzyme succinate dehydrogenase (Figure 1).

Since reduced cell viability can be caused by activation of caspase-dependent apoptosis, we investigated the activity of an effector caspase, caspase-3, following exposure to 1 mM NMDA and 0.3 μM STS for 6 hours. NMDA did not increase the enzymatic activity of caspase-3, whereas STS, which has been previously shown to activate caspase 3 (Bijur et al., 2000), increased the activity of caspase-3 four-fold (Figure 2).

Another hallmark of "classic" apoptosis is the presence of fragmented DNA (Fiedorowicz et al., 2001). To investigate whether NMDA caused apoptosis through a caspase-independent pathway we used TUNEL staining to visualize fragmented DNA. Hippocampal neurons were exposed to 1 mM NMDA or 0.3 μM STS for 6 hours. STS treatment resulted in the presence of TUNEL-positive nuclei in rat hippocampal neurons, whereas NMDA did not cause DNA fragmentation (Figure 3).

## DISCUSSION

We have previously shown that rat hippocampal neurons exposed to NMDA have dramatic increases in cytoplasmic vacuolization, which is not associated with any significant reduction in cell viability. In this study, we investigated whether longer exposure (6 hours) to NMDA could affect cell viability and whether this cell death was the result of caspase-mediated (or classic) apoptotic pathways. Rat hippocampal neurons exposed to NMDA for 6 hours had a significant reduction in cell viability, which was not associated with activation of caspase 3 or DNA fragmentation, two hallmark characteristics of apoptosis (Kerr et al., 1972). Staurosporine, a known activator of caspase-mediated apoptosis showed reduced cell viability, with activation of caspase 3 and DNA fragmentation. These results suggest that NMDA causes cell death via some other mechanism than those events described for the classical apoptosis continuum.

Prolonged exposure of rat hippocampal neurons to NMDA significantly reduced cell viability, as determined by measuring the cleavage of MTT into the blue-coloured formazan. NMDA has been shown in a number of different studies to cause cell death (Almli et al., 2001; Prendergast et al., 2001; Tai et al., 2001). A sudden influx of Ca2+ caused by NMDA receptor activation is thought to be the initial event that eventually leads to cell death. Much of the $Ca^{2+}$ that enters the cell following NMDA-receptor activation is sequestered in the mitochondria, until the mitochondria reaches threshold (Wang and Thayer, 1996). This accumulation will eventually lead to depolarization of the mitochondria membrane and activation of the permeability transition pore (PTP), which returns $Ca^{2+}$ back to the cytoplasm (Schild et al., 2001). In addition to releasing

$Ca^{2+}$ in the cytoplasm, reactive oxygen species are also created and along with cytochrome c are released into the cytoplasm (Budd et al., 2000; Mora et al., 1999). The liberation of these mitochondrial factors results in a number of damaging events, however, the most critical event for activation of caspase-mediated apoptotic pathway is the release of cytochrome c (Junn and Mouradian, 2001). Cytochrome c, when released into the cytoplasm, forms a heterodimer with apoptosis protease activating factor-1 (Apaf-1) causing the activation of caspase 9 (Junn and Mouradian, 2001). Activated caspase 9 subsequently cleaves the cysteine protease pro-caspase 3 into active caspase 3.

We next studied the activity of caspase 3, following exposure of rat hippocampal neurons to NMDA. Since initiation of the caspase cascade can occur through the mitochondria, endoplasmic reticulum or via activation of death receptors, studying caspase 3 activity allows for detection of apoptosis regardless of which initiation pathway was activated (Brecht et al., 2001). The activation of caspase 3 results in the cleavage of various substrates within the cytoplasm (cytoskeletal molecules, PKC, phospholipase, CaMKII) and the nucleus (CaMKIV, ADPRT/PARP, caspase 6) (Budd et al., 2000; Mora et al., 1999; Wang, 2000). Following 6 hours of exposure to 1 mM NMDA, we could not detect any significant activation of caspase 3, whereas STS, a potent inhibitor of phospholipid/$Ca^{2+}$ dependent protein kinase C (Tamaoki et al., 1986), could significantly increase the activity caspase 3, which is in agreement with other studies (Bijur et al., 2000). Studies have shown that activation of caspase 3 by NMDA might depend on the concentration of NMDA in the media, the length of exposure, as well as the cell line being examined. A recent study by Thomas and Mayle (2000) showed that rat cortical

neurons exposed to 0.5 mM NMDA for 20 hours resulted in neuronal death without caspase 3 activation. In contrast, Tenneti and Lipton (Tenneti and Lipton, 2000) showed that rat cortical neurons exposed to 0.3 mM NMDA from 20 minutes to 24 hours caused cell death via caspase 3 activation. A study by Bhave et al. (1999) showed that rat cerebellar granule neurons treated with 100 μM NMDA for 24 hours reduced the amount of caspase 3 activity and appeared to protect neurons against $K^+$-induced apoptosis. These studies show that NMDA can have significant and varied effects on cell viability, depending on the system being studied.

A cytopathological hallmark of apoptosis is the appearance of fragmented DNA. Activation of caspase 3, in neuronal cells, causes activation of caspase-activated deoxyribonuclease (CAD), which in turns degrades DNA. Although DNA fragmentation is commonly associated with caspase 3 activation, some studies have shown that DNA laddering, or fragmentation, can occur independent of caspase 3 activity (Hamabe et al., 2000). To determine whether NMDA exposure of rat hippocampal neurons resulted in caspase 3-independent DNA fragmentation, we used TUNEL staining to identify fragmented DNA. The results show that NMDA treatment did not result in DNA fragmentation, whereas STS significantly increased the number of TUNEL-positive cells. A recent study by Volbracht et al. (2001) showed that blocking caspase activity in rat cerebellar granule cells can still result in DNA fragmentation following cytotoxic insult. The authors suggest that one of the possible explanations for their observations is that blocking caspase activation could result in activation of other proteases such as calpains, cathepsins, or proteosome and serine proteases. It is unknown within our system whether

NMDA is activating one or more of these caspase-independent factors, which are responsible for cell death without any biochemical characteristics of apoptosis.

The results of this study would propose that exposure of primary rat hippocampal neurons to NMDA for 6 hours results in cell death not characterized by classical markers of apoptosis, i.e. caspase 3 activation and DNA fragmentation. The most likely explanation for these results would be that NMDA exposure causes a necrotic type of cell death. However, two morphological characteristics associated with necrotic cell death, membrane blebbing and cell rupture (Lemasters et al., 1983; Lemasters et al., 1987) are not present following both acute and chronic exposure to NMDA. Using classic models of necrosis, oxygen deprivation and metabolic inhibition, Lemasters et al. (Lemasters et al., 1983) showed that one of the earliest events of necrosis was characterized by plasma membranes containing membrane protrusions or blebs. They later showed that membrane blebbing was reversible, but cell death resulted once one of the blebs ruptured (Lemasters et al., 1987). In a previous study, we used electron microscopy to characterize the ultrastructural appearance in rat hippocampal neurons following exposure to 0.5 mM NMDA for 10 minutes (Bown et al., manuscript submitted). We did not observe any changes in plasma membrane integrity following NMDA exposure. Moreover, cell viability assays that rely on the loss of membrane integrity did not show any change in membrane permeability following NMDA treatment (Bown et al., manuscript submitted). Even after exposing rat hippocampal neurons to 1 mM NMDA for 6 hours, no signs of cell rupture could be detected in neurons counterstained with methyl green in the TUNEL staining experiment. Although further studies using flow

cytometry and confocal microscopy will likely have to be conducted to unequivocally show that NMDA is not causing necrotic cell death, preliminary results would suggest that NMDA is not causing necrotic cell death.

The many faces of cell death following excitotoxic insults has led many researchers to suggest that the classic events of necrosis and apoptosis are the two ends of a spectrum of events that can lead to cell death (Leist and Nicotera, 1997). Therefore, glutamate-mediated cell death could be a separate entity that encompasses mechanisms from both necrotic and apoptotic cell death (reviewed by Choi, 1992). In this view of cell death, $Ca^{2+}$ plays a central role in determining the mechanism of cell death. During initial stimulation, $Ca^{2+}$ enters the cell and triggers many of the events associated with the early events of apoptosis (i.e. mitochondrial $Ca^{2+}$ overload, and activation of $Ca^{2+}$-activated enzymes). Recovery of the cell from the insult often results once the stimulus is removed. However, if the stimulus remains, then completion of apoptosis could result, with all of its characteristics. Choi (1992) proposes a three-stage model to describe this phenomenon. In the first stage, induction, intracellular ionic concentrations are disturbed as the result of glutamate stimulation. During this stage, if the stimulus is removed from the media, there is a greater likelihood of cell survival. The next stage, amplification, results in those events of induction translating into activation of certain enzymes and release of $Ca^{2+}$ from intracellular stores. Full cellular recovery from this stage is also possible. During the final stage, expression, recovery is unlikely, as proteases are activated and reactive oxygen species are generated. The events proposed in this model are loosely based on the events of apoptosis and therefore hallmarks of apoptosis may be

identifiable. In contrast, prolonged or high glutamate stimulation may result in an overload of $Ca^{2+}$ that cannot be processed in an systematic manner, and thus causes the cell to show some characteristics of necrosis. Interestingly, the critical concentration or exposure length may be controlled by a number of factors, including the cell type, the age of the cell, and extracellular ionic concentrations (Sastry and Rao, 2000).

A possible explanation for the results we observed in our system are that rat hippocampal neurons grown in culture respond to NMDA stimulation by a mechanism that falls somewhere in between classical apoptosis and necrosis. Cell death may be the result of activation of other proteases, such as calpain and calpathesin, causing degradation of cytoplasmic substrates and disruption of normal cellular function independent of activation of caspase 3, DNA fragmentation, membrane blebbing and rupture. Further studies, examining the time course of cell death following NMDA exposure in rat hippocampal neurons, activation of other proteases and a closer investigation of membrane integrity will have to be conducted to show that NMDA does not activate classic apoptotic or necrotic pathways.

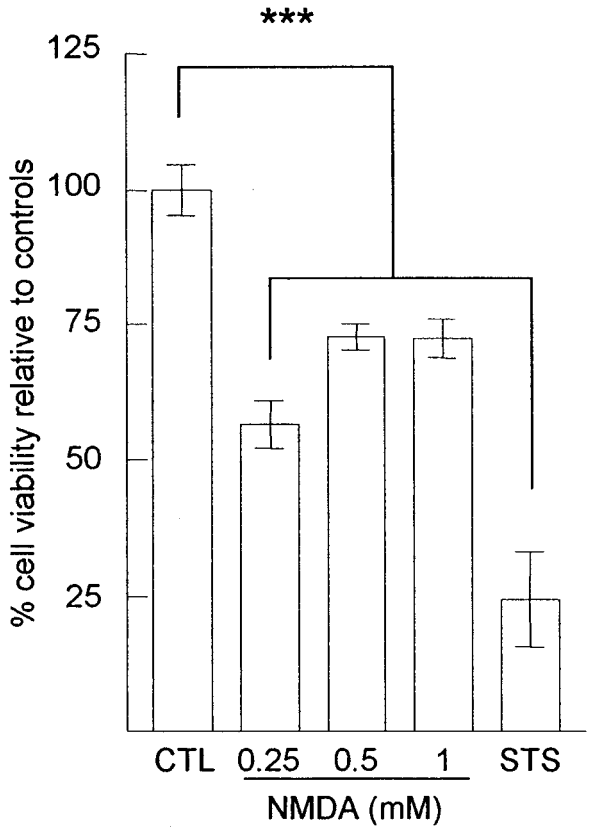

***
125
100
75
50
25
% cell viability relative to controls
CTL
0.25
0.5
1
STS
NMDA (mM)

**Figure 3.6.6.1.** Prolonged exposure to NMDA reduces cell viability in rat hippocampal neurons. Rat hippocampal neurons were grown in culture for 13 days and then exposed to 0.25, 0.5, 1 mM NMDA, or 0.3 μM staurosporine (STS) for 6 hours. Control (CTL) neurons did not receive NMDA or STS exposure. Cell viability was assessed following the treatment period using the MTT assay for cell viability. The absorbance of formazan at 540 nm was measured as an indication of mitochondrial function. All values are expressed as percent cell viability relative to the control samples and are expressed an average of at least 6 samples ± S.E.M. *** $p < 0.001$ significantly different from controls as determined by one-way analysis of variance followed by Dunnett's test for multiple comparisons comparing 0.25, 0.5 and 1 mM NMDA to CTL. Statistical significance between CTL and STS was determined using Student's t-test.

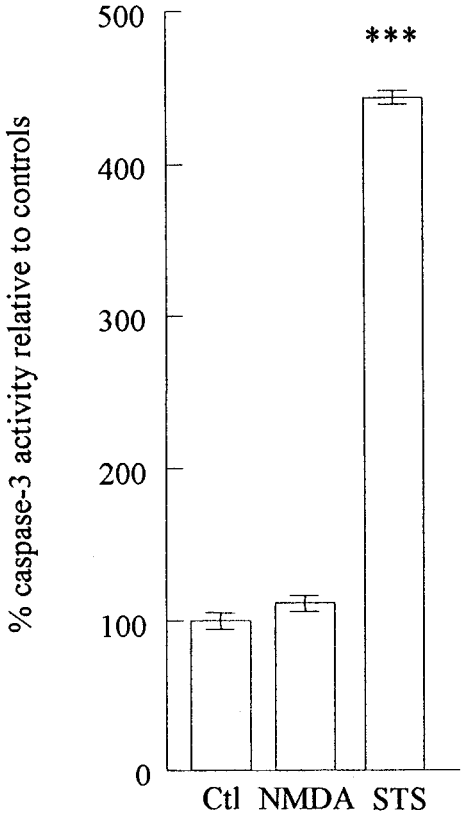

500
400
300
200
100
0
% caspase-3 activity relative to controls
***
Ctl
NMDA
STS

**Figure 3.6.6.2.** Staurosporine, but not NMDA, increases caspase-3 activity in rat hippocampal neurons. Rat hippocampal neurons were grown in culture for 13 days and then exposed to 1 mM NMDA or 0.3 μM STS for 6 hours. Caspase-3 activity was determined by measuring the emission of Ac-DEVD-AMC, a fluorescently tagged substrate of caspase-3 (ex/em: 350/450 nm). All values are expressed as percent caspase-3 activity relative to the control samples and are shown as the mean of 4 samples ± S.E.M. *** $p < 0.001$ significantly different from controls as determined by Student's t-test.

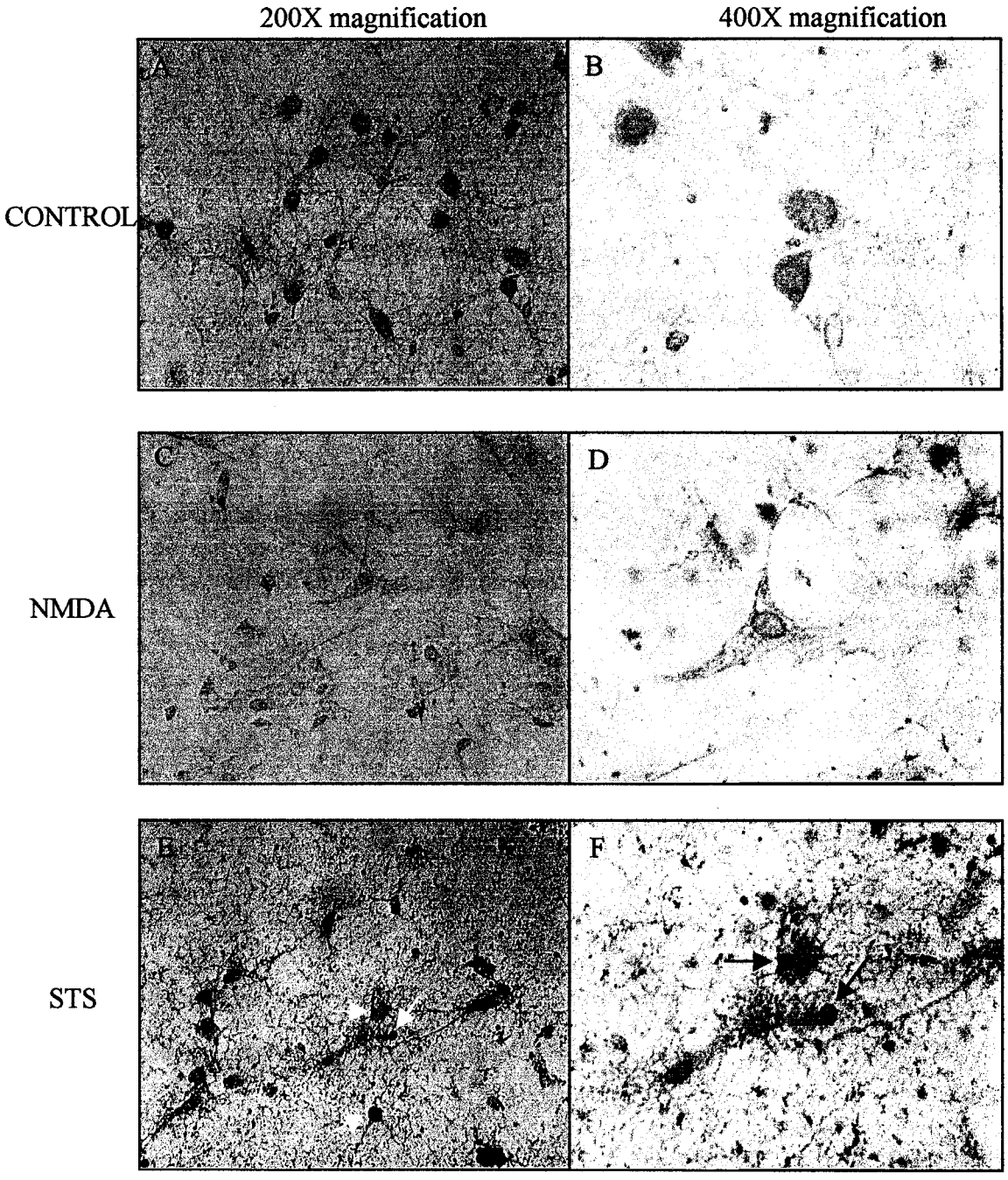

200X magnification
400X magnification
CONTROL
NMDA
STS
A
B
C
D
E
F

**Figure 6.3.3.3.** Staurosporine, but not NMDA, induces DNA fragmentation in rat hippocampal neurons. Rat hippocampal neurons were grown in culture for 13 days and then exposed to 1 mM NMDA (C, D) or 0.3 μM STS (E, F) for 6 hours. Control (CTL; A, B) neurons did not receive NMDA or STS exposure. DNA fragmentation as detected by TUNEL staining is shown by arrows. Neurons were counterstained with 0.5% methyl green to detect cell structure.

