IDENTIFICATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES FOLLOWING CHRONIC LITHIUM TREATMENT IN RAT BRAIN

By

Len Van Hua

A thesis submitted in conformity with the requirements
for the Degree of Doctor of Philosophy,
Department of Pharmacology, in the
University of Toronto

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ABSTRACT

The precise mechanisms underlying the therapeutic actions of lithium in bipolar disorder remain unclear, however, substantial evidence suggests alterations in gene expression may be critical to its long-term mood stabilization. The primary objectives of this study were to screen and identify genes, whose expression is altered in rat frontal cortex following chronic lithium treatment, by mRNA differential display. The pharmacospecificity of lithium's effects on gene expression was subsequently evaluated in comparison to those of anticonvulsant mood stabilizers, valproate and carbamazepine, and non-mood stabilizing psychotropic drugs, haloperidol and imipramine.

Analyses by mRNA differential display and northern blotting identified two lithium regulated genes (LRG), LRG1 and LRG2. In addition, a third LRG (LRG3) was serendipitously identified by rapid amplification of cDNA ends. Molecular cloning and DNA sequence analyses of LRG1, LRG2 and LRG3 revealed their identities as aldolase A, diphosphoinositol polyphosphate phosphohydrolase 2 (DIPP2) and CD151, respectively. The mRNA levels of aldolase A (and C isozyme) were significantly reduced by chronic (5 weeks) lithium or carbamazepine treatment. The DIPP2 mRNA levels were increased only by lithium, whereas CD151 mRNA levels were reduced by chonic lithium, valproate or carbamazepine treatment. No significant effect on the mRNA levels of any of these genes was evident following either short-term (1-week) lithium treatment or chronic administration of haloperidol and imipramine, demonstrating time dependence and pharmacological specificity of the lithium's effects. Chronic

lithium treatment also reduced the cytosolic, but not particulate, protein levels of aldolase in rat frontal cortex.

Although the pharmacotherapeutic relevance of the effects of lithium on the expression of these three genes remains to be further investigated, the observations of a potential role of these gene products in the inositol phosphoinositide metabolism and signaling suggest that lithium may act, at the level of gene expression, on different components of the phosphoinositide signaling cascades, thereby producing its therapeutic efficacy. This suggestion is consistent with the current notion that abnormalities in intracellular signaling, including phosphoinositide signaling, may contribute, at least in part, to the pathophysiology of bipolar disorder.

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ABBREVIATIONS

A_{260}	absorbance at 260nm
Ab	antibody
AC	adenylyl cyclase
ADP	adenosine 5'-diphosphate
Ag	antigen
AKAP	protein kinase A-anchoring protein
ANOVA	analysis of variance
Ap_5A	diadenosine 5',5'''-P^1,P^5-pentakisphosphate
Ap_6A	diadenosine 5',5'''- P^1,P^6-hexakisphosphate
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
BD	bipolar disorder
bis	N,N'-methylene-bisacrylamide
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CaM	calmodulin
cAMP	cyclic adenosine 3',5'-monophosphate
CBZ	carbamazepine
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine 3',5'-monophoshate
CRE	cAMP responsive element

CNS	central nervous system
CO_2	carbon dioxide
cpm	counts per minute
CREB	CRE binding protein
CsCl	cesium chloride
CTX	cholera toxin
Da	dalton
dATP	2'-deoxyadenosine-triphosphate
dCTP	2'-deoxycytosine-triphosphate
ddNTP	dideoxynucleoside triphosphate
ddPCR	differential display polymerase chain reaction
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DIPP	diphosphoinositol polyphosphate phosphohydrolase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DAG	1,2-diacylglycerol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
g	*gravity*
GDP	guanosine diphosphate
GIT	guanidine isothyiocyanate
Gpp(NH)p	guanylylimidodiphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)

GTPase	guanosine triphosphatase
h	hour
5-HT	serotonin
$InsP_6$	inositol hexakisphosphate
IP	inositol monophosphate
IPTG	isopropyl-β-D-thiogalactoside
IP_3	inositol 1,4,5 triphosphote
k	kilo
Li_2CO_3	lithium carbonate
LB	luria broth
M	molar
MAP	microtubule associated protein
β-ME	β-mercaptoethanol
$MgCl_2$	magnesium chloride
min	minute
µl	microliter
µM	micromolar
ml	milliliter
mM	millimolar
MOPS	3-(4-morpholinyl) 1-propanesulfonic acid
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
PBS	phosphate buffer saline
KCl	potassium chloride
NaCl	sodium chloride

NaOAc	sodium acetate
NaOH	sodium hydroxide
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE	phosphodiesterase
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
$[PP]_2$-$InsP_4$	bis-diphosphoinositol tetrakisphosphate
PP-$InsP_5$	diphosphoinositol pentakisphosphate
PTX	pertussis toxin
RNA	ribonucleic acid
rpm	revolution per minute
RE	restriction endonuclease
RT	reverse transcriptase
rt	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
SSC	salt sodium citrate
TAE	tris-acetate-EDTA

TCA	trichloracetic acid
TBE	tris-borate-EDTA
TEMED	N,N,N',N'-tetramethyl ethylene diamine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volts
VPA	valproate
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

OVERVIEW

1.1 INTRODUCTION

Lithium, an alkali metal and the lightest of the solid elements, was discovered in 1817. Over the ensuing period of time, it has been used in various formulations as a remedy for a multitude of maladies (Johnson, 1984). When its overall benefits were found not to hold up to the hype, however, lithium fell out of favor and came to be regarded as another blunder in medicinal chemistry. It was not until 1949 when Australian psychiatrist, John Cade, first reported lithium had antimanic effects and subsequent clinical studies by Mogen Schou in the early 1950s, that interest was renewed in lithium's potential as an antimanic treatment and a prophylactic agent in the therapy for bipolar affective disorder (Cade, 1949; Schou, 1979).

Bipolar affective disorder (BD) comprises a group of episodic illnesses characterized by at least one manic or hypomanic episode and recurrent major depressive episodes (Goodwin and Jamison, 1990). The lifetime prevalence is about 1% (Weissman et al, 1988). During the manic phase of the illness, patients experience a distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting for at least one week, with three or more of the following symptoms: inflated self-esteem or grandiosity, decreased need for sleep, more talkative than usual, flight of ideas, distractibility, increase in goal-directed activity either socially, at work or school, or sexually, psychomotor agitation, excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g. unrestrained buying sprees, sexual indiscretions, or foolish business investments). In contrast, the depressive phase of the illness is marked by five or more of the following symptoms during the same 2-week period: depressed mood or diminished interest or pleasure in activities, significant weight loss or gain, decrease or increase in appetite, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthlessness, diminished ability to think or concentrate, recurrent thoughts of death or suicide. Suicide is the cause of death in 10-20% of individuals with BD (Simpson and Jamison, 1999). In addition to suicide, the economic burden of direct (inpatient

burden of direct (inpatient and outpatient care) and indirect costs (loss of productivity) associated with BD is estimated to be in the tens of billions of dollars annually in United States alone (Wyatt and Henter, 1995). The effect on the broader community is highlighted by one estimation that the use of lithium saved the United States $4 billion in the period 1969 – 1979 by reducing associated medical costs and restoring productivity (Reifman and Wyatt, 1980). Furthermore, according to a Global Burden of Disease Study, BD is one of the leading causes of disability worldwide (Goldberg and Harrow, 1999). Despite well-established genetic diatheses and extensive research, the biochemical abnormalities underlying the predisposition to, and the pathophysiology of, BD remain to be fully elucidated.

The discovery of lithium's efficacy as a mood-stabilizing agent revolutionized the treatment of BD. Long-term lithium treatment not only reduces the excess mortality from suicide observed in the illness but also reduces cardiovascular mortality (Baldessarini et al, 1999). After more than half a century since its rediscovery, lithium continues to be widely used for the treatment of BD, both for the acute manic phase and as prophylaxis for recurrent manic and depressive episodes (Lenox and Manji, 1998). It has been estimated that in most industrialized countries, one in every thousand people in the population is undergoing lithium treatment (Schou, 1991).

Although lithium is regarded as one of the important pharmacotherapies in psychiatry, this drug has a narrow therapeutic window and a number of disconcerting side effects. Appreciable side effects occur in 35%-93% of lithium-treated patients (Lenox et al, 1998). Most common side effects include excessive thirst, polyuria, memory problems, tremor, weight gain, drowsiness and diarrhea, and these can often lead to noncompliance. Additionally, it is increasingly appreciated that a significant number of patients, up to 40%, respond poorly to lithium therapy, while many others are helped but continue to suffer significant morbidity (Chou, 1991). Furthermore, the specific cellular and molecular mechanisms underlying its mood-

stabilizing properties remain unclear. In these respects, the study of the mechanisms by which lithium exerts its mood-stabilizing effects is important not only for the development of new therapeutic agents that work potently and specifically, with fewer side effects, but also for the potential to improve understanding of the etiology and pathophysiology of BD.

Major progress has been made over the past few decades with accumulating evidence suggesting that lithium may exert its mood-stabilizing effects by modulating the functional balance between various interacting neurotransmitter systems thought to be dysregulated in BD (Manji et al, 1995; Jope, 1999b). In this context, it is noteworthy that recent biochemical, molecular, and cellular studies have revealed the action of lithium on various cellular signaling processes that are critical to the normal functioning of cells, including phosphoinositide (PI), cyclic adenosine monophosphate (cAMP), and calcium signaling, among others (Manji et al 1995; Jope, 1999b; Lenox and Hahn, 2000; Li et al, 2000).

Many of the cellular signaling cascades are known to induce long-term cellular responses by means of regulating gene expression (Karin and Hunter, 1995). Moreover, the therapeutic effect of lithium is delayed in onset, generally a week or more for definite changes to be observed (Goodwin and Jamison, 1990). The dependence of biochemical changes on prolonged drug administration, such as with lithium, is most compatible with the notion that altered expression of critical neuronal genes is essential to its therapeutic action. Therefore, regulation of gene expression has been postulated as one of the potential mechanisms by which lithium exerts its long-term mood stabilizing effects. Indeed, a growing body of evidence to date supports the notion that lithium, at clinically effective doses, is capable of regulating the expression of a number of genes in rat brain and cultured cell models (Sivam et al, 1988; Li et al, 1991; Colin et al, 1991; Chen et al, 1998). However, the identification of lithium regulated genes has been primarily undertaken with the "candidate gene' approach, which only permits the examination of one or a few known genes based on specific hypotheses derived from earlier findings. The

transcriptional changes of other genes, especially novel genes that could not be readily linked on a conceptual base to earlier findings, might be overlooked. Furthermore, lithium has been shown to modulate the DNA binding activity of transcription factors, cAMP responsive element binding (CREB) protein and activator protein 1 (AP-1) (Ozaki and Chuang, 1997; Yuan et al, 1998), both of which have been shown to regulate the expression of numerous genes (Hughes and Dragunow, 1995). Since many genes contain cAMP responsive element (CRE) and/or AP1 sites in their promoter regions, it is likely that many other genes are affected by lithium that would not be considered by the candidate gene approach. The identification of such lithium-regulated genes (LRGs) is important to obtaining a better understanding of the mechanisms by which lithium exerts its therapeutic and/or side effect(s) in BD, particularly if the products of these genes are important in central nervous system function.

In recent years, new methods have evolved to identify differential expression of genes between two populations of cells or tissues. One such method that is being increasingly used is the reverse transcription polymerase chain reaction mRNA differential display (dd-PCR) (Liang and Pardee, 1992). The primary objective of this thesis was to use this technique to screen, identify and characterize lithium-regulated genes in brain of rats treated chronically with lithium.

In the following sections the major signaling pathways and their roles in regulating gene expression are briefly reviewed. This overview focuses on those signaling cascades that have been implicated as targets for lithium's action. Subsequently, the current state of knowledge regarding the mode of action of lithium is discussed. The specific objectives and the hypotheses in this thesis are then detailed.

1.2 MAJOR SIGNALING PATHWAYS

Cellular signaling is initiated generally outside of the cell by extracellular molecules, including odorants, antigens, neurotransmitters, neuromodulators or hormones. The majority of

these signals are hydrophilic and do not permeate lipid bilayer membranes. In most cases, the signals are transmitted via membrane receptors to initiate intracellular cascades of different enzymatic reactions by various signaling molecules, resulting in short-term cellular effects in the cytoplasm and at membranes, and long-term effects via regulation of gene expression. Major signaling pathways that have been implicated to be modulated by lithium include the cAMP, PI, calcium, phosphoinositide 3-kinase (PI3-K)/protein kinase B (PKB) and Wnt/glycogen synthase kinase (GSK) signaling pathways.

1.2.1 cAMP Signaling Pathway

This signaling system is initiated by the action of neurotransmitters, neuromodulators, or hormones on G-protein coupled receptors (GPCR) that stimulate or inhibit adenylate cyclase (AC) via G proteins. GPCRs are heptahelical proteins. The amino terminal domain and three intervening segments between the membrane-spanning domains are extracellular, whereas three additional intervening segments as well as the carboxyl terminal reside intracellularly (Strader et al, 1994). The ligand-binding domain for small molecules is generally formed by sites in the transmembrane domains, whereas those for large ligands are located in the extracellular N-terminal segment (Ji et al, 1998). Upon binding of ligand, the conformation of the receptor changes to allow the intracellular loops of the receptor to interact with G proteins (Gether and Kobilka, 1998). G proteins are members of an ubiquitous superfamily of GTPases that serve as the first intracellular signal relaying molecules of the GPCR signaling pathways (Gilman, 1987). They are heterotrimers consisting of α -, β- and γ-subunits. β and γ subunits exist for the most part as tightly bound dimers. In the basal state, G proteins exist in the undissociated heterotrimer form with GDP bound to the α-subunit, and associated with receptors. Upon activation, α-subunits undergo conformational changes that induce the release of GDP and promote the binding of GTP. GTP-bound α-subunits dissociate from both the receptor and $\beta\gamma$ subunits. Both

α- and βγ-subunits are subsequently free to affect a variety of effector enzymes, such as AC and phospholipase C-β (PLCβ). The intrinsic GTPase activity of the α-subunits hydrolyzes GTP to GDP and allows the α-subunit to reassociate with βγ-dimer, thus terminating the activity of effector enzymes (Gilman, 1987). The GTPase activity of Gα and its association with βγ subunits are regulated by accessory proteins, such as regulator of G protein signaling (RGS) and phosducin, respectively. RGS functions to accelerate the inactivation rate of GTP-bound Gα subunits (Berman and Gilman, 1998). Phosducin tightly binds to Gβγ, thus preventing their interaction with Gα and effectors (Bluml et al, 1997).

Another mode of negative regulation is mediated by phosphorylation of GPCR resulting in the uncoupling of the receptor from its G-protein, also known as receptor desensitization. Receptor desensitization can be divided into two processes: homologous and heterologous desensitization (Lefkowitz, 1993; Lohse, 1993; Sterne-Marr and Benovic, 1995). Homologous desensitization occurs when the binding of agonist to the receptor induces conformational changes in the receptor that unmask the phosphoacceptor sites in the C-terminal tail of the receptor for phosphorylation by receptor kinases, such as β-adrenergic receptor kinase (β-ARK) for β-adrenoceptors (Bouvier et al, 1988; Fredericks et al, 1996). In contrast, heterologous desensitization is induced by a downstream second messenger in the pathway that stimulates a respective protein kinase to phosphorylate serine and threonine residues in the intracellular region of the activated receptor as well as other receptor subtypes (Fishman and Perkins, 1988; Palczewski and Benovic, 1991; Grady et al, 1997). The time course for homologous desensitization is rapid, within seconds of receptor stimulation, whereas heterologous desensitization is relatively slow, occurring within minutes rather than seconds of agonist stimulation (Roth et al, 1991).

Adenylate cyclase activity is regulated by two groups of G proteins with opposing effects. $G\alpha_s$ and $G\alpha_{olf}$ stimulate AC activity, while $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$ inhibit the enzyme. Activation of AC catalyzes the conversion of Mg^{2+}-ATP to cAMP that, in turn, activates protein kinase A (PKA). In the absence of cAMP, PKA consists of two regulatory and two catalytic subunits (Beebe and Corbin 1986). Two cAMP molecules bind to each regulatory subunit inducing a conformational change that releases the catalytic subunit. The free PKA catalytic subunit rapidly phosphorylates cytoplasmic or membrane proteins, thereby changing their activity and causing immediate alterations in the biological activity of the cell. PKA may also mediate delayed chronic effects when the active catalytic subunit enters the nucleus and phosphorylates transcription factors, such as CREB. This can affect the transcription of genes containing CRE consensus sequences in their promoter regions (Hoeffler et al, 1990). The duration of cAMP stimulation of PKA is short lived, because of its rapid hydrolysis to 5'-AMP by one or more cAMP-dependent phosphodiesterase(s) (Conti and Jin, 1999). Finally, the effect of PKA on protein phosphorylation is negatively regulated by the action of different protein phosphatases, which dephosphorylate and terminate the activity of phosphorylated proteins (Brindley and Waggoner, 1996).

1.2.2 Phosphoinositide Signaling Pathway

This signaling system represents another pathway in which G-protein coupled transduction mechanisms operate. In this instance, extracellular signals interact with receptors coupled to $G\alpha_{q/11}$, which in turn activates PLCβ (Taylor et al, 1986). PLCβ catalyzes the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2) generating two second-messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG). $InsP_3$ releases calcium (Ca^{2+}) from intracellular stores by binding to an $InsP_3$ receptor, which comprises an $InsP_3$-gated Ca^{2+} channel (Berridge, 1987). Ca^{2+} signals in cells are transduced via Ca^{2+}-dependent enzymes, such

as protein kinase C (PKC) (Nishizuka, 1992), or via Ca^{2+}-binding proteins, such as calmodulin (CaM) (Means et al, 1991). The Ca^{2+}/CaM complex in turn interacts and activates different cellular effectors including Ca^{2+}/CaM-dependent protein kinases (CaMK) (Cruzalegui et al, 1992; Knighton et al, 1992). The other second messenger derived from PI hydrolysis, DAG, activates Ca^{2+} and lipid-dependent PKC isozymes (Nishizuka, 1992). Similar to PKA, CaMK- and PKC-mediated phosphorylation of substrate proteins can elicit both rapid and long-term physiological effects. Upon activation, CaMK and PKC rapidly phosphorylate cytoplasmic or membrane proteins to achieve acute changes in the biological activity in the cell. In many cases, these kinases also phosphorylate transcription factors (i.e. CREB, serum response factor, etc.), thus regulating gene expression (Nishizuka, 1986; Cruzalegui et al, 1992; Misra et al, 1994) (Fig 1). The activity of these phosphorylated proteins is also negatively regulated by the action of different protein phosphatases (Brindley and Waggoner, 1996). Although CaMK, PKC and PKA are serine/threonine protein kinases, the amino acid sequences that they recognize in their target substrates generally differ (Kemp and Pearson, 1990).

The levels of $InsP_3$ and DAG are tightly regulated by different intracellular enzymes. DAG either is hydrolyzed by lipases to monoacylglycerol, and then to free arachidonate and glycerol, or is phosphorylated by diacylglycerol kinase to form phosphatidic acid, which is then used in the synthesis of phosphatidylinositol (Majerus et al, 1986). $InsP_3$ is rapidly dephosphorylated to one of several isomers of inositol bisphosphates ($InsP_2$), and subsequently to inositol monophosphates (InsP). These are further dephosphorylated by inositol monophosphatase (IMPase) to free myo-inositol for resynthesis of phosphatidylinositols (Berridge, 1997). On the other hand, $InsP_3$ can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), which can be phosphorylated to multiple higher order phosphorylated inositol isomers, including inositol pentakisphosphate ($InsP_5$), inositol hexakisphosphate ($InsP_6$),

and diphosphoinositol polyphosphates (PP-$InsP_5$ and PP-$InsP_6$), the functions of which are less clear (Michell, 1997; Shears, 1998) (Fig 1).

1.2.3 Wnt Signaling Pathway

Wnt proteins are a group of cysteine-rich secreted glycoproteins, usually 300-400 amino acids in length, that act as developmentally significant signaling molecules, controlling cell fate specification, cell migration and cell polarity (Cadigan and Nusse, 1997). Wnt signaling is transduced by members of the Frizzled (Fz) gene family that can function as Wnt receptors (Bhanot et al, 1996, 1999). Structurally, the Fz receptors have seven transmembrane domains, an amino-terminal extension acting as a ligand binding domain, and a cytoplasmic C-terminal tail (Bhanot et al, 1996). Although all previously identified heptahelical receptors utilize G protein for signaling, thus far there is no genetic or biochemical evidence for direct interaction between G proteins and Fz in the Wnt signal transduction pathway. In response to activation of Fz by Wnt, Dishevelled (Dsh), a cytoplasmic protein, is hyperphosphorylated and activated, leading to suppression of the activity of GSK3α/β, a serine/threonine kinase. This suppression of GSK3α/β activity probably also involves PKC acting either downstream or in parallel to Dsh (Cook et al, 1996). When the Wnt signal is absent, GSK3α/β in concert with the APC (adenomatous polyposis coli)/Axin complex phosphorylates β-catenin and promotes its degradation via ubiquitination. Inhibition of GSK3α/β activity leads to increased intracellular levels of β-catenin that enters the nucleus and forms a complex with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to regulate expression of target genes (Willert and Nusse, 1998). In addition GSK3α/β is also known to modulate the function of other transcription factors, including AP1, CREB and nuclear factor-kappa B (NF-κB) (de Groot et al, 1993a; Lin et al, 1993; Fiol et al, 1994; Pulverer et al, 1994; Hoeflich et al, 2000).

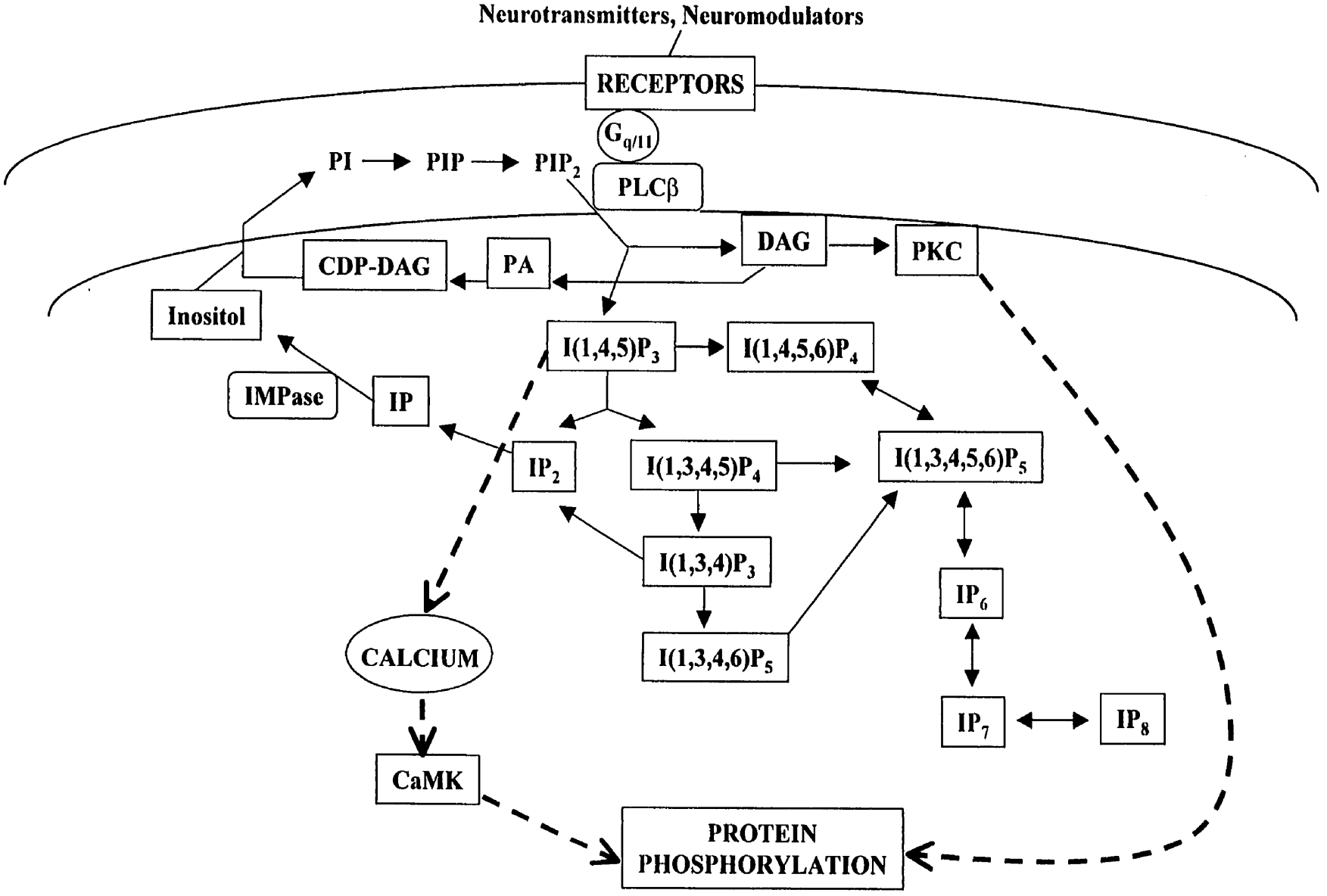


Figure 1 Overview of the phosphoinositol signaling system. Neurotransmitters/neuromodulators mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) produces the second messenger diacylglycerol (DAG), which activates protein kinase C (PKC), and myo-inositol 1,4,5-trisphophate (IP_3), which releases calcium from intracellular stores. Inositol polyphosphate metabolic pathways are also depicted.

1.2.4 PI3-K/PKB Signaling

Growth factors and neurotrophins bind to a family of receptors with intrinsic protein tyrosine kinase activity. These receptors, known as receptor tyrosine kinases (RTKs), consist of an extracellular region, membrane-spanning region and kinase domain (van der Geer et al, 1994). Upon ligand binding at the extracellular domain, these receptors dimerize and activate the intracellular protein tyrosine kinase domain. This kinase can phosphorylate itself at specific tyrosine residues. One of the signaling events that occurs following the activation of RTKs by growth factors is the recruitment and activation of PI3-K, via the interaction of its regulatory p85 subunit to phosphorylated RTK. Activated PI3-K phosphorylates inositol phospholipids at the D3 position resulting in the production of phosphatidylinositol (3,4,5)-trisphoshate (PIP_3) and phosphatidylinositol (3,4) bisphosphate [$PI(3,4)P_2$]. These lipids bind to the pleckstrin-homology (PH) domain of serine/threonine PKB (also known as Akt) and recruit PKB to the membrane, whereupon 3-phosphoinositide-dependent kinases (PDKs) phosphorylate and activate PKB (Franke et al, 1995, 1997; Burgering and Coffer, 1995; Alessi et al, 1996). The activated PKB phosphorylates GSK3α/β leading to inactivation of this kinase and stimulation of glycogen synthesis among numerous other cellular functions regulated by GSK3α/β, including modulation of transcription factor (β-catenin, CREB, AP-1, NF-κB) activity and subsequent gene expression (de Groot et al, 1993a; Cross et al, 1995; Pende et al, 1997; Hoeflich et al, 2000).

1.2.5 Complexity of Cellular Signaling

The preceding intracellular signaling pathways have been presented in a linear fashion as an initial step in understanding the cascade of events underlying signal transduction. Within cells, however, the different signaling pathways interact to regulate one another. Certain kinases can act on more than one substrate leading to divergence in the signaling pathway, whereas other kinases can act on the same substrate leading various pathways to converge. This contributes to

an intricate web for regulation of cellular function (Fig. 2). One example is the crosstalk between G-protein-coupled signaling pathways and mitogen-activated protein kinase (MAPK) pathways; PKC can activate the MAPK cascade directly via activating Raf-1, whereas PKA can repress the activation of this pathway by inhibiting Raf-1 (Burgering and Bos, 1995). In addition, PKC/PKA can phosphorylate and inhibit GSK3β, and Ras can also phosphorylate GSK3β via its activation of PI3-K (Rodriguez-viciana et al, 1996) (Fig 2).

In addition, the existence of multiple isoforms for each signaling molecule and their differential regulation further contributes to the complexity of signaling cascades. For instance, in the G-protein linked model of signal transduction, there are at least 16 known Gα subunits, 5 Gβ, and 11 Gγ subunits (Bourne, 1997), permitting different combinations of αβγ heterotrimeric G-proteins to exist. Individual receptors can activate different combinations of αβγ complexes, and the various Gα and Gβγ can activate different effector enzymes (Helpler and Gilman, 1992; Clapman and Neer, 1997; Morris and Malbon, 1999). Thus, the multiplicity of G proteins provides a mechanism for multiplexing varying messages in cellular processes and responses. Multiple isoforms also exist for different effector enzymes, including adenylate cyclase, protein kinases, protein phosphatases and others. For example, nine different types of AC (AC1 – AC9) with distinct regulatory characteristics have been reported (Defer et al, 1994; Krupinski, 1991; Mons et al, 1995). AC1, AC3 and AC8 are sensitive to Ca^{2+}/CaM and are thus stimulated by submicromolar concentration of Ca^{2+} (Chetkovich and Sweatt, 1993; Mons et al, 1995; Hanoune et al, 1997). On the other hand, it has been found that AC5 and AC6 are inhibited by micromolar concentrations of Ca^{2+} (Mons and Cooper, 1994; Wayman et al, 1995; Smit and Iyengar, 1998).

Subcellular localization of different signaling molecules provides an additional level of regulation for many signaling pathways. Anchoring an effector enzyme in a specific location

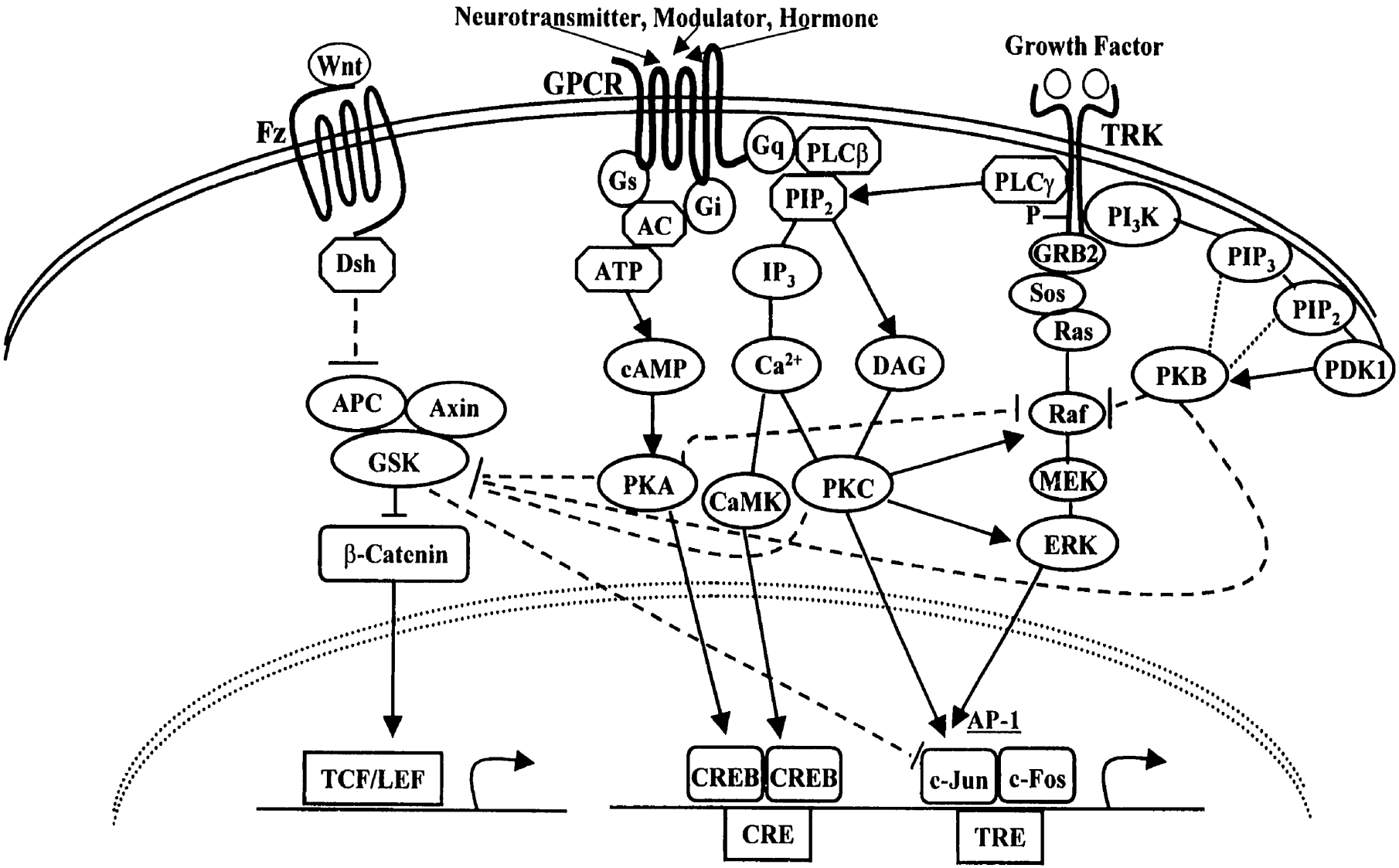


Figure 2 A simpified schematic representation of intracellular signaling pathways from extracellular signals to the nucleus. Solid arrows denote stimulatory pathways. Broken arrows indicate inhibitory pathways. Fz, fizzled receptor; GPCR, G-protein-coupled-receptor; TRK, tyrosine receptor kinase; Dsh, dishevelled; GSK, glycogen synthase kinase. For other abbreviations and detailed description of the pathways, see texts under respective subheading.

positions it in close proximity to its substrate and provides a stable framework in a signaling cascade. This subcellular localization can also limit the extent of activation of signaling enzymes. Examples of regulation by localization include components of the MAPK cascade that complexe with scaffolding proteins, which provide a stable framework between the cell membrane and the nucleus (Kyriakis and Avruch, 1996). The anchoring protein, AKAP 79, binds PKA, PKC and the type 2B protein phosphatase and coordinates their cellular actions (Klauck et al, 1996). In addition, many transducing enzymes are localized to cell adhesion complexes and cytoskeletal elements (Yamada and Geiger, 1997).