## 4.0 Discussion

The chronicity of bipolar disorder (BD) and the need for long-term prophylactic pharmacotherapy in the majority of patients have focussed our attention on the study of processes such as the regulation of gene expression. The work of our group and others has employed novel techniques to identify potential gene targets for mood stabilizing drugs. Our lab has extensively used differential display polymerase chain reaction (DD-PCR) to identify targets of sodium valproate and lithium (Wang and Young, 1996; Wang et al., 1999a; Wang et al., 1999b). Using this method we identified a new target for valproate and possibly other mood stabilizers, the ER stress proteins, especially the 78 kDa glucose regulated protein (GRP78).

Rats treated for 21 days with 300 mg/kg valproate revealed differential expression of 6 mRNA fragments in the cerebral cortex compared to saline treated rats (section 3.1). One of these fragments had 100% homology to the rat GRP78 gene. A similar increase in expression was also observed in rat C6 glioma cells treated with valproate. GRP78 expression was shown to be both dose- and time-dependent, with significant increases measured following 7 days of treatment with 1 mM valproate. Expression of GRP78 returned to basal levels within 6 hours following removal of the drug from the culture media, suggesting that drug treatment did not result in irreversible damage to the ER. This increase in GRP78 mRNA was accompanied by a dose-dependent increase in GRP78 protein expression and increased transcription of the GRP78 gene. Treatment of rat C6 glioma cells with lithium and carbamazepine revealed that this effect on GRP78

expression was not shared by lithium and may only occur after treatment with higher concentrations of carbamazepine.

The regulation of GRP78 by the mood stabilizing drug, valproate, is quite interesting in light of the important functions of GRP78 in neurons. GRP78, also known as immunoglobulin binding protein (BiP), was originally discovered in response to glucose deprivation in cultured cells (Shiu et al., 1977). Later studies showed that the upregulation of GRP78 by glucose deprivation served to protect the endoplasmic reticulum (ER) against the deleterious effects of damaged proteins which accumulate under these conditions either by preventing their aggregation or aiding in their solubilization (Munro and Pelham, 1986). The role of GRP78 as a molecular chaperone in the ER was further advanced by Brostrom et al. (1990). These investigators showed that when $Ca^{2+}$ was removed from the culture media, essentially all new synthesis of proteins was halted until the expression of GRP78 was increased. Increased GRP78 expression proved to be sufficient for protein translation to proceed without delay (Brostrom et al., 1990). These results suggest that cells require GRP78 not only to continue processing existing proteins, but its expression may also be required to properly process those proteins that may be damaged from a cytotoxic insult. Similar observations were also made when other chemical insults, such as tunicamycin, which inhibits N-linked glycosylation, and the $Ca^{2+}$ ionophore A23187 were added to culture media (Dorner et al., 1992a; Drummond et al., 1987; Dorner et al., 1992b).

Localization of GRP78 to the lumen of the ER, and its regulation by agents known to disrupt $Ca^{2+}$ homeostasis within the cell, has led investigators to examine the

$Ca^{2+}$-binding ability of GRP78. GRP78 has high capacity but low affinity for this bivalent cation (Koch et al., 1986). The $Ca^{2+}$-binding ability of GRP78 may be vitally important in protecting cells against cell damage and death. In epithelial cells exposed to *tert*-butylhydroperoxide (TBHP), cells that overexpressed GRP78 were able to maintain $Ca^{2+}$ homeostasis and prevent cell death (Liu et al., 1998a). This is further supported by a more recent study by Yu and colleagues (1999) who showed that blocking GRP78 expression with antisense techniques increased the sensitivity of primary hippocampal cultured neurons to glutamate-mediated changes in intracellular $Ca^{2+}$ and cell damage. A recent biochemical study by Rao and colleagues (2002) reported that the cytoprotective role of GRP78 may in part be due to its ability to form a complex with caspase-7 and capase-12, thus preventing the release of caspase-12 from the ER.

In addition to GRP78, another glucose-regulated protein with a molecular weight of 94 kDa (GRP94) has been identified (Lee et al., 1981). GRP94 has been shown to have many of the same characteristics as the 78-kilodalton form (Lee et al., 1984), with three notable exceptions. First, it has the unusual property of exisiting as both a transmembrane and luminal protein (Kang and Welch, 1991) Second, unlike GRP78, which associates transiently with nascent proteins and at an early stage of processing, GRP94 forms a stable complex and assists at a more advanced step in folding and assembly (Melnick et al., 1992). Finally, GRP94 may not be as effective as GRP78 in protecting a cell against damage from certain insults. Studies using antisense GRP94 constructs showed that vulnerability of the cell to toxic agents was not affected (Yu et al., 1999). In addition to the GRPs, calreticulin, another ER luminal protein known to have

both $Ca^{2+}$ binding and molecular chaperone functions, has also been identified. Although originally described as a major ER $Ca^{2+}$ binding protein, more recent evidence has shown that calreticulin also has molecular chaperone properties (reviewed by (Krause and Michalak, 1997a). Since all three of these proteins, GRP78, GRP94 and calreticulin, respond to cellular stress, they are commonly referred to as the ER stress proteins (Liu et al., 1998a).

Since we had identified GRP78 as a valproate-regulated gene, we were interested in examining whether valproate could also regulate other members of the ER stress protein family, GRP94 and calreticulin (section 3.2). Indeed, rat C6 glioma cells treated with valproate had significantly higher expression of GRP94 and calreticulin compared to controls. All three proteins, GRP78, GRP94 and calreticulin, showed very similar concentration-and time-dependent increases in expression, and all three returned to basal expression 6 hours following the removal of valproate from the media.

Transcriptional activation of the ER stress proteins is a highly regulated process. The promoter sequences of grp78, grp94 and calreticulin genes are highly conserved and contain numerous consensus consequences that can regulate the transcription of these genes following intracellular disruptions in either $Ca^{2+}$ homeostasis or ER stress (McCauliffe et al., 1992; Yoshida et al., 1998a; Yoshida et al., 1998b). One such consensus sequence shown to be present in all three genes is the ER stress element (ERSE) (Yoshida et al., 1998a). The discovery of the ERSE sequence suggested that all three ER stress proteins could be co-ordinately regulated. The transcription factor ATF-6 was identified as the trans-acting element that binds to the ERSE sequence in the

promoter of the ER stress proteins (Yoshida et al., 1998a). In addition to the activation of ERSE by ATF-6, novel consensus sequences may exist in the promoters of ER stress proteins that become activated in response to specific chemicals, such as ethanol. Miles et al. (1997) showed that ethanol could increase the expression of a variety of genes, including GRP78 and GRP94, in a different pattern than those observed with classical inducers of the GRPs. These investigators went on to show in a later study that a unique ethanol-responsive pathway probably exists to increase the expression of GRP78 and GRP94 (Hsieh et al., 1996). Analogous processes might exist causing valproate to regulate the transcription of the ER stress proteins.

Studies in which members of the ER stress protein family have been overexpressed, reveal a potential neuroprotective role for these proteins (Lee, 2001). Interestingly, a post-mortem brain study conducted by Hamos et al. (1991) showed that in Alzheimer's brain there was a significant increase in the expression of GRP78 in healthy brain tissue surrounding neurofibillary plaques and tangles. The authors propose that this upregulation was associated with the increased requirement of those healthy cells to process malfolded or damaged proteins resulting from disease pathogenesis. These results would suggest that the brain may be capable of upregulating the expression of ER stress proteins in order to compensate for abnormal $Ca^{2+}$ homeostasis or protein aggregation as part of disease pathology.

To study whether ER stress proteins are dysregulated in various psychiatric illnesses compared to controls, we examined the expression of GRP78, GRP94 and calreticulin in post-mortem temporal cortex from age- and sex-matched subjects with a

history of bipolar disorder, major depression, and schizophrenia compared to non-psychiatric, non-neurologic controls (n=15 in each group)(section 3.3). Immunoblot analysis revealed a significant increase in the expression of all three proteins in temporal cortex from major depressive subjects who died by suicide compared to non-suicide major depressives and controls. The results of this study suggests that induction of ER stress proteins in major depression may be an attempt to compensate for the toxic effect of prolonged stress and glucocorticoid release on vulnerable brain regions such as the temporal cortex.

Since ER stress proteins appear to be dysregulated in the brains of major depressives who commit suicide and some or all members of the ER stress proteins family may regulate glucocorticoid-mediated gene transcription (Michalak et al., 1996), we questioned whether other psychotropic drugs could also regulate the expression of GRP78 and calreticulin (section 3.4). Therefore, we treated rat C6 glioma cells with a tricyclic antidepressant (desipramine), a serotonin-norepinephrine reuptake inhibitor (venlafaxine) and monoamine oxidase inhibitor (tranylcypromine) and measured the mRNA levels of GRP78 and calreticulin. No significant dose- or time-dependent increases in either GRP78 or calreticulin could be measured following treatment with any of the antidepressants.

Since the psychotropic drugs we tested appear not to significantly regulate the expression of ER stress proteins, the results from this study suggest that the regulation of ER stress proteins by valproate may be either drug- or drug class-specific. In a previous study we showed that lithium was unable to regulate the expression of GRP78, whereas

carbamazepine at higher doses could significantly regulate its expression. Since both valproate and carbamazepine belong to the same class of mood stabilizers (i.e. mood stabilizing anticonvulsants), it would be interesting to examine the effect of other mood stabilizing anticonvulsants (i.e. lamotrigine and gabapentin) on the expression of ER stress proteins. In our study, we could not detect any consistent changes in ER stress protein levels following treatment with desipramine, venlafaxine or tranylcypromine, however, it cannot be ruled out that antidepressants do regulate the expression of GRP78 and calreticulin. The major classes of antidepressants were synthesized to either inhibit the reuptake of norepinephrine (tricyclic antidepressants) and serotonin (serotonin reuptake inhibitors) or the catabolism of monoamines (monoamine oxidase inhibitors), and newer antidepressants such as venlafaxine inhibit the reuptake of both norepinephrine and serotonin (atypical antidepressants). Therefore, if rat C6 glioma cells do not produce serotonin and norepinephrine then they would unlikely be a major target of antidepressant treatment. However, we do not anticipate this being the case, since the effects of desipramine treatment on the expression of G proteins has been extensively studied in rat C6 glioma cells, suggesting these drugs do elicit a response in this cell line (Chen and Rasenick, 1995; Donati et al., 2001; Tork, 1990). It unknown whether venlafaxine is capable of regulating gene expression in rat C6 glioma cells. However, since desipramine and fluoxetine, through inhibition of norepinephrine and serotonin reuptake, respectively, have been shown to cause changes in gene expression in C6 glioma cells (Chen and Rasenick, 1995; Donati et al., 2001; Tork, 1990) (Hisaoka et al., 2001), it is possible that venlafaxine will also elicit a response in these cells. The effects

of tranylcypromine and other monoamine oxidase inhibitors have been shown in C6 glioma cells, however, much of the work suggests that treatment may result in damage to the cells (Abakumova et al., 1998; Slamon and Pentreath, 2000; Slamon et al., 2001).

Since we have shown that valproate can regulate the expression of a family of proteins that have specific subcellular localization and provide neuroprotection when overexpressed, we were interested in studying the ultrastructural appearance of rat hippocampal neurons treated with mood stabilizing drugs before and after exposure to NMDA. Electron microscopy showed that mood stabilizers do not alter the ultrastructural appearance of hippocampal neurons, but do provide protection against NMDA-mediated cytoplasmic vacuolization (section 3.5). These observations would suggest that mood stabilizers share a common mechanism of action in preventing cytoplasmic vacuolization caused by $Ca^{2+}$ influxes through NMDA receptor operated-channels.

Although the significance of mood stabilizers preventing cytoplasmic vacuolization is not fully understood, these observations likely indicate that these drugs are capable of preventing or decreasing the cytotoxic effects of NMDA. Other studies have also shown that lithium can protect against NMDA-mediated cell damage. Nonaka et al. (1998) showed that pre-treatment of rat cerebellar granule cells with lithium could inhibit glutamate-mediated $Ca^{2+}$ influx, however, our studies are the first to show the ultrastructural appearance of neurons following pretreatment with lithium and exposure to NMDA. Interestingly, valproate and carbamazepine could also effectively block NMDA-mediated cytoplasmic vacuolization. It is currently unknown whether all three

mood stabilizers have the same mechanism of action in preventing NMDA-mediated cytoplasmic vacuolization, or whether each has a different mechanism of action that produces the same protective outcome. Nonaka and colleagues (1998) propose that lithium may regulate NMDA receptor function by inhibiting glycogen synthase kinase 3β (GSK-3β), which could in turn decrease the phosphorylation state of NMDA subunits. This group has recently shown that lithium reduces phosphorylation at Tyr1472 of the NR2B subunit, which may decrease activity of the receptor (Hashimoto et al., 2002). This particular mechanism of action on NMDA receptor subunits may be shared between both lithium and valproate, since other studies have shown that valproate can also inhibit the activity of GSK-3β (Chen et al., 1999b) and decrease NMDA-mediated excitotoxicity in rat cerebral cortical neurons (Hashimoto et al., 2002).

The results of our study show the utility of electron microscopy to identify early ultrastructural changes following cytotoxic insults, and changes that are not necessarily identifiable using biochemical assays. Although the results of this study clearly show that mood stabilizers prevent NMDA-mediated vacuolization, a few issues should be kept in mind when interpreting the results. First, we only tested reported therapeutic serum concentrations (Birkhimer et al., 1985; Bowden et al., 1996; Hopkins and Gelenberg, 2000) for each of the mood stabilizers in preventing cytoplasmic vacuolization. It is currently unknown whether prevention of cytoplasmic vacuolization by these drugs is dose dependent. Second, the expected loss of intravacuolar material during the preparation of samples for electron microscopy prevented any conclusions to be made about the origin of the vacuoles. Finally, we are unable to conclude unequivocally

whether pre-treatment with mood stabilizers completely prevented NMDA-induced cytoplasmic vacuolization or whether the drugs delayed the presentation of such vacuoles. Further studies using confocal microscopy will have to be conducted to fully understand the magnitude of this protection by mood stabilizers.

Although the morphological appearance of the rat hippocampal neurons following NMDA exposure would appear to suggest that the neurons have compromised cell viability, we could not in fact measure any significant loss of cell viability following NMDA exposure. However, we are not the first to observe this phenomenon, since numerous other studies have reported similar findings in various cell lines (Freeman and Goldberg, 1994; Henics et al., 1993; Henics and Wheatley, 1997; Henics and Wheatley, 1999). Moreover, in most cases vacuolization is fully reversible once the toxic agent has been removed from the culture media (Pollanen et al., 1990). This would suggest that cytoplasmic vacuolization, on its own, is not a measure of cell death but instead is likely an indication that the neuron is under cytotoxic stress. If the stress, in this case NMDA, is not removed from the culture media, cytoplasmic vacuolization may continue to a point where cell viability is compromised.