Despite the complexity of intracellular signaling, one element common to all the pathways considered here is the activation of protein kinases that subsequently mediate relatively rapid cellular effects by changes in the activity of cytoplasmic proteins, as well as long term effects by changes in gene transcription.

1.3 REGULATION OF GENE EXPRESSION BY CELLULAR SIGNALING

Cellular signaling leading to long term changes in biological activity involves the transduction of signals to the nucleus to modulate changes in gene transcription via various transcription factors that bind to specific short DNA regulatory sequence. The general mechanisms of gene transcription mediated by transcription factors via binding to their respective regulatory elements are discussed.

1.3.1 General Gene Transcription Mechanisms

The transcription of genes involves an intricate series of molecular events occurring in the nuclei of cells, which lead to the translation of genetic information encoded in the DNA into the proteins that mediate cellular functions. These molecular events are orchestrated by a highly specialized group of nuclear proteins in addition to DNA transcribing enzymes, RNA

polymerases. In eukaryotes, several types of RNA polymerases produce RNA molecules with a broad range of functions. However, only the type responsible for the synthesis of messenger RNAs (mRNA), RNA polymerase II (RNA pol II) (Darnell and Lodish, 1996), and the nuclear proteins that are involved in mRNA transcription will be discussed here.

The binding of RNA pol II to the promoter of a gene is a necessary initial step in the process of transcription. Promoters are short DNA sequences or motifs, such as the TATA box and initiator element (Inr) located upstream of the transcriptional start site, which are highly conserved among thousands of different genes (Latchman, 1990). In addition to RNA pol II, initiation of gene transcription requires various basal transcription factors (bTF), housekeeping proteins that participate in forming the pre-initiation complex (Roeder, 1996); and transcriptional activation factors (TAF), a group of inducible proteins that regulate the rate of gene transcription in response to cellular signals. In the absence of cellular signals for gene transcription, both the TATA box and the Inr are unoccupied, and the DNA upstream and downstream is tightly wound in a double helix (Roeder, 1996). Upon the reception of cellular signals to commence gene transcription, the pre-initiation complex begins to assemble in a series of steps. First, TAFs and bTFIIA activate TATA box binding protein (TBP) that subsequently binds to the TATA box (Conaway and Conaway, 1993). The binding of TBP to the TATA box distorts the helix, creating a binding site for bTFIIB, the key protein that facilitates the full assembly of the complex and initiation of gene transcription (Nikolov et al, 1995). Once the bTFIIB is in position, RNA pol II is recruited into the complex. Since bTFIIF exists in a complex with RNA pol II in the resting state, bTFIIF is also recruited to a specific position within the growing complex (Zawel and Reinberg, 1995). bTFIIF contains interacting sites for bTFIIE, which it recruits into the complex. bTFIIE in turn attracts and anchors the final component of the complex, bTFIIH (Maxon et al, 1994), which is a helicase whose function is to unwind the DNA helix (Svejstrup et al, 1996). Upon the completion of the pre-initiation complex, bTFIIH unwinds a short stretch of 10

nucleotides at the transcription start site and activates the RNA pol II molecule to begin transcription (Fig 3). RNA pol II and its associated bTFIIF use "phosphate energy" from ATP to sustain the transcription of DNA into mRNA, whereas other proteins in the pre-initiation complex dissociate soon after the transcription begins (Reines et al, 1996). The elongation of the nascent mRNA chain continues until the RNA pol II reaches a termination signal sequence on the DNA template, at which point it falls off the template, thus terminating the transcription (Fig. 3).

These processes constitute the basic molecular events that result in the synthesis of an mRNA molecule from its DNA template. In eukaryotes, transcriptional initiation is the most tightly regulated process in the production of a protein. Many genes contain multiple DNA elements (promoters) upstream of the TATA box and Inr that accommodate the binding of different transcription factors (TF) to modulate the rate of transcription (Jones et al, 1988; Davidson et al, 1990). In addition, other DNA elements, known as enhancers, increase the rate of transcription. While promoters are restricted to the upstream region of the gene, enhancers can be located almost anywhere in the gene (Brenner et al, 1961). Many transcription factors exist as dimers, comprising DNA binding and transcriptional activating domains (Latchman, 1992). In the following sections, the properties of the different DNA elements and their respective transcription factors are considered.

1.3.2 cAMP Responsive Element (CRE)

Transcription factors responsive to the activation of the cAMP signaling pathway include CREB, cAMP response element modulator (CREM), and activating transcription factor-1 (ATF-1) (Foulkes et al, 1991). These factors bind to and modulate target gene expression via a conserved CRE, an eight base-pair palindromic sequence (*TGACGTCA*) typically found between nucleotide -30 to -180 upstream of the gene. CREB, CREM, and ATF-1 share a conserved

(A)

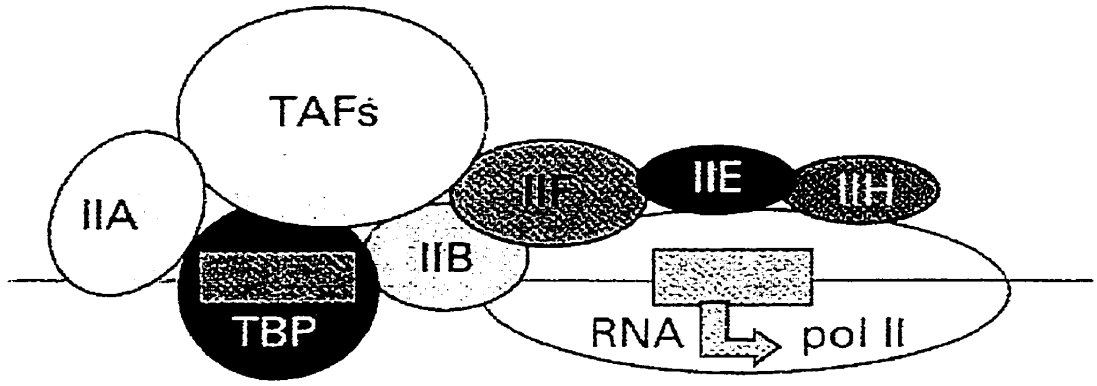


Figure 3A Components of the preinitiation complex. TAFs, transcriptional activation factors; TBP, TATA binding protein. Respective basal transcription factors for RNA polymerase II (adapted from Macfarlane, 2000).

(B)

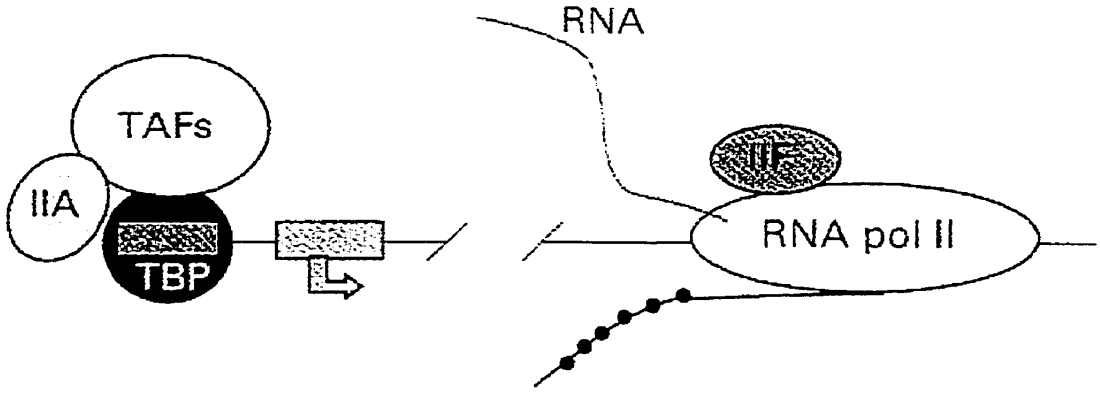


Figure 3B Elongation of the new mRNA molecule. The closed circles represent phosphate groups. TAFs, transcriptional activation factors; TBP, TATA binding protein (adapted from Macfarlane, 2000).

structure with carboxyl terminal leucine zippers region, an amino proximal terminal kinase-inducible domain (KID) also known as phosphorylation box (P-box), and glutamine-rich transactivation domains flanking the KID/P-box (Habener et al, 1995). Site-directed mutagenesis studies have revealed that PKA phosphorylates CREB at Ser-133 (Gonzalez and Montminy, 1989). Additional consensus sites for other serine/threonine protein kinases exist within the KID of CREB. Several of these sites are phosphorylated by hierarchical kinases, including GSK3β. Phosphorylation of serine 129 by GSK3β requires the initial phosphorylation of serine 133 by PKA (Roach, 1991; Sheng et al, 1991). Phosphorylation of CREB at serine 133 results in its association with the transcriptional co-activator CREB binding protein (CBP), thereby augmenting cAMP-induced pol II-directed gene transcription (Kwok et al, 1994).

The CREM gene is closely related to CREB. PKA also stimulates CREM by phosphorylation at a similar, functionally equivalent site (de Groot et al, 1993b). Many CREM isoforms exist that function as both activators and repressors of cAMP-mediated transcription and have a characteristic cell- and tissue-specific pattern of expression (Molina et al, 1993). ATF-1 was originally cloned as one of an extended family of CREB homologs (Hai et al, 1989). It binds CREs and forms heterodimers with CREB. Despite its similarity to CREB, the role of ATF-1 in PKA-dependent transactivation has not been demonstrated unequivocally.

1.3.3 TPA-responsive element (TRE)

The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, JunD) and Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) proteins (Karin et al, 1997). Although Jun proteins form very stable heterodimers with Fos family members, they can also homodimerize among themselves. The members of Fos family, however, do not form homodimers. Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-

13-acetate (TPA)-responsive element (TRE), which has the consensus sequence *TGACTCA* (Angel and Karin, 1991).

Different mechanisms are known to regulate AP-1 activity. The abundance of AP-1 proteins is regulated by controlling the transcription of their respective genes and by modulation of their stability (Karin, 1995). Phosphorylation of serine and threonine residues at the amino-terminal activation domain of c-Jun by MAPK reduces the ubiquitination of the protein and hence its degradation (Musti et al, 1997). Phosphorylation of Jun proteins by Jun amino-terminal kinase (a serine/threonine kinase and a member of the extensive family of MAPK, also known as stress-activated protein kinase) on serines 63 and 73 increases its stability to a certain extent (Musti et al, 1997). The function of c-Jun is also known to be regulated by Wnt signaling. In resting cells, c-Jun is highly phosphorylated by GSK3β on three sites close to its DNA-binding domain preventing c-Jun from binding to DNA. Activation of Wnt pathway inhibits GSK3β leading to the dephosphorylation of these sites and subsequent activation of gene transcription via AP-1 (de Groot et al, 1993a). Regarding c-*fos*, stimulation of cells by different stimuli induces rapid, transient c-*fos* transcription (Piechaczyk and Blanchard, 1994). Proximal to the c-*fos* TATA box is a CRE that is responsive to both cAMP- and Ca^{2+}-dependent signaling pathways.

1.3.4 High Mobility Group (HMG) Responsive Element

LEF/TCF transcription factors mediate a nuclear response to the Wnt signal by interacting with β-catenin. This group of proteins share a virtually identical DNA-binding domain, referred to as high mobility group (HMG) domain, which bind to a *(A/T)(A/T)CAA(A/T)GG* DNA motif (Clevers and van de Wetering, 1997). The exact mechanism by which β-catenin activates transcription is not known. However, β-catenin is known to contain two activation domains that are important for transcriptional responses to Wnt (Hsu et al, 1998).

Nuclear β-catenin has been suggested to displace corepressors from DNA-bound LEF-1/TCF proteins (Waltzer and Bienz, 1998).

1.4 A BRIEF HISTORY OF LITHIUM THERAPY

In this section, the use of lithium in medicine since its discovery as an element in 1817 by Johan August Arfwedson is briefly reviewed. A detailed account of this topic has been given by Johnson (Johnson, 1984).

Lithium was discovered in 1817 by Arfweson in analyzing the metal petalite. It constitutes about 20 parts per million of the igneous rocks of the earth's crust, so it is sparsely but widely distributed. Lithium salts were first introduced into medicine in the 1850s by the English physician Garrod in his book *Gout and Rheumatic Gout* (Garrod, 1859) following the observation that gouty deposits disappeared from a bone suspended in lithium carbonate solution. The rationale was that gout was caused by an excess of uric acid in the body, and lithium urate was known to be the most soluble of the urates. Thus administration of lithium salts could convert the insoluble uric acid to the highly soluble lithium urate to be excreted. This idea was generally accepted for almost a century. It was not until the twenty-second edition of this same text, published in 1941, that a clear statement appeared declaring that soluble urate could not exist in the body or the urine in the presence of sodium or potassium ions, and that there was no rational foundation for the use of lithium in the treatment of gout (Martindale, 1941). So the introduction of lithium into medicine was essentially the result of a simplistic interpretation of an empirical observation. Unfortunately, there were other misconceptions about the ions that lead to disastrous consequences. By the late 1940s lithium chloride was being used *ad libidum* as a salt substitute for flavoring low-sodium diets for patients suffering from edema due to congestive heart failure. Severe lithium chloride poisoning led to some deaths, and by March 1949, lithium was effectively removed from the pharmacopea as therapeutic substance in the USA.

Interestingly, in the same year, an Australian psychiatrist published a short paper suggesting that lithium salts may be useful in the treatment of psychotic excitement (Cade, 1949). This work apparently was not highly regarded because of the bitter experience with lithium in the USA. Fortunately, the paper came to the attention of Mogens Schou in Denmark who validated and extended the original observations in the following decade (Schou et al, 1976). The appreciation of the benefits of lithium in medicine in the USA was understandably slow given the skepticism that developed because of its toxicity. It was not until the 1970s that lithium was reintroduced as a therapeutic drug by the US Food and Drug Administration. After more than half a century since the seminal work by Cade, lithium still remains the "gold standard" for the treatment of bipolar disorder.

1.5 CURRENT UNDERSTANDING OF THE MECHANISM OF ACTION OF LITHIUM

Extensive research has been undertaken over the past few decades on the mechanism of therapeutic action of lithium in BD and while the literature is voluminous, the results can generally be categorized into those actions at pre- as compared to post-receptor mechanisms. Progress in understanding the mechanism of therapeutic action of lithium seems to parallel advances in understanding of cellular signaling mechanisms. Lithium, once administered, is distributed throughout the body affecting cellular signaling pathways from extracellular signals (neurotransmitters) to intracellular signaling components, and ultimately leading to long-term effects in the nucleus (gene expression). Current understanding of the mechanism(s) by which lithium exerts its action at different levels of cellular signaling is discussed in the following sections.

1.5.1 Lithium and Neurotransmitters

Various neurotransmitter systems have been extensively studied in the search for a potential mechanism underlying the therapeutic action of lithium in BD. Earlier studies focused on the presynaptic events, including synthesis, release, turnover and reuptake of the neurotransmitters. Most of these works were done in the 60s and 70s and have been reviewed in detail elsewhere (Murphy, 1974; Goodnick and Gershon, 1985; Bunney and Garland-Bunney, 1987). Therefore, only a synopsis of the current status in this field is presented.

1.5.1.1 Lithium and Norepinephrine (NE)

Lithium appears to exert differential effects on presynaptic levels of NE over time. Acute lithium treatment induces an increase in NE uptake and synthesis, which is normalized following chronic treatment (El-Mallakh, 1996). Investigation of the effect of lithium on adrenergic receptor binding or receptor number has been generally inconclusive (El-Mallakh, 1996). However, it has been shown that chronic lithium treatment prevents β-adrenoceptor supersensitivity induced by reserpine (Treiser and Kellar, 1979; Hermoni et al, 1980). A consistent observation *in vivo*, as well as *in vitro*, is that β-adreneroceptor-mediated accumulation of cAMP is decreased by lithium (Mork et al, 1990, 1992). In the absence of consistent changes in β-adrenoceptor density and/or affinity, these observations suggest that modulation of the NE system by lithium may be mediated via post-receptor mechanisms. However, the presynaptic α_2 autoreceptors might also be affected, since chronic lithium treatment has been shown to facilitate the release of norepinephrine (Manji et al, 1991).

1.5.1.2 Lithium and Dopamine (DA)

Chronic administration of lithium has little or no consistent effect upon the synthesis, release, turnover, and the level of DA in different rat brain regions (Wood and Goodwin, 1987).

However, lithium modifies adaptive changes in the dopaminergic system in response to neuroleptic administration. For example, lithium has been reported to prevent haloperidol-induced supersensitivity of dopamine receptors (Pert et al, 1978; Gallagher et al, 1978). Lithium also appears to block amphetamine-induced behavioral changes in both animals and humans (Goodnick and Gershon, 1985). These effects are likely mediated through post-receptor mechanisms, because lithium treatment has not been shown to significantly alter the parameters for ligand binding for DA receptors (Bunney and Garland, 1983).

1.5.1.3 Lithium and Serotonin (5-HT)

Although the effects of lithium on serotonergic function have been also studied extensively, no clear conclusion has been reached for the effect of chronic administration of lithium on the synthesis, release, turnover or receptor binding of 5HT, since much of the work has resulted in conflicting findings (Price et al, 1990). However, there is a general consensus amongst investigators that lithium tends to cause a net enhancement of 5-HT function, which has lead to the use of lithium for potentiation of antidepressants (Muller-Oerlingerhausen, 1985; Bunney and Garland-Bunney 1987; Waldmeier, 1987; Wood and Goodwin, 1987).

1.5.1.4 Acetylcholine (ACh)

Increased rates of synthesis and whole-brain ACh concentrations in rats were reported after chronic treatment with lithium (Jope, 1979). However, the effect of lithium upon the binding of ligands to muscarinic receptors in brain has been reported to increase (Tollefson et al, 1982), decrease (Hrushka et al, 1984) or remain unchanged after lithium(Maggi and Enna, 1980). Cumulative evidence over the years, nevertheless, suggests that chronic lithium treatment enhances the synaptic processing of ACh in rat brain (Dilsaver and Coffman, 1989). Similar to the effects observed in dopaminergic and β-adrenergic systems, lithium blocks the development

of receptor supersensitivity in the cholinergic system without altering the muscarinic cholinergic receptor binding (Lerer and Stanley 1985; Ellis and Lennox, 1990). These findings are also consistent with a post-receptor mechanism of action of lithium.

1.5.1.5 Lithium and γ-Aminobutyric acid (GABA)

GABA is the major inhibitory amino acid neurotransmitter in the CNS. Abnormal levels of GABA in the brain have been postulated as a possible etiological factor in affective disorders (Squires and Saederup, 1991). Lithium treatment has been associated with an increase in both plasma and CSF GABA levels in bipolar patients as well as various regions of rat brain (Berrettini et al, 1983; Motohashi, 1993). Chronic administration of lithium has been found to markedly reduce GABA-binding sites in the rat striatum and hypothalamus (Maggi and Enna, 1980). However, later studies using specific agonists for $GABA_A$ and $GABA_B$ receptors reported a reduction of $GABA_A$ binding in frontal cortex (Hetmar et al, 1983), whereas an increase in $GABA_B$ binding was found in hippocampus of rats treated chronically with lithium (Motahashi, 1992). The effects of lithium on GABAergic receptor function remain to be further investigated.

1.5.1.6 Lithium and Glutamate

More recently, a number of studies have stimulated increasing interest in the possible link between the effect of lithium on the glutamatergic system and its therapeutic action in bipolar disorder. Acute exposure of monkey and mouse brain tissue slices to lithium *in vitro* inhibits synaptic glutamate uptake leading to an increase in activation of N-methyl-D-aspartate (NMDA) receptors and subsequently intracellular IP_3 levels (Dixon et al, 1994; Hokin et al, 1996). In contrast, chronic lithium treatment significantly increased glutamate uptake *ex vivo* by stabilizing the glutamate uptake transporter (Dixon and Hokin, 1998). This increase in glutamate uptake following chronic lithium treatment has been proposed to play, at least in part, a role in

neuroprotection. In line with this supposition, lithium has been shown to reduce by 56% ischemic infarct size measured 24 hours after occlusion of the left middle cerebral artery (Nokana and Chuang, 1998). Since glutamate excitotoxicity may participate in ischemic brain damage (Nokana and Chuang, 1998), these findings suggest that neurodegeneration induced by the excitatory glutamatergic stimulus could be attenuated by lithium. However, the significance of such a neuroproprective effect of lithium in relation to its therapeutic efficacy in BD must await further investigation.

In summary, lithium has been shown to affect various neurotransmitter systems, however the implications of these effects with respect to its therapeutic action in BD remain unclear. Nevertheless, cumulatively these findings have inspired the view that multiple interacting and overlapping systems are involved in regulating mood, rather than any one specific neurotransmitter system in the pathogenesis of depression or mania. Similarly, most effective drugs in affecting mood do not work on any particular neurotransmitter system in isolation but rather affect the functional balance between interacting neurotransmitter systems (Hsiao et al, 1987; Janowsky et al, 1972; Meltzer, 1992). Therefore, the ability of lithium to affect various neurotransmitter systems at multiple levels may be necessary for its efficacy as a mood stabilizer.

1.5.2 Post-Receptor Pharmacological Effects of Lithium

Cumulative experimental data from studies of lithium's presynaptic effects indicated that lithium affected various neurotransmitter systems without consistent effects at the receptor level; however, these findings could not fully explain the mechanisms of lithium's therapeutic action. Hence, the focus of research has shifted to intracellular signaling mechanisms in the past two decades in the hope of obtaining information about lithium's cellular action. Accordingly, the

following sections will present some of the key findings on the effects of lithium on intracellular signaling pathways.

1.5.2.1 Lithium and cAMP Signaling Pathway

The first study on the effect of lithium on cAMP signaling reported an attenuation of AC activity stimulated by hormones in rabbit brain tissue (Dousa and Hechter, 1970). However, this study was done with rather high concentrations (25-50 mM) of lithium. Forn and Valdecasas (1971) later demonstrated that lithium, at a more therapeutically relevant concentration (2 mM), antagonizes the NE- and histamine- induced formation of cAMP in rabbit and rat brain cortical slices. Numerous studies in rats and humans have since corroborated that lithium, at therapeutically relevant concentrations, inhibits cAMP accumulation induced by various neurotransmitters and hormones, *in vitro* and *ex vivo* (Mork et al, 1992).

The mechanisms by which lithium modulates AC function have been extensively investigated over the years. *In vitro* studies have shown that lithium inhibits AC activity stimulated by the guanosine triphosphate (GTP) analogue, guanylyl-5'-imidodiphosphate (Gpp(NH)p) or forskolin, which directly stimulates AC (Anderson and Geisler, 1984; Mork and Geisler, 1987). Of mechanistic relevance, this *in vitro* inhibitory effect of lithium on AC was found to be competitively attenuated by increasing concentrations of Mg^{2+}, suggesting that lithium might compete for a Mg^{2+} binding site on the AC catalytic subunit (Newman and Belmaker, 1987; Mork and Geisler, 1989). In contrast, findings from studies of cortical membranes from rats treated chronically with lithium indicated that the attenuation of AC activity by lithium was not antagonized by Mg^{2+}, but reversed by increasing concentrations of GTP (Newman and Belmaker, 1987). The latter finding suggests that chronic lithium treatment may modulate AC activity through effects at the level of G proteins.

In addition to the differential mechanisms suggested for acute versus chronic lithium administration on AC activity, a number of findings have also indicated that lithium acts differently on the basal as compared with the stimulated AC activity in cells. Agonist-mediated activation of AC and accumulation of cAMP was attenuated by chronic lithium treatment, whereas basal cAMP formation was elevated (Ebstein et al, 1980; Newman and Belmaker, 1987). The increased basal level of cAMP in rats was accompanied by enhanced pertussis toxin-catalyzed [^{32}P] adenosine 5'-diphosphate (ADP) ribosylation of $G\alpha_i$ *in vitro* (Masana et al, 1992), suggesting that lithium might attenuate the basal inhibitory tone of $G\alpha_i$ on AC, thereby increasing basal cAMP levels. Apart from its effects on $G\alpha_i$, lithium may also increase basal cAMP level through an upregulation of the mRNA and protein levels of AC types I and II (Colin et al, 1991). Thus, lithium appears to exert dual effects on the accumulation of cAMP, dependent on the state of activation. Dual effects of lithium have been postulated in a *bimodal model* as possible mechanisms by which lithium is able to normalize opposite poles of the disorder, mania and depression (Jope, 1999a).

Cyclic AMP regulates an array of cellular functions, in most cases through activation of PKA. Several studies found that lithium can modulate stimulated PKA-mediated protein phosphorylation, the protein levels of PKA, as well as its translocation from cytosol to nuclear fractions. For example, lithium reduced the cAMP-stimulated phosphorylation of microtubule associated protein 2 (MAP2) in the microtubule fraction of rat cerebral cortex in vitro (Mori et al, 1996), suggesting a direct effect of lithium on PKA activity. Similarly, chronic lithium treatment reduced cAMP-dependent phosphorylation of several endogenous proteins (42, 54 and 71 kDa) in the particulate fractions of rat hippocampus or cerebral cortex (Casebolt and Jope 1991; Jensen and Mork, 1997). The protein level of a major PKA substrate, the 32-kDa dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32), was increased in the frontal

cortex of rats treated chronically with lithium (Guitart and Nestler, 1992). Since protein phosphatase 1 activity is regulated by DARPP-32 (Hemmings et al, 1987), the phosphorylation status of other cellular proteins also may conceivably be affected by lithium. Moreover, chronic lithium treatment significantly increased the immunoreactive levels of the regulatory and catalytic subunits of PKA in the soluble fraction of rat hippocampus and frontal cortex (Mori et al, 1998), and induced translocation of PKA from cytosol to nucleus in fractionated preparations from frontal cortex (Guitart and Nestler, 1992). The altered levels of nuclear PKA may mediate changes in the phosphorylation of specific nuclear transcription factors (e.g. CREB) and subsequently the expression of different genes. In line with this notion, chronic lithium treatment has been shown to increase the levels of phosphorylated CREB and to induce a concentration-dependent increase in CRE binding activities at therapeutically relevant concentrations (0.5-1.0 mM) in both cerebellar granule cells and various rat brain regions (frontal cortex, hippocampus, amygdala and cerebellum) (Ozaki and Chuang, 1997). In contrast, chronic treatment with lithium at therapeutically relevant concentrations decreased stimulation of CREB phosphorylation and its DNA binding to the CRE consensus sequence by AC activator, forskolin, in cultured human SH-SY5Y neuroblastoma cells (Wang et al, 1999a), and decreased CREB phosphorylation in rat cerebral cortex and hippocampus (Chen et al, 1999a). Although further experiments are required to explain the somewhat conflicting results observed in these studies, these findings, nevertheless, suggest that chronic lithium treatment has the potential to modulate cAMP-stimulated gene expression in neuronal tissue through CREB. Of note in this regard, studies of postmortem brain from BD patients with bipolar illness have shown changes in cAMP binding, as well as in PKA activity in temporal cortex (Rahman et al, 1997; Fields et al, 1999), thus further supporting the pathophysiological relevance of cAMP signaling disturbances in BD and the potential importance of modulation of PKA-mediated events in lithium's therapeutic efficacy.

1.5.2.2 Lithium and Phosphoinositide Signaling Pathway

The first suggestion that lithium administration might affect the PI signaling pathway was the findings that lithium reduced the level of *myo*-inositol in rat brain (Allison and Stewart, 1971). This was accompanied by a large increase in the levels of InsP (Allison et al, 1976). It was later shown that lithium is a potent uncompetitive inhibitor of IMPase (Haller and Sherman 1980) and to a lesser extent, of inositol polyphosphate-1-phosphatase (Inhorn and Majerus, 1987). The recycling of *myo*-inositol from InsP is crucial for the resynthesis of PIP_2, since it is the main source of *myo*-inositol in the brain (Sherman, 1991). Thus, inhibition of IMPase by lithium could lead to inositol depletion in cells. Moreover, since the mode of inhibition is uncompetitive, the inhibitory effect of lithium has been postulated to be most pronounced in neurons undergoing the highest rate of PIP_2 hydrolysis. These findings and their implications led to the "inositol depletion" hypothesis, postulating that the therapeutic consequence of lithium's action is mediated by the depletion of inositol in cells (Berridge, 1989). However, a number of studies to date have provided evidence conflicting with this hypothesis. For example, chronic lithium administration was found in some studies to increase (Renshaw et al, 1986) or exhibit no significant effect on the activity of IMPase in rat cerebral cortex (Honchar et al, 1989). Moreover, concentrations of $InsP_3$ were increased (Whitworth et al, 1990) or remained unaltered (Jope et al, 1992; Ishima et al, 1993) in rat cerebral cortex following chronic lithium treatment. Furthermore, the levels of PI, PIP, and PIP_2 were not altered in rat brain following chronic lithium treatment (Ishima et al, 1993). Studies of the effects of acute and chronic administration of lithium on agonist-stimulated PIP_2 hydrolysis in rat brain are also equivocal; the observed effects have often been small and inconsistent (Song and Jope 1992; Jope and Williams 1994; Lenox and Manji, 1998).

In an attempt to test the "inositol depletion" hypothesis more directly, the effect of exogeneous inositol on cellular function has been examined in several studies. Although findings

of some studies suggested that addition of exogenous *myo*-inositol can prevent and reverse the effects of lithium on receptor-coupled PI hydrolysis (Kendall and Nahorski, 1987; Kennedy et al., 1990), others showed that addition of *myo*-inositol only reduced and did not fully reverse lithium-induced behavioral effects (Kofman et al, 1991; Tricklebank et al, 1991). Furthermore, the stereoisomer *epi*-inositol, which was not incorporated into PI, was also effective in attenuating lithium-induced behavior effects (William and Jope, 1995). Therefore, the validity of the 'inositol depletion' hypothesis remains to be further established. Of note, a recent magnetic resonance spectroscopy study (Moore et al, 1999) has provided some interesting *in vivo* evidence on the effect of lithium on *myo*-inositol levels in the brain of BD patients. These investigators found that lithium significantly reduced *myo*-inositol levels in BD patients in selective brain regions. This effect, however, occurred at a time when the patient's clinical state had not yet improved, suggesting that *myo*-inositol reduction *per se* was not associated with therapeutic response. These data, however, suggest that lithium may cause a relative reduction in neuronal *myo*-inositol level leading to an accumulation of the second messenger DAG, which in turn initiates a cascade of secondary changes mediated by other downstream signaling molecules, such as PKC, in the PI signaling pathway (Manji and Lenox, 1999).

Protein kinase C (PKC), an immediate downstream effector of DAG and Ca^{2+}, is known to play a major role in regulating presynaptic and postsynaptic processes in neurotransmission and gene expression (Huang, 1989; Stabel and Parker, 1991; Karin and Hunter, 1995). Numerous studies have suggested that lithium's effects may be mediated via PKC-dependent pathways in the brain (reviewed in Manji and Lenox, 1994; 1999). Acute treatment with lithium enhanced PKC-mediated 5-HT release, whereas chronic treatment attenuated both phorbol ester-mediated PKC activation and 5HT release in rat hippocampus (Sharp et al, 1991; Wang and Friedman, 1989). Chronic lithium treatment has been shown to modulate PKC-mediated phosphorylation of several endogenous substrate proteins in soluble and particulate fractions of rat hippocampus

(Casebolt and Jope, 1991). Activation of PKC by phorbol esters in rat brain tissue slices was inhibited by chronic lithium treatment (Friedman and Wang, 1988). This inhibitory effect may be mediated, in part, by the downregulation of membrane-associated PKCα and PKCε isozymes in rat brain following prolonged lithium treatment (Manji and Lenox, 1994). Furthermore, the protein levels of myristoylated alanine rich C kinase susbstrate (MARCKS), a major phosphoprotein substrate for PKC, were dramatically reduced in the hippocampus after chronic lithium exposure (Lenox et al, 1992). The effect of lithium on MARCKS regulation was dependent upon both the *myo*-inositol concentration and the level of receptor-mediated activation of PI hydrolysis (Watson and Lenox, 1996). These observations suggest that the effects of lithium on PKC and MARCKS levels may be mediated by its inhibition of IMPase resulting in an increase of DAG levels, activation of PKC, and long-term changes in regulation of MARCKS expression. Because prolonged PKC activation results in its proteolytic degradation (Kishimoto et al, 1989; Young et al, 1987), a sustained increase in DAG levels as a result of lithium treatment may lead to increased membrane translocation and subsequent degradation of PKC (Manji et al, 1993, 1995). Such a mechanism is consistent with findings that acute lithium stimulates PKC, whereas chronic exposure attenuates PKC-mediated responses (Manji et al, 1995). These effects of lithium on PKC take on even more importance when viewed against the backdrop of pathophysiological changes in PKC protein levels and activity implicated in BD. For example, the ratio of platelet membrane bound PKC to cytosolic PKC activity was higher in unmedicated BD patients compared with control subjects (Friedman et al, 1993). In postmortem brain, higher brain membrane PKC activity was found in cerebral cortical areas from BD patients compared with control subjects (Wang and Friedman, 1996). These observations strongly suggest that PKC-mediated events are important targets in the mood stabilizing action of lithium.

PKC-mediated gene expression is linked to this enzyme's ability to modulate the binding activity of transcription factor AP-1 to the TPA-response element and, in turn, to regulate the

expression of genes with this 5'-upstream response element (Karin and Hunter, 1995). Recognizing this, a number of recent studies have examined the effect of lithium on the function of transcription factor AP-1 in addition to CREB. Several studies reported that lithium affected not only the expression of c-fos (Mathe et al, 1995; Miller et al, 1997), but also significantly increased AP-1 binding activity both in cell lines and rat brain (William and Jope, 1995; Jope and Song, 1997; Ozaki and Chuang, 1997; Unlap and Jope, 1997). Therefore, these findings strongly suggest that chronic lithium treatment affects expression of genes by modulating the activity of transcription factors, such as AP-1.