To address the question of prolonged NMDA treatment on cell survival, we treated rat hippocampal neurons with 1 mM NMDA for 6 hours and measured cell viability using the MTT assay (section 3.6). A significant dose-dependent decrease in cell viability was measured following prolonged NMDA exposure. Since cytoplasmic vacuolization has been suggested to be a characteristic of apoptotic cell death in some cells (Clarke, 1990), and prolonged NMDA exposure resulted in loss of cell viability, we

questioned whether rat hippocampal neurons exposed to NMDA were dying via an apoptotic mechanism. Caspase 3 activation and DNA fragmentation are classic hallmarks of apoptotic cell death, so we measured both following NMDA exposure. Interestingly, neither caspase 3 activity nor DNA fragmentation was shown to be elevated following NMDA treatment in these cells, suggesting that NMDA initiates atypical apoptotic cell death pathways (section 3.6).

*Since mood stabilizers blocked NMDA-mediated cytoplasmic vacuolization, and NMDA-mediated cytoplasmic vacuolization eventually leads to decreased cell viability through a caspase 3 independent mechanism, these results would suggest that mood stabilizers can provide protection against both traditional apoptotic and atypical cell death pathways. Indeed, when we treated rat hippocampal neurons with lithium for 7 days and then exposed the cells to 1mM NMDA for 6 hours, lithium could significantly reduce the loss of cell viability caused by NMDA (data not shown). Since rat hippocampal neurons exposed to 1 mM NMDA for 6 hours did not activate caspase 3 or increase DNA fragmentation, these results would indicate that these cells die through some other mechanism (i.e. calpain activation or necrosis).* In view of this evidence, it is interesting to consider the implications of these findings for the action of mood stabilizers and pathophysiology of BD (reviewed by Bown et al., 2002a). There are several key issues that may be relevant to this discussion. First, a number of studies have consistently found increased intracellular $Ca^{2+}$ levels in blood cells from patients with BD. Second, numerous abnormalities in PI signalling have been identified in tissues from subjects with BD which have been extensively reviewed elsewhere (Atack et al., 1995). Supporting

these data is the fact that lithium and possibly other mood stabilizers regulate the level and activity of a number of key components of this pathway, including: inhibition of the key enzyme inositol monophosphatase and levels of inositol polyphosphates which mobilize ER $Ca^{2+}$ stores in addition to protein kinase C activity and levels. Third, an increasing number of target genes for mood stabilizers have demonstrated or putative roles in neuroprotection. Finally, cellular loss or reduction in cell density in several key brain regions with BD subjects has recently been reported. It is interesting to consider the possibility that any or all of these processes might be targets of increased levels of ER stress proteins due to chronic treatment with mood stabilizers

Studies that have measured intracellular $Ca^{2+}$ levels in platelets from BD subjects have consistently shown that elevated $Ca^{2+}$ levels are associated with the disorder (Dubovsky et al., 1992; Emamghoreishi et al., 1997a; Emamghoreishi et al., 1997b). Although the consequence of this elevation remains unclear, increased intracellular $Ca^{2+}$ has been associated with such adverse cellular events as apoptosis and necrosis (Cebers et al., 1997; Goldberg and Bateman, 1993; Lin et al., 1997a; Velasco and Tapia, 2000a; Goldberg and Choi, 1993; Lin et al., 1997b; Velasco and Tapia, 2000b). It is interesting to speculate that mood stabilizers such as VPA and carbamazepine may be able to regulate intracellular $Ca^{2+}$ levels by increasing the expression of ER stress proteins. Numerous studies have shown that if ER stress proteins are overexpressed, then the $Ca^{2+}$ buffering ability of the ER increases. Mery et al. (Mery et al., 1996) showed that overexpression of calreticulin in *L* fibroblasts leads to increased storage of $Ca^{2+}$ within the ER, possibly without any change in intraluminal free $Ca^{2+}$. They also showed similar

results if the ER $Ca^{2+}$ stores were depleted by the addition of thapsigargin, an irreversible inhibitor of the ER $Ca^{2+}$-ATPase pump. Following thapsigargin exposure, there was less $Ca^{2+}$ flow into the cytosol from the ER in cells overexpressing calreticulin. Increasing the $Ca^{2+}$ binding ability of the ER may be a protective mechanism that allows cells to process cellular stresses that disrupt intracellular $Ca^{2+}$ homeostasis. Brostrom and colleagues (1990) showed that induction of GRP78 and GRP94 restores protein synthesis under conditions where intracellular $Ca^{2+}$ homeostasis is disrupted.

Studies of tissues from BD subjects have shown that the PI-generated second messenger system is likely involved in the pathophysiology of the disorder. Moreover, lithium has been shown to have numerous inhibitory effects on components of the PI-generated pathway, which should theoretically decrease the binding of $IP_3$ to its receptor on the ER and prevent ER $Ca^{2+}$ release. Interestingly, overexpression of calreticulin has been shown to prevent ER $Ca^{2+}$ stores depletion following activation of the PI-generated second messenger system with saturating concentrations of $IP_3$ (Xu et al., 2000). These results indicate that overexpression of calreticulin, and possibly GRP78 and 94, causes more $Ca^{2+}$ to be stored within the ER and prevents its release following activation of the PI-generated second messenger system. Therefore, in BD in which the PI generated second messenger system may be upregulated resulting in increased release of ER $Ca^{2+}$, VPA treatment may result in increased expression of ER stress proteins that could bind ER $Ca^{2+}$ (see Figure 4.0.1). Recently, Hough et al. (1999) reported that lymphocytes and platelets from BD subjects had greater concentrations of intracellular $Ca^{2+}$ compared to

controls following exposure to thapsigargin. These results show that ER $Ca^{2+}$ stores may be more sensitive to neurochemical changes in BD compared to control subjects.

Investigators have also identified that mood stabilizers can provide protection against cell death, possibly through regulation of anti-apoptotic genes. Nonaka and colleagues (1998) were the first to show that chronic lithium treatment could protect rat cerebellar granule cells from N-methyl-D-aspartate (NMDA) mediated excitotoxicity. It was later shown by Chen and Chuang (1999) that lithium was capable of suppressing expression of the pro-apoptotic genes p53 and Bax, while increasing the expression of the anti-apoptotic gene bcl-2. Increased Bcl-2 expression has also been shown in the frontal cortex of rats treated chronically with VPA (Chen et al., 1999c). More recently, Bijur et al. (2000) reported that lithium could inhibit the activity of GSK-3β and attenuate the activation of the pro-apoptotic enzyme caspase-3, thus protecting the cell from death. These processes (i.e. neuroprotection or reduction of apoptosis) may also be downstream targets of increased ER stress proteins after treatment with VPA. Studies have clearly illustrated that if GRP78, GRP94 or calreticulin expression is increased prior to a cellular insult then the cell has a better chance of recovery. Yu et al. (1999), found that pretreatment of hippocampal neurons with 2-deoxy-D-glucose caused overexpression of GRP78, which could protect neurons against excitotoxic and oxidative insults. They further

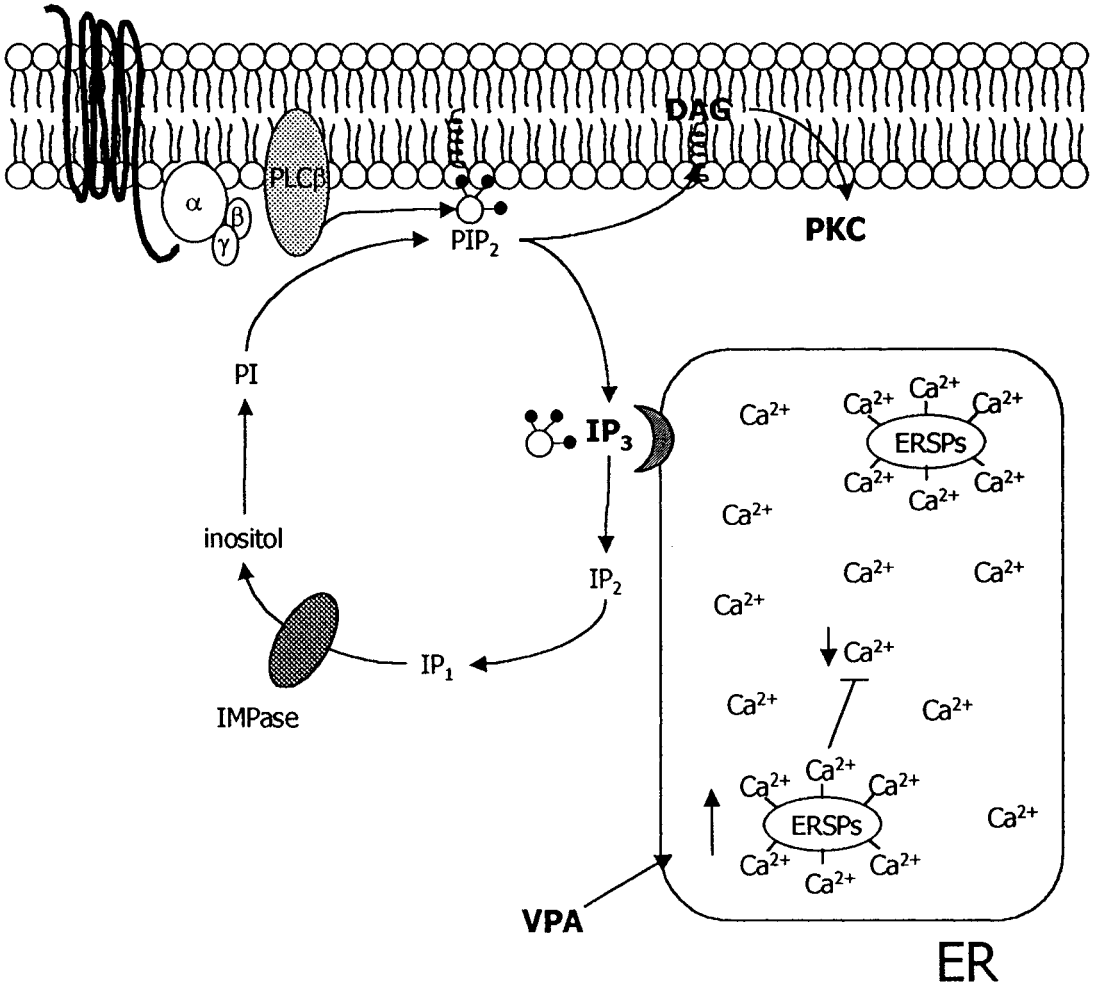

DAG
PLCβ
α
β
γ
PIP2
PKC
PI
IP3
inositol
IP2
IP1
IMPase
Ca2+
ERSPs
VPA
ER

**Figure 4.0.1.** *Possible implication of increased ER stress proteins on the PI-generated second messenger system.* The phosphatidylinositol (PI) second messenger system causes myo-inositol to be converted into inositol 1,4,5-triphosphate ($IP_3$), which binds to its receptor on the surface of the ER and causes stored $Ca^{2+}$ to be released from the ER. Increased $IP_3$ turnover has been suggested in the pathophysiology of BD, which could cause free $Ca^{2+}$ to be released from ER stores. This might contribute to increased intracellular $Ca^{2+}$ concentrations measured in platelets from BD subjects. Increased ER stress proteins (ERSPs) by valproate could possibly increase the $Ca^{2+}$-binding capacity of the ER, thus buffering the amount of free $Ca^{2+}$ in the ER. Therefore, if there is increased $IP_3$ turnover within the cytosol, the ER will not be depleted of $Ca^{2+}$ at the same rate as those cells with basal expression of the ER stress proteins (modified from Bown et al., 2002a).

showed that those neurons transfected with antisense GRP78 transcripts were unable to protect against the same excitotoxic and oxidative injuries. Similar results have also been obtained in other cells and models of cellular insult (Liu et al., 1997; Liu et al., 1998a; van De Water et al., 1999) .

The regulation of neuroprotective agents by mood stabilizing drugs may be important in the pathophysiology of BD. Researchers have shown decreased cell density and volume in several brain regions of BD patients (Altshuler et al., 2000; Benes et al., 1998a; Drevets et al., 1997a; Strakowski et al., 1999; Benes et al., 1998b; Drevets et al., 1997b; Krause and Michalak, 1997b; Moore et al., 2000). The ability of the ER stress proteins to process aberrant proteins may also provide protection against damage or death to a cell. Although there is no direct evidence to suggest that BD is associated with increased production of malfolded or damaged proteins, this may occur as part of the emerging neuropathologic process in this patient group. The idea that an accumulation of aberrant proteins can lead to neurodegenerative disorders is not novel and has been fundamentally important in the understanding of Alzheimer's disease (Selkoe, 1986; Zhang et al., 1989a; Zhang et al., 1989b). The results shown by the Hamos et al. (1991) study indicate that GRP78 may be involved in protecting the Alzheimer's brain from neurofibrillary tangles and plaques.

The results described in this dissertation show that the mood stabilizing agent valproate regulates the expression of a family of resident endoplasmic reticulum proteins, the ER stress proteins. The regulation of ER stress proteins by valproate appears to be drug- or drug class-specific, since a variety of other psychotropic drugs, desipramine,

venlafaxine and tranylcypromine, and lithium did not significantly regulate their expression. Only carbamazepine, another mood stabilizing anticonvulsant, at high doses could increase the expression of the ER stress proteins. All these studies were conducted in cultured C6 glioma cells, since this cell line has been previously shown to regulate gene expression in response to both mood stabilizer and antidepressant treatments (Chen et al., 1996; Chen and Rasenick, 1995). Moreover, previous studies have shown that all ER stress proteins, GRP78, GRP94 and calreticulin are constitutively expressed in rat C6 glioma cells (Brostrom et al., 1991), making them a suitable line to study. It would be interesting to study the effect of these different psychotropic drugs and newer mood stabilizing anticonvulsants on different cell lines and in primary culture. Two relevant cell lines to study would be a rat embryonic serotonergic raphe neuronal cell line (RN46A) and a rat hippocampal neuronal cell line (H19-7) (Storring et al., 1999; Wang et al., 2000). As described in section (1.3.1), serotonin appears to have a fundamental role in depression. Since a very limited amount of serotonin crosses the blood-brain barrier, the brain must synthesize its own supply of serotonin. The midbrain, in particular the raphe nuclei, is responsible for production of serotonin, which is projected to key brain regions involved in mood, emotion and memory, including the frontal cortex and the hippocampus. Studying the regulation of ER stress proteins in a hippocampal cell line is important for several reasons. Hippocampal cells, in vivo, are known to be vulnerable to excitotoxic stress and glucocorticoid-mediated gene regulation, both of which have been shown to be involved in mood disorders, thus in part explaining why hippocampal remodelling is associated with mood disorders (Brown et al., 1999). Moreover, studies

measuring the expression of ER stress proteins following treatment with psychotropic drugs in hippocampal cell lines compared to RN46A cells would provide insight into whether the regulation of these proteins is an important pre- or post-synaptic event.

The fact that valproate can regulate the expression of ER stress proteins, and these proteins are known to bind calcium within the endoplasmic reticulum, is really quite interesting in light of what is known about calcium and bipolar disorder. Intracellular calcium levels have been shown to be consistently elevated in platelets from bipolar subjects. Moreover, in lymphocytes and platelets from BD subjects, ER $Ca^{2+}$ stores may be more sensitive to neurochemical changes (Hough et al., 1999). These results really question whether bipolar disorder is in part associated with decreased ER $Ca^{2+}$ storage, and whether mood stabilizers act to return ER $Ca^{2+}$ storage to basal levels. Therefore, it would be relevant to examine $Ca^{2+}$ retention after challenge with agents that disrupt $Ca^{2+}$ homeostasis (i.e. NMDA, thapsigargin, A23187) using radiolabelled $Ca^{2+}$ in antidepressant and mood stabilizer treated cells. In addition, the use of live cell imaging techniques and fluorescent dyes would allow for studies examining the rate and level of $Ca^{2+}$ sequestration following mood stabilizer treatment to be conducted. If these studies were successful, conducting a similar set of experiments using transformed lymphoblasts from bipolar disorder subjects could provide clinical evidence to the relationship between ER $Ca^{2+}$ storage and the regulation of ER stress proteins by valproate.