1.5.2.3 Lithium and Calcium

An important mechanism regulating free intracellular Ca^{2+} concentrations involves its release from intracellular stores in response to receptor-stimulated PI hydrolysis (Clapham, 1995). Ca^{2+} regulates the synthesis and release of neurotransmitters, neuronal excitability and long-term neuroplastic events through the activation of intracellular enzymes including PKC and CaMK (Berridge, 1998). Thus, it is not surprising that numerous studies have also examined the effect of lithium on intracellular Ca^{2+} homeostatis. In this regard, chronic lithium treatment was found to decrease the basal intracellular Ca^{2+} concentration and attenuate NE-induced increases in intracellular Ca^{2+} concentration in mouse astrocytes (Chen and Hertz, 1996). Chronic lithium treatment also attenuated the 5-HT-induced increase in intracellular Ca^{2+} concentrations in C6 rat glioma cells (Yamaji et al, 1997), and reduced NMDA receptor-evoked Ca^{2+} response in rat cerebellar granule cells (Nonaka et al, 1998a). Of pathophysiological relevance, increased baseline Ca^{2+} levels have been found in platelets and leukocytes of unmedicated bipolar patients compared with normal control subjects (Dubovsky et al, 1992b), and higher basal intracellular Ca^{2+} concentrations were observed in T-lymphocytes and transformed B-lymphoblasts from subjects with BD (Emamghoreishi et al, 1997). Furthermore, preliminary data suggested that

calcium channel blockers such as nimodipine and verapamil were clinically effective in the treatment of mania (Chou, 1991; Pazzaglia et al, 1993). These observations suggested that modulation of Ca^{2+} homeostasis by lithium might account for some aspects of long-term mood stabilization. These long-term adaptive responses are likely to involve changes in gene transcription mediated by Ca^{2+} activation of different protein kinases (Hardingham and Bading, 1999).

1.5.2.4 Lithium and G proteins

As mentioned above, mounting evidence has shown that lithium modulates receptor-coupled cAMP and PI signaling. Because G proteins play a pivotal role in these two second-messenger systems, it has been hypothesized that G proteins may be the molecular targets for the therapeutic effect of lithium. A variety of experiments have been performed to probe the function and/or levels of G proteins in an attempt to establish a unified postreceptor mechanism of lithium's action. The first line of evidence indicating that G proteins may be the targets of lithium's action was provided by Avissar and colleagues, who reported that lithium, at therapeutic concentrations (0.6 - 1.0mM), completely blocked adrenergic and cholinergic agonist-induced increases in [^{3}H]GTP binding to membranes from rat cortex, both *in vitro* and *ex vivo*. These findings suggested the function of several of these G-proteins, such as $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$, is modulated by lithium (Avissar et al, 1988; Avissar and Schreiber, 1989). The interaction of lithium with G proteins was shown to be mediated, in part, by its competition with magnesium ions (Mg^{2+}) on Mg^{2+} low-affinity sites essential for guanine nucleotide exchange on these proteins (Avissar et al, 1991). Since then, findings from numerous studies reported to date using various techniques support the involvement of G proteins in lithium's mechanism of action (Lenox and Manji, 1998; Jope, 1999b, Wang and Friedman 1999; Avissar and Schreiber, 2000).

However, the precise mechanism(s) underlying the effect of lithium on G protein function is less clear.

A number of investigators examined the effect of chronic lithium treatment on the expression of G proteins at both protein and mRNA levels in brain in an attempt to elaborate the mechanisms by which lithium modifies G protein function. Colin et al (1991) reported a decrease in the protein and mRNA levels of $G\alpha_{i1}$ and $G\alpha_{i2}$ in the rat frontal cortex following chronic lithium treatment. In contrast, Masana et al (1992) and Li et al (1993) noted no alterations in the immunoreactive levels of $G\alpha_s$, $G\alpha_{i1}$ and $G\alpha_{i2}$, although a significant reduction in $G\alpha_s$, $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNA levels was reported by Li et al (1993). While the effects of lithium on the protein or mRNA levels of G proteins in rat brain remain unclear, examination of the G protein levels in postmortem brain has shown increased $G\alpha_s$ levels in frontal, temporal, and occipital regions in BD patients compared with control subjects (Young et al, 1993). In addition, $G\alpha_{q/11}$ level has been found increased in occipital cortex from BD (Mathews et al, 1997). These findings suggest that abnormalities in G-proteins levels may be involved in the pathophysiology of BD (Warsh and Li, 1996, 2000).

The effects of lithium on posttranslational modifications of G proteins as potential mechanisms for its modulation of G protein function have also been examined over the years. Chronic lithium treatment was found to significantly increase PTX-catalyzed [^{32}P]ADP-ribosylation of $G\alpha_{i/o}$ in rat frontal cortex and hippocampus, but no change was observed in the amount of ^{32}P incorporated into $G\alpha_s$ by ADP ribosylation catalyzed by cholera toxin (Masana et al, 1992; Wang and Friedman, 1999). Because PTX is known to selectively catalyze ADP ribosylation of the undissociated, inactive heterotrimeric form of $G\alpha_{i/o}$ (Ui, 1990), these findings suggest that lithium might inactivate $G\alpha_{i/o}$ by stabilizing the inactive conformation of the G protein. This would attenuate the basal inhibitory tone of $G\alpha_{i/o}$ on AC, thereby increasing basal

cAMP levels (Masana et al, 1992). Furthermore, the levels of endogenous ADP-ribosylation in rat brain (Nestler et al, 1995) and in C6 glioma cells (Young and Woods, 1996) was altered by chronic lithium treatment, suggesting another possible mechanism by which chronic lithium may indirectly regulate the function of G proteins.

The above observations suggest several possible mechanisms by which lithium can affect G protein function. While it is still not clear whether one or more of these effects account for its therapeutic effect in BD, the changes induced in this critical upstream node of signal transduction can have major consequences on downstream targets of these signaling mechanisms. For example, these effects could have significant long-term effects via regulation of gene expression mediated by PKC or PKA, the downstream effectors of G protein signaling pathways.

1.5.2.5 Lithium and Glycogen Synthase Kinase 3β

The profound effects of lithium on the development of various organisms, including *Dictyostelium*, *Xenopus*, zebrafish and sea urchin (Maeda, 1970; Kao and Elinson, 1988; Livingston and Wilt, 1989; Stachel et al, 1993) have been known for some time, but it is only in recent years with the elucidation of the molecular mechanisms underlying cell-fate determination that the basis for such effects has emerged. In *Xenopus*, lithium's effect was found to be remarkably similar to that observed in the ectopic expression of wingless related genes (*Wg*) (McMahon and Moon, 1989), suggesting that common mechanisms may be involved. Moreover, GSK-3β, an intermediary enzyme in the *Wnt* pathway that was demonstrated to play a central role in the development of *Dictyostelium* and *Xenopus* (Harwood et al, 1995; Pierce and Kimelman, 1995; He et al, 1995), was subsequently found to be inhibited *in vitro* at therapeutic lithium concentrations. This suggested GSK3β may represent another potential target for the action of lithium (Klein and Melton, 1996). GSK3β, originally characterized in mammalian cells

as an enzyme phosphorylating glycogen synthase, also phosphorylates several other proteins, including the microtubule-associated proteins (MAPs), *tau* and MAP-1B, which are known to regulate the neuronal cytoskeletal network (Woodgett, 1991). Later studies reported that inhibition of GSK3β by lithium reduced phosphorylation of *tau* protein promoting microtubule assembly (Hong et al, 1997; Munoz-Montano et al, 1997). Moreover, lithium treatment also decreased phosphorylation of MAP-1B reducing its ability to bind to microtubules (Lucas et al, 1998; Garcia-Perez et al, 1998). Thus, inhibition of GSK3β by lithium may induce significant changes in microtubule assembly resulting in cytoskeletal rearrangement and neuroplastic alterations in critical regions of the brain. In addition to regulating cytoskeletal processes, GSK3β can exert long-term nuclear events via phosphorylation of c-jun, CREB and β-catenin (Dale, 1998; Willert and Nusse, 1998), which in turn can modulate expression of different genes.

These findings raise the question of whether this enzyme and its substrates are altered in the brain of subjects with bipolar disorder. Recently, a preliminary study has found no significant differences in GSK3β, β-catenin and tau levels in prefrontal cortex from BD compared with matched controls subjects, although there was a tendency for the tau isoform profile to be modified in bipolar tissue (Lesort et al, 1999). Although no significant changes were observed in the total levels of each these proteins between bipolar and matched control samples, additional studies of the activity of GSK3β, the cytosolic and particulate levels of β-catenin may extend these findings.

1.5.2.6 Lithium and Protein Kinase B/Akt

As alluded to earlier in section 1.5.1.5, lithium was found to robustly protect cultured neurons against glutamate-induced apoptosis mediated by NMDA receptors (Nonaka et al, 1998a), and *in vivo* lithium was observed to protect rats against focal ischemia-induced brain

damage (Nokana and Chuang, 1998). However, the molecular mechanisms underlying this neuroprotective effect remain unknown. Similar neuroprotective actions have been observed with insulin growth factor 1 (IGF-1) in earlier studies. For example, IGF-1 has been reported to reduce brain damage induced by hypoxic-ischemic injury (Gluckman et al, 1992) and rescue rat cerebral cortical neurons from NMDA receptor mediated cell death in a PI3-K-dependent manner (Tagami et al, 1997). The similarity of the protective actions elicited by IGF-1 and lithium suggested the potential role of the PI3-K/Akt signaling pathway in lithium-induced neuroprotection. Using a rat cerebellar granule cell preparation, Chalecka-Franaszek and Chuang (1999) found that lithium induced a rapid increase in PI3-K activity and Akt-1 phosphorylation with accompanying kinase activation, all of which were blocked by PI3-K inhibitors. These findings suggested that modulation of Akt-1 activity might mediate the neuroprotective effect of lithium in addition to other possible effects on gene expression, since among a number of different substrate for Akt is GSK3β, a modulator of gene transcription (Pende et al, 1997). Figure 4 summarizes the signaling components implicated as targets for lithium action that are presented.

1.5.3 Lithium and Neuroprotection

In recent years, a number of exciting developments have suggested a novel therapeutic action for lithium, namely neuroprotection (Manji et al, 1999). Lithium has been demonstrated to robustly protect cerebellar granule cell against the toxic effects of excessive receptor activation or insults, including glutamate, NMDA receptor activation, low potassium, or toxic concentration of anticonvulsants (D'Mello et al, 1994; Nonaka et al, 1998a,b). The protective effects of lithium against the excitotoxic effects of glutamate and NMDA receptor activation have also been demonstrated in primary cultures of hippocampal and cortical neurons (Nonaka et al, 1998b). Chronic lithium treatment has been shown to exert dramatic protective effects against middle

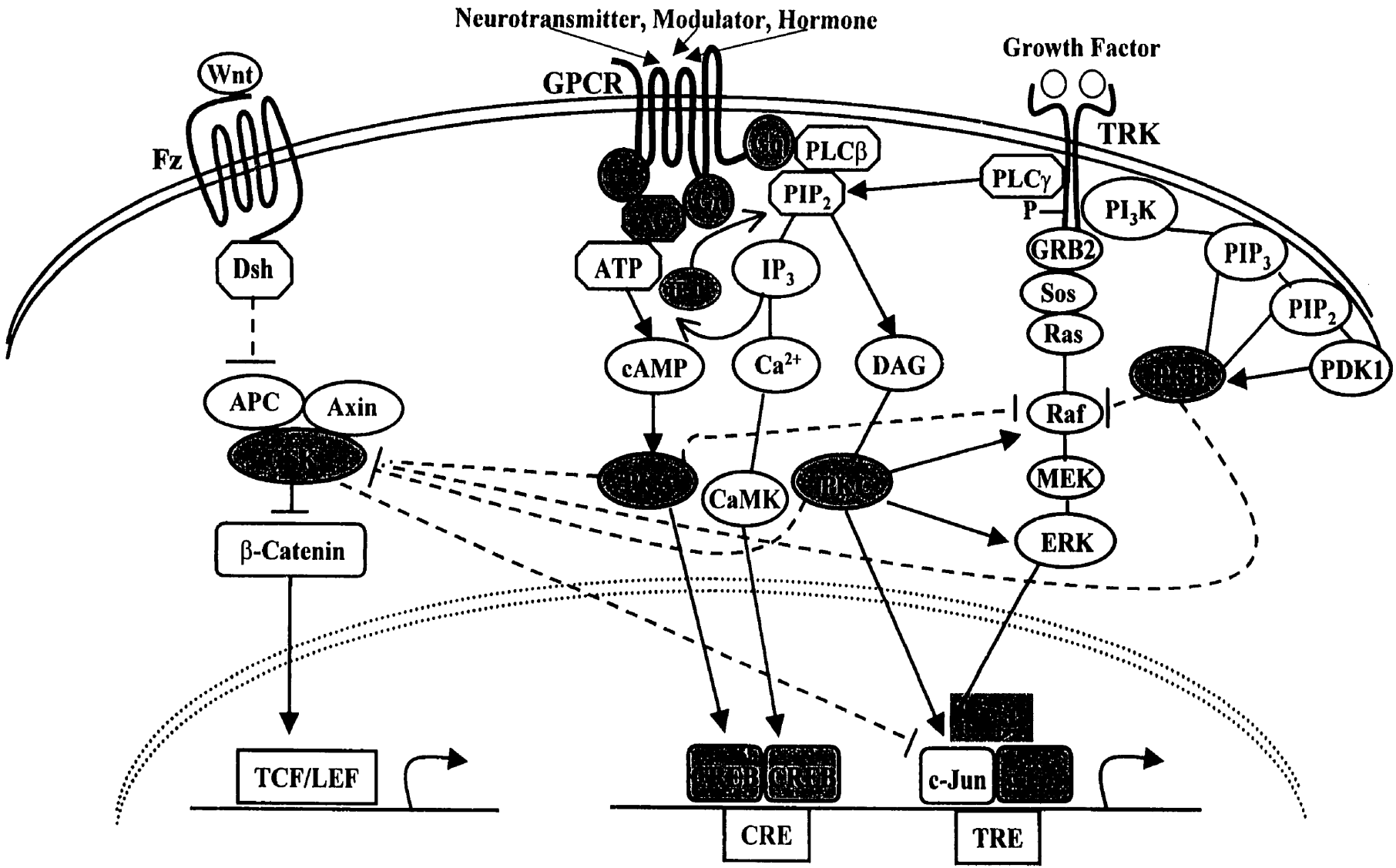


Figure 4 A simplified schematic representation of major signaling pathways and sites of lithium's action (shaded). Lithium, at therapeutically relevant concentrations, inhibits activity of inositol monophosphatases, affects the function of G proteins, modulates adenylate cyclase activity, interferes with protein phosphorylation by protein kinases, and regulates expression of different genes via transcription factors. Solid arrows denote stimulatory pathways. Broken arrows indicate inhibitory pathways. Fz, fizzled receptor; GPCR, G-protein-coupled-receptor; TRK, tyrosine receptor kinase; Dsh, dishevelled; GSK, glycogen synthase kinase. For other abbreviations and detailed description of the pathways see text under respective subheading.

cerebral artery occlusion, reducing not only the infarct size (by 56%), but also the neurologic deficits (abnormal posture and hemiplegia) (Nonaka and Chuang, 1998). In addition, chronic administration with lithium has been shown to markedly increase the levels of the anti-apoptotic protein, bcl-2, in rat frontal cortex, dentate gyrus and striatum (Manji et al, 1999), in rat cerebellar granule cells (Chen and Chuang, 1999), and in human neuroblastoma SH-SY5Y cells (Manji et al, 1999). Furthermore, chronic lithium treatment has been found to reduce the levels of the pro-apoptotic protein p53 in rat cerebellar granule cells (Chen and Chuang, 1999) and SH-SY5Y cells (Lu et al, 1999). As bcl-2 also play a role in regeneration of CNS axons (Adams and Cory, 1998), the effect of lithium on neurogenesis has recently been examined. Chronic lithium treatment has been found to increase BrdU- (bromodeoxyuridine, a thymidine analog that is incorporated into the DNA of dividing cells) labeled neurons in the hippocampus of adult mice (Chen et al, 2000a), suggesting an increase in neurogenesis. Cumulatively these findings suggest that the therapeutic action of lithium in BD may be mediated in part by its neuroprotective/neurotrophic properties and that neuroanatomical abnormalities may underlie the pathophysiology of bipolar disorder. Indeed, a number of recent neuroimaging, magnetic resonance spectroscopy, and postmortem morphometric studies have supported this possibility (reviewed in Manji et al, 2000b). The findings from these studies have shown distinct neuroanatomical differences in BD prefrontal cortex, limbic system, third ventricle, cerebellum, temporal lobe, basal ganglia and subcortical white matter compared with control (Soares and Mann, 1997; Drevets, 1998; Pearlson, 1999; Manji et al, 2000b). Of note, the regulation of cell survival and cell death is a complex process involving multiple interacting signaling pathways, transcription factors, and gene expression. Thus, the effects of lithium on signaling pathways and transcription factors may also contribute to its neuroprotective/neurotrophic effects.

1.5.4 Lithium and Gene Expression

Any relevant mechanism postulated to explain its therapeutic actions must account for the delayed therapeutic onset and course of action (Goodwin and Jamison, 1990). Biochemical changes requiring such a prolonged administration of a drug suggest the potential for induction of new programs of gene and subsequent protein expression (Hyman and Nestler, 1996). Furthermore, many of the signaling transduction cascades are known to induce long-term changes in neuronal function by regulating gene expression via protein phosphorylation (Hunter and Karin, 1992). Based on these considerations, a number of studies have been conducted over the past decade examining the long-term effect of lithium on gene expression (Table 1). As has been discussed in the section 1.5.2.4, chronic, but not short-term, lithium treatment in rats has been shown to modulate the mRNA levels of various Gα subunits (Li et al, 1991, 1993; Colin et al, 1991; McGowan et al, 1996; Jakobsen and Wiborg, 1998). Chronic lithium treatment increased mRNA levels for AC types I and II in cerebral cortex (Colin et al, 1991), glucocorticoid receptor in hippocampus (Semba et al, 2000), and decreased D2 dopamine receptor mRNA in striatum of rats (Dziedzicka-Wasylewska and Wedzony, 1996). Chronic lithium treatment also decreased the basal and agonist stimulated expression of c-fos mRNA in rat cerebral cortex (Mathe et al, 1995; Miller et al, 1997). Attenuation of c-fos expression by lithium may be one of the mechanisms by which lithium affects the expression of critical neuronal genes that are regulated by transcription factor Fos or AP-1.

More recently, several studies have examined the potential direct effects of lithium on transcription factor activity. A number of findings have confirmed that lithium affects not only the expression of c-fos, but also significantly increased AP-1 and CRE binding activity both in cultured cell lines and rat brain (William and Jope, 1995; Jope and Song, 1997; Ozaki and Chuang, 1997; Unlap and Jope, 1997). To further test whether the lithium-induced increase in AP-1 activity has functional consequences, human neuroblastoma SH-SY5Y cells and rat C6

Table 1: Effects of Chronic Lithium on Gene Expression in Rodent Brains by 'Candidate Gene' Approach

LITHIUM TREAMENT PARADIGMS	LITHIUM EFFECTS	COMMENTS	REFERENCE
1.5g/kg lithium diet for 21 days (Fisher 344 rats)	↑ (51%) prodynorphin mRNA in striatum	Chronic lithium augments striatal dynorphin synthesis	Sivam et al, 1988
0.24% lithium diet for 4 weeks (Sprague Dawley rats)	↑ (50 - 60%) AC_I and AC_{II} mRNA; ↓ (20%) $G\alpha_{i1}$ and $G\alpha_{i2}$ mRNA in cerebral cortex	No significant effects with 6 days treatment.	Colin et al, 1991
0.2% lithium diet for 21 days (Wistar rats)	↓ (25 - 30%) $G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$ mRNA in cerebral cortex	These effects were not found with chronic CBZ.	Li et al, 1991 Li et al, 1993
0.1% lithium carbonate i.p. for 14 days (Sprague Dawley rats)	↑ hippocampal $G\alpha_o$ (19%), $G\alpha_{i2}$ (17%)	G protein dysfunction might play a role in BD.	McGowan et al, 1996
0.2% lithium diet for 28 days (Wistar rats)	↑ $G\alpha_o$, $G\alpha_{i1}$ mRNA in neostriatum; $G\alpha_s$ mRNA ↓ in frontal cortex and ↑ in thalamus	Chronic CBZ also exert similar effects.	Jakobsen and Wiborg, 1998
0.22% lithium diet for 4 weeks (Sprague Dawley rats)	↓ (16%) basal c-fos mRNA in cortical, hippocampal and thalamic areas	↓ PKC activity by lithium might serve to inhibit basal c-fos mRNA levels.	Mathe et al, 1995
0.22% lithium diet for 6 weeks (Sprague Dawley rats)	↓ basal and agonist induced c-fos expression in frontal cortex.	One week after discontinuation of lithium treatment, basal c-fos expression remained lower.	Miller et al, 1997
6mEq/kg lithium carbonate p.o. for 14 days (Wistar rats)	↑ (31%) D2 mRNA in striatum	7 days treatment also produce significant effect (14%)	Dziedzicka-Wasylewska and Wedzony, 1996
1.5mEq/kg lithium chloride i.p. 14 days (Wistar rats)	↑ (30%) glucocorticoid receptor (GR) mRNA in hippocampus	Acute treatment did not change the expression of GR	Semba et al, 2000

glioma cells were transfected with a reporter gene vector driven by SV40 promoter containing two AP-1 sites (Yuan et al, 1998). Lithium was found to increase the activity of the reporter gene in a time- and concentration-dependent manner; furthermore, mutations in the AP-1 sites of the reporter gene markedly attenuated the stimulatory effect of lithium (Yuan et al, 1998). In addition, chronic treatment of rats with lithium increased the frontocortical, hippocampal and striatal protein levels of tyrosine hydroxylase (Chen et al, 1998), a gene that is known to be regulated by transcription factor AP-1 (Kumer and Vrana, 1996). This also suggests that lithium could regulate gene expression via AP-1 *in vivo*. In contrast, chronic lithium treatment was found to attenuate nerve growth factor-induced and carbachol-stimulated activation of AP-1 DNA binding activity (Unlap and Jope, 1997; Jope and Song, 1997). These seemingly contradictory findings, however, are consistent with the bimodal effects of lithium, i.e. raises basal activities and diminishes stimulated activities, thereby stabilizing signaling activities within an optimal range (Jope, 1999a).

Another possible mechanism by which lithium may modulate gene expression is via its effect on GSK3β (Hedgepeth et al, 1997). In addition to phosphorylating β-catenin and regulating gene expression via the Wnt pathways, GSK3β can phosphorylate c-jun at three sites adjacent to the DNA binding domain thereby reducing the DNA binding activity of AP-1 . Similarly, GSK3β can phosphorylate CREB at Ser 129 and modulate CRE-mediated transcriptional activity (Roach 1991; Fiol et al, 1994; Bullock and Habener, 1998). Cumulatively, these findings suggest that gene regulation through AP-1 and CREB may represent one of the molecular targets for lithium's action.

1.5.5 Lithium versus Valproate and Carbamazepine

Although the two anticonvulsants, CBZ and VPA, structurally are highly dissimilar to each other and to lithium, these agents are effective in treating BD (Bowden, 1996; Dunn et al,

1998). Similar to lithium, their mood stabilizing properties are delayed in onset suggesting they also may act through similar effect such as modulating gene expression. A number of studies have been conducted to identify whether there are indeed common molecular targets and shared mechanisms of therapeutic action among mood-stabilizing agents. Similar to lithium, VPA has been reported to decrease the activity of GSK3β and PKC, and the levels of membrane-associated PKCα in C6 glioma cells and rat brain (Chen et al, 1994; Manji et al, 1999; Chen et al, 1999c). Moreover, the levels of MARCKS were dramatically decreased in the immortalized hippocampal cells by VPA, but unlike lithium, this downregulation was independent of any effect on the PI signaling pathway (Watson et al, 1998). The latter finding suggests that while chronic lithium and valproate both appear to modulate PKC isozyme(s) and its substrate, they do so through different mechanisms. In contrast to lithium and VPA, CBZ does not appear to affect PKC signaling pathway. However, it has been shown to inhibit both basal and forskolin-stimulated cAMP accumulation in C6 glioma cells and rat cerebral cortex (Chen et al, 1996a) suggesting that it modulates cAMP signaling pathways. As discussed, chronic lithium treatment also appears to attenuate agonist stimulation of AC (Lenox and Hahn, 2000), and valproate has similar effects as lithium on cAMP signaling (Chen et al, 1996c). Therefore, while lithium and VPA exert similar effects on GSK3β and/or PKC mediated events, all three mood stabilizers affect the cAMP signaling system.

The potential effects of these mood stabilizers on transcription factor activity and in neuroprotection have also been studied. Lithium and CBZ, but not VPA, inhibited AP-1 activation stimulated by carbachol in human neuroblastoma SH-SY5Y cells (Pacheco and Jope, 1999), whereas lithium and VPA robustly increased the levels of the neuroprotective protein bcl-2 in the CNS (Chen et al, 1999b). On the other hand, chronic treatment with lithium, but not VPA, decreased CREB phosphorylation in rat cerebral cortex (Chen et al, 1999a). Chronic VPA and CBZ treatment have also been shown to affect gene expression. Chronic VPA administration

to rats increased the expression of 78-kDa glucose-regulated protein (GRP78) (Wang et al, 1998), GRP94 and calreticulin (Bown et al, 2000; Chen et al, 2000b), and tyrosine hydroxylase (Sands et al, 2000). Chronic CBZ treatment significantly suppressed methamphetamine-induced Fos expression in the core of nucleus accumbens and the caudate/putamen (Lee et al, 2000). Although the findings indicate that there are common as well as distinct effects of these mood stabilizers, they all are capable of modulating transcription factor activity, and subsequent gene expression. This suggests that the long-term effects of all mood stabilizers may be mediated by changes in the expression of critical neuronal genes in regulating mood.

The observations of distinct as well as common biochemical/molecular effects among these mood stabilizing agents are consistent with the clinical observations that some patients respond to VPA or CBZ, while others are more responsive to lithium, and additive effects are often seen when two agents are co-administered (Freeman and Stoll, 1998). Therefore, it appears that while these agents are effective in the treatment of BD, they do not act on precisely the same targets, yet may share some common targets that contribute to their mood-stabilizing properties.

In summary, major progress has been made over the past decades with a burgeoning body of evidence indicating that modulation of intracellular signaling transduction pathways and subsequent regulation of expression of critical neuronal genes may be crucial to lithium's delayed therapeutic onset and long term mood stabilization and/or neuroprotection. Despite this progress, the precise mechanisms underlying its long-term mood stabilizing properties are still to be fully elucidated.

1.6 RESEARCH STRATEGIES, OBJECTIVES AND HYPOTHESIS

As reviewed in the previous sections, long-term neuronal adaptation mediated through changes in gene expression may be essential to the therapeutic effects of lithium. Previous studies examining the effect of lithium on gene expression have used a "candidate gene"

approach, whereby one or a few known gene candidates were targeted based on specific hypotheses formulated based on earlier findings. Therefore, the transcriptional changes of other genes, especially novel genes that could not be linked to earlier findings, might be overlooked. In recent years, new methods have evolved for the simultaneous examination of many genes and the subsequent identification of differentially expressed genes from cells or tissues under different treatment conditions. One such method that has been increasingly used in different fields of research is the mRNA differential display (Liang and Pardee, 1992). Accordingly, the research strategy for this project was to use mRNA differential display to identify candidate gene transcripts that are differentially expressed after prolonged administration of lithium.

mRNA differential display methodology involves a series of steps: (1) reverse transcription (RT) using a set of 3'-anchored primers, (2) PCR amplification of cDNA fragments using arbitrary (upstream) primers and anchored (downstream) primers, and (3) radiolabeled, PCR-amplified cDNA fragments are separated on denaturing sequencing gel for comparison on autoradiograms. Differentially displayed bands can be excised from the gel and amplified with the same primers used for the original display, and the resulting PCR product can be used to probe Northern blots of total RNA to confirm the identification of a differentially expressed mRNA. A summary of the overall strategy for discovery of pharmacologically relevant targets using mRNA differential display is illustrated in Figure 5. This technique was developed to provide a simpler and more efficient technique over available conventional methods such as differential screening and subtractive hybridization (Rebagliati et al, 1985; Watson and Margulies, 1993) for the identification of known and novel genes that are differentially expressed in biological systems. Compared with the conventional methods, the mRNA differential display technique has several advantages: (1) simplicity in all key approaches (primarily RT-PCR); (2) sensitivity due to PCR amplification; (3) versatility in detecting genes that are either upregulated or downregulated under various conditions, and the ability to perform a side-by-side comparison

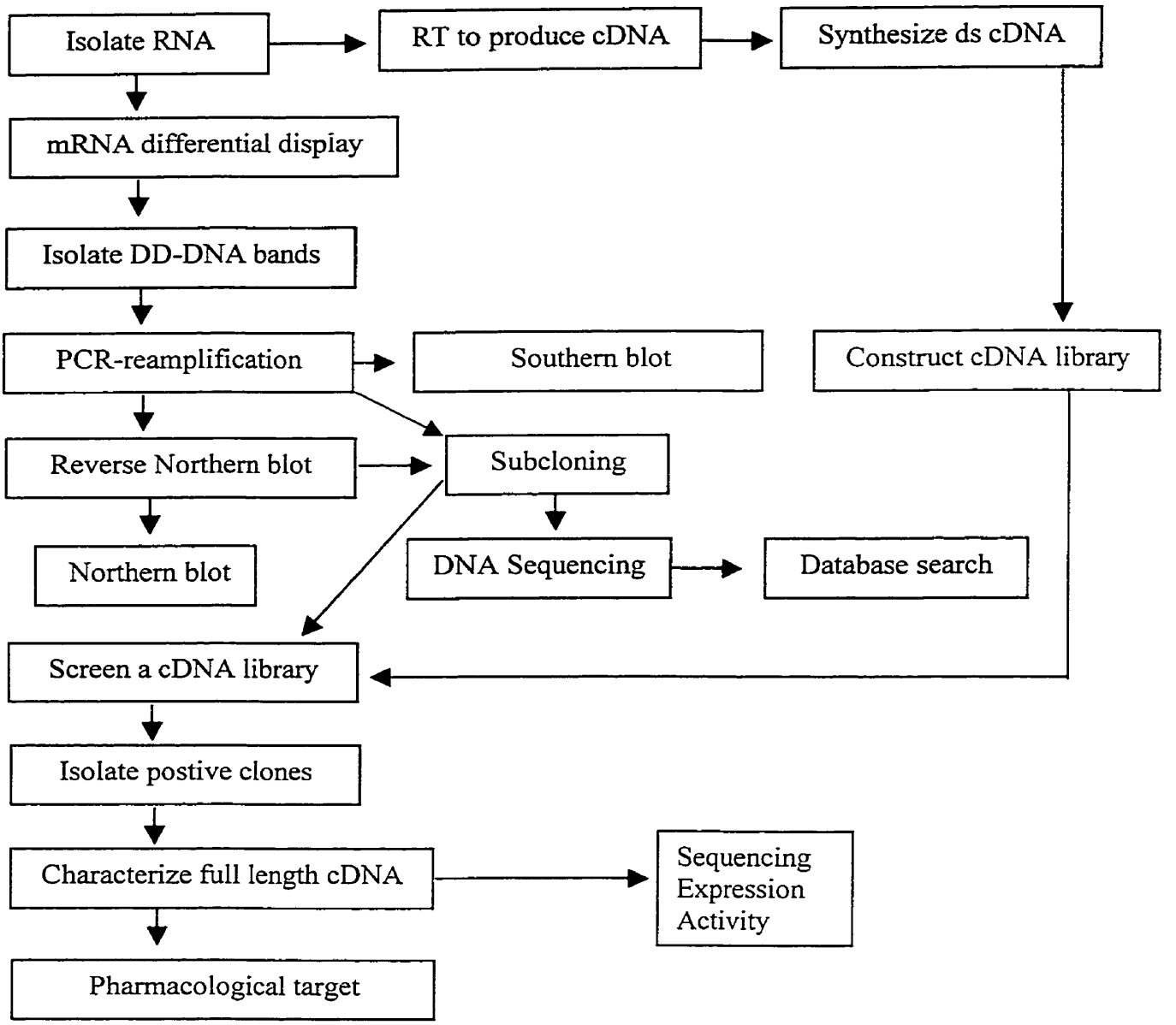


Figure 5 Overall strategy for discovery of pharmacological target(s) using mRNA differential display. All the procedures are described in detail in this chapter. RT, reverse transcription; ds, double stranded; DD, diffentially displayed (Adapted from Wang et al, 1996).

of more than two samples; (4) rapidity in identifying a candidate cDNA fragment and confirming the result (e.g. northern blot); (5) the requirement of smaller amounts of RNAs; and (6) reproducibility. These characteristics have rendered this technique increasingly popular for the discovery of differentially expressed genes in many areas of biological research (Sunday, 1995; Harris, 1996; Wang and Feuerstein, 1997; Flanigan and Leslie, 1997; Fislage, 1998).

Most of the previous studies on the chronic effects of lithium on gene expression have been done in rat brain and cultured cells including rat pheochromocytoma PC12 cells and C6 glioma cells. While cultured cells provide a simple cellular model with a homogeneous population of genes for mRNA differential display, they do not manifest the cell-cell interactions that occur in CNS and may fail to exhibit adaptive changes in gene expression that are often dependent on intercellular signaling. Therefore, whole animal models are essential for the detection of certain long-term treatment regulated genes that are dependent on interacting neuronal networks. In the absence of a suitable experimental model of BD, investigators have used normal animals treated chronically with lithium in many studies, with the assumption that the pharmacological effects of lithium are conserved and are relevant to its therapeutic action in BD. Although it can be argued that the effect of lithium treatment on "normal" experimental systems could not be extrapolated to possible therapeutically relevant effects in BD patients, cumulative experimental evidence to date suggests that lithium-treated animal model has been useful for advancing understanding of the molecular mechanism of action of this drug and in identifying potential biochemical substrates dysregulated in BD (Ikonomov and Manji, 1999; Jope, 1999b; Li et al, 2000; Warsh and Li, 1996; 2000).

In this work, inbred male Wistar rats were selected to minimize individual genetic differences, because individual animals within an inbred strain are as identical as monozygotic twins (Lipman, 1997). Frontal cortex was chosen for study because aberrant intracellular signaling in this area has been implicated in the pathophysiology of BD (Ketter et al, 1997) and

lithium has been shown to exert specific pharmacological effects in this region (Goodwin and Jamison, 1990; Manji et al., 1995; Jope, 1999b; Li et al, 2000).

Because VPA and CBZ have been shown to be effective in the treatment of certain subgroups of BD that are less responsive to lithium (Goodwin and Jamison, 1990; Bowden, 1996; Dunn et al, 1998), animals treated with these drugs were included in this study to identify common, as well as contrasting differentially regulated gene targets of mood stabilizing drugs. Haloperidol (HAL), a typical antipsychotic, and imipramine (IMI), a typical tricyclic antidepressant, were used as negative control treatments with the rationale that the pharmacological effects of these two classes of drug were likely distinct from those of the mood stabilizing drugs.

Since the therapeutic onset of mood-stabilizing drugs usually takes several weeks (Goodwin and Jamison, 1990), any experimental design used in searching for biological factors relevant to the therapeutic action of these drugs must account of this latency in onset. Therefore, a chronic drug treatment paradigm was used in this study to mirror the clinical time course of mood stabilizer action, whereas short-term treatment with lithium was included to rule out acute or transient effects that are unlikely to relate to its therapeutic mechanism in BD.

The objectives of the studies undertaken in this thesis were:

(1) to identify and characterize lithium-regulated genes in rat frontal cortex using mRNA differential display;

(2) to examine the pharmacological specificity of the lithium's effect.

The hypotheses addressed in this thesis were:

(1) chronic lithium treatment differentially regulates the expression of a specific panel of genes in a time-dependent manner;

(2) the effects of lithium on gene expression may be shared by VPA and CBZ; and

(3) the effects of lithium on gene expression are not shared by typical antidepressant or antipsychotic drugs.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Reagents

Ammonium acetate, citric acid, ethylenediaminetetraacetic acid (EDTA), 3-(4-morpholinyl) 1-propanesulfonic acid (MOPS), boric acid, glucose, sodium dodecyl sulfate (SDS), sodium phosphate, sodium chloride, tris(hydroxymethyl)aminomethane (tris), urea, potassium chloride, Sigmacote, ficoll, dextran sulfate, bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl-β-D-galactoxide (X-gal), ampicillin, diethylpyrocarbonate (DEPC), 2-mercaptoethanol (β-ME), formaldehyde, salmon sperm DNA, lithium carbonate (Li), carbamazepine (CBZ), sodium valproate (VPA), haloperidol (HAL) and imipramine hydrochloride (IMI) were obtained from Sigma Chemical Co. (St. Louis, MO). Guanidine isothyiocyanate, cesium chloride, phenol, and agarose were purchased from GIBCO BRL (Grand Island, NY). Xylene cyanol, acrylamide, N, N'-bisacrylamide (bis) and ammonium persulfate (APS) were obtained from Bio-Rad Laboratories (Hercules, CA). Sodium hydroxide and N,N,N',N'-tetramethyl ethylene diamine (TEMED) were acquired from ACP Chemical Incorporated (Montreal, QB). Hydrochloric acid, glacial acetic acid, and isopropanol were from Caledon (Georgetown, ON). Ethanol and methanol were purchased from Commercial Alcohols Inc. (Toronto, ON). Bacto-agar, yeast extract and tryptone were obtained from DIFCO Laboratories (Detroit, MI).

2.1.2 Enzymes

Restriction endonucleases were purchased from either Canadian Life Technologies Inc. (Burlington, ON) or Boehringer Mannheim (Laval, QB). Proteinase K was obtained from Life Technologies Inc. (Gaithersburge, MD). Taq DNA polymerase was obtained from QIAGEN (Mississauga, ON) or Perkin Elmer (Mississauga, ON). T4 Ligase was purchased from Promega Corp. (Madison, WI). DNase I was purchased from GenHunter (Nashville, TN).

2.1.3 Isotopes

[α-^{32}P]-dCTP (~3000 Ci/mmol) and [α-^{33}P]-dATP (~2000Ci/mmol) were purchased from DuPont, New England Nuclear (Boston, MA). [α-^{33}P]-ddNTP was obtained from Amersham Phamacia Biotech (Baie d'Urfe, QB).

2.1.4 Oligonucleotides

All oligonucleotides used for the PCR and DNA sequencing were synthesized and salt-purified by ACGT (Toronto, ON).

2.1.5 Kits

RNeasy mini kit for total RNA extraction, QIAprep spin Miniprep kit for plasmid DNA preparation, and QIAquick Gel Extraction kit for extraction of DNA from agarose gel were purchased from QIAGEN (Mississauga, ON). RNAmap and RNAimage kits for mRNA differential dispaly were obtained from GenHunter. Strip-EZ DNA kit for radiolabeling of DNA was obtained from Ambion (Austin, TX). Nick Spin Sephadex G-50 columns for purification of radiolabeled DNA probes was purchased from Amersharm Pharmacia Biotech. The original TA cloning kit was acquired from Invitrogen (Carlsbad, CA). The pGEMT vector system kit for cloning was obtained from Promega. Marathon cDNA amplification kit was obtained from Clontech (Palo Alto, CA). The Thermo Sequenase radiolabeled terminator cycle sequencing kit was purchased from USB (Cleveland, OH). The Rapid-Screen cDNA library kit for cloning of full length cDNA was purchased from OriGene Technologies Inc. (Rockville, MD). Positively charged nylon membranes were obtained from Dupont. The pET system kit for protein expression was purchased from Novagen (Madison, WI).

2.1.6 Plasmids and cDNA Library

Plasmid vector pCR2.1 was purchased from Invitrogen. pGEMT vector was obtained from Promega. Adult rat pCMV6-XL4 cDNA library was obtained from Origene.

2.1.7 Antibodies

Antibody for aldolase was purchased from Rockland (Gilbertsville, PA) and for β-tubulin was from Santa Cruz Biotechnology (Santa Cruz, CA). Antigoat IgG-HRP secondary antibody was obtained from Sigma. Anti-DIPP2 antibody raised against the C-terminal peptide (CVTAAPPSGVPSSIR) of mouse DIPP2 was a gift from Dr. Stephen Shears (NIEHS, Research Triangle Park, NC).

2.2 EXPERIMENTAL DESIGN

2.2.1 Animal and Drug Treatments

Male Wistar rats (Charles River, St. Constant, Quebec), weighing 200 - 225 g (44 - 48 days old) were housed individually and allowed free access to food and water. The animals were maintained in a light- and temperature-controlled room (12 hr light cycle, 21 ± 1 °C) and allowed to acclimatize for at least a week prior to treatments. At least ten rats were used in each treatment group. Drug containing diets were prepared by Bioserve Ltd (Frenchtown, NJ).

The experimental treatment paradigm is illustrated in figure 6. For the control group, rats were fed regular chow, whereas treatment groups were fed chow containing 0.22% Li_2CO_3 for 1 week (short term) or 5 weeks (chronic treatment) (Li et al, 1993; Kofman et al, 1998). Both short-term and chronic lithium treatment paradigms produced a plasma lithium concentration of 0.5-1.0 mEq/L, closely approximating the therapeutic levels used in human. Separate bottles of water and hypertonic saline (2.6%) were provided *ad lib* for all animals to prevent dehydration. In chronic studies with CBZ, rats were fed chow containing 0.25% CBZ for the first 4 days, followed by chow containing 0.5% CBZ for the next 31 days. This paradigm was used to achieve plasma levels of CBZ and its metabolites similar to the therapeutic levels in humans (Daval et al, 1989; Kofman et al, 1998). In chronic studies with VPA, animals were fed chow containing 0.4% VPA for 5 weeks. Assuming 25 g of chow were consumed by each rat

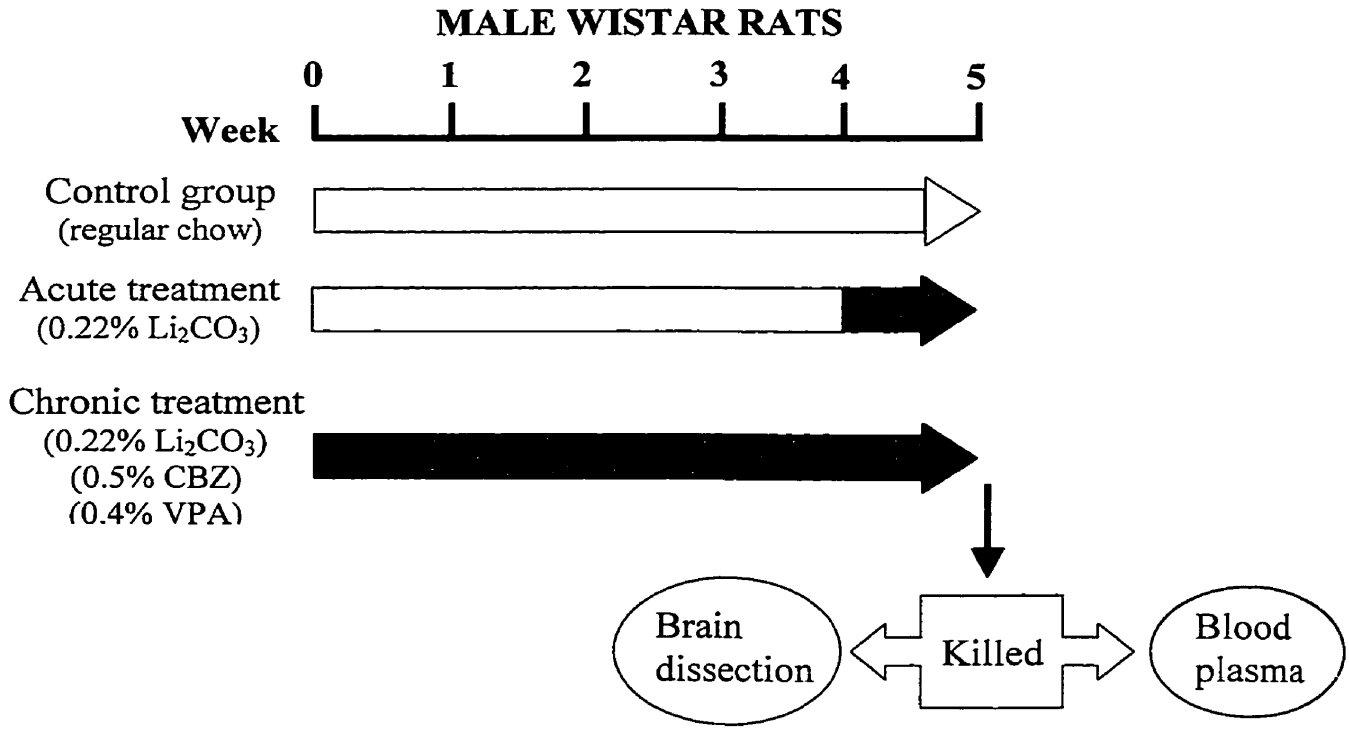


Figure 6 Animal treatment paradigm. Male Wistar rats (200 - 225 g) were fed with rat chow in pellet form containing lithium, valproate, or carbamazepine as described in section 2.2, whereas the control group was given regular chow for 5 weeks. In addition, a separate group of animals was given chow containing lithium for only one week. For haloperidol and imipramine groups, animals were injected intraperitoneally once daily; the control group received an equivalent volume of vehicle for 5 weeks as described in section 2.2. At the end of experiments, animals were killed by decapitation. Brain was rapidly removed, and dissected over ice, frozen and stored at -70°C until use. Blood samples were collected immediately after decapitation from the cervical trunk into heparinized test tubes for determination of plasma drug concentration.

(~250 mg) per day, this would be equivalent to an intake of 100 mg VPA per day, or ~400 mg/kg/day, the dose commonly used by other investigators in studying the chronic effect of VPA. In separate experiments, animals were injected intraperitoneally with VPA (300 mg/kg/day) or 0.9% saline (control) once daily for 35 days (Wang et al, 1999b).

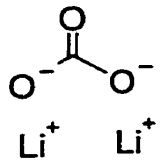
To determine the pharmacological specificity of the lithium's effect, rats were treated chronically with representative antidepressant and antipsychotic drugs. Following acclimatization, groups of 9 rats were injected intraperitoneally once daily with imipramine (10 mg/kg) or haloperidol (1 mg/kg) for 5 weeks. Control animals received equivalent volumes of vehicle [0.9% (w/v) NaCl; 0.05% (v/v) acetic acid]. The drug doses and duration of treatment are comparable to those that have been shown in previous studies to lead to chronic effects of these drug treatments (Morinobu et al, 1995). The chemical structures of the drugs used in this thesis are shown in figure 7.

At the end of experiments, animals were killed by decapitation. Blood samples were collected from the cervical trunk of the rats into tubes containing 500 µl heparin. Plasma was separated by centrifugation (900 g, 20 min) for subsequent determination of drug levels. Immediately after decapitation, brains were rapidly excised from the crania and rinsed in ice-cold saline. After removal of the pineal and meninges, frontal cortex was dissected over ice using natural lines of demarcation (Thomas et al, 1980), frozen on dry ice and stored at –70 °C until experimentation.

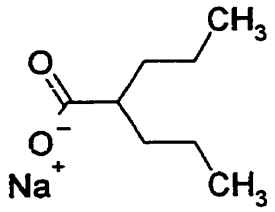
2.2.2 Determination of Plasma Drug Concentration

Plasma lithium levels were determined using the *Vitros* Li Slides (Johnson & Johnson, Mississauga, ON), a colorimetric assay for lithium-crown-ether dye complex. Plasma carbamazepine concentrations were determined by the *Vitros* CRBM Slides (Johnson & Johnson), an immunoassay using anti-CBZ antibody. Plasma valproate levels were quantitated by the TDxFLx valproic acid assay system using fluorescence polarization

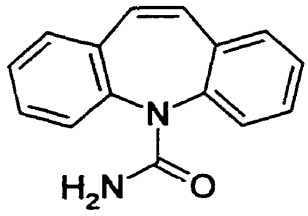
Mood Stabilizers



Lithium Carbonate



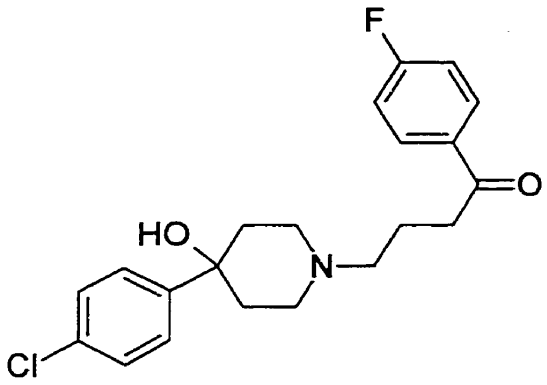
Sodium Valproate



Carbamazepine

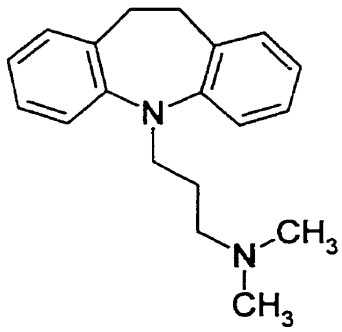
Figure 7 (A) Chemical structure of the three mood stabilizers.

Antipsychotic



Haloperidol

Antidepressant



Imipramine

Figure 7 (B) Chemical structure of haloperidol and imipramine.

immunoassay (Abbott Lab., Abbott Park, IL). The assays were performed in the clinical laboratory at Queen Street Mental Health Centre (CAMH, Toronto, ON)

2.3 METHODS

2.3.1 Decontamination and Inhibition of Ribonucleases for RNA Work

Sterile disposable plastic wares and autoclaved Eppendorf tips were used when necessary. Metal tools wrapped in aluminum foil and glasswares were baked at 180 °C for 8 hr prior to usage. RNase free solutions were prepared by DEPC treatment [0.1%(v/v)], stirring overnight, and autoclaving for one hour to hydrolyze any remaining DEPC into CO_2 and ethanol.

2.3.2 Total RNA Isolation

Brain tissues, up to 600 mg, were homogenized in 7 mL GIT buffer [4 M guanidinium isothiocyanate; 25 mM sodium acetate, pH 6.0; 1% ß-mercaptoethanol] using a polytron Kinematic CH-6010 (Brinkmann Instruments, Rexdale, ON). Homogenates were layered on to 3.3 mL of CsCl buffer [5.7 M CsCl; 25 mM sodium acetate, pH 6.0] in polyallomer centrifuge tubes (Beckman Instruments, Palo Alto, CA) and centrifuged using a SW41ti rotor at 30,000 rpm at room temperature for 23 hr in L8-M ultracentrifuge (Beckman Instruments). Total RNA pellets settled at the bottom of the tube were rinsed with 1 mL of 70% ethanol and resuspended in 360 µl of TES buffer [10 mM Tris.HCl, pH7.4; 5 mM EDTA; 1% SDS].

One-tenth volume of 3 M NaOAc pH 5.2 and 2.5 volumes of ice-cold absolute ethanol were added to the RNA sample in a microcentrifuge tube, which was then incubated on dry ice for 30 min. To recover the precipitated RNA, the tube was centrifuged at 12,000 *g* for 5 min at 4°C. The supernatant was discarded, and the RNA pellet was rinsed with 1 mL of 70% ethanol solution. After a second centrifugation, the supernatant again was discarded, and the RNA pellet was dried in a Speed Vac SC110. The RNA pellet was then dissolved in DEPC-treated water and stored in -20 °C till further use.

2.3.3 Extraction of Genomic DNA from Rat Tissue

Rat frontal cortex (200 mg) was homogenized in 5 volumes of extraction buffer [100 mM Tris-base, pH 8.0; 50 mM Na_2EDTA; 200 mM NaCl; 1 mg/mL proteinase K] using an Ultra Turrax polytron (Interscience, Markham, ON). The homogenate was incubated at 65 °C for 20 min and separated into two equal aliquots. 0.5 volumes of phenol (pH 8.0) was added to the homogenate, which was then mixed, and centrifuged at 3500 *g* for 5 min. The aqueous layer was transferred to new tube and 0.5 volumes of 4% (v/v) isoamyl alcohol in chloroform was added, and the samples mixed and centrifuged at 3500 g for 5 min, 2.5 volumes of 100% ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) were added to the DNA sample in a microcentrifuge tube, which was then incubated at room temperature for 10 minutes. To recover the precipitated DNA, the tube was centrifuged in an IEC Micromax centrifuge (International Equipment Company, Needham Hts, MA) at 12,000 *g* at 4 °C for 10 min. The supernatant was discarded, and the DNA pellet was rinsed with 1 mL of 75% ethanol solution. After a second centrifugation, the supernatant was again discarded, and the DNA pellet was dried under vacuum. The DNA pellet was dissolved in TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 8.0) and stored in -20 °C till further use.

2.3.4 Spectrophotometric Determination of DNA and RNA

DNA and RNA absorbance were measured at 260 nm against TE buffer or H_2O and concentrations calculated based on dilution factor. The ratio at absorbance of A_{260}/A_{280} was used to estimate the purity of the nucleic acid. An OD of 1 corresponds to approximately 50 μg/mL for double-stranded DNA or 40 μg/mL of single-stranded DNA and RNA.

2.3.5 Removal of DNA Contaminant from RNA

Total RNA (10 – 50 μg) was incubated with 10 units of DNase I (GenHunter) in 1X reaction buffer for 30 min at 37 °C. DNase I was removed by phenol/chloroform (3:1) extraction.

DNase-treated RNA was precipitated with ethanol and stored at -20 °C for further experimentation.

2.3.6 Formaldehyde-Agarose Gel Electrophoresis of RNA

Gel electrophoresis apparati were rinsed with DEPC-treated dH_2O. Agarose [1%(w/v)] was added to 1X MOPS buffer [20 mM 3-(N-morpholino)propanesulphonic acid; 5 mM sodium acetate; 1 mM EDTA] and dissolved by heating in a microwave for 2 min. The solution was allowed to cool to about 55°C and 0.5 μg/mL ethidium bromide (EtBr) and 0.66M formaldehyde were added before pouring the solution into a sealed gel tray with comb. The comb was removed and gel tray was submerged in 1X MOPS buffer in an electrophoresis chamber. RNA samples were dissolved in a solution containing 50% (v/v) formamide, 2.2 M formaldehyde and 0.5X MOPS. Samples were heated at 70 °C for 5 min and cooled on ice for 5 min prior to loading. 15-20 μL of RNA solution was loaded per well. The gel was electrophoresed at 1 - 5V/cm until the Bromophenol Blue (BPB) marker dye migrated three-quarters of the length of the gel. EtBr-stained RNA bands were visualized and photographed on a UV light source at 312 nm (Vilber Lourmat, Cedex2, France).

2.3.7 Agarose Gel Electrophoresis of DNA

Agarose [0.5% - 1.5% (w/v)] was added to 1X TBE buffer [89 mM Tris base; 89 mM boric acid; 2 mM EDTA] or TAE buffer [40 mM Tris base; 40 mM glacial acetic acid; 1 mM EDTA (pH8.0)] and dissolved by microwaving for 2 min. The solution was allowed to cool to about 55 °C, 0.5 μg/mL EtBr was added before pouring the solution into a sealed gel tray with comb. The gel was allowed to solidify for at least 20 minutes at room temperature. The comb was removed and gel tray was submerged in 1 X TBE or TAE buffer in an electrophoresis chamber. DNA samples were prepared with 1 X gel loading buffer. 10-20 μL of DNA solution was loaded per well. The gel was electrophoresed at 70-120 V until the BPB dye migrated ¾ of

the length of the gel. EtBr stained DNA bands were visualized and photographed on a UV light box at 312 nm.

2.3.8 Purification of DNA from Agarose Gels

DNA was extracted from agarose gel using QIAquick gel extration kit (QIAGEN) according to manufacturer's instruction. Briefly, EtBr-stained DNA fragments were excised from gels with a clean and sharp scalpel. Gel slices were incubated in 3 volumes of Buffer QG at 50 °C for 10 min with intermittent vortexing until the gel slice has completely dissolved. One gel volume of isopropanol was added and loaded onto a QIAquick column which was then centrifuged at 10 000 g for 1 min, followed by a 1 min centrifuge wash with 0.75 mL of Buffer QG. DNA was then eluted with 50 μL of 10 mM TrisHCl, pH 8.5 and frozen at -20 °C until further use. The concentration of DNA extracted from agarose gel was estimated by the EtBr spot test (Sambrook et al, 1989), which estimates DNA concentration with a sensitivity in nanogram (ng) range. A standard curve was constructed with increasing concentrations of salmon sperm DNA (0, 5, 10, 20, 30, 40, 50, 100 ng/mL) in water. Reference standards and DNA samples were prepared in 1:2 dilutions in EtBr (0.05 μg/mL) solution. Standard curve and DNA samples were spotted over a sheet of parafilm on top of an UV transilluminator. The concentrations of DNA samples were estimated by comparison to the standard DNA samples.

2.3.9 First-Strand cDNA Synthesis for RT-PCR

All incubations were performed in a Peltier thermal cycler PTC-200 (MJ Research, Watertown, MA) using thin-walled reaction tubes. 0.2 – 2 μg of total RNA was denatured at 65 °C for 5 min and chilled on ice for 2 min before mixing with first-strand synthesis reagents [50 mM Tris-HCl (pH 8.3 at 25 °C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 0.5 μg oligo$(dT)_{12\text{-}18}$, 200 units Superscript II reverse transcriptase] to a final volume of 20 μL. The

reaction was incubated at 42 °C for 50 min and terminated by heating at 70 °C for 15 min . Samples were then stored at -20 °C until use.

2.3.10 Polymerase Chain Reaction

All incubations were performed in a Peltier thermal cycler PTC-200 (MJ Research) using thin-walled reaction tubes. PCR reactions were comprised of: 2 μL of first strand cDNA or ≤1 μg of plasmid DNA, 0.1 – 0.5 μM of each set of primers, PCR buffer [50 mM KCl; 10 mM Tris-HCl, pH 8.3], 2 mM $MgCl_2$, 200 μM of each dNTP and 2.5 units of HotStarTaq DNA Polymerase (QIAGEN) or Taq DNA polymerase (Perkin Elmer) in a reaction volume of 20 – 100 μL. The amplification conditions were: Taq activation and/or DNA denaturation at 95 °C for 15 min, followed by 30 –35 cycles of denaturation at 94 °C for 1 min, annealing at 50-65 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min.

2.3.11 mRNA Differential Display

Differential display was carried out essentially as described by Liang and Pardee (1992) using the RNAmap or RNAimage kits (GenHunter, Nashville, TN). DNase-treated total RNA (0.2 μg) was reverse transcribed to cDNA using superscript II reverse transcriptase (GIBCO-BRL) and one of the 3' composite anchor primers $T_{12}VN$ (V = A, C, or G and N = A, C, G, or T) or H-T_{11}V (H = Hind III recognition sequence, V = A, C, or G). Two μL of the cDNA was subjected to PCR amplification in duplicate in 20 μl of PCR buffer [10 mM Tris-HCl, (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin] with 2 μM each of dNTP, 0.2 μM of various arbitrary primers, 0.2 μM of the respective anchor primer, 0.3 μL of [α-^{33}P]dATP (2000 Ci/mmol, New England Nuclear, Boston, MA) and 1 U of AmpliTaq (Perkin-Elmer, Branchburg, NJ). The PCR was conducted using the following program on a MJ Research thermal cycler (Model PTC-200): an initial denaturation at 95 °C for 2 min followed by 40 cycles at 94 °C for 30 sec, 40 °C for 2 min and 72 °C for 30 sec, then a final extension at 72 °C

for 5 min, and rapid cooling at 4 °C. The radiolabeled amplified cDNA fragments were then separated on a 6% (w/v) denaturing polyacrylamide sequencing gel. The gel was vacuum- dried at 80 °C for 2 h and autoradiographed (24 - 48 h). Autoradiographic bands that were quantitatively different in densitometric intensity in 2 - 5 independent reactions from control versus lithium-treated tissues were excised from the dried gel, rehydrated, and the DNA was reamplified by PCR using the appropriate set of primers under the PCR conditions described above, except that the dNTP concentration was 25 μM and no radioisotope was included. Figure 8 shows a schematic outline of the described procedures.

2.3.12 Ligation of DNA Fragments and Transformation of Plasmid DNA

The reamplified DNA was electrophoresed on a 1.5% agarose gel and bands of interest were excised and purified using a QIAEX Gel Extraction Kit (Qiagen, Chatsworth, CA) as described in section 2.3.8. PCR-amplified DNA fragments were ligated into pGEM-T or pCR2.1 vector using T4 DNA ligase in 1l X Rapid Ligation Buffer (Promega or Invitrogen). Molar ratio of insert: vector was 3:1. The reactions were incubated at 4 °C overnight. Two microliters of each ligation reaction and 2 μL of β-mercaptoethanol (β-ME) were added to a 50 μL vial of Invitrogen competent INVαF' *E. coli* cells. The reactions were incubated on ice for 30 min, heat-shocked for 30 sec in a 42 °C water bath and permitted to grow at 37 °C for 1 h with the addition of 250 μL of SOC medium. Fifty to 200 μL from each transformation vial were spread on separate LB agar plates containing 1.6 mg X-Gal and 50 μg/mL of ampicillin. The plates were then placed in a 37°C incubator for at least 18 h. Plates were subsequently kept in a 4 °C incubator for 2-3 h to allow for proper color development. Insert containing plasmids were verified by restriction enzyme digestion using sites on the vector flanking the cloning sites or PCR screening using primers across the cloning sites.

mRNA DIFFERENTIAL DISPLAY

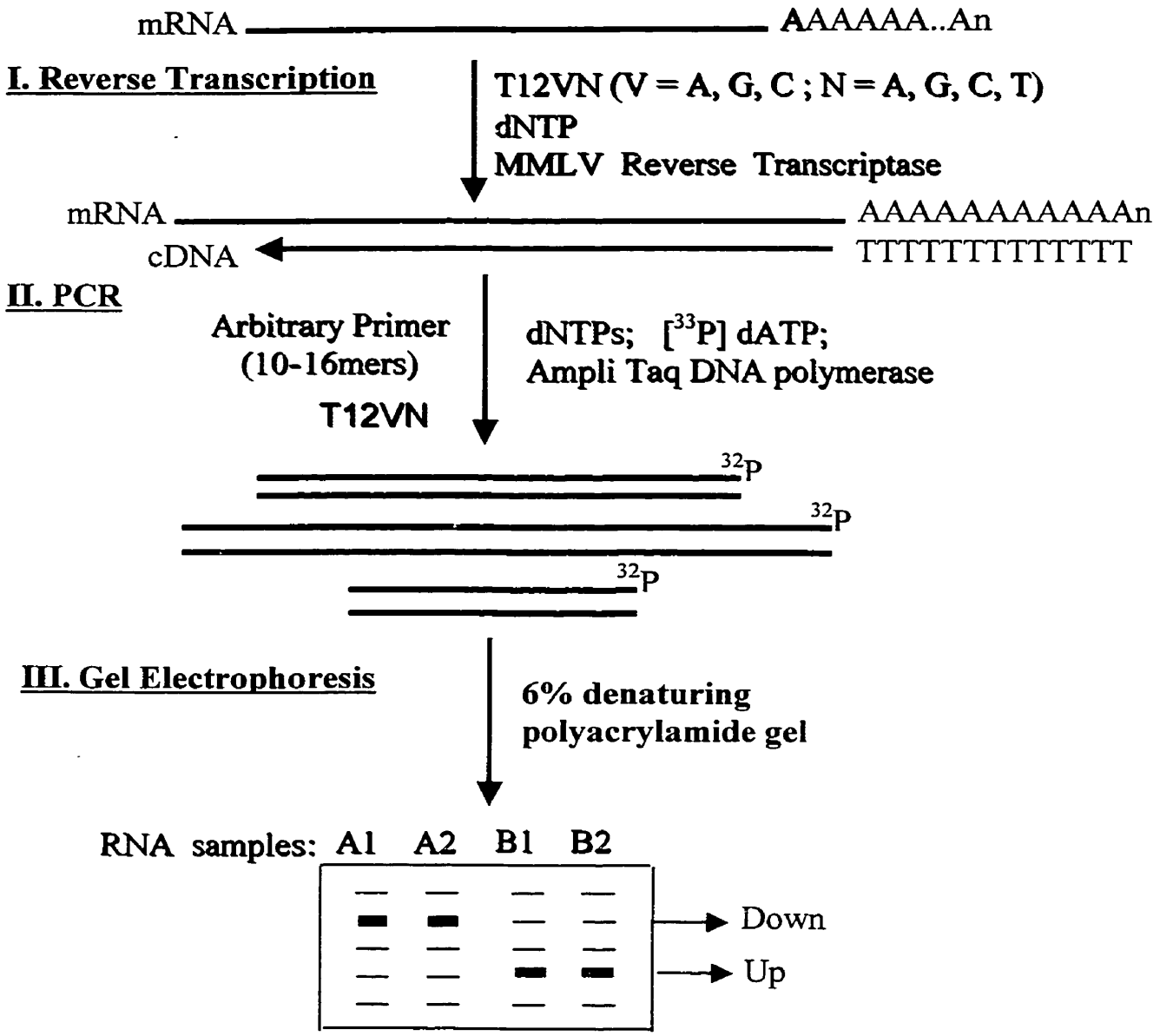


Figure 8 Schematic representation of mRNA differential display. The methodology involves a series of three steps: reverse transcription, PCR and gel electrophoresis.

2.3.13 Minipreparation of Plasmid DNA

A single bacterial colony was inoculated in 2 mL LB medium containing 50 μg/mL ampicillin and grown at 37 °C in a water bath with shaking at 225 rpm overnight, following which an 1.5 mL aliquot was removed and centrifuged at 6000 *g* for 20 sec. The bacterial pellet was lysed in 100 μL glucose solution [50 mM glucose; 25 mM Tris, pH 8.0; 10 mM EDTA] by vortexing vigorously and incubated at room temperature for 5 min. Two hundred μL of alkaline SDS solution [0.2 N NaOH; 1% SDS] was added with mixing by inverting 3-6 times to obtain a clear suspension. The cleared lysate was then incubated on ice for 5 min. 150 μL of 3 M NaAc, pH 4.8, was then added, the lysate was mixed gently by inverting 5 times, and then was left on ice for 10 min to precipitate most of the protein, chromosomal DNA and high molecular weight RNA. The precipitate was sedimented by centrifugation at 12 000 *g* for 3 min. Plasmid DNA in the supernatant was transferred to another tube for precipitation with 0.3 M NaAc, pH 5.2 and 2.5 volume of ethanol at -20 °C. The final plasmid DNA pellet was dissolved in TE buffer.

2.3.14 Restriction Digestion of DNA

Restriction enzyme digestions were performed by incubating plasmid DNA samples with an appropriate amount of restriction enzyme in its respective buffer, as recommended by the supplier, and at the optimal temperature for that specific enzyme. Typical digestions included one unit of enzyme per microgram of DNA in 1X assay buffer at a final volume of 20-100 μL, and were incubated for 1-3 hours at the optimal temperature, most often 37 °C.

2.3.15 PCR-based Colony Screening for The Presence of Cloned Inserts.

Overnight cultures (200 μL) were pelleted and lysed in 0.1% Tween 20 pH 8.0 with heating for 10 min at 95 °C and centrifuging (1 min at 10 000 g). Two μL of supernatant containing plasmid from each colony was amplified in a 20 μL PCR reaction volume consisting of 1X PCR buffer, 200 μM dNTP, 0.5 μM T_7 primer, 0.5 μM M_{13} reverse primer and 0.5 U of

Taq polymerase. The sample was denatured at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 51 °C for 30 sec, 72 °C for 45 sec and a final extention of 72 °C for 5 min. PCR products were then analyzed on EtBr-stained agarose gels.