Recent studies have suggested that one possible common mechanism of action shared between the different mood stabilizers is neuroprotection. Our results show that valproate is capable of regulating the expression of the neuroprotective ER stress

proteins. Moreover, when we studied the ultrastructural appearance of rat hippocampal neurons exposed to NMDA, a significant increase in cytoplasmic vacuolization was quantified, which was completely prevented when these cells were pretreated with mood stabilizers. These results suggest that mood stabilizers can protect against excitotoxic injury mediated by NMDA receptors *in vitro*. To fully understand the importance of these results and how they may relate to the pathophysiology of bipolar disorder, a similar *in vivo* study would have to be conducted. Using transmission electron microscopy, ultrathin sections of rat hippocampus would be studied in animals pretreated for 21 days with lithium, valproate or carbamazepine, and then exposed to intrathecal NMDA application. It would be interesting to know whether the morphological changes observed *in vitro* would also be seen in rat brain slices. It has been suggested that, *in vitro,* cells destined to undergo apoptosis revert to other forms of cell death, since macrophages are not present in the culture that would normally engulf these apoptotic cells *in vivo*. Therefore the results of *in vivo* studies would provide a more relevant representation of the actual events of NMDA-mediated cell damage or death and the possible roles mood stabilizers have in preventing this death.

We have shown that NMDA-mediated cytoplasmic vacuolization does not initially result in loss of cell viability, however if NMDA is left in the media for 6 hours, a significant reduction in cell viability occurs that is not associated with caspase 3 activation or DNA fragmentation. Given that NMDA-mediated cytoplasmic vacuoles are very large (~ 2 μm) in size, they could be studied using confocal microscopy. Using a fluorescently-tagged antibody to a cytoplasmic marker (i.e. MAP2), vacuolization could

be studied at various time points following exposure to NMDA. Since we examined only one time point following NMDA exposure with transmission electron microscopy, we cannot rule out that cytoplasmic vacuolization is a transient event following NMDA exposure. Also, we do not know if mood stabilizer treatment acts to completely prevent the onset of NMDA-mediated cytoplasmic vacuolization or whether these drugs act to delay the onset of vacuolization. Technical difficulties and cost dissuade the use of transmission electron microscopy to study the time course of vacuole formation, and therefore confocal microscopy is a better alternative. Real-time imaging would also be useful in studying NMDA-mediated cytoplasmic vacuolization, since a precise time course could be established. These studies could also be correlated with markers of cell death such as mitochondrial depolarization using the JC-1 fluorescent marker. Early stages of cell death are often associated with rapid mitochondrial depolarization (Zhu et al., 2000). JC-1 allows for visual and quantitative measures of mitochondrial depolarization by producing a green colour at normal resting mitochondrial potentials, and red when the mitochondria are undergoing depolarization. Therefore, correlating changes in mitochondrial potential to cytoplasmic vacuolization following NMDA exposure would allow for a better understanding of the role cytoplasmic vacuolization plays in the health of the cell.

Advances in brain imaging techniques have allowed researchers to identify neuroanatomical changes associated with mood disorders. The commonality between these studies is that mood disorders are associated with reductions in brain volume and density in areas of the brain associated with mood and emotion. Therefore, it seems

plausible that the drugs used to treat these disorders act to protect neurons against damage or death. The delayed response of patients to psychotropic drugs suggests that changes in gene expression may be in part responsible for neuroprotection. Our discovery of the ER stress proteins as valproate-regulated genes suggests that this particular family of proteins may contribute to the neuroprotective properties of the drug. Since other psychotropic drugs (i.e. lithium, desipramine, venlafaxine and tranylcypromine) did not show an effect on the expression of ER stress proteins, the regulation of these proteins by valproate may be an effect unique to this drug or drug-class. Moreover, when primary rat hippocampal neurons were challenged with NMDA, mood stabilizers could completely prevent any ultrastructural changes caused by the excitotoxic insult. These results suggest that the mood stabilizers, lithium, valproate and carbamazepine may have a common role in mediating NMDA receptor activation.

These results, together with other published reports, give strong evidence to support the hypothesis that mood disorders are associated with vulnerability of certain brain regions to damage and death, and psychotropic drugs used to treat these disorders regulate the expression of neuroprotective genes. These findings should advance the development of both diagnostic techniques for the disorders, as well as development of drugs that are more effective in a wider number of mentally ill patients.

5.0 References

(1987). Diagnostic and Statistical Manual of Mental Disorders - DSMIII. Washington, DC: American Psychiatric Association.

(1994). Diagnostic and Statistical Manual of Mental Disorders - DSMIV.Washington, DC: American Psychiatric Association

Abakumova,O.Y., Podobed,O.V., Tsvetkova,T.A., Yakusheva,I.V., Moskvitina,T.A., Kondakova,L.I., Navasardyantz,D.G., and Medvedev,A.E. (1998). Modulation of glutamate neurotoxicity in the transformed cell culture by monoamine oxidase inhibitors, clorgyline and deprenyl. J. Neural Transm. Suppl *52*, 87-91.

Adams,J.M. and Cory,S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*, 1322-1326.

Ahrens,B., Linden,M., Zaske,H., and Berzewski,H. (2000). Suicidal behavior--symptom or disorder? Compr. Psychiatry *41*, 116-121.

Ainsworth,K., Smith,S.E., and Sharp,T. (1998). Repeated administration of fluoxetine, desipramine and tranylcypromine increases dopamine D2-like but not D1-like receptor function in the rat. J. Psychopharmacol. *12*, 252-257.

Akin,L.K. (2001). Pediatric and adolescent bipolar disorder: medical resources. Med. Ref. Serv. Q. *20*, 31-44.

Almli,L.M., Hamrick,S.E., Koshy,A.A., Tauber,M.G., and Ferriero,D.M. (2001). Multiple pathways of neuroprotection against oxidative stress and excitotoxic injury in immature primary hippocampal neurons. Brain Res. Dev. Brain Res. *132*, 121-129.

Altschul,S.F., Gish,W., Miller,W., Myers,E.W., and Lipman,D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Altshuler,L.L., Bartzokis,G., Grieder,T., Curran,J., Jimenez,T., Leight,K., Wilkins,J., Gerner,R., and Mintz,J. (2000). An MRI study of temporal lobe structures in men with bipolar disorder or schizophrenia. Biol. Psychiatry *48*, 147-162.

Ambrosio,A.F., Silva,A.P., Malva,J.O., Soares-da-Silva,P., Carvalho,A.P., and Carvalho,C.M. (1999). Carbamazepine inhibits L-type Ca2+ channels in cultured rat hippocampal neurons stimulated with glutamate receptor agonists. Neuropharmacology *38*, 1349-1359.

Andrews,J.M., Ninan,P.T., and Nemeroff,C.B. (1996). Venlafaxine: a novel antidepressant that has a dual mechanism of action. Depression. *4*, 48-56.

Angst,J. and Sellaro,R. (2000). Historical perspectives and natural history of bipolar disorder. Biol. Psychiatry *48*, 445-457.

Arborelius,L., Nomikos,G.G., Hertel,P., Salmi,P., Grillner,P., Hook,B.B., Hacksell,U., and Svensson,T.H. (1996). The 5-HT1A receptor antagonist (S)-UH-301 augments the increase in extracellular concentrations of 5-HT in the frontal cortex produced by both acute and chronic treatment with citalopram. Naunyn Schmiedebergs Arch. Pharmacol. *353*, 630-640.

Ascher,J.A., Cole,J.O., Colin,J.N., Feighner,J.P., Ferris,R.M., Fibiger,H.C., Golden,R.N., Martin,P., Potter,W.Z., and Richelson,E. (1995). Bupropion: a review of its mechanism of antidepressant activity. J. Clin. Psychiatry *56*, 395-401.

Asghari,V., Wang,J.F., Reiach,J.S., and Young,L.T. (1998). Differential effects of mood stabilizers on Fos/Jun proteins and AP-1 DNA binding activity in human neuroblastoma SH-SY5Y cells. Brain Res. Mol. Brain Res. *58*, 95-102.

Atack,J.R., Broughton,H.B., and Pollack,S.J. (1995). Inositol monophosphatase--a putative target for Li+ in the treatment of bipolar disorder. Trends Neurosci. *18*, 343-349.

Atack,J.R., Levine,J., and Belmaker,R.H. (1998). Cerebrospinal fluid inositol monophosphatase elevated activity in depression and neuroleptic-treated schizophrenia. Biol Psychiatry *44*: 433-437.

Avissar,S., Schreiber,G., Danon,A., and Belmaker,R.H. (1988). Lithium inhibits adrenergic and cholinergic increases in GTP binding in rat cortex. Nature *331*, 440-442.

Avissar,S., Barki-Harrington,L., Nechamkin,Y., Roitman,G., and Schreiber,G. (1996). Reduced beta-adrenergic receptor-coupled Gs protein function and Gs alpha immunoreactivity in mononuclear leukocytes of patients with depression. Biol. Psychiatry *39*, 755-760.

Avissar,S., Nechamkin,Y., Barki-Harrington,L., Roitman,G., and Schreiber,G. (1997a). Differential G protein measures in mononuclear leukocytes of patients with bipolar mood disorder are state dependent. J. Affect. Disord. *43*, 85-93.

Avissar,S., Nechamkin,Y., Roitman,G., and Schreiber,G. (1997b). Reduced G protein functions and immunoreactive levels in mononuclear leukocytes of patients with depression. Am. J. Psychiatry *154*, 211-217.

Axelrod,J., Whitby,L., and Hertting,G. (1961). Effect of psychotropic drugs on the uptake $^{3}$H-Norepinephrine by tissues. Science *133*, 383-384.

Baf,M.H., Subhash,M.N., Lakshmana,K.M., and Rao,B.S. (1994a). Sodium valproate induced alterations in monoamine levels in different regions of the rat brain. Neurochem. Int. *24*, 67-72.

Baf,M.H., Subhash,M.N., Lakshmana,K.M., and Rao,B.S. (1994b). Alterations in monoamine levels in discrete regions of rat brain after chronic administration of carbamazepine. Neurochem. Res. *19*, 1139-1143.

Ballenger,J.C. and Post,R.M. (1980). Carbamazepine in manic-depressive illness: a new treatment. Am. J. Psychiatry *137*, 782-790.

Banker,G.A. and Cowan,W.M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res. *126*, 397-42.

Bartlett,W.P. and Banker,G.A. (1984). An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. J. Neurosci. *4*, 1944-1953.

Bel,N. and Artigas,F. (1993). Chronic treatment with fluvoxamine increases extracellular serotonin in frontal cortex but not in raphe nuclei. Synapse *15*, 243-245.

Benes,F.M., Kwok,E.W., Vincent,S.L., and Todtenkopf,M.S. (1998). A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. Biol. Psychiatry *44*, 88-97.

Benes,F.M., Vincent,S.L., and Todtenkopf,M. (2001). The density of pyramidal and nonpyramidal neurons in anterior cingulate cortex of schizophrenic and bipolar subjects. Biol. Psychiatry *50*, 395-406.

Bhave,S.V., Ghoda,L., and Hoffman,P.L. (1999). Brain-derived neurotrophic factor mediates the anti-apoptotic effect of NMDA in cerebellar granule neurons: signal transduction cascades and site of ethanol action. J. Neurosci. *19*, 3277-3286.

Bijur,G.N., De Sarno,P., and Jope,R.S. (2000). Glycogen synthase kinase-3beta facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. J. Biol. Chem. *275*, 7583-7590.

Birkhimer,L.J., Curtis,J.L., and Jann,M.W. (1985). Use of carbamazepine in psychiatric disorders. Clin. Pharm. *4*, 425-434.

Bole,D.G., Hendershot,L.M., and Kearney,J.F. (1986). Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. *102*, 1558-1566.

Bonifacino,J.S. and Lippincott-Schwartz,J. (1991). Degradation of proteins within the endoplasmic reticulum. Curr. Opin. Cell Biol. *3*, 592-600.

Borre,L. and Kanner,B.I. (2001). Coupled, but not uncoupled, fluxes in a neuronal glutamate transporter can be activated by lithium ions. J. Biol. Chem. *276*, 40396-40401.

Bowden,C.L., Brugger,A.M., Swann,A.C., Calabrese,J.R., Janicak,P.G., Petty,F., Dilsaver,S.C., Davis,J.M., Rush,A.J., and Small,J.G. (1994). Efficacy of divalproex vs lithium and placebo in the treatment of mania. The Depakote Mania Study Group. JAMA *271*, 918-924.

Bowden,C.L. (1996). Role of newer medications for bipolar disorder. J. Clin. Psychopharmacol. *16*, 48S-55S.

Bowden,C.L., Janicak,P.G., Orsulak,P., Swann,A.C., Davis,J.M., Calabrese,J.R., Goodnick,P., Small,J.G., Rush,A.J., Kimmel,S.E., Risch,S.C., and Morris,D.D. (1996). Relation of serum valproate concentration to response in mania. Am. J. Psychiatry *153*, 765-770.

Bown,C., Wang,J.F., MacQueen,G., and Young,L.T. (2000a). Increased temporal cortex ER stress proteins in depressed subjects who died by suicide. Neuropsychopharmacology *22*, 327-332.

Bown,C.D., Wang,J.F., and Young,L.T. (2000b). Increased expression of endoplasmic reticulum stress proteins following chronic valproate treatment of rat C6 glioma cells. Neuropharmacology *39*, 2162-2169.

Bown,C.D., Wang,J.F., Azzam,J.E., Sun,X.J., and Young,L.T. (2001). Protective effect of the mood stabilizers lithium and valproate. Soc. Neurosci. Abstr. *27*, 872.9.

Bown,C.D., Wang,J.F., Chen,B., and Young,L.T. (2002a). Regulation of ER stress proteins by valproate: therapeutic implications. Bipolar Disorders *4*, 145-151.

Bown,C.D., Wang,J.F., and Young,L.T. (2002b). Attenuation of NMDA-mediated cytoplasmic vacuolization in primary rat hippocampal neurons by mood stabilizers. Manuscript submitted to Neuroscience.

Bradford,M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248-254.

Brecht,S., Gelderblom,M., Srinivasan,A., Mielke,K., Dityateva,G., and Herdegen,T. (2001). Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity. Brain Res. Mol. Brain Res. *94*, 25-34.

Bremner,J.D., Narayan,M., Anderson,E.R., Staib,L.H., Miller,H.L., and Charney,D.S. (2000). Hippocampal volume reduction in major depression. Am. J. Psychiatry *157*, 115-118.

Brewer,G.J., Torricelli,J.R., Evege,E.K., and Price,P.J. (1993). Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. *35*, 567-576.

Brewer,G.J. (1999). Regeneration and proliferation of embryonic and adult rat hippocampal neurons in culture. Exp. Neurol. *159*, 237-247.

Brodmann,K. (1909). Vergleichende Lokalisationslehre der Grosshirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues. Leizig: Barth.

Brostrom,C.O. and Brostrom,M.A. (1998). Regulation of translational initiation during cellular responses to stress. Prog Nucleic Acid Res. Mol. Biol *58*, 79-125.

Brostrom,M.A., Cade,C., Prostko,C.R., Gmitter-Yellen,D., and Brostrom,C.O. (1990). Accommodation of protein synthesis to chronic deprivation of intracellular sequestered calcium. A putative role for GRP78. J. Biol. Chem. *265*, 20539-20546.

Brostrom,M.A., Prostko,C.R., Gmitter-Yellen,D., Grandison,L.J., Kuznetsov,G., Wong,W.L., and Brostrom,C.O. (1991). Inhibition of translational initiation by metalloendoprotease antagonists. Evidence for involvement of sequestered Ca2+ stores. J. Biol. Chem. *266*, 7037-7043.

Brown,E.S., Rush,A.J., and McEwen,B.S. (1999). Hippocampal remodeling and damage by corticosteroids: implications for mood disorders. Neuropsychopharmacology *21*, 474-484.

Bruckheimer,E.M., Cho,S.H., Sarkiss,M., Herrmann,J., and McDonnell,T.J. (1998). The Bcl-2 gene family and apoptosis. Adv. Biochem. Eng Biotechnol. *62*, 75-105.

Brustovetsky,N. and Dubinsky,J.M. (2000). Dual responses of CNS mitochondria to elevated calcium. J. Neurosci. *20*, 103-113.

Budd,S.L., Tenneti,L., Lishnak,T., and Lipton,S.A. (2000). Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. Proc. Natl. Acad. Sci U. S. A *97*, 6161-6166.

Budihardjo,I., Oliver,H., Lutter,M., Luo,X., and Wang,X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu. Rev Cell Dev. Biol. *15*, 269-290.

Burns,K., Duggan,B., Atkinson,E.A., Famulski,K.S., Nemer,M., Bleackley,R.C., and Michalak,M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature *367*, 476-480.

Cameron,G.R. (1952). Pathology of the cell. London: Oliver & Boyd.

Cao,G., Pei,W., Lan,J., Stetler,R.A., Luo,Y., Nagayama,T., Graham,S.H., Yin,X.M., Simon,R.P., and Chen,J. (2001). Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures. J. Neurosci. *21*, 4678-4690.

Cebers,G., Zhivotovsky,B., Ankarcrona,M., and Liljequist,S. (1997). AMPA neurotoxicity in cultured cerebellar granule neurons: mode of cell death. Brain Res. Bull. *43*, 393-403.

Chen,A.C., Shirayama,Y., Shin,K.H., Neve,R.L., and Duman,R.S. (2001). Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. Biol. Psychiatry *49*, 753-762.

Chen,B., Wang,J.F., Hill,B.C., and Young,L.T. (1999a). Lithium and valproate differentially regulate brain regional expression of phosphorylated CREB and c-Fos. Brain Res. Mol. Brain Res. *70*, 45-53.

Chen,B., Wang,J.F., and Young,L.T. (2000). Chronic valproate treatment increases expression of endoplasmic reticulum stress proteins in the rat cerebral cortex and hippocampus. Biol. Psychiatry *48*, 658-664.

Chen,G., Manji,H.K., Hawver,D.B., Wright,C.B., and Potter,W.Z. (1994). Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon in vitro. J. Neurochem. *63*, 2361-2364.

Chen,G., Manji,H.K., Wright,C.B., Hawver,D.B., and Potter,W.Z. (1996). Effects of valproic acid on beta-adrenergic receptors, G-proteins, and adenylyl cyclase in rat C6 glioma cells. Neuropsychopharmacology *15*, 271-280.

Chen,G., Yuan,P., Hawver,D.B., Potter,W.Z., and Manji,H.K. (1997). Increase in AP-1 transcription factor DNA binding activity by valproic acid. Neuropsychopharmacology *16*, 238-245.

Chen,G., Yuan,P.X., Jiang,Y.M., Huang,L.D., and Manji,H.K. (1998). Lithium increases tyrosine hydroxylase levels both in vivo and in vitro. J. Neurochem. *70*, 1768-1771.

Chen,G., Huang,L.D., Jiang,Y.M., and Manji,H.K. (1999b). The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. J. Neurochem. *72*, 1327-1330.

Chen,G., Zeng,W.Z., Yuan,P.X., Huang,L.D., Jiang,Y.M., Zhao,Z.H., and Manji,H.K. (1999c). The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. J. Neurochem. *72*, 879-882.

Chen,G., Yuan,P.X., Jiang,Y.M., Huang,L.D., and Manji,H.K. (1999d). Valproate robustly enhances AP-1 mediated gene expression. Brain Res. Mol. Brain Res. *64*, 52-58.

Chen,J. and Rasenick,M.M. (1995a). Chronic treatment of C6 glioma cells with antidepressant drugs increases functional coupling between a G protein (Gs) and adenylyl cyclase. J. Neurochem. *64*, 724-732.

Chen,J. and Rasenick,M.M. (1995b). Chronic antidepressant treatment facilitates G protein activation of adenylyl cyclase without altering G protein content. J. Pharmacol. Exp. Ther. *275*, 509-517.

Chen,R.W. and Chuang,D.M. (1999e). Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. J. Biol. Chem. *274*, 6039-6042.

Choi,D.W. (1992). Excitotoxic cell death. J. Neurobiol. *23*, 1261-1276.

Chotai,J. and Asberg,M. (1999). Variations in CSF monoamine metabolites according to the season of birth. Neuropsychobiology *39* , 57-62.

Clarke,P.G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol. (Berl) *181*, 195-213.

Colin,S.F., Chang,H.C., Mollner,S., Pfeuffer,T., Reed,R.R., Duman,R.S., and Nestler,E.J. (1991). Chronic lithium regulates the expression of adenylate cyclase and Gi- protein alpha subunit in rat cerebral cortex. Proc. Natl. Acad. Sci U. S. A *88*, 10634-10637.

Compton,M.T. and Nemeroff,C.B. (2000). The treatment of bipolar depression. J. Clin. Psychiatry *61 Suppl 9*, 57-67.

Coppen,A. (1967). The biochemistry of affective disorders. Br. J. Psychiatry *113*, 1237-1264.

Cotariu,D., Zaidman,J.L., and Evans,S. (1990). Neurophysiological and biochemical changes evoked by valproic acid in the central nervous system. Prog. Neurobiol. *34*, 343-354.

Cotter,D., Mackay,D., Landau,S., Kerwin,R., and Everall,I. (2001). Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. Arch. Gen. Psychiatry *58*, 545-553.

Cotter,D., Mackay,D., Chana,G., Beasley,C., Landau,S., and Everall,I.P. (2002). Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. Cereb. Cortex *12*, 386-394.

Couture,L., Elie,R., and Lavoie,P.A. (2001). Effect of antidepressants on ATP-dependent calcium uptake by neuronal endoplasmic reticulum. Can. J. Physiol Pharmacol. *79*, 946-952.

Cowburn,R.F., Marcusson,J.O., Eriksson,A., Wiehager,B., and O'Neill,C. (1994). Adenylyl cyclase activity and G-protein subunit levels in postmortem frontal cortex of suicide victims. Brain Res. *633*, 297-304.

Dailey,J.W., Reith,M.E., Steidley,K.R., Milbrandt,J.C., and Jobe,P.C. (1998). Carbamazepine-induced release of serotonin from rat hippocampus in vitro. Epilepsia *39*, 1054-1063.

Dardennes,R., Even,C., Bange,F., and Heim,A. (1995). Comparison of carbamazepine and lithium in the prophylaxis of bipolar disorders. A meta-analysis. Br. J. Psychiatry *166*, 378-381.

Davies,S. and Ramsden,D.B. (2001). Huntington's disease. Mol. Pathol. *54*, 409-413.

Dawson,L.A., Nguyen,H.Q., and Geiger,A. (1999). Effects of venlafaxine on extracellular concentrations of 5-HT and noradrenaline in the rat frontal cortex: augmentation via 5-HT1A receptor antagonism. Neuropharmacology *38*, 1153-1163.

Dixon,J.F. and Hokin,L.E. (1998). Lithium acutely inhibits and chronically up-regulates and stabilizes glutamate uptake by presynaptic nerve endings in mouse cerebral cortex. Proc. Natl. Acad. Sci U. S. A *95* , 8363-8368.

Donati,R.J., Thukral,C., and Rasenick,M.M. (2001). Chronic treatment of C6 glioma cells with antidepressant drugs results in a redistribution of Gsalpha. Mol. Pharmacol. *59*, 1426-1432.

Doris,A., Ebmeier,K., and Shajahan,P. (1999). Depressive illness. Lancet *354*, 1369-1375.

Dorner,A.J., Wasley,L.C., and Kaufman,R.J. (1992). Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. EMBO J. *11*, 1563-1571.

Doutheil,J., Gissel,C., Oschlies,U., Hossmann,K.A., and Paschen,W. (1997). Relation of neuronal endoplasmic reticulum calcium homeostasis to ribosomal aggregation and protein synthesis: implications for stress- induced suppression of protein synthesis. Brain Res. *775*, 43-51.

Dowlatshahi,D., MacQueen,G.M., Wang,J.F., and Young,L.T. (1998). Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. Lancet *352*, 1754-1755.

Dowlatshahi,D., MacQueen,G.M., Wang,J.F., Reiach,J.S., and Young,L.T. (1999). G Protein-coupled cyclic AMP signaling in postmortem brain of subjects with mood disorders: effects of diagnosis, suicide, and treatment at the time of death. J. Neurochem. *73*, 1121-1126.

Dowlatshahi,D. and Young,L.T. (2000). Abnormalities in brains of depressed patients. The Neuroscientist *6*, 401-410.

Drevets,W.C., Price,J.L., Simpson,J.R., Jr., Todd,R.D., Reich,T., Vannier,M., and Raichle,M.E. (1997). Subgenual prefrontal cortex abnormalities in mood disorders. Nature *386*, 824-827.

Drevets,W.C., Ongur,D., and Price,J.L. (1998). Neuroimaging abnormalities in the subgenual prefrontal cortex: implications for the pathophysiology of familial mood disorders. Mol. Psychiatry *3*, 220-221.

Drevets,W.C. (2000). Functional anatomical abnormalities in limbic and prefrontal cortical structures in major depression. Prog. Brain Res. *126*, 413-431.

Drummond,I.A., Lee,A.S., Resendez,E., Jr., and Steinhardt,R.A. (1987). Depletion of intracellular calcium stores by calcium ionophore A23187 induces the genes for glucose-regulated proteins in hamster fibroblasts. J. Biol. Chem. *262*, 12801-12805.

Dubovsky,S.L., Christiano,J., Daniell,L.C., Franks,R.D., Murphy,J., Adler,L., Baker,N., and Harris,R.A. (1989). Increased platelet intracellular calcium concentration in patients with bipolar affective disorders. Arch. Gen. Psychiatry *46*, 632-638.

Dubovsky,S.L., Murphy,J., Thomas,M., and Rademacher,J. (1992). Abnormal intracellular calcium ion concentration in platelets and lymphocytes of bipolar patients. Am. J. Psychiatry *149*, 118-120.

Duchen,M.R. (2000). Mitochondria and calcium: from cell signalling to cell death. J. Physiol *529 Pt 1*, 57-68.

Duman,R.S., Heninger,G.R., and Nestler,E.J. (1997). A molecular and cellular theory of depression. Arch. Gen. Psychiatry *54*, 597-606.

Dwivedi,Y., Janicak,P.G., and Pandey,G.N. (1998). Elevated [3H]inositol 1,4,5-trisphosphate binding sites and expressed inositol 1,4,5-trisphosphate receptor protein level in platelets of depressed patients. Psychopharmacology (Berl) *138*, 47-54.

Eastwood,S.L. and Harrison,P.J. (2000). Hippocampal synaptic pathology in schizophrenia, bipolar disorder and major depression: a study of complexin mRNAs. Mol. Psychiatry *5*, 425-432.

Eaton,M.J., Staley,J.K., Globus,M.Y., and Whittemore,S.R. (1995). Developmental regulation of early serotonergic neuronal differentiation: the role of brain-derived neurotrophic factor and membrane depolarization. Dev. Biol. *170*, 169-182.

Ebstein,R.P., Moscovich,D., Zeevi,S., Amiri,Z., and Lerer,B. (1987). Effect of lithium in vitro and after chronic treatment on human platelet adenylate cyclase activity: postreceptor modification of second messenger signal amplification. Psychiatry Res. *21*, 221-228.

Ebstein,R.P., Lerer,B., Shapira,B., Shemesh,Z., Moscovich,D.G., and Kindler,S. (1988). Cyclic AMP second-messenger signal amplification in depression. Br. J. Psychiatry *152*, 665-669.

Emamghoreishi,M., Schlichter,L., Li,P.P., Parikh,S., Sen,J., Kamble,A., and Warsh,J.J. (1997). High intracellular calcium concentrations in transformed lymphoblasts from subjects with bipolar I disorder. Am. J. Psychiatry *154*, 976-982.

Emilien,G., Maloteaux,J.M., Seghers,A., and Charles,G. (1996). Lithium compared to valproic acid and carbamazepine in the treatment of mania: a statistical meta-analysis. Eur. Neuropsychopharmacol. *6*, 245-252.

Engidawork,E., Gulesserian,T., Seidl,R., Cairns,N., and Lubec,G. (2001). Expression of apoptosis related proteins in brains of patients with Alzheimer's disease. Neurosci. Lett. *303*, 79-82.

Entsuah,A.R., Huang,H., and Thase,M.E. (2001). Response and remission rates in different subpopulations with major depressive disorder administered venlafaxine, selective serotonin reuptake inhibitors, or placebo. J. Clin. Psychiatry *62*, 869-877.

Extein,I., Tallman,J., Smith,C.C., and Goodwin,F.K. (1979). Changes in lymphocyte beta-adrenergic receptors in depression and mania. Psychiatry Res. *1*, 191-197.

Fatemi,S.H., Earle,J.A., and McMenomy,T. (2000). Reduction in Reelin immunoreactivity in hippocampus of subjects with schizophrenia, bipolar disorder and major depression. Mol. Psychiatry *5*, 654-63, 571.

Fatemi,S.H., Earle,J.A., Stary,J.M., Lee,S., and Sedgewick,J. (2001). Altered levels of the synaptosomal associated protein SNAP-25 in hippocampus of subjects with mood disorders and schizophrenia. Neuroreport *12*, 3257-3262.

Fawcett,D.M. (1994). Bloom and Fawcett, a textbook of histology. New York: Chapman & Hall.

Feinberg,A.P. and Vogelstein,B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Fiedorowicz,A., Figiel,I., Kaminska,B., Zaremba,M., Wilk,S., and Oderfeld-Nowak,B. (2001). Dentate granule neuron apoptosis and glia activation in murine hippocampus induced by trimethyltin exposure. Brain Res. *912*, 116-127.

Fishman,P.H. and Finberg,J.P. (1987). Effect of the tricyclic antidepressant desipramine on beta-adrenergic receptors in cultured rat glioma C6 cells. J. Neurochem. *49*, 282-289.

Fliegel,L., Burns,K., MacLennan,D.H., Reithmeier,R.A., and Michalak,M. (1989). Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. *264*, 21522-21528.

Fowler,C.J. and Brannstrom,G. (1990). Reduction in beta-adrenoceptor density in cultured rat glioma C6 cells after incubation with antidepressants is dependent upon the culturing conditions used. J. Neurochem. *55*, 245-250.

Frazer,A. (2001). Serotonergic and noradrenergic reuptake inhibitors: prediction of clinical effects from in vitro potencies. J. Clin. Psychiatry *62 Suppl 12*, 16-23.

Freeman,H. (1993). Moclobemide. Lancet *342*, 1528-1532.

Freeman,J.K. and Goldberg,M.P. (1994). Confocal microscopic visualization of MK-801-induced cytoplasmic vacuoles in vitro. Psychopharmacol. Bull. *30*, 541-547.

Freemantle,N. and Mason,J. (2000). The importance of achieving additional drug benefits at a reasonable cost. A review of the fluoxetine years. Pharmacoeconomics. *17*, 319-324.

Friedman,E. and Wang,H.Y. (1996). Receptor-mediated activation of G proteins is increased in postmortem brains of bipolar affective disorder subjects. J. Neurochem. *67*, 1145-1152.

Fuller,R.W., Perry,K.W., and Molloy,B.B. (1974). Effect of an uptake inhibitor on serotonin metabolism in rat brain: studies with 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine (Lilly 110140). Life Sci *15*, 1161-1171.

Garcia-Sevilla,J.A., Escriba,P.V., Ozaita,A., La Harpe,R., Walzer,C., Eytan,A., and Guimon,J. (1999). Up-regulation of immunolabeled alpha2A-adrenoceptors, Gi coupling proteins, and regulatory receptor kinases in the prefrontal cortex of depressed suicides. J. Neurochem. *72*, 282-291.

Geracioti,T.D., Jr., Loosen,P.T., Ekhator,N.N., Schmidt,D., Chambliss,B., Baker,D.G., Kasckow,J.W., Richtand,N.M., Keck,P.E., Jr., and Ebert,M.H. (1997). Uncoupling of serotonergic and noradrenergic systems in depression: preliminary evidence from continuous cerebrospinal fluid sampling. Depress. Anxiety. *6*, 89-94.

Gething,M.J. and Sambrook,J. (1992). Protein folding in the cell. Nature *355*, 33-45.

Gething,M.J. (1997). Guidebook to Molecular Chaperones and Protein-Folding Catalysts. Oxford: Oxford Univeristy Press.

Glowinski,J. and Axelrod,J. (1964). Inhibition of uptake and tritiated-noradrenaline in the intact rat brain by imipramine and strucutrally related compounds. Nature 1318-1319.

Goldberg,M.P. and Bateman,M.C. (1993). Taxol attenuates glutamate receptor-mediated neurite swelling in vitro. Soc. Neurosci. Abstr. *19*, 26.

Goldstein,B.J. and Goodnick,P.J. (1998). Selective serotonin reuptake inhibitors in the treatment of affective disorders--III. Tolerability, safety and pharmacoeconomics. J. Psychopharmacol. *12*, S55-S87.

Goodwin,F.K. and Jamison,K.R. (1990). Manic Depressive Illness. (Oxford: Oxford University Press).

Grimes,C.A. and Jope,R.S. (2001a). CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. J. Neurochem. *78*, 1219-1232.

Grimes,C.A. and Jope,R.S. (2001b). The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog. Neurobiol. *65*, 391-426.

Gross-Isseroff,R., Biegon,A., Voet,H., and Weizman,A. (1998). The suicide brain: a review of postmortem receptor/transporter binding studies. Neurosci. Biobehav. Rev *22*, 653-661.

Hamabe,W., Fukushima,N., Yoshida,A., and Ueda,H. (2000). Serum-free induced neuronal apoptosis-like cell death is independent of caspase activity. Brain Res. Mol. Brain Res. *78*, 186-191.

Hamos,J.E., Oblas,B., Pulaski-Salo,D., Welch,W.J., Bole,D.G., and Drachman,D.A. (1991). Expression of heat shock proteins in Alzheimer's disease. Neurology *41*, 345-350.