2.3.16 Reverse Northern Blot Analysis

Plasmids containing the cDNAs of interest or cyclophilin were denatured by boiling in 200 μL of 0.4 M NaOH, 10 mM EDTA for 10 min, and neutralized with an equal volume of 2 M ammonium acetate, pH 7.0. Each plasmid was spotted onto two replicas of nylon membrane (GeneScreen Plus, NEN) at three concentrations ranging from 0.2 to 1.8 μg using the Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA). The duplicate blots were UV-crosslinked in an UV-Stratalinker 2400 (Stratagene, La Jolla, CA) for prehybridization and hybridization overnight. Plasmid containing cyclophilin was used to normalize for unequal loading. Self-annealing plasmid was included as a negative control on each blot.

2.3.17 Prehybridization and Hybridization of DNA/RNA

Prior to hybridization, the blots were washed in 2 X SSC for 5 min at room temperature, then prehybridized with constant rotation in a Hybaid oven (Interscience, Markham, ON) at 42 °C in the presence of 10 mL hybridization buffer for 4 hr. cDNA probes were radiolabeled to a specific activity of 4-10 × 10^8 cpm/μg DNA. Probes (1 × 10^6 cpm/mL) were denatured by boiling for 10 min and then chilled on ice for 5 min before adding to the hybridization mixture. Blots were hybridized with constant rotation overnight at 42 °C and washed with agitation at room temperature (twice, 5 min each in 2 X SSC), at 60 °C (twice, 30 min each in 2 X SSPE, 2% SDS), at 60 °C (twice, 30 min each in 0.2X SSPE, 1% SDS), and finally at room temperature (twice, 15 min each in 0.2 X SSPE). The blots were dried with Kimwipe paper prior to autoradiography.

2.3.18 Northern Blot Analysis

Ten μg of heat-denatured total RNA from individual control and experimental animals were separated by electrophoresis on 1% agarose gels containing 0.66 M formaldehyde. After electrophoresis, the gel was washed 20 min in each of the following: DEPC-treated H_20, 50 mM NaOH/10 mM NaCl, 0.1 M Tris, pH 7.5, and 10 X SSC. RNA was transferred by capillary action onto GeneScreen Plus nylon membranes (NEN) overnight at room temperature in the presence of 10 X SSC, and crosslinked onto the membrane on an UV transilluminator for 1 min and stored at 4 °C until further use. The blots were subjected to prehybridization and overnight hybridization procedures. After stringency washing, hybridization signals were obtained by autoradiography on Kodak Biomax films (Interscience, Markham, ON) or on phosphor screens (STORM, Molecular Dynamics, Sunnyvale, CA). The hybridization signals on phosphor screens were quantitated using the ImageQuant software (Molecular Dynamics), whereas hybridization signals on films were analyzed using an MCID Imaging Analysis Sytem (St. Catharines, Ontario). The bound radiolabeled probes on the blots were stripped (see section 2.3.20) and the blots were rehybridized with ^{32}P-labeled rat cyclophilin cDNA to normalize for RNA loading and transfer.

2.3.19 StripAble DNA Probe Synthesis

The radiolabeled cDNA probe was prepared by random primed probe synthesis using the Strip-EZ DNA kit from Ambion. Linearized DNA template (~25 ng) was added with dH_2O to a final volume of 9 μL, denatured by boiling in a water bath for 5 minutes, and snap-frozen in dry-ice/ethanol. The denatured DNA templates were mixed with 2.5 μL (10 X Decamer Solution), 5.0 μL (5X Buffer -dATP/-dCTP), 2.5 μL (10X dCTP), 5.0 μL ([α-^{32} P]dATP (3000 Ci/mmol, 10 mCi/mL)), and 1.0 μL Exonuclease-free Klenow. The reactions were incubated at 37 °C for 10 - 30 minutes and stop by adding 1 μL of 0.5 M EDTA. Probes were separated from unincorporated ^{32}P-labeled nucleotides by passage through a NICK Spin Column (Pharmacia)

containing Sephadex G-50 equilibrated with TE buffer. Probes were eluted by centrifugation for 4 min at ~ 500 *g*.

2.3.20 Removal of Hybridized StripAble DNA Probe from Northern Blots

Following detection of the hybribidized probe, the blots were placed into a container consisting of 20 mL of 1 X Probe Degradation Dilution Buffer and DNA Probe Degradation Buffer in nuclease-free water and incubated for 30 min at 68 °C. The solution was discarded and replaced by 20 mL of 1 X Blot Reconstitution Buffer and 0.1% SDS in DEPC-treated water. It was then incubated for 30 minutes at 68 °C, followed by incubation with 1% SDS in nuclease-free water for 10 min at 68 °C. The solution was then discarded and the blots were kept at 4 °C for reprobing. All reagents were part of the Ambion kit.

2.3.21 Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from rat brain as described in section 2.3.2. Poly(A) mRNA was isolated by using FastTrack 2.0 Kit (Invitrogen, San Diego, CA) and subjected to 5'-RACE or 3'-RACE to obtain the full-length cDNA. RACE was performed with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) under the conditions suggested by the manufacturer. Double-stranded cDNAs were synthesized from the poly(A) RNA and were ligated to the Marathon cDNA adaptor (see Appendix, Table 2). The resulting adaptor-ligated cDNAs were subjected to PCR amplification with adaptor primer and different gene-specific primers (see Appendix, Table 2). The RACE products were extracted from agarose gels and cloned into pGEM-T vectors. Inserts were sequenced by the dideoxy chain-termination as described in Section 2.3.24.

2.3.22 cDNA Dot Blotting

Plasmid DNA (50 – 200 ng) was denatured in 400 mM NaOH and 10 mM EDTA, pH 8.0 in 200 μL by boiling for 10 min and neutralized by adding an equal volume of ice-cold ammonium acetate, pH 7.0. DNA samples were loaded onto a prewetted GeneScreen Plus

membrane (NEN) using a Bio-Dot Apparatus (BioRad) and washed with 400 μL of 400 mM NaOH via gentle vacuum suction. The membrane was rinsed in 2X SSC for 2 min, UV crosslinked in an UV Stratalinker 1800 (Stratagene, La Jolla, CA) for 45 sec, and stored at 4 °C.

3.3.23 Arrayed cDNA Library Screening

The master plate of a rat brain cDNA library (OriGene Technologies, Rockville, MD) was screened by PCR with a vector-specific primer (5'-GCAGAGCTCGTTTAGTGAACC-3') and a ddPCR fragment specific primer (LRG2: 5'-CCATTTCTTTACGCCGCCACACAAGTC-3'; or LRG3: 5'-GAAGACCTGCACACAGGCGA-3') in accordance to manufacturer's protocol. The cycling conditions were 10 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 65 °C min, 3.5 min at 72 °C, with a final extension step of 5 min at 72 °C. Positive subplates were re-screened by PCR with the same set of primers to identify positive subwells. Positive subwell stocks were plated out onto LB-Ampicillin (100 μg/mL) plates, and colonies were screened to identify positive clones. DNA sequencing of these cDNA inserts was done using the dye-terminator sequencing kit or on an ABI Model Prism 377 DNA sequencer (PE Applied System, Foster City, CA). Primers were synthetic oligonucleotides that were either vector (pCMV-XL4) specific or derived from previously determined sequence information. The cDNA sequence was confirmed through the sequencing of both strands.

2.3.24 DNA Sequencing

Cloned cDNA was sequenced using a Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, Ohio) according to the manufacturer's instructions. Each reaction tube contained 200 ng plasmid DNA, 1 X reaction buffer [26 mM Tris.HCl, pH 9.5; 6.5 mM $MgCl_2$], 2.1 μM dNTP, 21 nM of one of the four radiolabeled terminators ([^{33}P]ddATP, [^{33}P]ddCTP, [^{33}P]ddGTP, or [^{33}P]ddTTP), 70 nM T_7 or M_{13} reverse primer, and 2 units of Thermosequenase DNA polymerase in a final volume of 7 μL. The

reactions were amplified for 30 cycles (95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 1 min). At the end of the PCR cycles, 4 µL of stop solution [5% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF] was added. Samples were heated at 70 °C for 5 min prior to loading to a 6% denaturing sequencing gel. Gels were run at 1500 V for 2 h and exposed onto Biomax MR film (Kodak, Rochester, NY) for 24 – 48 h. Sequences were read manually.

2.3.25 Analyses of DNA and Protein Sequences

DNA and protein sequences analyses were done on-line using BLAST algorithms (Altschul et al, 1990) provided by the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/] and other analysis programs hosted by ExPASy Molecular Biology Server [http://www.expasy.ch/].

2.3.26 Southern Blotting

Thirty µg of rat genomic DNA was digested with 40 U of restriction enzyme (Hind III, EcoR I, BamH I or Pst I) at 37 °C overnight. Digested DNA was precipitated with 0.1 volume of 3 M NaOAC, pH 6.0 and 2 volumes of cold absolute ethanol at –80 °C for 30 min and pelleted by centrifugation at 16, 000 g for 10 min at 4 °C. The pellet was washed with 70% ethanol and centrifuged again. The clean DNA pellet was dissolved in 20 µL of loading dye [80% glycerol, 0.02% bromophenol blue, 1 X TBE] and loaded onto a 0.8% agarose gel in TBE. The gel was electrophoresed at 100V for 4 h and then stained with 5 µg/mL EtBr for visualization. DNA on gels to be transferred was first partially depurinated by soaking the gel in a 0.25 N HCl solution for 15 minutes and then transferred overnight onto Genescreen Plus membrane (NEN) in transfer buffer [0.4 M NaOH, 0.6M NaCl]. The membrane was neutralized the next day by immersing in a solution of 0.5 M Tris-HCl (pH 7.5) and 1 M NaCl for 15 minutes at room temperature. DNA was then UV-crosslinked to the membrane and subjected to prehybridization and hybridization conditions.

2.3.26 Western Blotting

Rat brains were homogenized by ultrasonication for 40 sec (30% duty cycle; output control = 4) at 4 °C with 3 volumes of homogenization buffer [50 mM Tris HCl, pH 8.5; 50 mM NaF, 1 mM EDTA, 10 mM DTT, 1 X protease cocktail (Calbiochem, San Diego, CA)]. The homogenates were centrifuged at 100,000 g (Beckman TL-100 ultracentrifuge) for 60 min at 4 °C. The supernatant was collected as cytosolic fraction, whereas the pellet was resuspended in homogenization buffer by ultrasonication as the particulate fraction. Protein concentrations in different samples were determined by the coomassie brilliant blue protein assay using bovine serum albumin (BSA) as standard (Bradford, 1976). UVmax microplate reader and SOFTmax PRO (Molecular Devices, Sunnyvale, CA) were utilized for protein concentration analyses.

Protein samples were diluted with gel-loading buffer [62.5 mM Tris-HCl, pH 6.8; 3% SDS; 20% glycerol; and 5% mercaptoethanol] to 10 μL and boiled for 3 min at 100 °C. Proteins were separated on a 10% acrylamide/0.25% bisacrylamide SDS-Slab gels with constant voltage of 100 V for 1.5 h. Following SDS-PAGE, proteins were transferred electrophoretically to 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in transfer buffer [25 mM Tris; 192 mM glycine; 20% (v/v) methanol (pH 8.3)] for 2 h on ice. After transfer, membranes were washed briefly twice with PBS-T solution [80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl, pH 7.5; 0.5% Tween 20] and incubated in milk solution [5% (w/v) skim milk in PBS-T] for 1 h at room temperature, and subsequently incubated with respective primary antibodies in milk solution overnight at 4 °C. Blots were washed 3 X for 5 min with PBS-T solution, and then incubated with respective secondary antibodies in milk solution at room temperature for 1 h, followed by another three 5 min washes with PBS-T solution. Proteins were detected by enhanced chemiluminescence using the ECL^+ reagent kit (Amersham, Oakville, ON) with a phosphoimaging detection system with ImageQuant program (Molecular Dynamics).

2.3.28 Protein Expression in Bacterial Host

The pET vector system (Novagen, Madison, MI) was used to express the recombinant rDIPP2 according to manufacturer's instructions. cDNA region coding for the rat DIPP2 protein was amplified from a rat DIPP2 plasmid DNA preparation by PCR using two gene-specific primers U1: 5'-CCG CTC GAG ATG AAG TTC AAG CCC AAC CA- 3' and L1: 5'-CGC GGA TCC TAT CTT ATA CTG GAT GGC AC-3'. These primers were designed such that the amplified gene product included an Xho I site at the 5' end and a BamH I site at the 3' end, to facilitate unidirectional ligation downstream from the T_7 promoter in the expression vector pET15b. The PCR product was analyzed on a 1% agarose gel, extracted from the gel, and subjected to sequential digestion by Xho I and BamH I. The digested product was then extracted from agarose gel for ligation into protein expression vector, pET15b, which was also sequentially digested with Xho I and BamH I and purified from agarose gel. Ligated products were transformed into competent DH5α cells and plated onto LB-Amp agar. Recombinant plasmid DNAs were prepared and digested with Xho I and BamH I to confirm for pET15b-rDIPP recombinant plasmid. Subsequently pET15-rDIPP was sequenced using primers along the flanking regions of the insert to confirm that the insert was in frame and contained the correct coding sequence. Once confirmed, the recombinant pET15b-rDIPP was transformed into the *E. coli* expression host BL21(DE3)pLysS (Novagen). A colony was inoculated in 3 mL LB-Amp (50 μg/mL) culture at 30 °C with shaking overnight. Two millilitres of the overnight culture were then inoculated with 200 mL BL-Amp, allowed to grow at 30 °C with shaking till the OD_{600} ~ 0.8, and induced by adding isopropyl-β-D-thiogalactpyranoside (IPTG) to a final concentration of 1 mM. The culture was grown for an additional 6 hours post-induction. The cells were harvested by centrifugation at 6500 g for 15 minutes at 4 °C. The pellet was resuspended with 4 mL of cold lysis solution [20 mM Tris-HCl, pH 8.0; 300 mM NaCl] and

lysed by sonication. Lysates were centrifuged at 14 000 × g for 30 minutes at 4 °C. The supernatant was passed through a 0.45 μm filter syringe prior to loading onto the nickel column. The protein was purified over "His•Bind" resin. The void volume of the column was 2.5 mL and all elution was performed under gravity flow. The resin was prepared by first allowing the resin to settle in the column, after which it was washed with 3 volumes of sterile water, 5 volumes 1 X Charge Buffer [50 mM $NiSO_4$], and 3 volumes of 1 X Binding Buffer [5 mM imidazole; 500 mM NaCl; 20 mM Tris-HCl pH 7.9]. The filtered lysate was loaded onto the column, and then the column washed with 10 volumes of 1 X Binding Buffer, then 6 volumes of 1 X Wash Buffer [60 mM imidazole; 500 mM NaCl; 200 mM Tris-HCl pH 7.9]. Bound protein was eluted with 6 volumes (15 mL) of 1× Elute Buffer (1 M imidazole; 500 mM NaCl; 20 mM Tris-HCl pH 7.9), which was collected in two 7.5 mL fractions. Eluted protein was dialyzed overnight in 2 liters of 20 mM Tris-HCl, 300 mM NaCl at 4 °C with gentle stirring. Protein was stored in a solution of 20 mM Tris-HCl, 300 mM NaCl, 15% glycerol at –70 °C. Expressed protein was analyzed by coomassie blue stained SDS-PAGE and immunoblotted with anti-mouse DIPP2.

2.3.29 Assay of Rat DIPP2

DIPP activity against PP-$InsP_5$ ($InsP_7$) was determined as previously reported (Safrany et al, 1998). Briefly, the assays were conducted at 37 °C 1 h in 100 μL assay buffer [50 mM KCl, 50 mM HEPES (pH7.2 with KOH), 4 mM CHAPS, 0.05 mg/mL BSA, 1 mM Na_2EDTA, 2 mM $MgSO_4$] containing 50 ng of recombinant rDIPP2 and 3.33 nCi [^{3}H]-$InsP_7$. The reactions were quenched by the addition of equal volume of ice-cold 8%(w/v) perchloric acid containing 1 mg/mL $InsP_6$, and neutralized by the addition of 55 μL of 1 M K_2CO_3 containing 5 mM Na_2EDTA. After being kept at 4 °C for 30 min, the perchlorate precipitate was removed by centrifugation at 10 000 g for 2 min. The supernatants were diluted with 3 volumes of 1 mM Na_2EDTA and loaded onto a 4.6 x 125 mm Partisphere 5 μm SAX HPLC column. Inositol

phosphates were eluted at 1 mL/min by following gradient generated by mixing buffer A (1 mM Na_2EDTA) and buffer B [buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.85 with H_3PO_4; total $[P_i] = 2.6$ M]: 0–10 min, 0%B; 10–25 min, 0–35% B; 25–105 min, 35–100% B; 105–115 min, 100% B. Fractions of 1 mL were collected, mixed with 3 volumes of Monoflow 4 scintillant, and radioactivity was determined using liquid scintillation spectrometry.

2.3.30 Statistical Analysis

Statistical analysis of the data was performed using either one-way ANOVA followed by post-hoc Tukey's HSD test to assess differences between cell means, or two-tailed unpaired Student's t-test. P values < 0.05 were considered statistically significant. All data were expressed as mean ± SEM.

CHAPTER 3

RESULTS

3.1 DRUG TREATMENTS AND BODY WEIGHT

Figure 9 shows the changes in body weight for the animals receiving mood stabilizing drugs in one of the several experiments that were conducted during the course of this project. At the beginning of the experiment, there was no difference in weights between control (323 ± 4 g, mean ± SEM, n = 12) and mood-stabilizing drug treatment groups (lithium, 329 ± 6 g; VPA, 325 ± 3 g; CBZ, 327 ± 8 g; n = 12 in each group) [$F(3,44) = 0.237$, $p = 0.87$]. Animals receiving mood-stabilizing drugs had weight gains [lithium, 120 ± 10 g; VPZ, 134 ± 4 g; CBZ, 111 ± 6 g] similar to those of control subjects (130 ± 5 g) [$F(3,44) = 1.77$, $p = 0.17$] during the treatment periods (Fig. 9A). Likewise, rats receiving either IMI or HAL had similar weight at the beginning of the experiment [control, 269 ± 5 g; HAL, 270 ± 3 g; IMI, 267 ± 6 g; $F(2,23) = 0.079$, $p = 0.924$], and similar weight gains [HAL, 179 ± 3 g; IMI, 178 ± 2 g; $F(2,23) = 2.48$; $p = 0.11$] compared with controls (193 ± 8 g), during the course of the experiment (Fig. 9B).

The plasma concentration in rats receiving lithium for 5 weeks ranged from 0.58 to 1.22 mM, with a mean ± SEM of 0.82 ± 0.02 mM (n = 46), whereas the acute lithium treatment group attained a plasma lithium concentration of 0.55 ± 0.05 mM (n = 8). Mean plasma CBZ concentrations were 13 ± 2 μM (n = 18). Plasma VPA levels were 27 ± 3 μM (n = 18) in the VPA-treated rats.

3.2 mRNA DIFFERENTIAL DISPLAY

With 128 combinations of the degenerate anchored oligo(dT) and arbitrary primers (Appendix, Table 3), differential display of mRNA from control versus lithium-treated groups revealed 38 putative differentially expressed bands or lithium-regulated genes (LRGs), designated as LRG 1 to LRG 38. Thirty-two of the bands were upregulated and six bands were down-regulated (Table 2A). These bands were excised from the DD-gels, reamplified and

(A)

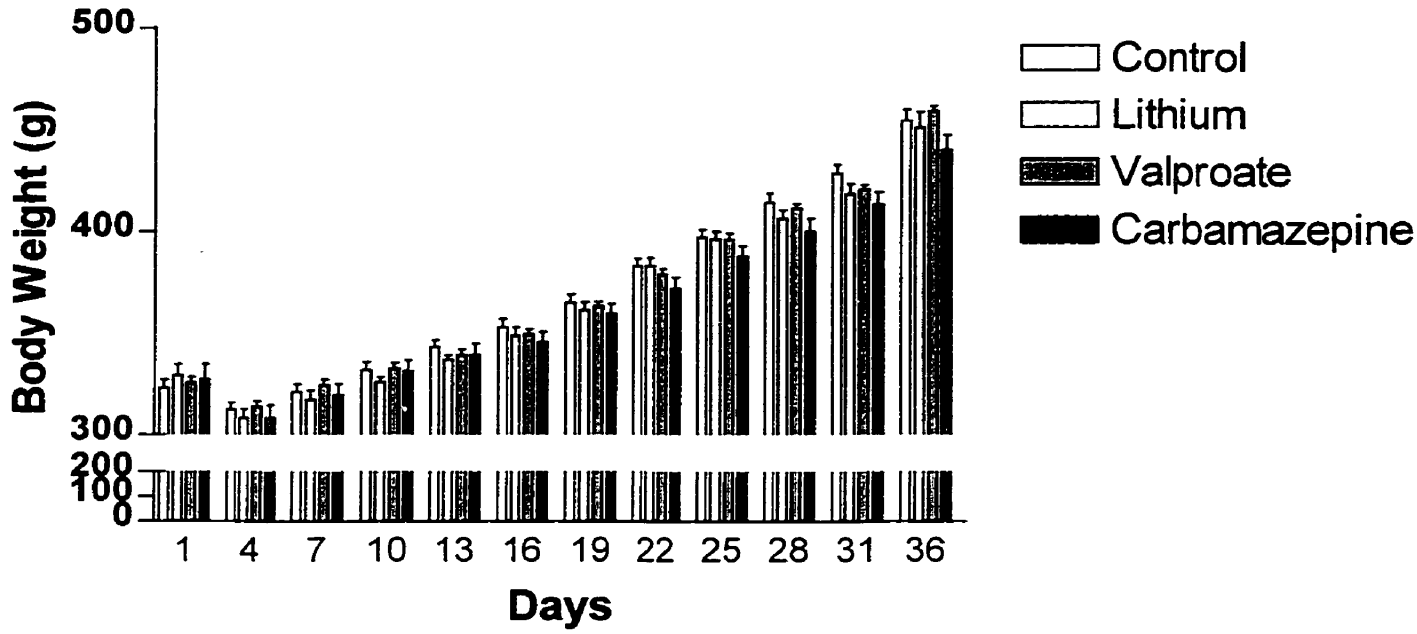


Figure 9 (A) A representative plot of the effect chronic mood stabilizers on body weight of rat. Animals were treated with mood stabilizing drugs as described in Methods. Rats were weighed twice a week for five weeks. n = 12 per group.

(B)

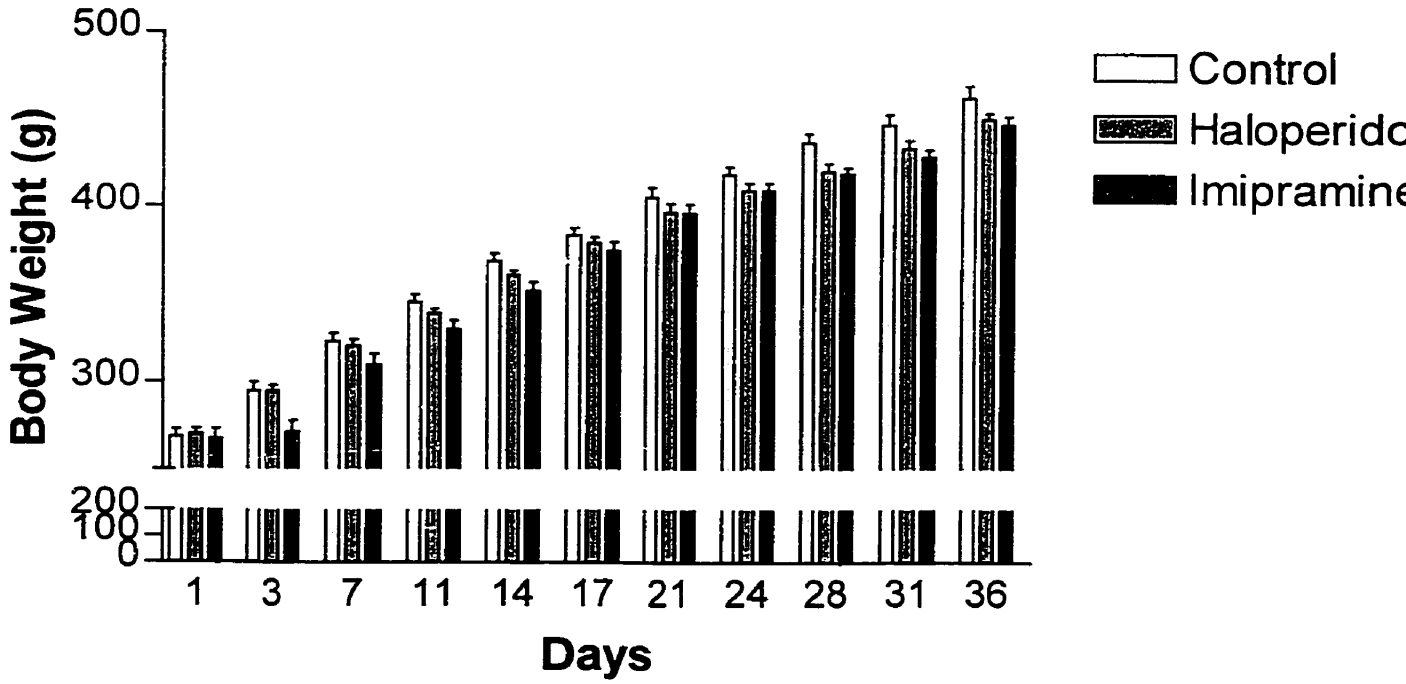


Figure 9 (B) Effect chronic haloperidol and imipramine on body weight of rat. Rats were treated with haloperidol or imipramine as described in Methods. Rats were weighed twice weekly for five weeks. n = 8 or 9 per group.

Table 2 (A) Summary of number of candidates identified by mRNA differential display.

EFFECTS	DIFFERENTIAL DISPLAY
UP	32
DOWN	6

Table 2 (B) Summary of the results of DNA sequence analysis. Lithium-regulated genes (LRG) were sequenced and searched against DNA sequences in the Genbank for matches. EST, expressed sequence tag.

DNA SEQUENCE ANALYSIS	UP	DOWN
KNOWN	12	4
EST	5	0
NO MATCH	10	2

subcloned. Thirty-three of these clones were successfully sequenced and analyzed against DNA sequences in the Genbank (Table 2B, and appendix for full details).

Twenty clones were screened by reverse northern blot using cDNAs labeled by reverse strand priming of the frontocortical RNA samples from control or lithium treated rats (Fig. 10). A subset of ten clones was found to be up-regulated by chronic lithium treatment, whereas a subset of four clones was down-regulated. No apparent differences were observed for the remaining six clones (Appendix I).

Fourteen candidate genes as revealed by reverse northern were subjected to northern blot analysis to confirm whether they represented true positives. There was a trend of up or down regulation for twelve of these clones, but the differences were not statistically significant between control and lithium-treated rats (two-tailed t-test, $p > 0.05$, $n = 9$) (Fig 11C). Two clones, however, showed statistically significant differences and were deemed to be true positives (Fig 11C and Appendix II). These two clones, designated as LRG1 and LRG2, were further characterized and will be presented in turn.

3.3 CHARACTERIZATION OF LRG1

3.3.1 Effects of Lithium and Other Drug Treatments on LRG1 mRNA Levels

LRG1 represented a reamplified cDNA fragment of 540 bp that showed a lower signal intensity in frontocortical samples from rats treated chronically with lithium compared with controls (Fig. 11A). This candidate fragment hybridized with high stringency to a mRNA of ~1.4 kb in rat frontal cortex (Fig. 11B). Northern blot analysis revealed that the frontocortical levels of LRG1 mRNA were significantly decreased ($t = 2.33$, $df = 16$, $p = 0.03$) following chronic lithium treatment in comparison to control (Fig. 12). In contrast, short-term (1-week) lithium treatment did not significantly affect ($t = 0.38$, $df = 10$, $p = 0.71$) the abundance of LRG1 mRNA (Fig. 12).

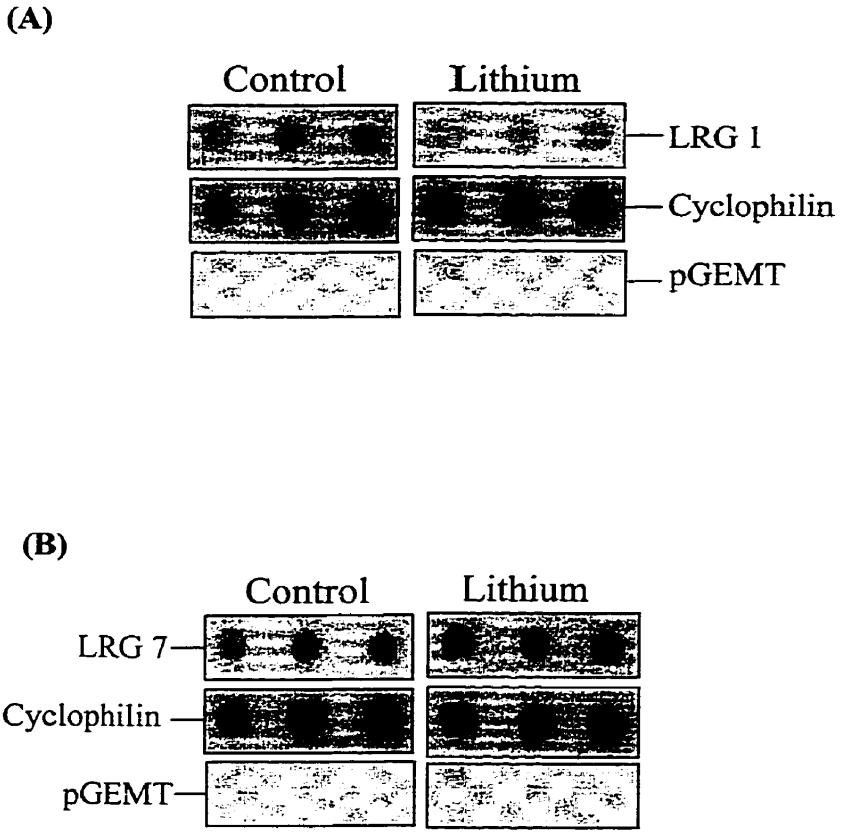


Figure 10 Two representative reverse northern blots. (A) shows that the level of LRG1 is reduced by lithium, whereas (B) shows that LRG7 is increased. Cyclophilin was used to control for differences in loading. pGEMT without insert serves as negative control. Samples were blotted in increasing DNA concentrations (0.2 – 1.8 μg).

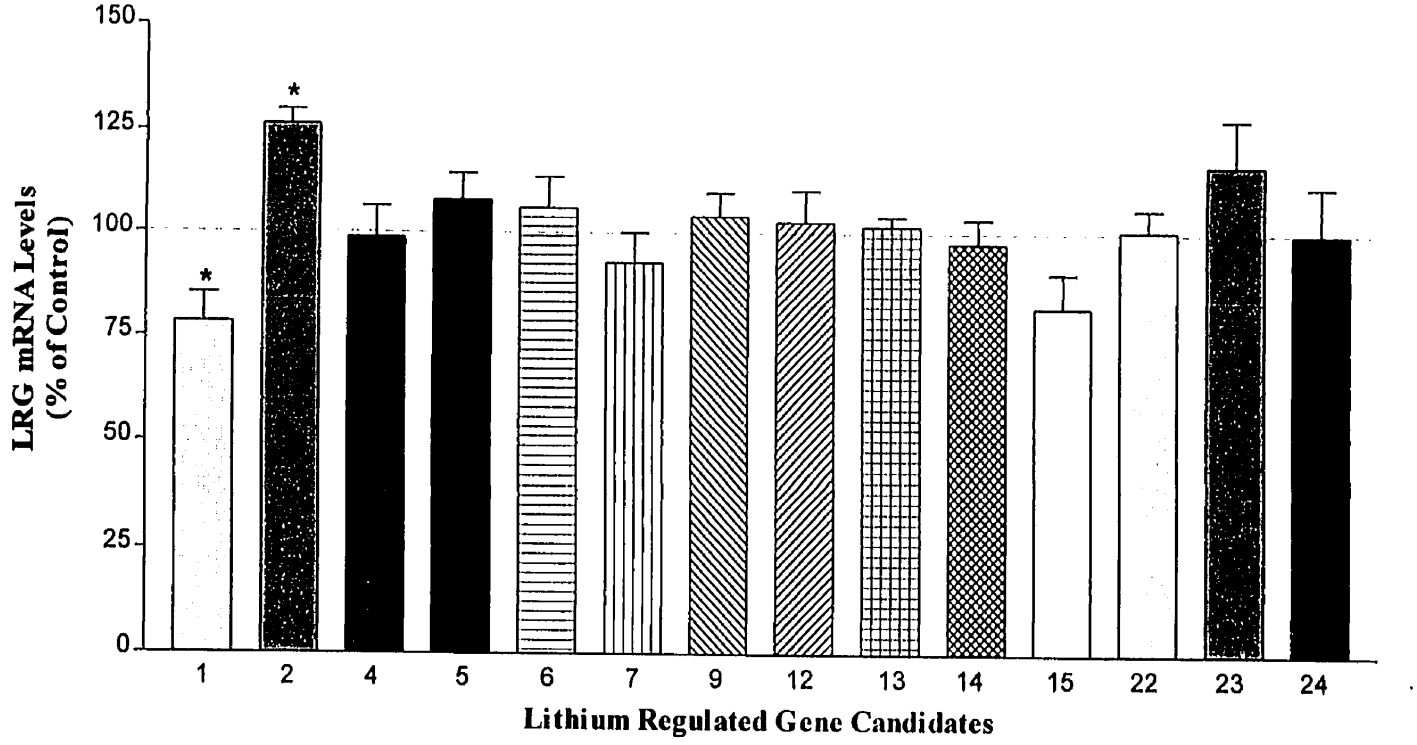


Figure 11C: Effects of chronic lithium administration on the expression of lithium regulated gene (LRG) candiates in rat frontal cortex. Rats were fed with 0.22% Li_2CO_3 for 5 weeks, and levels of LRG mRNAs were determined by northern blot analysis. The signals for LRG mRNAs were normalized to those of cyclophilin mRNA. The results (mean + SEM) are expressed as percentage of control. * $p < 0.05$, $n = 9$ for each group (Student's t- test, two-tailed).