Harms,H.H. (1983). The antidepressant agents desipramine, fluoxetine, fluvoxamine and norzimelidine inhibit uptake of [3H]noradrenaline and [3H]5- hydroxytryptamine in slices of human and rat cortical brain tissue. Brain Res. *275*, 99-104.

Hartmann,A. and Hirsch,E.C. (2001). Parkinson's disease. The apoptosis hypothesis revisited. Adv. Neurol. *86*, 143-153.

Hashimoto,R., Hough,C., Nakazawa,T., Yamamoto,T., and Chuang,D.M. (2002). Lithium protection against glutamate excitotoxicity in rat cerebral cortical neurons: involvement of NMDA receptor inhibition possibly by decreasing NR2B tyrosine phosphorylation. J. Neurochem. *80*, 589-597.

Henics,T., Koszegi,T., Szucs,G., Kellermayer,M.S., Jr., and Kellermayer,M. (1993). Transient cytoplasmic vacuolization in cultured normal and neoplastic cells treated with low molecular weight human serum ultrafiltrate: is out inside? Physiol Chem. Phys. Med. NMR *25*, 227-236.

Henics,T. and Wheatley,D.N. (1997). Vacuolar cytoplasmic phase separation in cultured mammalian cells involves the microfilament network and reduces motional properties of intracellular water. Int. J. Exp. Pathol. *78*, 343-354.

Henics,T. and Wheatley,D.N. (1999). Cytoplasmic vacuolation, adaptation and cell death: a view on new perspectives and features. Biol. Cell *91*, 485-498.

Hisaoka,K., Nishida,A., Koda,T., Miyata,M., Zensho,H., Morinobu,S., Ohta,M., and Yamawaki,S. (2001). Antidepressant drug treatments induce glial cell line-derived neurotrophic factor (GDNF) synthesis and release in rat C6 glioblastoma cells. J. Neurochem. *79*, 25-34.

Hopkins,H.S. and Gelenberg,A.J. (2000). Serum lithium levels and the outcome of maintenance therapy of bipolar disorder. Bipolar Disord. *2*, 174-179.

Hough,C., Lu,S.J., Davis,C.L., Chuang,D.M., and Post,R.M. (1999). Elevated basal and thapsigargin-stimulated intracellular calcium of platelets and lymphocytes from bipolar affective disorder patients measured by a fluorometric microassay. Biol. Psychiatry *46*, 247-255.

Hough,C.J., Irwin,R.P., Gao,X.M., Rogawski,M.A., and Chuang,D.M. (1996). Carbamazepine inhibition of N-methyl-D-aspartate-evoked calcium influx in rat cerebellar granule cells. J. Pharmacol. Exp. Ther. *276*, 143-149.

Hsieh,K.P., Wilke,N., Harris,A., and Miles,M.F. (1996). Interaction of ethanol with inducers of glucose-regulated stress proteins. Ethanol potentiates inducers of grp78 transcription. J. Biol. Chem. *271*, 2709-2716.

Hyman,S.E. and Nestler,E.J. (1996). Initiation and adaptation: a paradigm for understanding psychotropic drug action. Am. J. Psychiatry *153*, 151-162.

Isaev,N.K., Zorov,D.B., Stelmashook,E.V., Uzbekov,R.E., Kozhemyakin,M.B., and Victorov,I.V. (1996). Neurotoxic glutamate treatment of cultured cerebellar granule cells induces Ca2+ -dependent collapse of mitochondrial membrane potential and ultrastructural alterations of mitochondria. FEBS Lett. *392*, 143-147.

Jamison,K.R. (2000). Suicide and bipolar disorder. J. Clin. Psychiatry *61 Suppl 9*, 47-51.

Johnson,R.J., Liu,N.G., Fishman,J.B., Dixon,J.D., and Fine,R.E. (1992). Isolation of a calreticulin-like calcium binding protein from bovine brain. Brain Res. Mol. Brain Res. *12*, 69-76.

Johnston,N.L., Cervenak,J., Shore,A.D., Torrey,E.F., Yolken,R.H., and Cerevnak,J. (1997). Multivariate analysis of RNA levels from postmortem human brains as measured by three different methods of RT-PCR. Stanley Neuropathology Consortium. J. Neurosci. Methods *77*, 83-92.

Jope,R.S., Song,L., Li,P.P., Young,L.T., Kish,S.J., Pacheco,M.A., and Warsh,J.J. (1996). The phosphoinositide signal transduction system is impaired in bipolar affective disorder brain. J. Neurochem. *66*, 2402-2409.

Jope,R.S. and Song,L. (1997). AP-1 and NF-kappaB stimulated by carbachol in human neuroblastoma SH- SY5Y cells are differentially sensitive to inhibition by lithium. Brain Res. Mol. Brain Res. *50*, 171-180.

Jope,R.S. and Bijur,G.N. (2002). Mood stabilizers, glycogen synthase kinase-3beta and cell survival. Mol. Psychiatry *7 Suppl 1*, S35-S45.

Josepho,S.A. and Plutchik,R. (1994). Stress, coping, and suicide risk in psychiatric inpatients. Suicide Life Threat. Behav. *24* , 48-57.

Junn,E. and Mouradian,M.M. (2001). Apoptotic signaling in dopamine-induced cell death: the role of oxidative stress, p38 mitogen-activated protein kinase, cytochrome c and caspases. J. Neurochem. *78*, 374-383.

Kandel,E.R., Schwartz,J.H., and Jessell,T.M. (1991). Principles of Neural Science. New York: Elsevier.

Kang,H.S. and Welch,W.J. (1991). Characterization and purification of the 94-kDa glucose-regulated protein. J. Biol. Chem. *266*, 5643-5649.

Karege,F., Bovier,P., Rudolph,W., and Gaillard,J.M. (1996). Platelet phosphoinositide signaling system: an overstimulated pathway in depression. Biol. Psychiatry *39*, 697-702.

Kerr,J.F., Wyllie,A.H., and Currie,A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Kim,Y.K., Kim,K.S., and Lee,A.S. (1987). Regulation of the glucose-regulated protein genes by beta- mercaptoethanol requires de novo protein synthesis and correlates with inhibition of protein glycosylation. J. Cell Physiol *133*, 553-559.

Kirwin,P.D., Anderson,G.M., Chappell,P.B., Saberski,L., Leckman,J.F., Geracioti,T.D., Heninger,G.R., Price,L.H., and McDougle,C.J. (1997). Assessment of diurnal variation of cerebrospinal fluid tryptophan and 5- hydroxyindoleacetic acid in healthy human females. Life Sci *60*, 899-907.

Klimek,V., Stockmeier,C., Overholser,J., Meltzer,H.Y., Kalka,S., Dilley,G., and Ordway,G.A. (1997). Reduced levels of norepinephrine transporters in the locus coeruleus in major depression. J. Neurosci. *17*, 8451-8458.

Koch,G., Smith,M., Macer,D., Webster,P., and Mortara,R. (1986). Endoplasmic reticulum contains a common, abundant calcium-binding glycoprotein, endoplasmin. J. Cell Sci. *86*, 217-232.

Korner,M., Rattner,A., Mauxion,F., Sen,R., and Citri,Y. (1989). A brain-specific transcription activator. Neuron *3*, 563-572.

Kraeplin,E. (1913). Psychiatire Ein Lehrbuch fur Studieride und Arzte. Barth, Germany: Leipzig.

Krause,K.H. and Michalak,M. (1997). Calreticulin. Cell *88*, 439-443.

Kreiss,D.S. and Lucki,I. (1995). Effects of acute and repeated administration of antidepressant drugs on extracellular levels of 5-hydroxytryptamine measured in vivo. J. Pharmacol. Exp. Ther. *274*, 866-876.

Lam,M., Dubyak,G., Chen,L., Nunez,G., Miesfeld,R.L., and Distelhorst,C.W. (1994). Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc. Natl. Acad. Sci U. S. A *91*, 6569-6573.

Law,A.J. and Deakin,J.F. (2001). Asymmetrical reductions of hippocampal NMDAR1 glutamate receptor mRNA in the psychoses. Neuroreport *12*, 2971-2974.

Lecrubier,Y. and Guelfi,J.D. (1990). Efficacy of reversible inhibitors of monoamine oxidase-A in various forms of depression. Acta Psychiatr. Scand. Suppl *360*, 18-23.

Lee,A.S., Delegeane,A., and Scharff,D. (1981). Highly conserved glucose-regulated protein in hamster and chicken cells: preliminary characterization of its cDNA clone. Proc. Natl. Acad. Sci. U. S. A *78*, 4922-4925.

Lee,A.S., Delegeane,A.M., Baker,V., and Chow,P.C. (1983). Transcriptional regulation of two genes specifically induced by glucose starvation in a hamster mutant fibroblast cell line. J. Biol. Chem. *258*, 597-603.

Lee,A.S., Bell,J., and Ting,J. (1984). Biochemical characterization of the 94- and 78-kilodalton glucose-regulated proteins in hamster fibroblasts. J. Biol. Chem. *259*, 4616-4621.

Lee,A.S. (2001). The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem. Sci *26*, 504-510.

Leist,M. and Nicotera,P. (1997). The shape of cell death. Biochem. Biophys. Res. Commun. *236*, 1-9.

Lemasters,J.J., Stemkowski,C.J., Ji,S., and Thurman,R.G. (1983). Cell surface changes and enzyme release during hypoxia and reoxygenation in the isolated, perfused rat liver. J. Cell Biol. *97*, 778-786.

Lemasters,J.J., DiGuiseppi,J., Nieminen,A.L., and Herman,B. (1987). Blebbing, free Ca2+ and mitochondrial membrane potential preceding cell death in hepatocytes. Nature *325*, 78-81.

Lenox,R.H., Watson,D.G., Patel,J., and Ellis,J. (1992). Chronic lithium administration alters a prominent PKC substrate in rat hippocampus. Brain Res. *570*, 333-340.

Lenox,R.H., McNamara,R.K., Watterson,J.M., and Watson,D.G. (1996). Myristoylated alanine-rich C kinase substrate (MARCKS): a molecular target for the therapeutic action of mood stabilizers in the brain? J. Clin. Psychiatry *57 Suppl 13*, 23-31.

Lenox,R.H., McNamara,R.K., Papke,R.L., and Manji,H.K. (1998). Neurobiology of lithium: an update. J. Clin. Psychiatry *59 Suppl 6*, 37-47.

Li,P.P., Young,L.T., Tam,Y.K., Sibony,D., and Warsh,J.J. (1993). Effects of chronic lithium and carbamazepine treatment on G-protein subunit expression in rat cerebral cortex. Biol. Psychiatry *34*, 162-170.

Li,X.M., Chlan-Fourney,J., Juorio,A.V., Bennett,V.L., Shrikhande,S., and Bowen,R.C. (2000). Antidepressants upregulate messenger RNA levels of the neuroprotective enzyme superoxide dismutase (SOD1). J. Psychiatry Neurosci. *25*, 43-47.

Liang,P., Averboukh,L., Keyomarsi,K., Sager,R., and Pardee,A.B. (1992). Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer Res *52*:6966-8.

Lievremont,J.P., Rizzuto,R., Hendershot,L., and Meldolesi,J. (1997). BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. J. Biol. Chem. *272*, 30873-30879.

Lin,S.Z., Yan,G.M., Koch,K.E., Paul,S.M., and Irwin,R.P. (1997). Mastoparan-induced apoptosis of cultured cerebellar granule neurons is initiated by calcium release from intracellular stores. Brain Res. *771*, 184-195.

Lingjaerde,O. (1963). Tetrabenazine (Nitroman) in the treatment of psychoses. Acta Psychiatr. Scand. *39 (Suppl 170)*, 1-109.

Little,E., Ramakrishnan,M., Roy,B., Gazit,G., and Lee,A.S. (1994). The glucose-regulated proteins (GRP78 and GRP94): functions, gene regulation, and applications. Crit Rev. Eukaryot. Gene Expr. *4*, 1-18.

Liu,H., Bowes,R.C., III, van De,W.B., Sillence,C., Nagelkerke,J.F., and Stevens,J.L. (1997). Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca2+ disturbances, and cell death in renal epithelial cells. J. Biol Chem. *272*, 21751-21759.

Liu,H., Miller,E., van De,W.B., and Stevens,J.L. (1998). Endoplasmic reticulum stress proteins block oxidant-induced Ca2+ increases and cell death. J. Biol Chem. *273*, 12858-12862.

Lofquist,A.K., Mondal,K., Morris,J.S., and Haskill,J.S. (1995). Transcription-independent turnover of I kappa B alpha during monocyte adherence: implications for a translational component regulating I kappa B alpha/MAD-3 mRNA levels. Mol. Cell Biol. *15*, 1737-1746.

Lopez,J.F., Young,E.A., Herman,J.P., Akil,H., and Watson,S.J. (1991). Regulatory Biology of the HPA Axis: An Integrative Approach. Washington, D.C.: American Psychiatric Press.

Lopez,J.F., Vazquez,D.M., Chalmers,D.T., and Watson,S.J. (1997). Regulation of 5-HT receptors and the hypothalamic-pituitary-adrenal axis. Implications for the neurobiology of suicide. Ann. N. Y. Acad. Sci *836*, 106-134.

Loscher,W. (1981). Anticonvulsant activity of metabolites of valproic acid. Arch. Int. Pharmacodyn. Ther. *249*, 158-163.

Lowenstein,D.H., Gwinn,R.P., Seren,M.S., Simon,R.P., and McIntosh,T.K. (1994). Increased expression of mRNA encoding calbindin-D28K, the glucose- regulated proteins, or the 72 kDa heat-shock protein in three models of acute CNS injury. Brain Res. Mol. Brain Res. *22*, 299-308.

MacQueen,G.M. and Young,L.T. (2001). Bipolar II disorder: symptoms, course, and response to treatment. Psychiatr. Serv. *52*, 358-361.

Magarinos,A.M., McEwen,B.S., Flugge,G., and Fuchs,E. (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. J. Neurosci. *16*, 3534-3540.

Malberg,J.E., Eisch,A.J., Nestler,E.J., and Duman,R.S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J. Neurosci. *20*, 9104-9110.

Manji,H.K., Chen,G.A., Bitran,J.A., Gusovsky,F., and Potter,W.Z. (1991a). Chronic exposure of C6 glioma cells to desipramine desensitizes beta-adrenoceptors, but increases KL/KH ratio. Eur. J. Pharmacol. *206*, 159-162.

Manji,H.K., Chen,G.A., Bitran,J.A., and Potter,W.Z. (1991b). Down-regulation of beta receptors by desipramine in vitro involves PKC/phospholipase A2. Psychopharmacol. Bull. *27*, 247-253.

Manji,H.K., Chen,G., Shimon,H., Hsiao,J.K., Potter,W.Z., and Belmaker,R.H. (1995). Guanine nucleotide-binding proteins in bipolar affective disorder. Effects of long-term lithium treatment. Arch. Gen. Psychiatry *52*, 135-144.

Manji,H.K., Moore,G.J., and Chen,G. (2000a). Clinical and preclinical evidence for the neurotrophic effects of mood stabilizers: implications for the pathophysiology and treatment of manic-depressive illness. Biol. Psychiatry *48*, 740-754.

Manji,H.K., Moore,G.J., and Chen,G. (2000b). Lithium up-regulates the cytoprotective protein Bcl-2 in the CNS in vivo: a role for neurotrophic and neuroprotective effects in manic depressive illness. J. Clin. Psychiatry *61 Suppl 9*, 82-96.

Manji,H.K., Drevets,W.C., and Charney,D.S. (2001a). The cellular neurobiology of depression. Nat. Med. *7*, 541-547.

Manji,H.K., Moore,G.J., and Chen,G. (2001b). Bipolar disorder: leads from the molecular and cellular mechanisms of action of mood stabilizers. Br. J. Psychiatry Suppl *41*, s107-s119.

Mann,J.J. (1998). The neurobiology of suicide. Nat. Med. *4*, 25-30.

Mann,J.J., Waternaux,C., Haas,G.L., and Malone,K.M. (1999). Toward a clinical model of suicidal behavior in psychiatric patients. Am. J. Psychiatry *156*, 181-189.

Mann,J.J., Brent,D.A., and Arango,V. (2001). The neurobiology and genetics of suicide and attempted suicide: a focus on the serotonergic system. Neuropsychopharmacology *24*, 467-477.

Martin,M.L. and Regan,C.M. (1991). The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. Brain Res. *554*, 223-228.

Mayr,B. and Montminy,M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev Mol. Cell Biol. *2*, 599-609.

McCauliffe,D.P., Yang,Y.S., Wilson,J., Sontheimer,R.D., and Capra,J.D. (1992). The 5'-flanking region of the human calreticulin gene shares homology with the human GRP78, GRP94, and protein disulfide isomerase promoters. J. Biol Chem. *267*, 2557-2562.