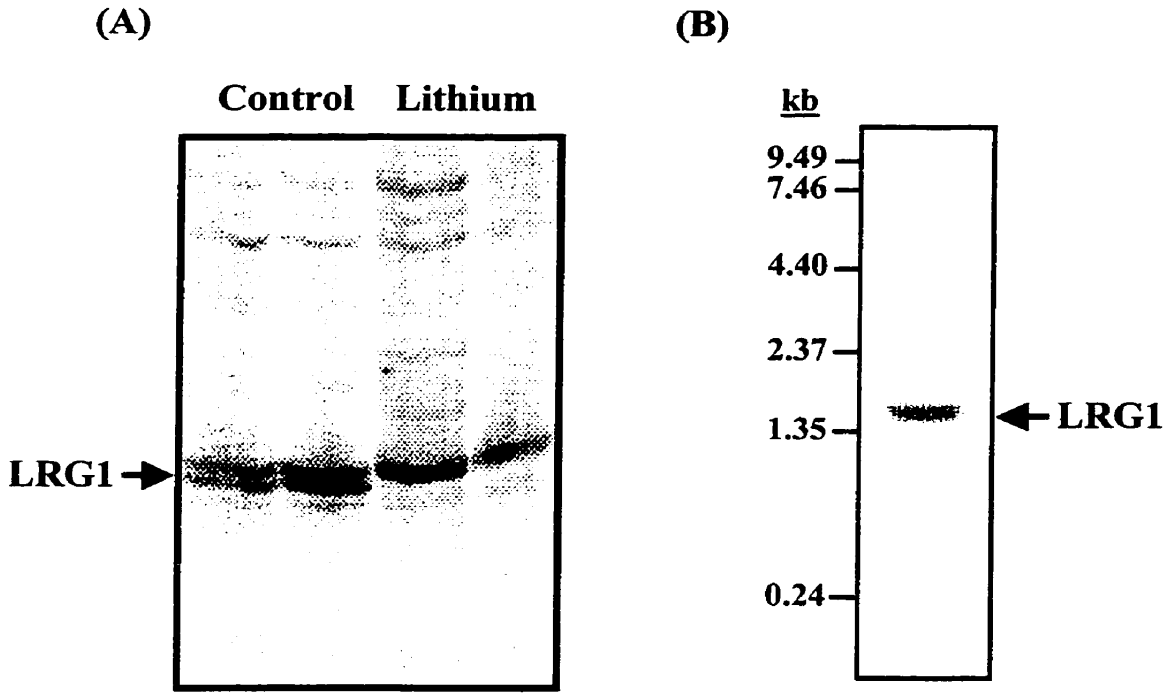


Figure 11 (A) Differential display of mRNA comparing gene expression in frontal cortex from representative control and lithium-treated rats. The autoradiogram of [α^{33}P]dATP-labeled differentially displayed products, using T_{12}MC as 3' primer and AP3 (AGGTGACCGT) as 5' primer, is shown. The band representing LRG1 is indicated by the arrow. (B) Analysis by Northern blot of LRG1 mRNA in rat frontal cortex. Total RNA (10 μg) from rat frontal cortex probed with the LRG1 cDNA fragment shows a single band estimated to be 1.4-kb message.

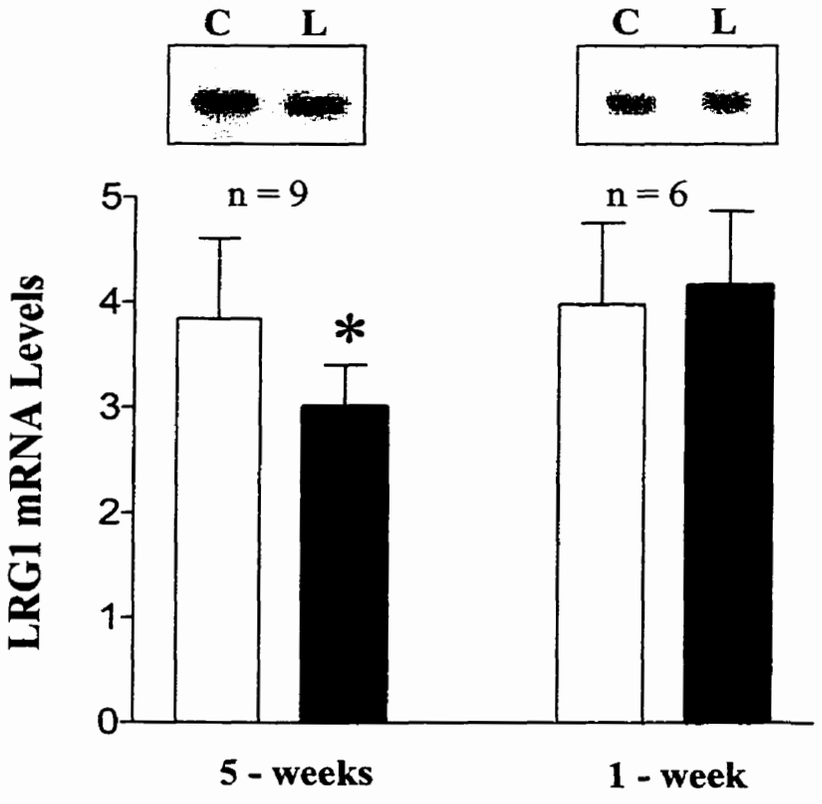


Figure 12 Effect of short-term and chronic lithium administration on the expression of LRG1 mRNA in rat frontal cortex. Rats were fed with 0.22% Li_2CO_3 for 1 or 5 weeks, and levels of LRG1 mRNA were determined by northern blot analysis. The signal for LRG1 mRNA was normalized to cyclophilin mRNA. Representative autoradiograms are shown in the top panels of each bar graph. The results are expressed as the mean $\pm$ SEM for six to nine animals. * $P = 0.03$ compared with controls (Student's t- test, two-tailed).

To assess whether the regulation of LRG1 mRNA level was unique to lithium or common to other mood stabilizing agents, the effects of chronic lithium, VPA and CBZ treatment on the levels of LRG1 mRNA were studied (Fig. 13A). ANOVA showed a significant main effect of drug on LRG1 mRNA levels ($F = 5.70$, $df = 3,16$; $p = 0.008$). Post hoc (Tukey HSD) analysis revealed that the levels of LRG1 mRNA were significantly reduced in rat frontal cortex following either chronic CBZ (-32%, $p = 0.006$) or lithium (-24%, $p = 0.04$) treatment. There was also a trend towards a decreased LRG1 mRNA levels following chronic VPA administration, however the small reduction was not statistically significant (-16%, $p = 0.36$).

To determine whether the LRG1 mRNA level was altered by other classes of psychotropic drugs, such as typical antidepressant or antipsychotic drug, LRG1 mRNA levels were measured following chronic imipramine or haloperidol treatment. Northern blot analysis revealed no significant effect [$F(2,18) = 0.182$, $p = 0.835$] of these drugs on LRG1 mRNA levels in rat frontal cortex, however (Fig. 13B).

3.3.2 Sequence Analysis and Homology off LRG1

Results from the DNA sequence analysis revealed that LRG1 contained 533 bp and had the expected primers at its 5' and 3' ends. LRG1 contained a putative polyadenylation signal, which was located 21 bp upstream from the poly A^+ tail, further supporting the identity of this cDNA fragment as the 3'-untranslated end of the corresponding transcript. Using the BLAST algorithms to search GenBank database, the sequence of LRG1 displayed a near perfect homology (>99%) to the nucleotide sequence (921-1441) for rat aldolase A mRNA (Fig. 14).

3.3.3 Effects of Chronic Lithium on Aldolase C mRNA Levels

In rat brain, aldolase A is known to exist as a heterotetramer with aldolase C (Penhoet et al, 1966; Lebherz and Rutter, 1969), which is considered to be the brain-specific isozyme (Kukita

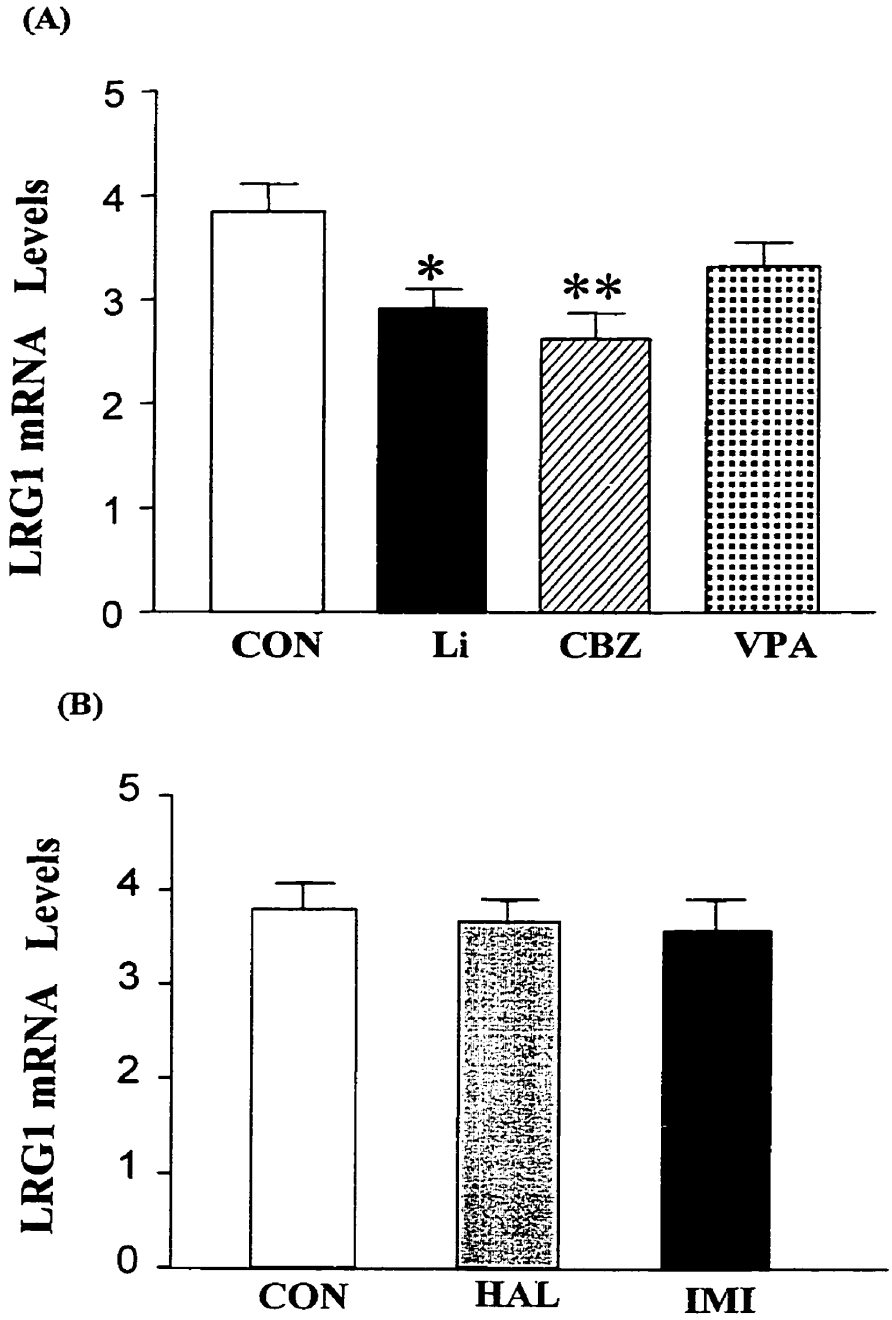


Figure 13 (A) Effect of chronic lithium, carbamazepine or valproate administration on the expression of LRG1 mRNA. Rats were fed with regular chow (CON), or chow containing lithium (Li), carbamazepine (CBZ) or valproate (VPA) for 5 weeks as described Methods. Levels of LRG1 mRNA were determined in the frontal cortex by northern blot analysis. The signal for LRG1 mRNA was normalized to cyclophilin mRNA The results are expressed as the mean $\pm$ SEM for five animals. * $P = 0.04$, ** $P = 0.006$ compared with controls (Tukey's test). (B) Effect of chronic haloperidol or imipramine administration on the expression of LRG1. Levels of LRG1 mRNA were determined as described in Methods. $n = 7$ for each group.

```
LRG1    6    accgtcacagcacttcgtcgaacagtgccccctgccgtccctggggtcactttcctgtct 65
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   921  accgtcacagcacttcgtcgaacagtgccccctgccgtccctggggtcactttcctgtct 980

LRG1    66   ggagggcagagtgaggaagaggcatccatcaacctcaatgctatcaacaagtgtcccctg 125
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   981  ggagggcagagtgaggaagaggcatccatcaacctcaatgctatcaacaagtgtcccctg 1040

LRG1    126  ctgaagccatgggccttgactttctcctatggccgagccctgcaggcctctgctctaaag 185
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   1041 ctgaagccatgggccttgactttctcctatggccgagccctgcaggcctctgctctaaag 1100

LRG1    186  gcttggggtgggaagaaggagaacctgaaggcagcccaggaggagtacatcaagcaagcc 245
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Ald A   1101 gcttggggtgggaagaaggagaacctgaaggcagcccaggaggagtacatcaagcgagcc 1160

LRG1    246  ctggccaacagcctcgcttgtcaaggaaagtacactccaagtggccagtctggagccgca 305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   1161 ctggccaacagcctcgcttgtcaaggaaagtacactccaagtggccagtctggagccgca 1220

LRG1    306  gccagtgaatctctcttcatctctaaccatgcctactaaccagagctgatctaaggctgc 365
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   1221 gccagtgaatctctcttcatctctaaccatgcctactaaccagagctgatctaaggctgc 1280

LRG1    366  tccatcgacactccaggcccctgcctacccacttgctattgaagaggggccttcaggctc 425
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   1281 tccatcgacactccaggcccctgcctacccacttgctattgaagaggggccttcaggctc 1340

LRG1    426  tttcccatcactcttgctgccctcgtgtgtgcagtgttgtctgtgaatgctaaatctgcc 485
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Ald A   1341 tttcccatcactcttgctgccctcgtgtgtgcagtgttgtctgtgaatgctaaatctgcc 1400

LRG1    486  atcccttccagcccactgccaataaacagctatttaagggg 526
             |||||||||||||||||||||||||||||||||||||||||
Ald A   1401 atcccttccagcccactgccaataaacagctatttaagggg 1441
```

Figure 14 Nucleotide sequence homology of LRG1 and rat aldolase A (Gene Bank Accession No. M12919). Identical residues are shown as | and the polyadenylation signal is underlined.

et al, 1988). In order to test whether lithium also affected the mRNA level of aldolase C, a cDNA probe was prepared by PCR using primer pairs [5RALDOC: 5'-ATA TGA AGG CAG TGG AGA TG-3' and 3RALDOC: 5'-GGA AAG GTT CTG TTC TGA CTT-3'] that were designed to allow amplification of nucleotides (nt)1131-1434 for aldolase C (Mukai et al, 1991). Northern blot analysis demonstrated that radiolabeled aldolase C cDNA probe hybridized to a single mRNA species of ~ 1.5 kb, the expected size of the rat aldolase C mRNA (Mukai et al, 1991). As reported for aldolase A, chronic administration of lithium significantly reduced the mRNA level of aldolase C by 30% ($t = 2.33$, $df = 14$, $p = 0.04$) in frontal cortex (Fig. 15).

3.3.3 Effects of Chronic Lithium Treatment on Protein Levels of Aldolase

Quantitative immunoblotting was used to examine whether lithium modulation of aldolase expression is also manifested at the protein level. On western blots, aldolase antisera reacted with a single ~ 41 kDa polypeptide, the mass expected for aldolase (Kao et al, 1999). Under the assay conditions, the aldolase signals from rat frontal cortex on western blot were linear from 2.5 – 10 μg for cytosolic, and 2.5 – 15 μg for particulate fraction, whereas the linear range for purified muscle aldolase (positive control) was from 10 – 100 ng. The signals for aldolase (7.5 μg for cytosolic, 10 μg for particulate fraction) from rat frontal cortex were normalized against the signals from purified aldolase (50 ng) run in quadruplicate on the same gels. The level of aldolase was significantly reduced ($t = 2.56$, $df = 14$, $p = 0.01$) in the cytosolic fraction, but unaltered ($t = 0.43$, $df = 14$, $p = 0.33$) in the particulate fraction of frontal cortex of rats treated chronically with lithium (Fig. 16).

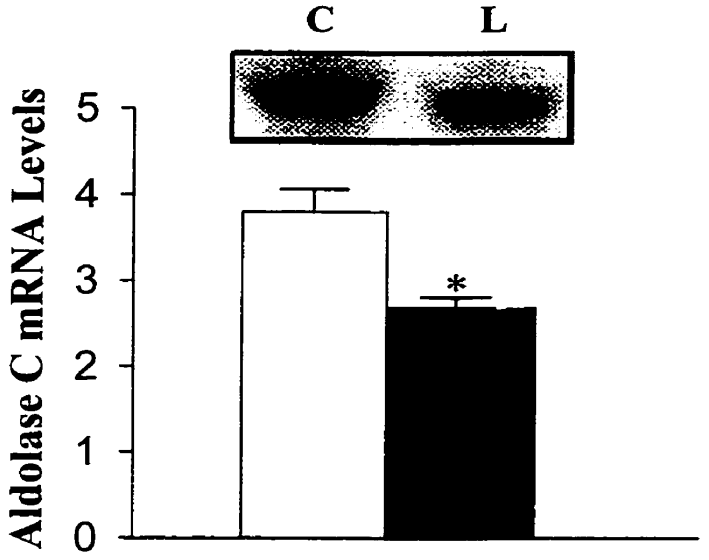


Figure 15 Northern blot analysis of aldolase C mRNA. Total RNA (10 μg) from frontal cortex of rats treated with chronic lithium (L) and control (C) were subjected to northern blotting procedure as described in Methods. Blot was hybridized with cDNA probe corresponding to nucleotides 1131-1434 of rat aldolase C mRNA. The signal for aldolase C mRNA was normalized to cyclophilin mRNA. $n = 9$, * $P < 0.05$.

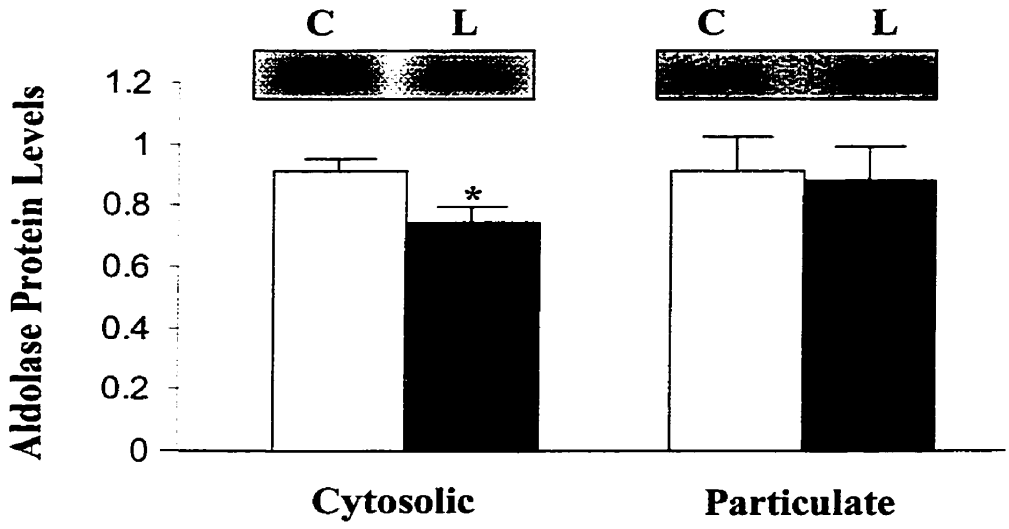


Figure 16 Western blot analysis of rat adolase protein level. Cytosolic (7.5 μg) and particulate (10 μg) protein from rat frontal cortex treated with lithium (L) or control (C) were resolved on a 10% SDS-PAGE and subjected to western blot procedures as described in Materials and Methods. Relative protein levels for cytosolic and particulate fractions were expressed as the levels of aldolase signal over the signal for purified aldolase protein (50 ng) that was used to control for loading and transfer. *P <0.03, n = 8 per group, two-tailed Student's t-test.

3.4 CHARACTERIZATION OF LRG2

3.4.1 Effects of Lithium and Other Psychotropic Drugs Treatment on LRG2 mRNA

A cDNA fragment of about 630 bp was amplified to a greater extent in the frontal cortex samples from rats treated chronically with lithium compared with control animals (Fig. 17A). This ~630 bp cDNA fragment (designated as LRG2) was excised, reamplified, radiolabeled, and used for northern blot analysis. A single mRNA species of ~3.0 kb was detected in rat frontal cortex (Fig. 17B). Figure 18 shows that the abundance of mRNA for LRG2 was significantly increased (26%) in the frontal cortex ($t = 2.79$, $df = 14$, $p = 0.02$) following chronic lithium treatment. In contrast, short-term (1-week) lithium treatment did not significantly affect ($t = 0.26$, $df = 10$, $p = 0.8$) frontocortical LRG2 mRNA levels.

To assess whether the regulation of LRG2 mRNA level was an effect unique to lithium or common to other mood stabilizing agents, the effects of VPA and CBZ treatment on the levels of LRG2 mRNA were compared with those of chronic lithium. Figure 19A shows that 5 weeks of VPA or CPZ administration did not influence the expression of LRG2 mRNA in the rat frontal cortex, in contrast to the increase that was observed in response to lithium treatment ($t = 3.11$, $df = 8$, $p = 0.02$). Likewise, chronic IMI or HAL treatment had no significant effect on the levels of LRG2 mRNA (Fig. 19B).

3.4.2 Sequence Analysis and Homology of LRG2

DNA sequencing of LRG2 identified a 633 bp fragment containing the expected primers at its 5' and 3' ends. LRG2 contained a putative polyadenylation signal that was located 21 bp upstream from the poly A^+ tail, supporting the identity of this cDNA fragment as the 3'-untranslated end of the corresponding gene. Using the BLAST algorithm to search GenBank database, the sequence of LRG2 displayed a near perfect homology (98%) to the nucleotide sequence for an expressed sequence tag (EST) (GenBank Accession U95001) (Fig 20).

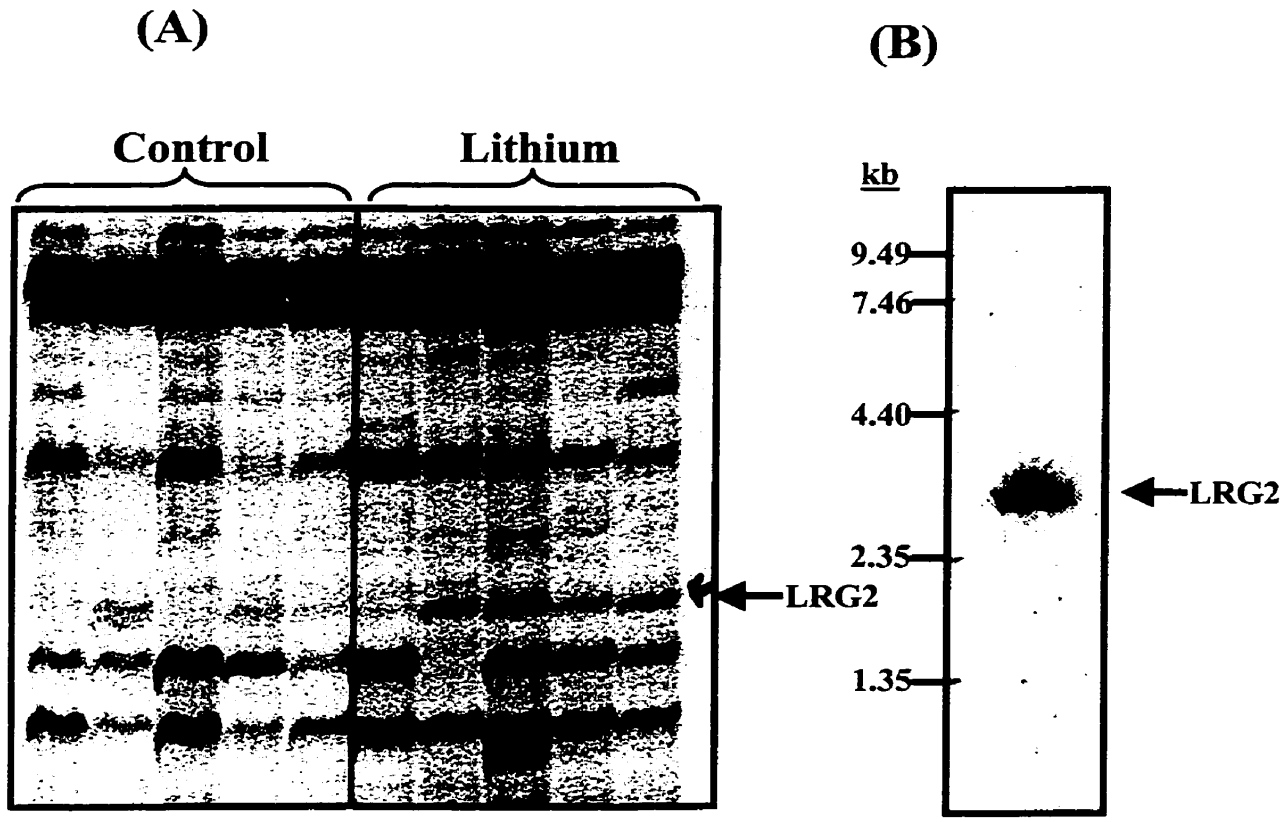


Figure 17 (A) Differential display of mRNA comparing gene expression in the frontal cortex from representative control and lithium-treated rats. The autoradiogram of [α-^{33}P]dATP-labeled differentially displayed products, using H-$T_{11}C$ as 3' primer and H-AP3 (5'-AGGCTTTGGTCAG-3') as 5' primer, is shown, and the cDNA fragment representing LRG2 is indicated by the arrow. (B) Analysis by northern blot of LRG2 mRNA in the rat frontal cortex. Northern blot of 10 μg total RNA from the rat frontal cortex probed with LRG2 cDNA fragment shows a single 3.0-kb message.

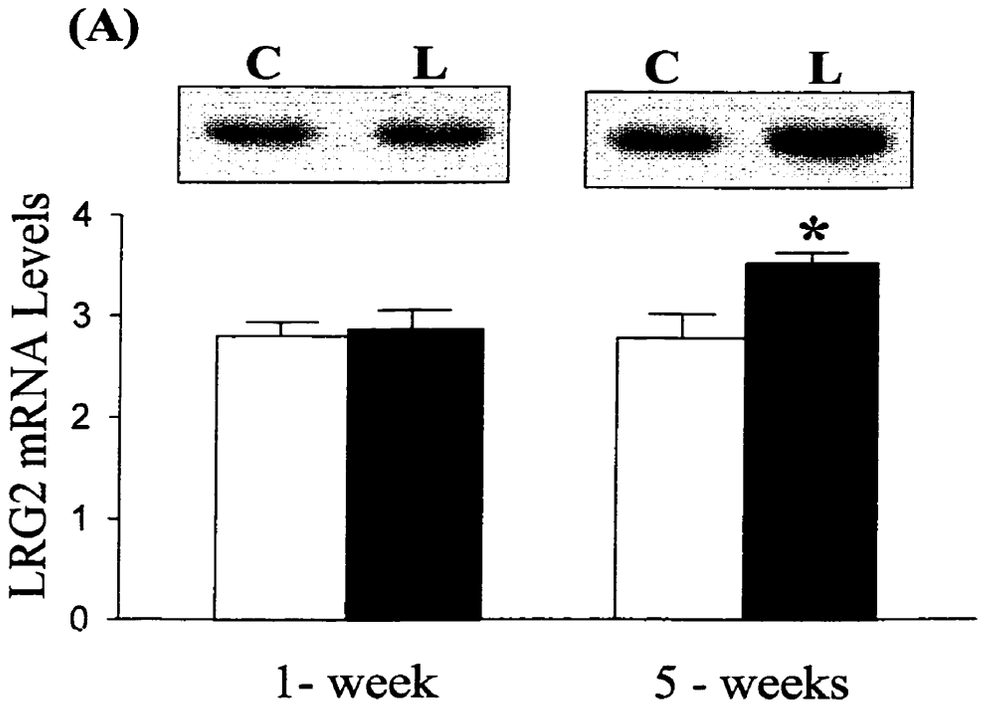


Figure 18 Effect of short-term and chronic lithium administration on the expression of LRG2 mRNA in the rat frontal cortex. Rats were fed with 0.22% Li_2CO_3 (filled columns) or regular rat chow (open columns) for 1 or 5 weeks, and levels of LRG2 mRNA were determined by northern blot analysis. The signal for LRG2 mRNA was normalized to cyclophilin mRNA. Representative autoradiograms are shown in the top panels of each bar graph. The results are expressed as the mean $\pm$ SEM for six to nine animals. * $P < 0.05$ compared with control animals (Student's t- test).

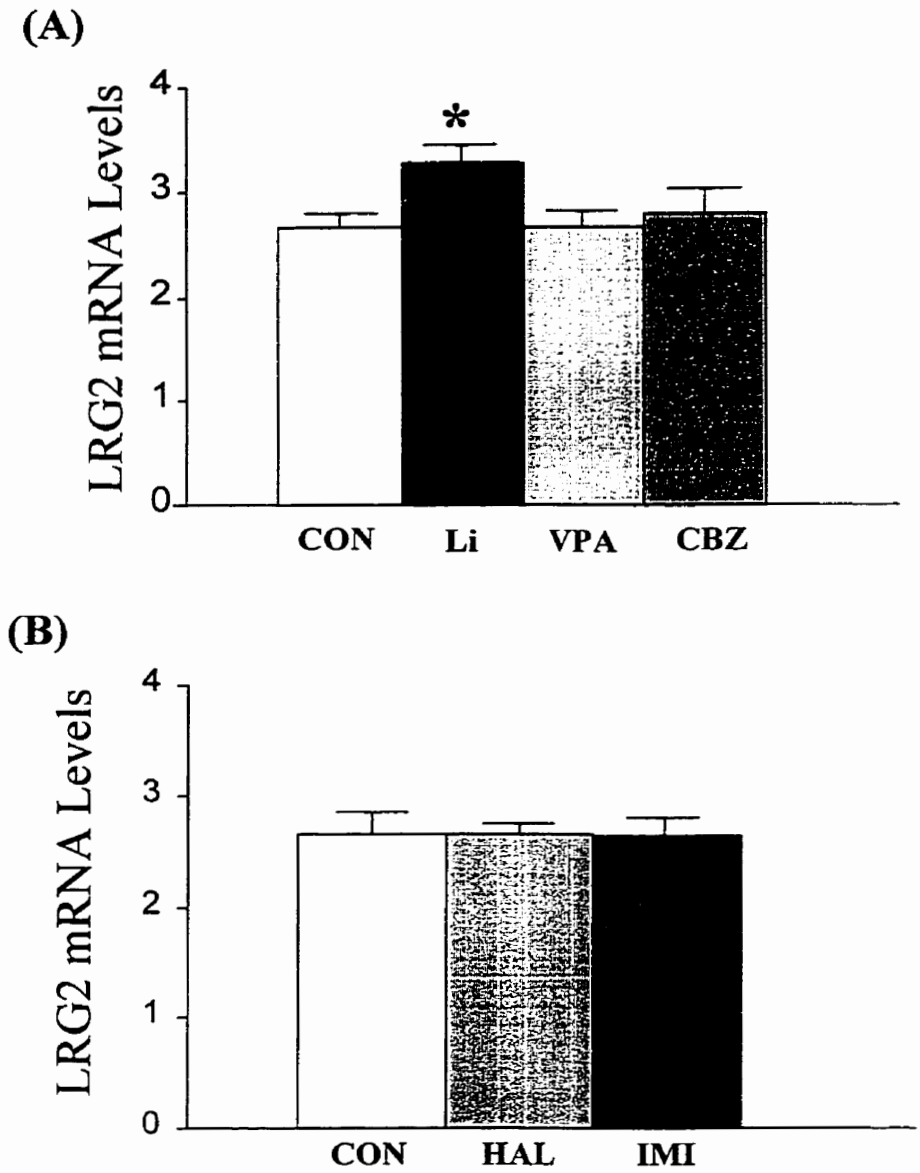


Figure 19 (A) Effect of chronic lithium (Li), valproate (VPA) or carbamazepine (CBZ) administration on the expression of LRG2 mRNA in the rat frontal cortex. Rats were fed with regular chow (CON), or chow containing lithium Li, VPA or CBZ for 5 weeks as described in Materials and Methods. (B) Effect of chronic intraperitoneal administration of haloperiodol (HAL) or imipramine (IMI) on the expression of LRG2 mRNA in rat frontal cortex. Levels of LRG2 mRNA were determined in the frontal cortex by northern blot analysis. The signal for LRG2 mRNA was normalized to cyclophilin mRNA. The results are expressed as the mean $\pm$ SEM for five animals (A), and for seven animals (B). * $P < 0.05$, compared with control animals (Student's t test).

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LRG2 : 14  aaacatttggactaagt-catgtcacctgggtcaggattttcttcaatgccgtgtagcaa 72
           ||||||||||||||||| ||||||||||||||||||||||||||||| ||||||||||||
EST  :  1  aaacatttggactaagttcatgtcacctgggtcaggattttcttcaacgccgtgtagcaa 60

LRG2 : 73  aactatctttagtctatgcaaatagcgagtcactgagtgtgacagaatgcaacttcactg 132
           |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 61  aactgtctttagtctatgcaaatagcgagtcactgagtgtgacagaatgcaacttcactg 120

LRG2 : 133 ttaaacttcacctgagggttcctcattctcctggaatccagactgcaagattataaagga 192
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 121 ttaaacttcacctgagggttcctcattctcctggaatccagactgcaagattataaagga 180

LRG2 : 193 aaagacctaaggcaattcagttctttttgcaaatcaattgaatccacgagagatgtctac 252
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 181 aaagacctaaggcaattcagttctttttgcaaatcaattgaatccacgagagatgtctac 240

LRG2 : 253 cagcgagatgtctaccagcccagccgcctgcagcctgctgtgtgtgcttatttgtgcgct 312
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 241 cagcgagatgtctaccagcccagccgcctgcagcctgctgtgtgtgcttatttgtgcgct 300

LRG2 : 313 gaataaaatggggcagctaaattctccagttccatatgcctccgaagttcaaag--aaaa 370
           |||||||||||||||||||||||||||||||||||||||||||||||||||  ||||
EST  : 301 gaataaaatggggcagctaaattctccagttccatatgcctccgaagttcaaagaaaaaa 360

LRG2 : 371 aaagcaaagtaacatgttagacttgacttgtgtggcggcgtaaagaaatggcatcttccc 430
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 361 aaagcaaagtaacatgttagacttgacttgtgtggcggcgtaaagaaatggcatcttccc 420

LRG2 : 431 actaagaacgaaccatccagttcttttgtcagtcacactatgaaacagggaaggtgaagg 490
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 421 actaagaacgaaccatccagttcttttgtcagtcacactatgaaacagggaaggtgaagg 480

LRG2 : 491 gaagaaatggttatgtgtgcacgaatcgctttgcatggtctcatgagatggctgcattcg 550
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 481 gaagaaatggttatgtgtgcacgaatcgctttgcatggtctcatgagatggctgcattcg 540

LRG2 : 551 aactgttttaagaattgtaaggatcttgacttttttacatttggaaacatcaaataaaaa 610
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EST  : 541 aactgttttaagaattgtaaggatcttgacttttttacatttggaaacatcaaataaaaa 600

LRG2 : 611 caaacataatctgaaaaaaaaaa 633
           ||||||||||||  ||||||||
EST  : 601 caaacataatctgtgaaaaaaaa 623
```

Figure 20 Nucleotide sequence homology of LRG2 and rat EST (GenBank Accession U95001). Identical residue ar shown as | and the polyadenylation signal is underlined.

3.4.3 Rapid Amplification of cDNA Ends (RACE)

RACE procedures were initially used in an attempt to clone the full-length cDNA encoding LRG2. A band of ~ 2.0 kb was identified from amplification of a cDNA library targeting the 5' end upstream from LRG2 (5'RACE) using an adaptor primer 1 [AP1 = 5' CCA TCC TAA TAC GAC TCA CTATAG GGC 3'] and a gene specific primer [5HC3-4 = 5' CCA TTT CTT TAC GCC GCC ACA CAA GTC 3']. A band of ~ 0.4 kb was revealed as an amplified product for 3' end of LRG2 (3'RACE) using adaptor primer 1 [AP1 = 5' CCA TCC TAA TAC GAC TCA CTATAG GGC 3'] and a second gene specific primer [3HC3-4 = 5' CGC CTG CAG CCT GCT GTG TGT GCT TA 3']. The RACE products were cloned. Plasmid DNA of several colonies from 5'RACE and 3'RACE products were subjected to Southern dot blotting and hybridized with radiolabeled LRG2 cDNA fragment. Most of the colonies hybridized to LRG2 suggesting the presence of inserts of interest (Fig. 21).

3.4.4 DNA Sequencing and Analysis of RACE Products

Colony 5 of the 5'RACE product (5'5) and colony 13 of 3'RACE (3'5) were chosen initially for DNA sequencing, which identified an insert of 2071 bp for 5'5 and 379 bp for 3'5. DNA sequence analysis of 5'5 and 3'5 revealed a combined sequence of 2491 bp with an open reading frame of 759 bp encoding a 253 amino acid polypeptide (Fig. 22). Based on this sequence information, a 504 bp EcoR I/BamH I (nt 187 – 690) fragment was excised from 5'5, and used as probe for northern blot analysis. Unlike LRG2 (Fig. 23B), this probe hybridized to three mRNA transcripts in rat frontal cortex: 1.6 kb, 2.0 kb, and 3.0 kb (Fig. 23A), suggesting that 5'5 might not be the clone of interest. Subsequent analysis of the 5'5 nucleotide sequence using VecScreen (National Center for Biotechnology Information, NIH) identified two stretches of cloning vector sequences that linked a portion of LRG2 cDNA fragment in the 3' end to another gene in the 5' end (Fig. 22). Consequently, plasmid DNA insert (~ 2.0 kb) from colony 2

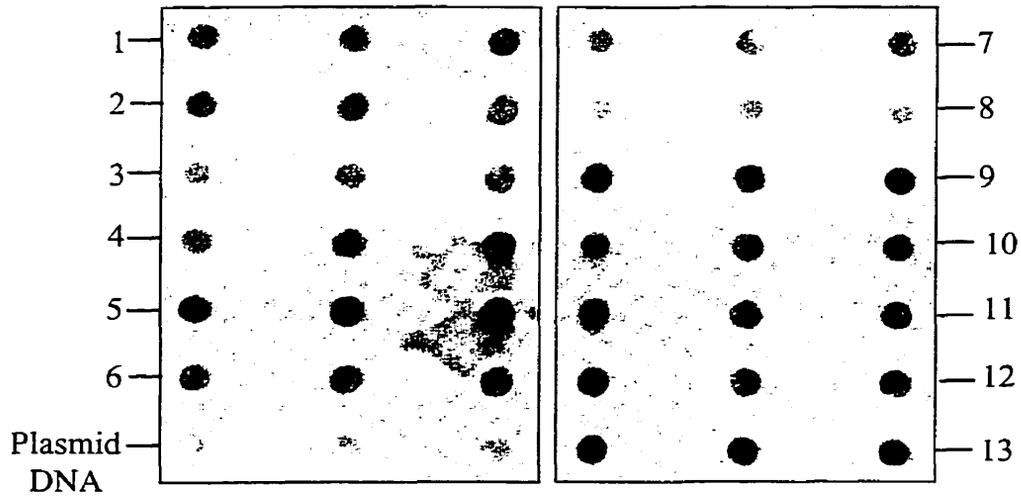

Figure 21 Southern dot blotting of RACE products. Different colonies of RACE recombinant plasmid DNA were dotted in triplicate onto the membrane and hybridized with radiolabeled LRG2. Eight colonies (1-8) from 5'RACE product and five colonies (9-13) from 3'RACE product are shown. Plasmid DNA without insert serves as negative control.

```
                    -183 GCGCTCCTACAGTGGCTGCCGGGGCTGGTATTTCAAAGCGTGGT
TGATCTGGTGGCTCTGAGAATCATTCTGATTACTGCTTCAGACACCATAGGAGTGGCAAGATCCCACCTG
CCAAGGTGAAGGGCGGGCAGATCAGTGACTTTGACCTGTACCTGCTGGTCCTGACTTCCAGAGGCCCAGG   -1

      ATGGGGGAATTCAAC GAGAAGAAGGCAACA TGTGGCACAGTCTGC CTCAAGTATCTGCTG     60
       M  G  E  F  N   E  K  K  A  T   C  G  T  V  C   L  K  Y  L  L      20
      TTCACCTACAACTGC TGCTTCTGGCTGGCT GGCCTGGCCGTCATG GCAGTGGGCATTTGG    120
       F  T  Y  N  C   C  F  W  L  A   G  L  A  V  M   A  V  G  I  W      40
      ACACTGGCCCTCAAG AGTGACTACATTAGT CTGCTGGCTTCAAGC ACCTACCTAGCAACA    180
       T  L  A  L  K   S  D  Y  I  S   L  L  A  S  S   T  Y  L  A  T      60
      GCCTACATCTTAGTG GTGGCTGGTGTTGTT GTCATGGTGACTGGA GTCCTGGGCTGCTGT    240
       A  Y  I  L  V   V  A  G  V  V   V  M  V  T  G   V  L  G  C  C      80
      GCCACTTTCAAGGAG CGACGGAACCTGTTA CGCTTGTACTTCATC CTGCTCCTCATCATC    300
       A  T  F  K  E   R  R  N  L  L   R  L  Y  F  I   L  L  L  I  I     100
      TTCCTTCTGGAGATC ATTGCTGGCATCCTG GCCTATGTCTACTAT CAGCAGCTCAACACC    360
       F  L  L  E  I   I  A  G  I  L   A  Y  V  Y  Y   Q  Q  L  N  T     120
      GAGCTCAAGGAGAAT CTGAAGGACACCATG ATCAAGAGATACCAC CAGTCAGGCCATAAG    420
       E  L  K  E  N   L  K  D  T  M   I  K  R  Y  H   Q  S  G  H  K     140
      GGTGTGACCAACGCT GTAGACAAGCTGCAG CAAGAGTTCCACTGC TGTGGCAGCAACAAC    480
       G  V  T  N  A   V  D  K  L  Q   Q  E  F  H  C   C  G  S  N  N     160
      TCTCGAGACTGGAGA GACGGCGGGTGGATC CGCTCTGGTGAGGCA GACAGCCGTGTGGTC    540
       S  R  D  W  R   D  G  G  W  I   R  S  G  E  A   D  S  R  V  V     180
      CCTGACAGCTGCTGT AAGACTGTGGTAACT GGCTGTGGTAAGCGG GAGCACGCCTCCAAC    600
       P  D  S  C  C   K  T  V  V  T   G  C  G  K  R   E  H  A  S  N     200
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       I  Y  K  V  E   G  G  C  I  T   K  L  E  S  F   I  Q  E  H  L     220
      CGGGTCATCGGGGCC GTGGGCATTGGCATC GCCTGTGTGCAGGTC TTCGGCATGATCTTC    720
       R  V  I  G  A   V  G  I  G  I   A  C  V  Q  V   F  G  M  I  F     240
      ACCTGCTGCTTGTAC AGAAGCCTCAAGCTG GAGCACTACTGA                       762
       T  C  C  L  Y   R  S  L  K  L   E  H  Y  *                       253

CCATGGTGCCAGGCCAGAAGGCCAGCCTCGCGCTGTGTCCCAGCCCCCCACTACTGAACACTACTGAGTT  832
AGACTGCTGACTGCCAGAGAGGTAGCTGCCCTCATGCCATTACCATGTCCCCTGGTGGCTCCACCTCCGT  902
GGTAGCTTCAAGTGCCTTTCCCTGCACACCAAAGTCCCAAAGCTTGAGGCAGCGTACTTGCCCCAGCATT  972
AACAAGAGGGGGCATGTATGCCAGTGTTGGGCTAGGCCTGGCTAGTAGAGTAGGGTTTAGATTACTTGAC 1042
AGAAGTGCCTCTTATGTAGACTGCTTCCCTTTGGGTAGCTGTGATCTGCAGTAAGTCTGGGGAGAATCCG 1112
AGCATACCACTACACGGTGGGTAAGAGCAGGTGGGAAGGGGCCTTTCTGGTTGGGCTAGGCACCTTTCAT 1182
GCCTGCTGCCAGGAACCCTCAGATAGGGCAAGCTTAGGGGAACTGGAATCCCACACTGTATGCTGTCCTT 1252
TCCACACAGGCCTCTTGCTCCTAACGGCCTCAGCCCTGGGCTTTTCCTACTACTGATAACACTATGTAAA 1322
TGTTCCATGTTCCTGTACACTGTCTCTTTTGGGAACATCTCTGAATGGAGCATGGCTGTTTTCATTCACT 1392
GCTGTATCCCAGATGCCCACAGATGCTCCTGCTGTGTGACAGGTGCTCAATAAATGTATAAGCAGTC**AAA** 1462
**AAAAAAAAAAAAAAAAAAAAAAAGCGGCCGCTGAATTC**CAAATTATCACATTTGCTCTTTGAGCAAAGTT 1532
GCTCTGTGTGGACAAGAAGACGGTCATGTACATGCCAGGAGAGCCCCAGACTGTACAACTGTGACAACAA 1602
AATAAAACGTTTTCTGTATG**AAAAAAAAAAAAAAAAAAAAAAAAAGCGGCATTCAGCGGCCGCTGAATTCT** 1672
**A**GGACTAAGTCATGTCACCTGGGTCAGGATTTTCTTCAATGCCGTGTAGCAAAACTGTCTTTAGTCTATG 1742
CAAATAGCGAGTCACTGAGTGTGACAGAATGCAACTTCACTGTTAAACTTCACCTGAGGGTTCCTCATTC 1812
TCCTGGAATCCAGACTGCAAGATTATAAAGGAAAAGACCTAAGGCAATTCAGTTCTTTTTGCAAATCAAT 1882
TGAATCCACGAGAGATGTCTACCAGCGAGATGTCTACCAGCCCAGCCGCCTGCAGCCTGCTGTGTGTGCT 1952
TATTTGTGCGCTGAATAAAATGGGGCAGCTAAATTCTCCAGTTCCATATGCCTCCGAAGTTCAAAGAAAA 2022
AAAGCAAAGTAACATGTTAGACTTGACTTGTGTGGCGGCGTAAAGAAATGGCATCTTCCCACTAAGAACG 2092
AACCATCCAGTTCTTTTGTCAGTCACACTATGAAACAGGGAAGGTGAAGGGAAGAAATGGTTATGTGTGC 2162
ACGAATCGCTTTGCATGGTCTCATGAGATGGCTGCATTCGAACTGTTTTAAGAATTGTAAGGATCTTGAC 2232
TTTTTTACATTTGGAAACATCAAATAAAAACAAACATAATCTGAAAAAAAAAAAAAAAAAAAAAAAAAA 2302
AAAAA                                                                   2307
```

Figure 22 The nucleotide and deduced amino acid sequence (single letter code) of 5'5 and 3'5. The positions of nucleotides and amino acids are on the right. The DNA sequence for LRG2 is shaded, whereas that for the 3'5 product is boxed. Contaminating cloning vector sequences (Clontech Marathon cDNA synthesis primer) are in bold.

(5'2) and colony 7 (5'7) were amplified and radiolabeled for northern blot analysis and compared with LRG2. The result from this blot verified that 5'2 , 5'7 and LRG2 hybridized to a transcript of the same size (Fig. 23C), suggesting that 5'2 and 5'7 might be the full length cDNA clones of LRG2. DNA sequencing of 5'2 revealed a 2147nt sequence containing a partial sequence of LRG2 at its 3' end. Similarly, DNA sequencing of 5'7 identified a cDNA fragment that was 23 bp shorter at the 5' end than 5'2, but had identical nucleotide sequence as 5'2 thereafter (Fig. 24). Sequence analysis of 5'2 identified a stretch of open reading frame of 216 bp encoding for 72 amino acids with no detectable initiation codon, suggesting that additional sequence information upstream of 5'2 is missing (Fig. 24).

3.4.5 Isolation and Analysis of a Putative Full Length Clone for LRG2 by Rapid Screen cDNA Library

With the RACE procedures, partial sequence information had been obtained on the cDNA encoding LRG2. Subsequently, screening of adult rat brain cDNA library by PCR was performed to isolate the full-length clone. This was done using a sense primer that is specific to the vector (pCMV-XL4) [VSP: 5'- GCA GAG CTC GTT TAG TGA ACC-3'] and an antisense primer [L2SP: 5'-CCA TTT CTT TAC GCC GCC ACA CAA GTC-3'] that is specific to the LRG2 cDNA fragment. Five positive clones with insert ~3.0 kb were isolated. Sequence analysis of one of the cloned cDNA (clone C10) revealed a 537-bp open reading frame (ORF) with an ATG start codon at nucleotide position 111 and a TAG termination codon at nucleotide position 648 (Fig. 25). The initiation of translation as indicated is presumed because this is the first methionine codon that is encountered in the coding sequence and it resides within the Kozak consensus sequence (Kozak, 1996). Examination of the 5'-untranslated region (UTR) showed it to be highly G + C rich (80%) with seventeen CpG doublets, and does not contain an in-frame stop codon upstream of the start codon. The 3'-UTR contained three polyadenylation signals

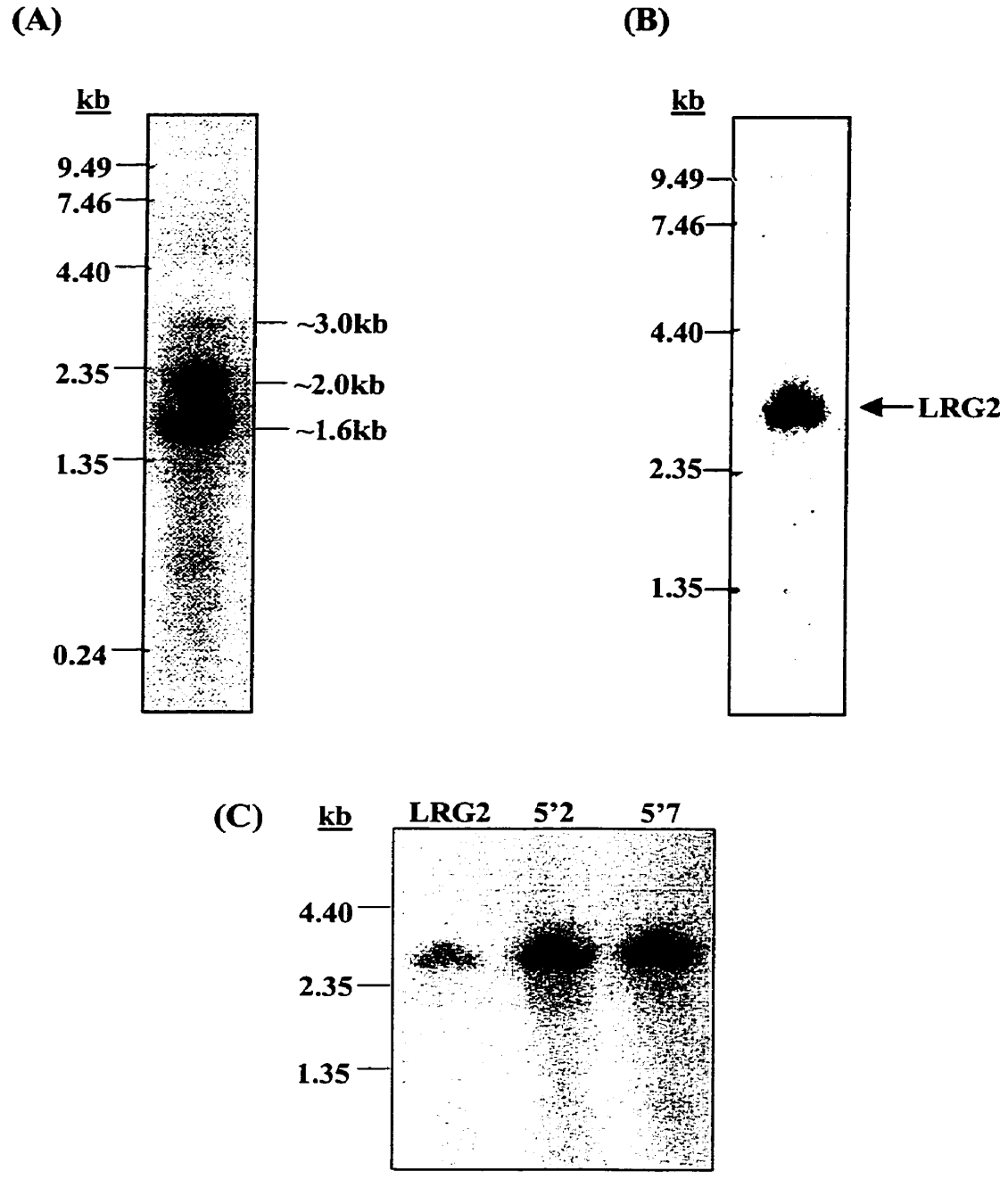

Figure 23 Northern blot analysis of rat total RNA with different cDNA probes. Total RNA (10μg) from rat frontal cortex was used for northern blot analysis as described in Methods. The membrane was probed with radiolabeled fragment of (A) 5'5EB; (B) LRG2; and (C) LRG2, 5'2 or 5'7

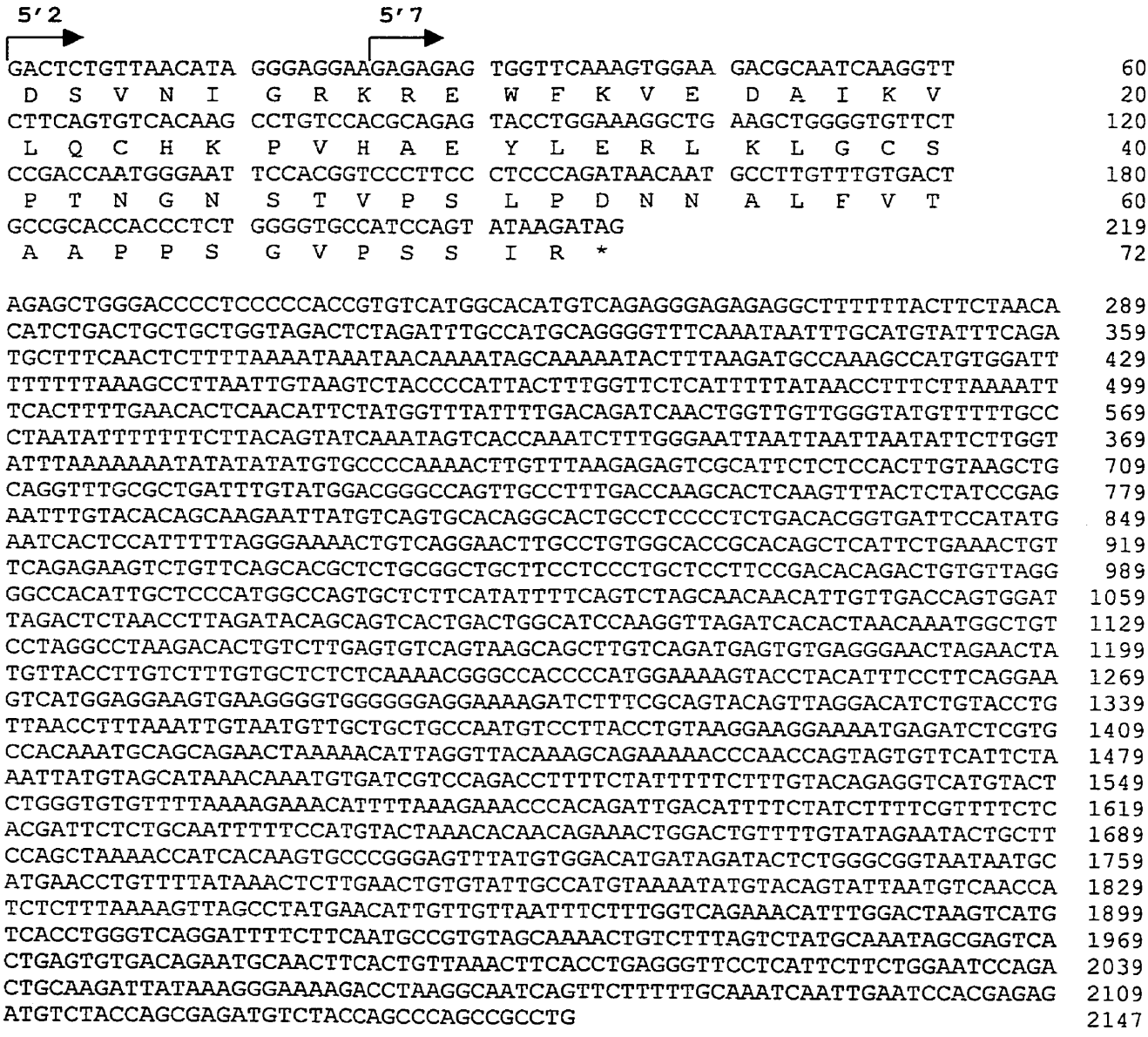

Figure 24 The nucleotide and deduced amino acid sequence (single letter code) of 5'2 and 5'7. 5'2 contains an extra 23 bp as shown with corresponding arrows. The positions of nucleotides and amino acids are on the right. DNA sequence for LRG2 is shaded.

```
                -110 CCGGCTCCGGAGCACCGCGACTTCGTTCGGTCCGACTCCCCGGCG
GGGTGGCGGCGGCCGGGTCCCCACGGTGGCGGCCGGAGCAGCGGCTGCAGGAGCCCGGCTCTAGG      -1

ATGAAGTTCAAGCCC AACCAGACGCGGACA TACGACCGCGAGGGC TTCAAGAAGCGGGCG        60
 M  K  F  K  P  (N) Q  T  R  T   Y  D  R  E  G   F  K  K  R  A         20
GCCTGCCTGTGCTTC CGCAGCGAGCAGGAG GACGAGGTGCTGTTG GTGAGCAGCAGTCGG       120
 A  C  L  C  F   R  S  E  Q  E   D  E  V  L  L   V  S  S [S] R         40
TACCCAGACCAATGG ATCGTGCCGGGAGGA GGGGTGGAGCCGGAG GAGGAGCCTGGCGGT       180
 Y  P  D  Q  W   I  V  P  G  G   G  V  E  P  E   E  E  P  G  G         60
GCTGCTGCAAGGGAA GTGTATGAAGAGGCT GGAGTCAAAGGAAAA TTAGGCAGACTCCTG       240
 A  A  A  R  E   V  Y  E  E  A   G  V  K  G  K   L  G  R  L  L         80
GGAATATTTGAGAAT CAAGACCGGAAGCAC AGAACATATGTTTAT GTTTTGACAGTCACT       300
 G  I  F  E  N   Q  D  R  K  H   R  T  Y  V  Y   V  L  T  V  T        100
GAAATATTGGAAGAC TGGGAAGACTCTGTT AACATAGGGAGGAAG AGAGAGTGGTTCAAA       360
 E  I  L  E  D   W  E  D  S  V   N  I  G  R  K   R  E  W  F  K        120
GTGGAAGACGCAATC AAGGTTCTTCAGTGT CACAAGCCTGTCCAC GCAGAGTACCTGGAA       420
 V  E  D  A  I   K  V  L  Q  C   H  K  P  V  H   A  E  Y  L  E        140
AGGCTGAAGCTGGGG TGTTCTCCGACCAAC GGGAATTCCACGGTC CCTTCCCTCCCAGAT       480
 R  L  K  L  G   C  S  P  T  N   G  (N) S  T  V   P  S  L  P  D        160
AACAATGCCTTGTTT GTGACTGCCGCACCA CCCTCTGGGGTGCCA TCCAGTATAAGATAG       540
 N  N  A  L  F   V  T  A  A  P   P  S  G  V  P   S [S] I  R  *         179