McElroy,S.L., Keck,P.E., Jr., Pope,H.G., Jr., and Hudson,J.I. (1992). Valproate in the treatment of bipolar disorder: literature review and clinical guidelines. J. Clin. Psychopharmacol. *12*, 42S-52S.

McEwen,B.S. (1998). Multiple ovarian hormone effects on brain structure and function. J. Gend. Specif. Med. *1*, 33-41.

Melnick,J., Aviel,S., and Argon,Y. (1992). The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. J. Biol. Chem. *267*, 21303-21306.

Mendels,J., Johnston,R., Mattes,J., and Riesenberg,R. (1993). Efficacy and safety of b.i.d. doses of venlafaxine in a dose-response study. Psychopharmacol. Bull. *29*, 169-174.

Mendlewicz,J. (1995). Pharmacologic profile and efficacy of venlafaxine. Int. Clin. Psychopharmacol. *10 Suppl 2*, 5-13.

Menkes,D.B., Rasenick,M.M., Wheeler,M.A., and Bitensky,M.W. (1983). Guanosine triphosphate activation of brain adenylate cyclase: enhancement by long-term antidepressant treatment. Science *219*, 65-67.

Mery,L., Mesaeli,N., Michalak,M., Opas,M., Lew,D.P., and Krause,K.H. (1996). Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx. J. Biol. Chem. *271*, 9332-9339.

Michalak,M., Burns,K., Andrin,C., Mesaeli,N., Jass,G.H., Busaan,J.L., and Opas,M. (1996). Endoplasmic reticulum form of calreticulin modulates glucocorticoid- sensitive gene expression. J. Biol. Chem. *271*, 29436-29445.

Mikuni,M., Kusumi,I., Kagaya,A., Kuroda,Y., Mori,H., and Takahashi,K. (1991). Increased 5-HT-2 receptor function as measured by serotonin-stimulated phosphoinositide hydrolysis in platelets of depressed patients. Prog. Neuropsychopharmacol. Biol. Psychiatry *15*, 49-61.

Mikuni,M., Kagaya,A., Takahashi,K., and Meltzer,H.Y. (1992). Serotonin but not norepinephrine-induced calcium mobilization of platelets is enhanced in affective disorders. Psychopharmacology (Berl) *106*, 311-314.

Miles,M.F., Wilke,N., Elliot,M., Tanner,W., and Shah,S. (1994). Ethanol-responsive genes in neural cells include the 78-kilodalton glucose-regulated protein (GRP78) and 94-kilodalton glucose-regulated protein (GRP94) molecular chaperones. Mol. Pharmacol. *46*, 873-879.

Mitchell,P.B., Manji,H.K., Chen,G., Jolkovsky,L., Smith-Jackson,E., Denicoff,K., Schmidt,M., and Potter,W.Z. (1997). High levels of Gs alpha in platelets of euthymic patients with bipolar affective disorder. Am. J. Psychiatry *154*, 218-223.

Mitchell,P.B. and Malhi,G.S. (2002). The expanding pharmacopoeia for bipolar disorder. Annu. Rev Med. *53*, 173-188.

Moore,G.J., Bebchuk,J.M., Wilds,I.B., Chen,G., Manji,H.K., and Manji,H.K. (2000). Lithium-induced increase in human brain grey matter. Lancet *356*, 1241-1242.

Mora,A., Gonzalez-Polo,R.A., Fuentes,J.M., Soler,G., and Centeno,F. (1999). Different mechanisms of protection against apoptosis by valproate and Li+. Eur. J. Biochem. *266*, 886-891.

Morinobu,S., Fujimaki,K., Okuyama,N., Takahashi,M., and Duman,R.S. (1999). Stimulation of adenylyl cyclase and induction of brain-derived neurotrophic factor and TrkB mRNA by NKH477, a novel and potent forskolin derivative. J. Neurochem. *72*, 2198-2205.

Mosmann,T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods *65*, 55-63.

Muller-Oerlinghausen,B., Berghofer,A., and Bauer,M. (2002). Bipolar disorder. Lancet *359*, 241-247.

Munro,S. and Pelham,H.R. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose- regulated protein and immunoglobulin heavy chain binding protein. Cell *46*, 291-300.

Nestler,E.J., Terwilliger,R.Z., and Duman,R.S. (1989). Chronic antidepressant administration alters the subcellular distribution of cyclic AMP-dependent protein kinase in rat frontal cortex. J. Neurochem. *53* , 1644-1647.

Nibuya,M., Morinobu,S., and Duman,R.S. (1995). Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J. Neurosci. *15*, 7539-7547.

Nibuya,M., Nestler,E.J., and Duman,R.S. (1996). Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. J. Neurosci. *16*, 2365-2372.

Nigam,S.K., Goldberg,A.L., Ho,S., Rohde,M.F., Bush,K.T., and Sherman,M.Y. (1994). A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca(2+)-binding proteins and members of the thioredoxin superfamily. J. Biol Chem. *269*, 1744-1749.

Nishio,Y., Aiello,L.P., and King,G.L. (1994). Glucose induced genes in bovine aortic smooth muscle cells identified by mRNA differential display. FASEB J. *8*, 103-106.

Noga,J.T., Vladar,K., and Torrey,E.F. (2001). A volumetric magnetic resonance imaging study of monozygotic twins discordant for bipolar disorder. Psychiatry Res. *106*, 25-34.

Nonaka,S., Katsube,N., and Chuang,D.M. (1998a). Lithium protects rat cerebellar granule cells against apoptosis induced by anticonvulsants, phenytoin and carbamazepine. J. Pharmacol. Exp. Ther. *286*, 539-547.

Nonaka,S., Hough,C.J., and Chuang,D.M. (1998b). Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting N-methyl-D-aspartate receptor-mediated calcium influx. Proc. Natl. Acad. Sci U. S. A *95*, 2642-2647.

O'Donnell,T., Rotzinger,S., Nakashima,T.T., Hanstock,C.C., Ulrich,M., and Silverstone,P.H. (2000). Chronic lithium and sodium valproate both decrease the concentration of myo-inositol and increase the concentration of inositol monophosphates in rat brain. Brain Res. *880*, 84-91.

Odagaki,Y., Garcia-Sevilla,J.A., Huguelet,P., La Harpe,R., Koyama,T., and Guimon,J. (2001). Cyclic AMP-mediated signaling components are upregulated in the prefrontal cortex of depressed suicide victims. Brain Res. *898*, 224-231.

Ongur,D., Drevets,W.C., and Price,J.L. (1998). Glial reduction in the subgenual prefrontal cortex in mood disorders. Proc. Natl. Acad. Sci U. S. A *95*, 13290-13295.

Ozaki,N. and Chuang,D.M. (1997). Lithium increases transcription factor binding to AP-1 and cyclic AMP- responsive element in cultured neurons and rat brain. J. Neurochem. *69*, 2336-2344.

Ozawa,H., Gsell,W., Frolich,L., Zochling,R., Pantucek,F., Beckmann,H., and Riederer,P. (1993). Imbalance of the Gs and Gi/o function in post-mortem human brain of depressed patients. J. Neural Transm. Gen. Sect. *94*, 63-69.

Pacheco,M.A., Stockmeier,C., Meltzer,H.Y., Overholser,J.C., Dilley,G.E., and Jope,R.S. (1996). Alterations in phosphoinositide signaling and G-protein levels in depressed suicide brain. Brain Res. *723*, 37-45.

Pandey,G.N., Dysken,M.W., Garver,D.L., and Davis,J.M. (1979). Beta-adrenergic receptor function in affective illness. Am. J. Psychiatry *136*, 675-678.

Pandey,G.N., Ren,X., Pandey,S.C., Dwivedi,Y., Sharma,R., and Janicak,P.G. (2001). Hyperactive phosphoinositide signaling pathway in platelets of depressed patients: effect of desipramine treatment. Psychiatry Res. *105*, 23-32.

Penry,J.K. and Dean,J.C. (1989). The scope and use of valproate in epilepsy. J. Clin. Psychiatry *50 Suppl*, 17-22.

Perez,J., Tinelli,D., Brunello,N., and Racagni,G. (1989). cAMP-dependent phosphorylation of soluble and crude microtubule fractions of rat cerebral cortex after prolonged desmethylimipramine treatment. Eur. J. Pharmacol. *172*, 305-316.

Perez,J., Zanardi,R., Mori,S., Gasperini,M., Smeraldi,E., and Racagni,G. (1995). Abnormalities of cAMP-dependent endogenous phosphorylation in platelets from patients with bipolar disorder. Am. J. Psychiatry *152*, 1204-1206.

Perez,J., Tardito,D., Mori,S., Racagni,G., Smeraldi,E., and Zanardi,R. (1999). Abnormalities of cyclic adenosine monophosphate signaling in platelets from untreated patients with bipolar disorder. Arch. Gen. Psychiatry *56*, 248-253.

Perez,J., Tardito,D., Mori,S., Racagni,G., Smeraldi,E., and Zanardi,R. (2000). Altered Rap1 endogenous phosphorylation and levels in platelets from patients with bipolar disorder. J. Psychiatr. Res. *34*, 99-104.

Peterfy,G., Pinter,E.J., and Pattee,C.J. (1976). Psychosomatic aspects of catecholamine depletion: comparative studies of metabolic, endocrine and affective changes. Psychoneuroendocrinology *1*, 243-253.

Placidi,G.P., Oquendo,M.A., Malone,K.M., Huang,Y.Y., Ellis,S.P., and Mann,J.J. (2001). Aggressivity, suicide attempts, and depression: relationship to cerebrospinal fluid monoamine metabolite levels. Biol. Psychiatry *50*, 783-791.

Pollanen,M.S., Quinn,B.A., Wollenberg,G.K., and Hayes,M.A. (1990). Reversible mitochondrial swelling in cultured rat hepatocytes exposed to 1,2-dimethylhydrazine. Exp. Mol. Pathol. *52*, 170-178.

Poolsup,N., Li Wan,P.A., and de Oliveira,I.R. (2000). Systematic overview of lithium treatment in acute mania. J. Clin. Pharm. Ther. *25*, 139-156.

Pope,H.G., Jr., McElroy,S.L., Keck,P.E., Jr., and Hudson,J.I. (1991). Valproate in the treatment of acute mania. A placebo-controlled study. Arch. Gen. Psychiatry *48*, 62-68.

Popoli,M., Mori,S., Brunello,N., Perez,J., Gennarelli,M., and Racagni,G. (2001). Serine/threonine kinases as molecular targets of antidepressants: implications for pharmacological treatment and pathophysiology of affective disorders. Pharmacol. Ther. *89*, 149-170.

Post,R.M., Uhde,T.W., Roy-Byrne,P.P., and Joffe,R.T. (1987). Correlates of antimanic response to carbamazepine. Psychiatry Res. *21*, 71-83.

Post,R.M. (1992a). Impact of affective illness on gene expression: implications for early and long-term prophylaxis. Clin. Neuropharmacol. *15 Suppl 1 Pt A*, 452A-453A.

Post,R.M. (1992b). Transduction of psychosocial stress into the neurobiology of recurrent affective disorder. Am. J. Psychiatry *149*, 999-1010.

Post,R.M., Weiss,S.R., and Chuang,D.M. (1992). Mechanisms of action of anticonvulsants in affective disorders: comparisons with lithium. J. Clin. Psychopharmacol. *12*, 23S-35S.

Potter,W.Z. and Bowden,C.L. (1992). Valproate and mood disorders: perspectives. Introduction. J. Clin. Psychopharmacol. *12*, 2S-6S.

Prendergast,M.A., Harris,B.R., Mayer,S., Holley,R.C., Pauly,J.R., and Littleton,J.M. (2001). Nicotine exposure reduces N-methyl-D-aspartate toxicity in the hippocampus: relation to distribution of the alpha7 nicotinic acetylcholine receptor subunit. Med. Sci Monit. *7*, 1153-1160.

Price,L.H., Charney,D.S., Delgado,P.L., and Heninger,G.R. (1990). Lithium and serotonin function: implications for the serotonin hypothesis of depression. Psychopharmacology (Berl) *100*, 3-12.

Puka-Sundvall,M., Hallin,U., Zhu,C., Wang,X., Karlsson,J.O., Blomgren,K., and Hagberg,H. (2000). NMDA blockade attenuates caspase-3 activation and DNA fragmentation after neonatal hypoxia-ischemia. Neuroreport *11*, 2833-2836.

Quetsch R.M., Achor R.W.P., Litin E.M., and Faucett R.L. (1959) Depressive reactions in hypertensive patients, a comparison of those treated with rauwolfia and those receiving no specific antihypertensive treatment. Circulation 19, 366-375.

Rahman,S., Li,P.P., Young,L.T., Kofman,O., Kish,S.J., and Warsh,J.J. (1997). Reduced [3H]cyclic AMP binding in postmortem brain from subjects with bipolar affective disorder. J. Neurochem. *68*, 297-304.

Rajkowska,G., Miguel-Hidalgo,J.J., Wei,J., Dilley,G., Pittman,S.D., Meltzer,H.Y., Overholser,J.C., Roth,B.L., and Stockmeier,C.A. (1999). Morphometric evidence for

neuronal and glial prefrontal cell pathology in major depression. Biol. Psychiatry *45*, 1085-1098.

Rajkowska,G. (2000). Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. Biol. Psychiatry *48*, 766-777.

Rajkowska,G., Halaris,A., and Selemon,L.D. (2001). Reductions in neuronal and glial density characterize the dorsolateral prefrontal cortex in bipolar disorder. Biol. Psychiatry *49*, 741-752.

Rao,R.V., Hermel,E., Castro-Obregon,S., del Rio,G., Ellerby,L.M., Ellerby,H.M., and Bredesen,D.E. (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J. Biol. Chem. *276*, 33869-33874.

Regan,R.F., Panter,S.S., Witz,A., Tilly,J.L., and Giffard,R.G. (1995). Ultrastructure of excitotoxic neuronal death in murine cortical culture. Brain Res. *705*, 188-198.

Regier,D.A., Farmer,M.E., Rae,D.S., Locke,B.Z., Keith,S.J., Judd,L.L., and Goodwin,F.K. (1990). Comorbidity of mental disorders with alcohol and other drug abuse. Results from the Epidemiologic Catchment Area (ECA) Study. JAMA *264*, 2511-2518.

Reiach,J.S., Li,P.P., Warsh,J.J., Kish,S.J., and Young,L.T. (1999). Reduced adenylyl cyclase immunolabeling and activity in postmortem temporal cortex of depressed suicide victims. J. Affect. Disord. *56*, 141-151.

Resendez,E., Jr., Wooden,S.K., and Lee,A.S. (1988). Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. Mol. Cell Biol *8*, 4579-4584.

Ressler,K.J. and Nemeroff,C.B. (1999). Role of norepinephrine in the pathophysiology and treatment of mood disorders. Biol. Psychiatry *46*, 1219-1233.

Rutter,J.J., Gundlah,C., and Auerbach,S.B. (1994). Increase in extracellular serotonin produced by uptake inhibitors is enhanced after chronic treatment with fluoxetine. Neurosci. Lett. *171*, 183-186.

Sanger,F., Nicklen,S., and Coulson,A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci U. S. A *74*, 5463-5467.

Sapolsky,R.M. (2001). Cellular defenses against excitotoxic insults. J. Neurochem. *76*, 1601-1611.

Sastry,P.S. and Rao,K.S. (2000). Apoptosis and the nervous system. J. Neurochem. *74*, 1-20.

Schatzberg,A.F. (1998). Noradrenergic versus serotonergic antidepressants: predictors of treatment response. J. Clin. Psychiatry *59 Suppl 14*, 15-18.

Schild,L., Keilhoff,G., Augustin,W., Reiser,G., and Striggow,F. (2001). Distinct Ca2+ thresholds determine cytochrome c release or permeability transition pore opening in brain mitochondria. FASEB J. *15*, 565-567.

Schildkraut,J.J. (1965). The catecholamine hypothesis of affective disorders: a review of supporting evidence. Am. J. Psychiatry *122*, 509-522.

Schreiber,G., Avissar,S., Danon,A., and Belmaker,R.H. (1991). Hyperfunctional G proteins in mononuclear leukocytes of patients with mania. Biol. Psychiatry *29*, 273-280.

Sclar,D.A., Robinson,L.M., Skaer,T.L., and Galin,R.S. (1998). Trends in the prescribing of antidepressant pharmacotherapy: office- based visits, 1990-1995. Clin. Ther. *20*, 871-884.

Selkoe,D.J. (1986). Altered structural proteins in plaques and tangles: what do they tell us about the biology of Alzheimer's disease? Neurobiol. Aging *7*, 425-432.

Sheline,Y.I., Wang,P.W., Gado,M.H., Csernansky,J.G., and Vannier,M.W. (1996). Hippocampal atrophy in recurrent major depression. Proc. Natl. Acad. Sci U. S. A *93*, 3908-3913.

Sheline,Y.I., Sanghavi,M., Mintun,M.A., and Gado,M.H. (1999). Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J. Neurosci. *19*, 5034-5043.

Sherwood,B.E., Suppes,T., Adinoff,B., and Rajan,T.N. (2001). Drug abuse and bipolar disorder: comorbidity or misdiagnosis? J. Affect. Disord. *65*, 105-115.

Shirayama,Y., Chen,A.C., Nakagawa,S., Russell,D.S., and Duman,R.S. (2002). Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J. Neurosci. *22*, 3251-3261.

Shiu,R.P., Pouyssegur,J., and Pastan,I. (1977). Glucose depletion accounts for the induction of two transformation-sensitive membrane proteinsin Rous sarcoma virus-transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. U. S. A *74*, 3840-3844.

Slamon,N.D. and Pentreath,V.W. (2000). Antioxidant defense against antidepressants in C6 and 1321N1 cells. Chem. Biol. Interact. *127*, 181-199.

Slamon,N.D., Ward,T.H., Butler,J., and Pentreath,V.W. (2001). Assessment of DNA damage in C6 glioma cells after antidepressant treatment using an alkaline comet assay. Arch. Toxicol. *75*, 243-250.

Slater,G.E. and Johnston,D. (1978). Sodium valproate increases potassium conductance in Aplysia neurons. Epilepsia *19*, 379-384.

Soares,J.C. and Mallinger,A.G. (1996). Abnormal phosphatidylinositol (PI)-signalling in bipolar disorder. Biol. Psychiatry *39*, 461-464.

Soares,J.C. and Mallinger,A.G. (1997). Intracellular phosphatidylinositol pathway abnormalities in bipolar disorder patients. Psychopharmacol. Bull. *33*, 685-691.

Soares,J.C., Dippold,C.S., Wells,K.F., Frank,E., Kupfer,D.J., and Mallinger,A.G. (2001). Increased platelet membrane phosphatidylinositol-4,5-bisphosphate in drug-free depressed bipolar patients. Neurosci. Lett. *299*, 150-152.

Son,S.E. and Kirchner,J.T. (2000). Depression in children and adolescents. Am. Fam. Physician *62*, 2297-2.

Song,L. and Jope,R.S. (1992). Chronic lithium treatment impairs phosphatidylinositol hydrolysis in membranes from rat brain regions. J. Neurochem. *58*, 2200-2206.

Southam,E., Kirkby,D., Higgins,G.A., and Hagan,R.M. (1998). Lamotrigine inhibits monoamine uptake in vitro and modulates 5- hydroxytryptamine uptake in rats. Eur. J. Pharmacol. *358*, 19-24.

Spleiss,O., van Calker,D., Scharer,L., Adamovic,K., Berger,M., and Gebicke-Haerter,P.J. (1998). Abnormal G protein alpha(s) - and alpha(i2)-subunit mRNA expression in bipolar affective disorder. Mol. Psychiatry *3*, 512-520.

Staley,J.K., Malison,R.T., and Innis,R.B. (1998). Imaging of the serotonergic system: interactions of neuroanatomical and functional abnormalities of depression. Biol. Psychiatry *44*, 534-549.

Stassen,H.H., Angst,J., and Delini-Stula,A. (1996). Delayed onset of action of antidepressant drugs? Survey of results of Zurich meta-analyses. Pharmacopsychiatry *29*, 87-96.

Stockmeier,C.A. (1997). Neurobiology of serotonin in depression and suicide. Ann. N. Y. Acad. Sci *836*, 220-232.

Storring,J.M., Charest,A., Cheng,P., and Albert,P.R. (1999). TATA-driven transcriptional initiation and regulation of the rat serotonin 5-HT1A receptor gene. J. Neurochem. *72*, 2238-2247.

Strakowski,S.M., DelBello,M.P., Sax,K.W., Zimmerman,M.E., Shear,P.K., Hawkins,J.M., and Larson,E.R. (1999). Brain magnetic resonance imaging of structural abnormalities in bipolar disorder. Arch. Gen. Psychiatry *56*, 254-260.

Sugawara,T., Noshita,N., Lewen,A., Gasche,Y., Ferrand-Drake,M., Fujimura,M., Morita-Fujimura,Y., and Chan,P.H. (2002). Overexpression of Copper/Zinc Superoxide Dismutase in Transgenic Rats Protects Vulnerable Neurons against Ischemic Damage by Blocking the Mitochondrial Pathway of Caspase Activation. J. Neurosci. *22*, 209-217.

Tai,K.K., Blondelle,S.E., Ostresh,J.M., Houghten,R.A., and Montal,M. (2001). An N-methyl-D-aspartate receptor channel blocker with neuroprotective activity. Proc. Natl. Acad. Sci U. S. A *98*, 3519-3524.

Tamaoki,T., Nomoto,H., Takahashi,I., Kato,Y., Morimoto,M., and Tomita,F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. Biochem. Biophys. Res. Commun. *135*, 397-402.

Tenneti,L. and Lipton,S.A. (2000). Involvement of activated caspase-3-like proteases in N-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. J. Neurochem. *74*, 134-142.

Thase,M.E. and Sachs,G.S. (2000). Bipolar depression: pharmacotherapy and related therapeutic strategies. Biol. Psychiatry *48*, 558-572.

Thies-Flechtner,K., Muller-Oerlinghausen,B., Seibert,W., Walther,A., and Greil,W. (1996). Effect of prophylactic treatment on suicide risk in patients with major affective disorders. Data from a randomized prospective trial. Pharmacopsychiatry *29*, 103-107.

Thomas,C.E. and Mayle,D.A. (2000). NMDA-sensitive neurons profoundly influence delayed staurosporine-induced apoptosis in rat mixed cortical neuronal cultures. Brain Res. *884*, 163-173.

Thome,J., Sakai,N., Shin,K., Steffen,C., Zhang,Y.J., Impey,S., Storm,D., and Duman,R.S. (2000). cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. J. Neurosci. *20*, 4030-4036.

Toki,S., Donati,R.J., and Rasenick,M.M. (1999). Treatment of C6 glioma cells and rats with antidepressant drugs increases the detergent extraction of G(s alpha) from plasma membrane. J. Neurochem. *73*, 1114-1120.

Tondo,L., Hennen,J., and Baldessarini,R.J. (2001). Lower suicide risk with long-term lithium treatment in major affective illness: a meta-analysis. Acta Psychiatr. Scand. *104*, 163-172.

Tork,I. (1990). Anatomy of the serotonergic system. Ann. N. Y. Acad. Sci *600*, 9-34.

Torrey,E.F., Webster,M., Knable,M., Johnston,N., and Yolken,R.H. (2000). The Stanley foundation brain collection and neuropathology consortium. Schizophr. Res. *44*, 151-155.

Uno,H., Tarara,R., Else,J.G., Suleman,M.A., and Sapolsky,R.M. (1989). Hippocampal damage associated with prolonged and fatal stress in primates. J. Neurosci. *9*, 1705-1711.

Utans,U., Liang,P., Wyner,L.R., Karnovsky,M.J., and Russell,M.E. (1994). Chronic cardiac rejection: identification of five upregulated genes in transplanted hearts by differential mRNA display. Proc. Natl. Acad. Sci. U. S. A *91*, 6463-6467.

Vaidya,V.A. and Duman,R.S. (2001). Depresssion--emerging insights from neurobiology. Br. Med. Bull. *57*, 61-79.

van De Water,B., Wang,Y., Asmellash,S., Liu,H., Zhan,Y., Miller,E., and Stevens,J.L. (1999). Distinct endoplasmic reticulum signaling pathways regulate apoptotic and necrotic cell death following iodoacetamide treatment. Chem. Res. Toxicol. *12*, 943-951.

Van Kammen,D.P. and Murphy,D.L. (1975). Attenuation of the euphoriant and activating effects of d- and l-amphetamine by lithium carbonate treatment. Psychopharmacologia. *44*, 215-224.

Vawter,M.P., Freed,W.J., and Kleinman,J.E. (2000). Neuropathology of bipolar disorder. Biol. Psychiatry *48*, 486-504.

Velasco,I. and Tapia,R. (2000). Alterations of intracellular calcium homeostasis and mitochondrial function are involved in ruthenium red neurotoxicity in primary cortical cultures. J. Neurosci. Res. *60*, 543-551.

Volbracht,C., Leist,M., Kolb,S.A., and Nicotera,P. (2001). Apoptosis in caspase-inhibited neurons. Mol. Med. *7*, 36-48.

Wang,G.J. and Thayer,S.A. (1996). Sequestration of glutamate-induced Ca2+ loads by mitochondria in cultured rat hippocampal neurons. J. Neurophysiol. *7*, 1611-1621.

Wang,J.F. and Young,L.T. (1996). Differential display PCR reveals increased expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase by lithium. FEBS Lett. *386*, 225-229.

Wang,J.F., Asghari,V., Rockel,C., and Young,L.T. (1999a). Cyclic AMP responsive element binding protein phosphorylation and DNA binding is decreased by chronic lithium but not valproate treatment of SH-SY5Y neuroblastoma cells. Neuroscience *91*, 771-776.

Wang,J.F., Bown,C., and Young,L.T. (1999b). Differential display PCR reveals novel targets for the mood-stabilizing drug valproate including the molecular chaperone GRP78. Mol. Pharmacol. *55*, 521-527.

Wang,J.F., Chen,B., and Young,L.T. (1999c). Identification of a novel lithium regulated gene in rat brain. Brain Res. Mol. Brain Res. *70*, 66-73.

Wang,J.F., Bown,C.D., Chen,B., and Young,L.T. (2001). Identification of mood stabilizer-regulated genes by differential- display PCR. Int. J. Neuropsychopharmacol. *4*, 65-74.

Wang,K.K. (2000). Calpain and caspase: can you tell the difference? Trends Neurosci. *23*, 20-26.

Wang,L., Watson,D.G., and Lenox,R.H. (2000). Myristoylation alters retinoic acid-induced down-regulation of MARCKS in immortalized hippocampal cells. Biochem. Biophys. Res. Commun. *276*, 183-188.

Wang,Y.C., Pandey,G.N., Mendels,J., and Frazer,A. (1974). Platelet adenylate cyclase responses in depression: implications for a receptor defect. Psychopharmacologia. *36*, 291-300.

Waser,M., Mesaeli,N., Spencer,C., and Michalak,M. (1997). Regulation of calreticulin gene expression by calcium. J. Cell Biol. *138*, 547-557.

Watson,D.G., Watterson,J.M., and Lenox,R.H. (1998). Sodium valproate down-regulates the myristoylated alanine-rich C kinase substrate (MARCKS) in immortalized hippocampal cells: a property of protein kinase C-mediated mood stabilizers. J. Pharmacol. Exp. Ther. *285*, 307-316.

Weizman,R. and Weizman,A. (2001). Use of atypical antipsychotics in mood disorders. Curr. Opin. Investig. Drugs *2*, 940-945.

Welch,S.S. (2001). A review of the literature on the epidemiology of parasuicide in the general population. Psychiatr. Serv. *52* , 368-375.

Welner,S.A., De Montigny,C., Desroches,J., Desjardins,P., and Suranyi-Cadotte,B.E. (1989). Autoradiographic quantification of serotonin1A receptors in rat brain following antidepressant drug treatment. Synapse *4*, 347-352.

Werner,E.D., Brodsky,J.L., and McCracken,A.A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc. Natl. Acad. Sci U. S. A *93*, 13797-13801.

Wheatley,D.P., van Moffaert,M., Timmerman,L., and Kremer,C.M. (1998). Mirtazapine: efficacy and tolerability in comparison with fluoxetine in patients with moderate to severe major depressive disorder. Mirtazapine- Fluoxetine Study Group. J. Clin. Psychiatry *59*, 306-312.

Williams,M.B. and Jope,R.S. (1995). Circadian variation in rat brain AP-1 DNA binding activity after cholinergic stimulation: modulation by lithium. Psychopharmacology (Berl) *122*, 363-368.

Williams,W.A., Shoaf,S.E., Hommer,D., Rawlings,R., and Linnoila,M. (1999). Effects of acute tryptophan depletion on plasma and cerebrospinal fluid tryptophan and 5-hydroxyindoleacetic acid in normal volunteers. J. Neurochem. *72*, 1641-1647.

Wolpert,E.A., Goldberg,J.F., and Harrow,M. (1990). Rapid cycling in unipolar and bipolar affective disorders. Am. J. Psychiatry *147*, 725-728.

Wooden,S.K., Li,L.J., Navarro,D., Qadri,I., Pereira,L., and Lee,A.S. (1991). Transactivation of the grp78 promoter by malfolded proteins, glycosylation block, and calcium ionophore is mediated through a proximal region containing a CCAAT motif which interacts with CTF/NF-I. Mol. Cell Biol *11*, 5612-5623.

Wyatt,R.J. and Henter,I. (1995). An economic evaluation of manic-depressive illness--1991. Soc. Psychiatry Psychiatr. Epidemiol. *30*, 213-219.

Xu,W., Longo,F.J., Wintermantel,M.R., Jiang,X., Clark,R.A., and DeLisle,S. (2000). Calreticulin modulates capacitative Ca2+ influx by controlling the extent of inositol 1,4,5-trisphosphate-induced Ca2+ store depletion. J. Biol. Chem. *275*, 36676-36682.

Yamaji,T., Kagaya,A., Uchitomi,Y., Yokata,N., and Yamawaki,S. (1996). Effects of carbamazepine and sodium valproate on 5-HT-induced calcium increase in individual C6 rat glioma cells. Neuropsychobiology *34*, 22-25.

Yau,J.L., Noble,J., Hibberd,C., and Seckl,J.R. (2001). Short-term administration of fluoxetine and venlafaxine decreases corticosteroid receptor mRNA expression in the rat hippocampus. Neurosci. Lett. *306*, 161-164.

Yoshida,H., Haze,K., Yanagi,H., Yura,T., and Mori,K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose- regulated proteins. Involvement of basic leucine zipper transcription factors. J. Biol. Chem. *273*, 33741-33749.

Young,L.T., Li,P.P., Kish,S.J., Siu,K.P., and Warsh,J.J. (1991). Postmortem cerebral cortex Gs alpha-subunit levels are elevated in bipolar affective disorder. Brain Res. *553*, 323-326.

Young,L.T., Li,P.P., Kish,S.J., Siu,K.P., Kamble,A., Hornykiewicz,O., and Warsh,J.J. (1993). Cerebral cortex Gs alpha protein levels and forskolin-stimulated cyclic AMP formation are increased in bipolar affective disorder. J. Neurochem. *61*, 890-898.

Young,L.T., Warsh,J.J., Kish,S.J., Shannak,K., and Hornykeiwicz,O. (1994a). Reduced brain 5-HT and elevated NE turnover and metabolites in bipolar affective disorder. Biol. Psychiatry *35*, 121-127.

Young,L.T., Li,P.P., Kish,S.J., and Warsh,J.J. (1994b). Cerebral cortex beta-adrenoceptor binding in bipolar affective disorder. J. Affect. Disord. *30*, 89-92.6

Young,L.T., Li,P.P., Kamble,A., Siu,K.P., and Warsh,J.J. (1994c). Mononuclear leukocyte levels of G proteins in depressed patients with bipolar disorder or major depressive disorder. Am. J. Psychiatry *151*, 594-596.

Yu,Z., Luo,H., Fu,W., and Mattson,M.P. (1999). The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Exp. Neurol. *155*, 302-314.

Yuan,P.X., Huang,L.D., Jiang,Y.M., Gutkind,J.S., Manji,H.K., and Chen,G. (2001). The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth. J. Biol. Chem. *276*, 31674-31683.

Zanardi,R., Racagni,G., Smeraldi,E., and Perez,J. (1997). Differential effects of lithium on platelet protein phosphorylation in bipolar patients and healthy subjects. Psychopharmacology (Berl) *129*, 44-47.

Zhang,H., Sternberger,N.H., Rubinstein,L.J., Herman,M.M., Binder,L.I., and Sternberger,L.A. (1989). Abnormal processing of multiple proteins in Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A *86*, 8045-8049.

Zhu,J., Mix,E., and Winblad,B. (2001). The antidepressant and antiinflammatory effects of rolipram in the central nervous system. CNS. Drug Rev *7*, 387-398.

Zhu,L.P., Yu,X.D., Ling,S., Brown,R.A., and Kuo,T.H. (2000). Mitochondrial Ca(2+)homeostasis in the regulation of apoptotic and necrotic cell deaths. Cell Calcium *28*, 107-117.

Zhu,M.Y., Klimek,V., Dilley,G.E., Haycock,J.W., Stockmeier,C., Overholser,J.C., Meltzer,H.Y., and Ordway,G.A. (1999). Elevated levels of tyrosine hydroxylase in the locus coeruleus in major depression. Biol. Psychiatry *46*, 1275-1286.