AGAGCTGGGGCCCCTCCCCCACCGTGTCATGGCACATGTCAGAGGGAGAGAGGCTTTTTTACTTC      605
TAACACATCTGACTGCTGCTGGCAGACTCTAGATTTGCCATGCAGGGGTTTCAAATAATTTGCAT      670
GTATTTCAGATGCTTTCAACTCTTTTAAAATAAATAACAAAATAGCAAAAATACTTTAAGATGCC      735
AAAGCCATGTGGATTTTTTTTAAAGCCTTAATTGTAAGTCTACCCCATTACTTTGGTTCTCATTT      800
TTATAACCTTTCTTAAAATTTCACTTTTGAACACTCAACATTCTATGGTTTATTTTGACAGATCA      865
ACTGGTTGTTGGGTATGTTTTTGCCCTAATATTTTTTTCTTACAGTATCAAATAGTCACCAAATC      930
TTTGGGAATTAATGAAAAATATTCTTGGTATTTAAAAAAATATATATGTGCCCCAAAACTTGTTT      995
AAGAGAGTCGCATTCTCTCCACTTGTAAGCTGCAGGTTTGCGCTGATTTGTATGGACGGGCCAGT     1060
TGCCTTTGACCAAGCACTCAAGTTTACTCTATCCGAGAATTTGTACACAGCAAGAATTATGTCAG     1125
TGCACAGGCACTGCCTCCCCTCTGACACGGTGATTCCATATGAATCACTCCATTTTTAGGGAAAA     1190
CTGTCAGGAACTTGCCTGTGGCACCGCACAGCTCATTCTGAAACTGTTCAGAGAAGTCTGTTCAG     1255
CACGCTCTGCGGCTGCTTCCTCCCTGCTCCTTCCGACACAGACTGTGTTAGGGGCCACATTGCTC     1320
CCATGGCCAGTGCTCTTCATATTTTCAGTCTAGCAACAACACTGTTGACCAGTGGATTAGACTCT     1385
AACCTTAGATACAGCAGTCACTGACTGGCATCCAAGGTTAGATCACACTAAACAAAATGGCTGTC     1450
CTAGGCCTAAGACACTGTCTTGAGTGTCAGTAAGCAGCTTGTCAGATGAGTGTGAGGGAACTAGA     1515
ACTATGTTACCTTGTCTTTGTGCTCTCTCAAAACGGGCCACCCCATGGAAAAGTACCTACATTTC     1580
CTTCAGGAAGTCATGGAGGAAGTGAAGGGGGAGGGGGGAGAGGAAAAGATCTTTCGCAGTACAGT     1645
TAGGACATCTGTAACCTGTTAACCTTTAAATTGTAATGTTGCTGCTGCCAATGTCCTTACCTGTA     1710
AGGAAGGAAAATGAGATCTCGTGCCCAAATGCAGCAGAACTAAAAACATTAGGTTACAAAGCAGA     1775
AAAACCCAACCAGTAGTGTTCATTCTAAATTATGTAGCGTAAACAAATGTGATCGTCCAGACCTT     1840
TTCTATTTTTCTTTGTACAGAGGTCATGTACTCTGGGTGTGTTTTAAAAGAAACATTTTAAAGAA     1905
ACCCACAGATTGACATTTTCTATCTTTTCGTTTTCTCACGATTCTCTGCAATTTTTCCATGTACT     1970
AAACACAACAGAAACTGGACTGTTTTGTATAGAATACTGCTTCCAGCTAAAACCATCACAAGTGC     2035
CCGGGAGTTTATGTGGACATGATAGATACTCTGGGCGGTAATAATGCATGAACCTGTTTTATAAA     2100
CTCTTGAACTGTGTATTGCCATGTAAAATATGTACAGTATTAATGTCAACCATCTCTTTAAAAGT     2165
TAGCCTATGAACATTGTTGTATAATTTCTTTGGTCAGAAACATTTGGACTAAGTCATGTCACCTG     2230
GGTCAGGATTTTCTTCAATGCCGTGTAGCAAAACTGTCTTTAGTCTATGCAAATAGCGAGTCACT     2295
GAGTGTGACAGAATGCAACTTCACTGTTAAACTTCACCTGAGGGTTCCTCATTCTGGAATCCAGA     2360
CTGCAAGATTATAAAGGAAAAGACCTAAGGCAATTCAAGTTCTTTTTGCAAATCAATTGAATCCA     2425
CGAGAGATGTCTACCAGCGAGATGTTTACCAGCCCCAGCCGCCTGCAGCCTGCTGTGTGTGCTTA     2490
TTTGTGCGCTGAATAAAATGGGGCAGCTAAATTCTCCAGTTCCATATGCCTCCGAAGTTCAAAGA     2555
AAAGAAAGCAAAGTAACATGTTAGACTTGACTTGTGTGGCGGCGTAAAGAAATGGCATCTTCCCA     2620
CTAAGAACGAACCATCCAGTTCTTTTGTCAGTCACACTATGAAACAGGGAAGGTGAAGGGAAGAA     2685
ATGGTTATGTGTGCACGAATCGCTTTGCATGGTCTCATGAGATGGCTGCATTCGAACTGTTTTAA     2750
GAATTGTAAGGATCTTGACTTTTTTACATTTGGAAACATCAAATAAAAACAAACATAATCTGTGA     2815
AAAAAAAAAAAAAAAAAAAAAAAAA                                             2840
```

Figure 25 The nucleotide and deduced amino acid sequence (single letter code) of the cDNA encoding rat DIPP2. Numbers indicate the positions of nucleotides or amino acid residues are relative to the ATG initiation codon. Deduced amino acid sequence is shown below the nucleotide sequence. The predicted termination codon is indicated by an asterisk. Potential phosphorylation sites for protein kinase C are boxed, whereas the consensus N-linked glycosylation sites are circled. The MutT/Nudix motif is underlined. Three potential polyadenylation signals are double underlined. The nucleotide sequence of the LRG2 cDNA fragment is noted in shading. This sequence has been deposited in the GenBank database (AF253473).

(AATAAA), a poly(A) tail, and a nucleotide sequence motif, ATTTA, which is important for the rapid mRNA degradation in immediate early genes. The complete ORF encodes a predicted protein of 179 amino acids in length with a deduced molecular mass of 20.1 kDa and a theoretical isoelectric point of 5.99. Analysis of the amino acid sequence of clone C10 predicts a predominantly soluble protein with two putative sites for N-linked glycosylation, and two consensus sites for phosphorylation by protein kinase C. Of particular interest, a single MutT/Nudix motif signature sequence ($Gx_5Ex_7REUxEExGU$, where U is one of the bulky hydrophobic amino acids I, L, or V (Bessman et al, 1996)) was identified.

Comparison of the cDNA and predicted amino acid sequences with those in the GenBank database demonstrated striking sequence similarity between clone C10 and members of the Nudix hydrolase family such as human diphosphoinositol polyphosphate phosphohydrolase 1 (hDIPP1) (Safrany et al, 1998), hDIPP2 (Caffrey et al, 2000), and the human unnamed protein (hUnP; Accession #AK001490). Figure 26A shows the comparison of the deduced amino acid sequence of C10 with those of other members of Nudix hydrolase family, which have recently been designated as NUDT (Nudix-type motif) on the website of the Human Gene Nomenclature Committee. The C10 sequence showed the greatest amino acid identity with the hDIPP2 (95%) and the hUnP (88%), and lower homology with the hDIPP1 (78%). Within the MutT motif, the amino acid sequence identity between C10, and hDIPP2 or hUnP was 91%, in contrast to the 78% identify between C10 and hDIPP1 or rDIPP1 (Fig. 26B). The MutT relationship between C10 and the other cloned NUDT members exhibited the following order of identity: NUDT6, 56%; NUDT2, 52%; NUDT5, 43%; and NUDT1, 39%. Based on these homologies, the protein encoded by clone C10 appears to be best classified as a species homolog of the human DIPP2 (designated as rDIPP2 or rNudT4).

The expression of rDIPP2 mRNA in rat tissues was examined by northern blot analysis using the radiolabeled C10 cDNA as a probe (Fig. 27). Corresponding to the size of cloned

A

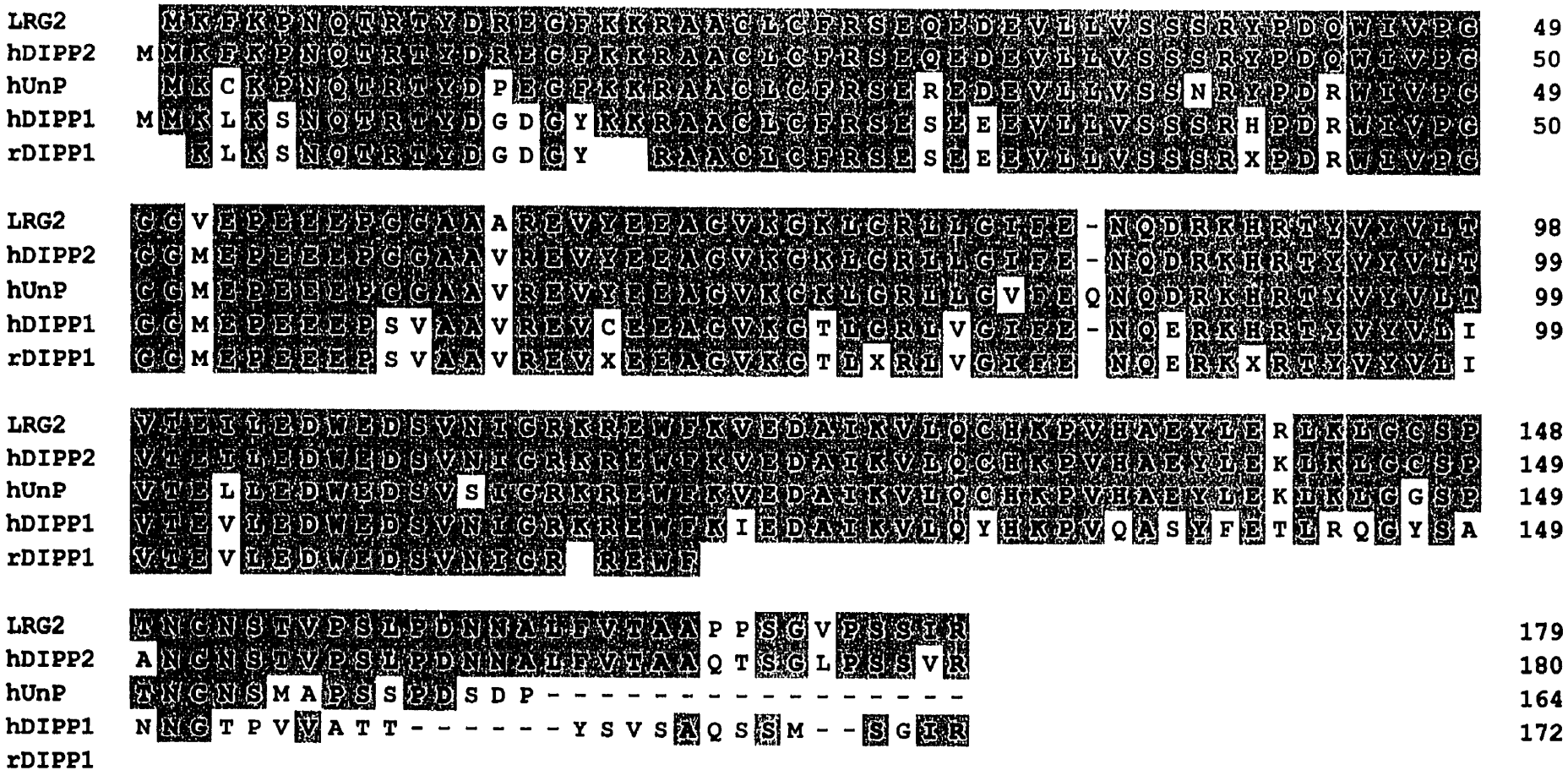

Figure 26 (A) Alignment of deduced amino acid sequences of the rat DIPP2 with other members of the DIPP family. The represented sequences are: human DIPP2 (Caffrey et al., 2000); human unnamed protein (GenBank accession #AK001490); human DIPP1 (Safrany et al., 1998); and the partial sequence of rat DIPP1 obtained from Lys-C and CNBr digests with 'X' indicates amino acid residues that could not be identified independently (Safrany et al., 1998).

B

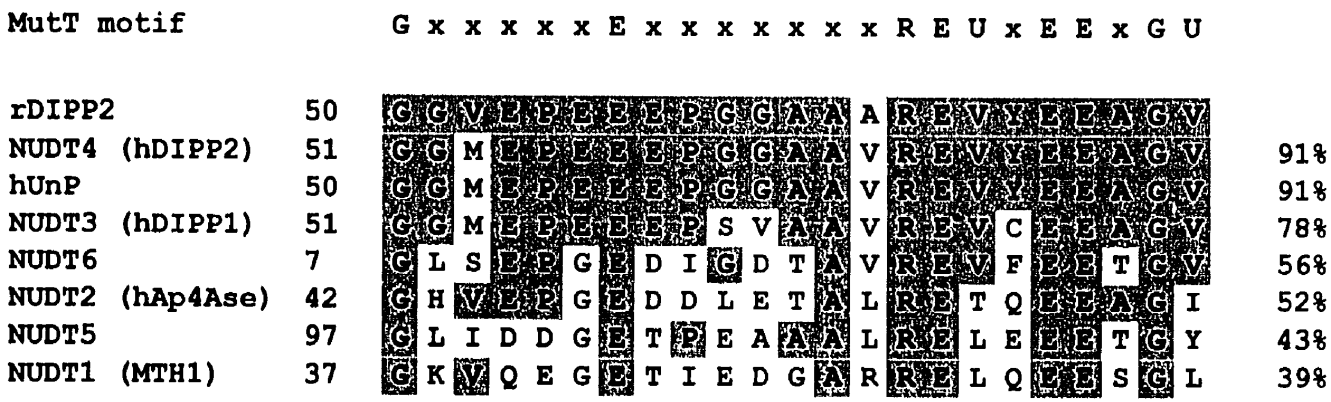

Figure 26 (B) Comparison of the MutT/Nudix domain ($Gx_5Ex_7REUxEExGU$, where 'x' represent any amino acid, and 'U' indicates either of the bulky aliphatic amino acids, I, L, or V) of the rat DIPP2 with corresponding regions from members of the human NUDT family. The represented sequences are from: NUDT1 (human MutT homologue gene MTH1; Accession #NM_002452); NUDT2 (human diadenosine tetraphosphate pyrophosphohydrolase, Accession #NM_001161); NUDT3 (human DIPP1, Safrany et al., 1998); NUDT4 (hDIPP2, Caffrey et al., 2000); NUDT5 (human ADP-sugar pyrophosphatase, Acession #AF218818); NUDT6 (human basic fibroblast growth factor antisense mRNA, Accession #L31408); and the human unnamed protein (GenBank accession #AK001490). Identical residues between rat DIPP2 and the other members are shaded. Single-letter amino acid code is used.

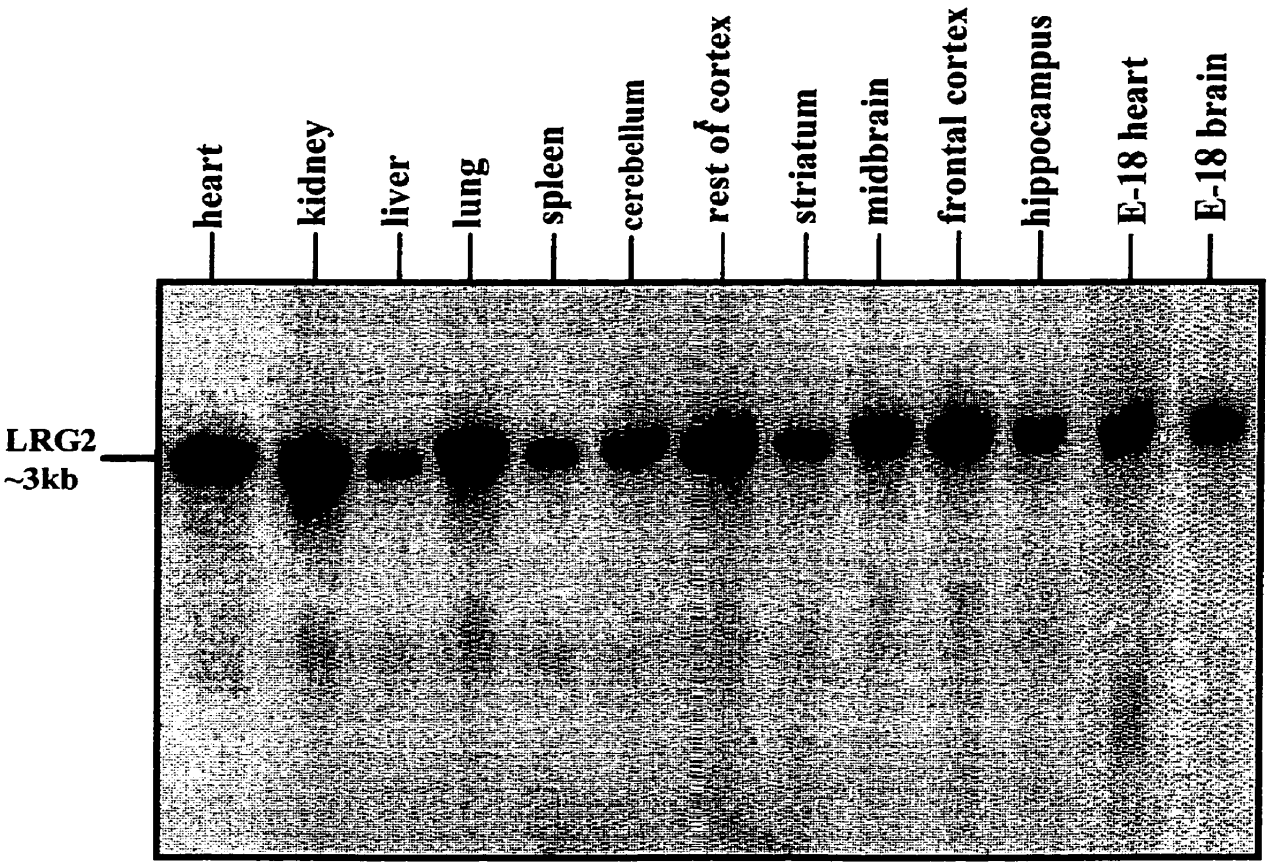

Figure 27 Tissue distribution of rDIPP2 mRNA in the rat. Northern blot containing 10 μg of total RNA from different adult rat tissues and the brain and heart of embryonic day (E) 18 rat, was hybridized with a [^{32}P]dCTP labeled rDIPP2 cDNA. A single mRNA species of ~3 kb is present from all tissues.

cDNA, a single transcript of ~3 kb was observed in a number of brain regions, with high abundance in the cerebral cortex and midbrain, and lower amounts found in the striatum, hippocampus, and cerebellum. In peripheral tissues, the strongest signal was observed in the kidney and lung, followed by heart, liver, and spleen. The rDIPP2 mRNA signal was also detectable in the heart and brain at embryonic day 18, with relatively higher amount in the heart (Fig. 27).

3.4.6 Functional Characterization of Rat DIPP2

To investigate whether the rat DIPP2 is functionally active in dephosphorylating diphosphoinositol polyphosphates, recombinant rDIPP2 protein was prepared by subcloning the full length cDNA of rat DIPP2 into the expression vector pET (called pET15b-DIPP2), transformed into the *E. coli* expression host BL21(DE3), and purified to apparent homogeneity (Fig. 28A, lane 5). The apparent molecular mass on the SDS-PAGE of purified recombinant rDIPP2 was around 25 kDa. Western blotting with an anti-mouse DIPP2 antibody also identified a protein of approximately 25 kDa (Fig. 28B, lane 5).

The phosphohydrolase activity of the purified recombinant hexahistidine-tagged rDIPP2 was analyzed using PP-[^{3}H]InsP$_5$ as substrate, followed by anion exchange high performance liquid chromatographic analysis of the dephosphorylated products (Safrany et al, 1998). Figure 29 shows that PP-[^{3}H]InsP$_5$ is dephosphorylated by rat DIPP2 to [^{3}H]InsP$_6$ by removal of the β-phosphate of the diphosphate group. Under the experimental conditions, there was no accumulation of either [^{3}H]InsP$_5$ or [^{3}H]PP-InsP$_4$.

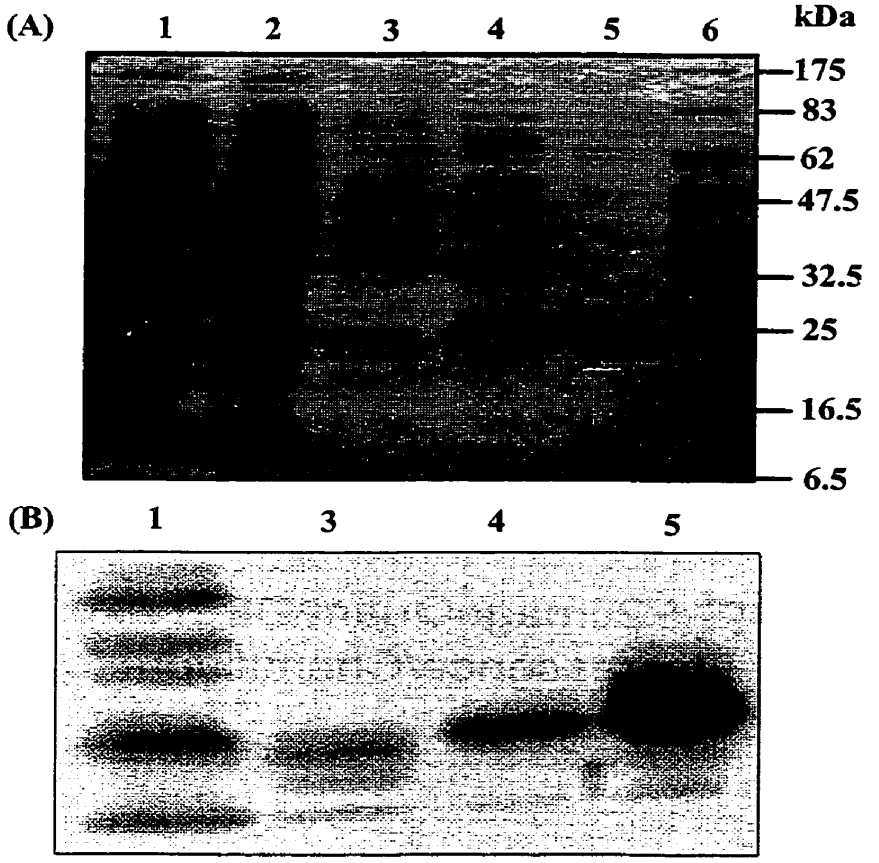

Figure 28 SDS-PAGE analysis of recombinant rDIPP2 protein. Expressed rDIPP2 was purified using a nickel column and eluted with increasing concentrations of imidazole as described in "Materials and Methods". The different flow through fractions were collected and analyzed (A) on a coomassie blue-stained 10% acrylamide gel or (B) by western blotting with anti-mDIPP2 antibody. Lane 1, soluble lysate; lane 2, flow through; lane 3, binding flow through; lane 4, washing flow through; lane 5, elutant, lane 6, molecular weight markers.

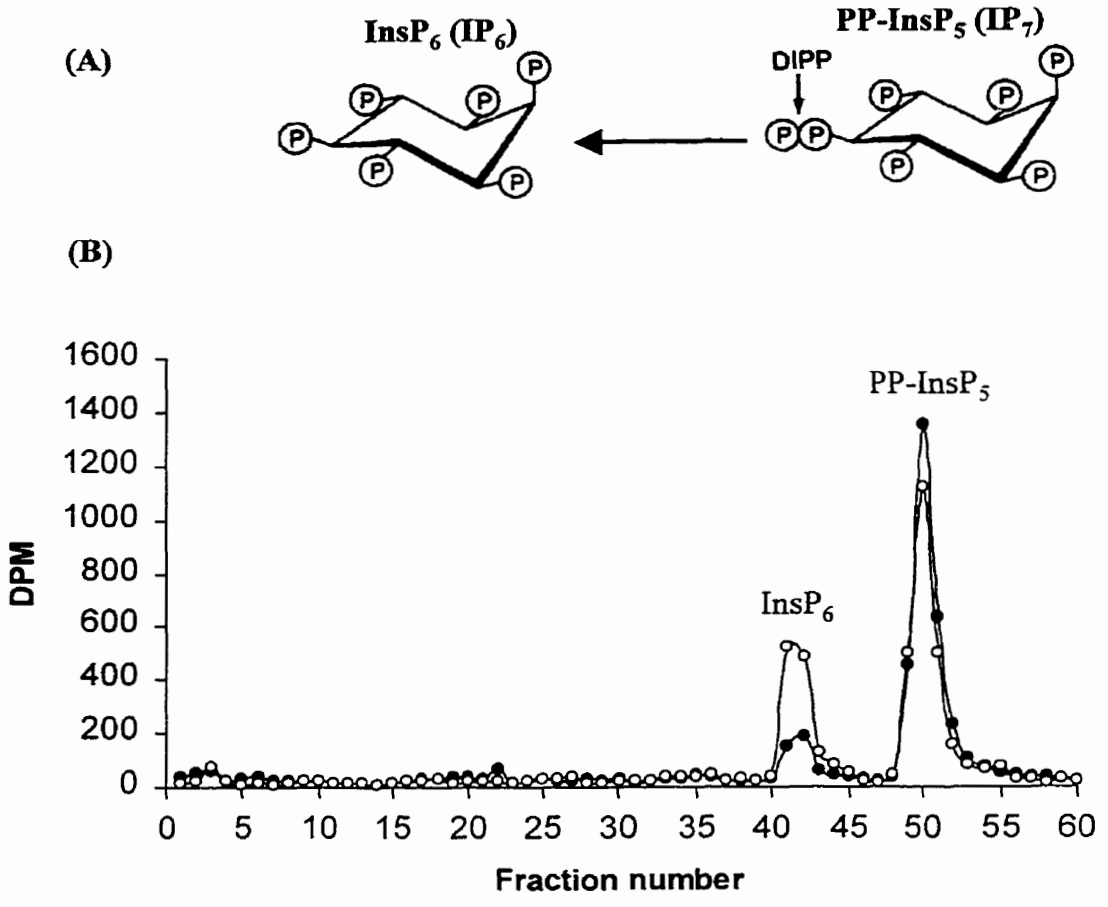

Figure 29 HPLC analysis of rat DIPP2-mediated hydrolysis of PP-$InsP_5$. (A) structure of PP-$InsP_5$ and $InsP_6$. (B) DIPP activity towards [^{3}H]PP-InsP5 was assayed and analyzed by HPLC as described in Materials and Methods. The HPLC profile from a zero time control (closed circle) and 2 hours incubation (open circle) are shown.

3.5 CHARACTERIZATION OF LRG3

3.5.1 Effects of Lithium and Other Psychotropic Drugs Treatment on LRG3 mRNA

As has been previously presented, 5'5 was an unexpected cloning artifact isolated as one of the 5' RACE products that encoded a full length cDNA for a protein of 253 amino acids. Out of curiosity, northern blot analysis was performed to test whether chronic lithium treatment also regulates the expression of these transcripts. Because the signals from the 3.0 kb species was weak and variable, only the more abundant transcripts, 2.0 and 1.6 kb bands, were quantitatively analyzed (Fig. 30A). The levels of the 1.6 kb mRNA transcript was significantly decreased (38%) in the frontal cortex ($t = 2.66$, $df = 14$, $p = 0.02$) following chronic lithium treatment. In contrast, short-term (1-week) lithium treatment did not significantly affect ($t = 1.01$, $df = 10$, $p = 0.33$) the amount of this species (Fig 30B). In comparison, the levels of the 2.0 kb mRNA transcript were not significantly affected by chronic lithium treatment ($t = 0.51$, $df = 14$, $p = 0.87$). Accordingly, the 1.6 kb transcript was designated as LRG3 and further characterization of this transcript was conducted.

To assess the pharmacospecificity of lithium's effect, the effects of chronic lithium, VPA and CBZ treatment on the levels of LRG3 mRNA were investigated (Fig. 31A). The ANOVA showed a significant main effect of drug on LRG3 mRNA levels ($F(3,23) = 5.89$, $p = 0.004$). Post hoc (Tukey HSD) analysis revealed that the levels of LRG3 mRNA were significantly reduced in rat frontal cortex following lithium (-51%, $p = 0.008$), VPA (-45%, $p = 0.017$), or CBZ (-52%, $p = 0.008$) treatment. In contrast, chronic administration of imipramine or haloperidol did not affect the levels of LRG3 mRNA in the frontal cortex ($F(2,18) = 0.413$, $p = 0.67$) (Fig. 31B).

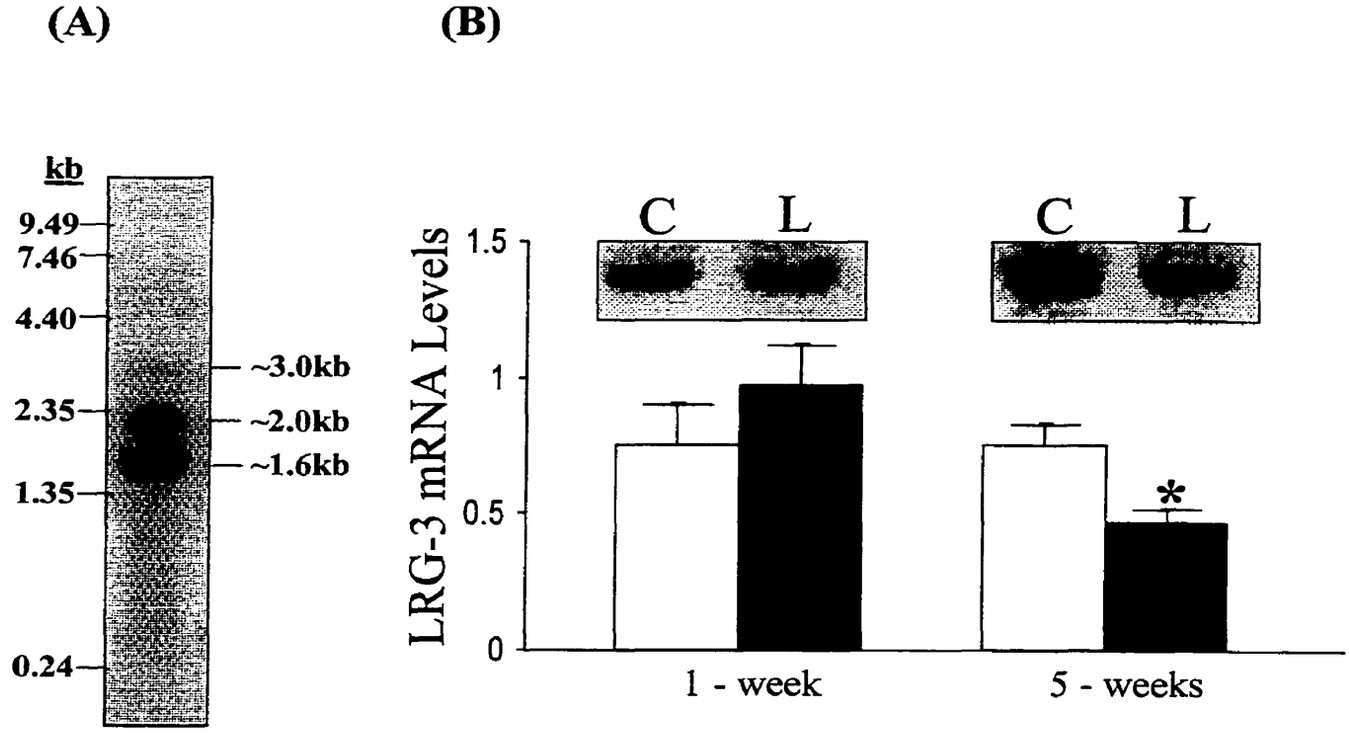

Figure 30 Northern blot analysis of LRG3 mRNA in the rat frontal cortex. (A) Northern blot of 10μg total RNA from rat frontal cortex probed with radiolabeled 5'5EB fragment identifies three species: 1.6 kb, 2.0 kb, and 3.0 kb. (B) Effect of short-term versus long-term lithium administration on the expression of LRG3 mRNA in rat frontal cortex. Representative mRNA message for control (C) and lithium (L) are shown in the top panels. The signal of the 1.6 kb species was normalized to cyclophillin mRNA. The results are expressed as the mean ± SEM for 6-9 animals. * $P < 0.05$ compared with control animals (Student's t-test).

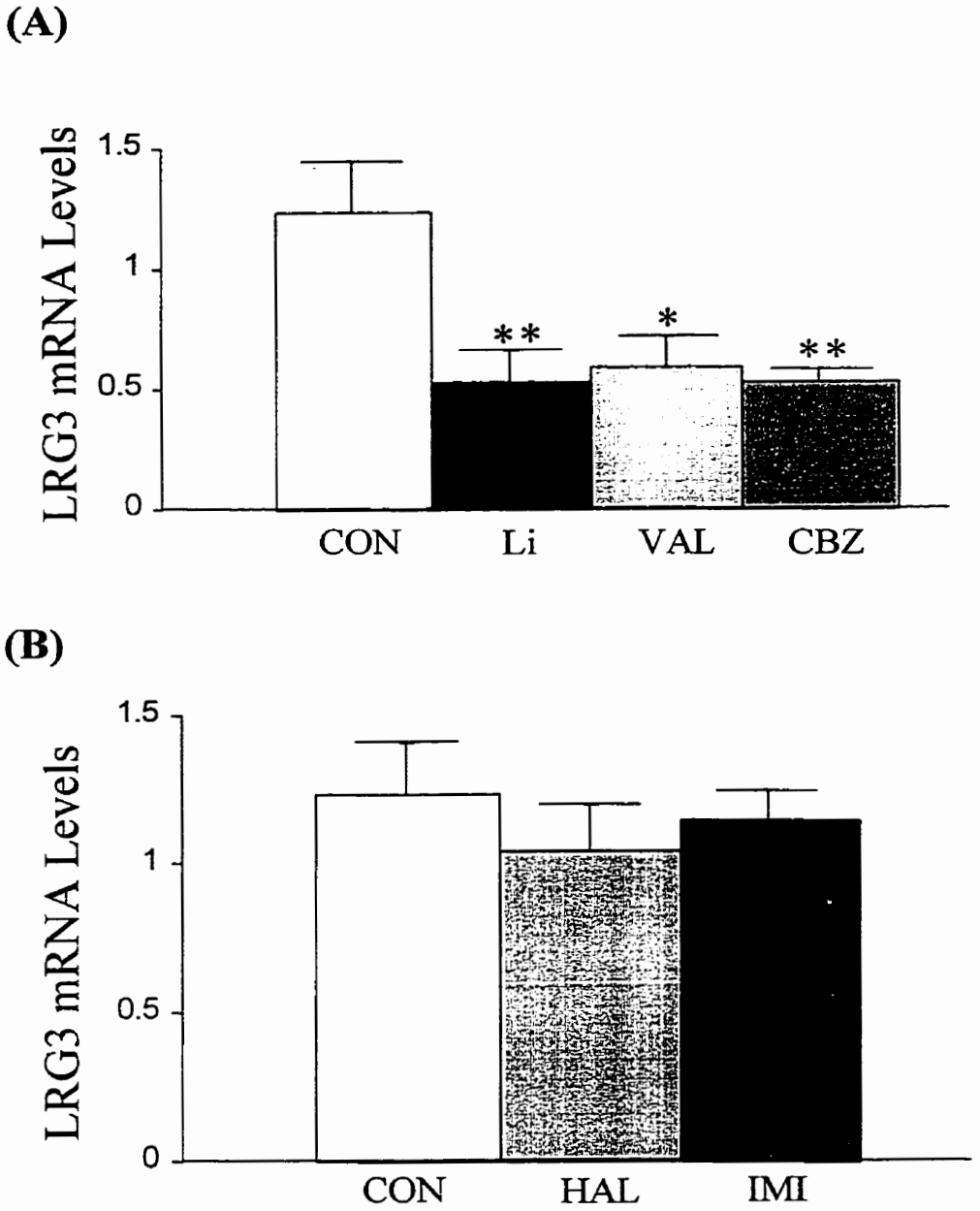

Figure 31 (A) Effect of chronic lithium (Li), valproate (VPA) and carbamazepine (CBZ) on the expression of LRG3 mRNA in the rat frontal cortex. Rats were fed with regular chow (CON), or chow containing Li, VPA or CBZ for 5 weeks. (B) Effect of chronic haloperidol (HAL) and imipramine (IMI) on the expression of LRG3 mRNA in the rat frontal cortex. Rats received intraperitoneal injection of HAL or IMI for 5 weeks. Levels of LRG3 mRNA were determined by northern blot analysis of the 1.6 kb mRNA signals in the frontal cortex normalized against cyclophillin signals. The results are expressed as the mean ± SEM for 6-7 animals. * $P < 0.05$ and ** $P < 0.01$ compared with control animals (Tukey HSD).

3.5.2 Isolation and Primary Sequence Analysis of Full Length Clone for LRG3

In addition to the RACE technique, an arrayed rat brain cDNA library was screened by PCR using a sense primer specific to the vector (pCMV-XL4) and an antisense primer specific to the 5'5 cDNA fragment as another independent method to isolate the full length cDNA encoding LRG3, as well as to identify possible splice variants. Only one positive clone (C1) was isolated, which contained a cDNA insert of ~1.6 kb. Sequence analysis of this clone revealed a 759-bp ORF encoding a protein of 253 amino acids, identical to amino acid sequence of 5'5, with a calculated molecular mass of 28.4 kDa (Fig. 32). The presumed initiation codon lies within the Kozak consensus sequence (Kozak, 1996), 39 base pairs downstream from an in-frame stop codon. Examination of the 5'-UTR showed it to have high G + C content (60%) with eighteen CpG doublets. The 3'-UTR contained a putative polyadenylation signal, AATAAA, which is located 12 bp upstream from the poly A^+ tail. Hydropathy analysis (Kyte and Doolittle, 1982) of the deduced amino acid sequence indicates the presence of four hydrophobic regions, predicted to represent putative transmembrane domains, and both the amino- and carboxyl termini located intracellularly. The predicted amino acid sequence reveals a single transmembrane 4 family (TM4SF) signature sequence (residue 69-91) overlapped with a leucine zipper like motif (residue 89-110). One consensus site for N-linked glycosylation is present in a region that may be a large extracellular loop (EC2). Potential sites for phosphorylation by protein kinase C are present on predicted intracellular domain and carboxyl terminus. There is also one possible site for phosphorylation by protein kinase A on the amino terminus.

Comparison of the cDNA and predicted amino acid sequences with those in the GenBank database demonstrates sequence similarity between clone C1 and members of the TM4SF superfamily (Wright and Tomlinson, 1994), particularly with a 96% amino acid identity to mouse CD151 (Hasegawa et al, 1997), 94% to monkey CD151 (Genbank accession #

```
                                                                -183 GCG
CTCCTACAGTGGCTGCCGGGGCTGGTATTTCAAAGCGTGGTTGATCTGGTGGCTCTGAGA
ATCATTCTGATTACTGCTTCAGACACCATAGGAGTGGCAAGATCCCACCTGCCAGGTGAA
GGGCGGGCAGATCAGTGACTTTGACCTGTACCTGCTGGTCCTGACTTCCAGAGGCCCAGG      -1

   ATGGGGGAATTCAAC GAGAAGAAGGCAACA TGTGGCACAGTCTGC CTCAAGTATCTGCTG   60
 1 M  G  E  F  N   E  K  K  A [T]  C  G  T  V  C   L  K  Y  L  L
   TTCACCTACAACTGC TGCTTCTGGCTGGCT GGCCTGGCCGTCATG GCAGTGGGCATTTGG  120
21 F  T  Y  N  C   C  F  W  L  A   G  L  A  V  M   A  V  G  I  W
   ACACTGGCCCTCAAG AGTGACTACATTAGT CTGCTGGCTTCAAGC ACCTACCTAGCAACA  180
41 T  L  A  L  K   S  D  Y  I  S   L  L  A  S  S   T  Y  L  A  T
   GCCTACATCTTAGTG GTGGCTGGTGTTGTT GTCATGGTGACTGGA GTCCTGGGCTGCTGT  240
61 A  Y  I  L  V   V  A  G  V  V   V  M  V  T  G   V  L  G  C  C
   GCCACTTTCAAGGAG CGACGGAACCTGTTA CGCTTGTACTTCATC CTGCTCCTCATCATC  300
81 A (T) F  K  E   R  R  N  L  L   R  L  Y  F  I   L  L  L  I  I
   TTCCTTCTGGAGATC ATTGCTGGCATCCTG GCCTATGTCTACTAT CAGCAGCTCAACACC  360
101 F  L  L  E  I   I  A  G  I  L   A  Y  V  Y  Y   Q  Q  L  N  T
   GAGCTCAAGGAGAAT CTGAAGGACACCATG ATCAAGAGATACCAC CAGTCAGGCCATGAG  420
121 E  L  K  E  N   L  K  D  T  M   I  K  R  Y  H   Q  S  G  H  E
   GGTGTGACCAACGCT GTAGACAAGCTGCAG CAAGAGTTCCACTGC TGTGGCAGCAACAAC  480
141 G  V  T  N  A   V  D  K  L  Q   Q  E  F  H  C   C  G  S (N) N
   TCTCGAGACTGGAGA GACAGCGAGTGGATC CGCTCTGGTGAGGCA GACAGCCGTGTGGTC  540
161 S  R  D  W  R   D  S  E  W  I   R  S  G  E  A   D  S  R  V  V
   CCTGACAGCTGCTGT AAGACTGTGGTAACT GGCTGTGGTAAGCGG GAGCACGCCTCCAAC  600
181 P  D  S  C  C   K  T  V  V  T   G  C  G  K  R   E  H  A  S  N
   ATCTATAAAGTGGAA GGTGGCTGCATCACT AAGCTGGAGTCCTTC ATTCAGGAGCACCTG  660
201 I  Y  K  V  E   G  G  C  I  T   K  L  E  S  F   I  Q  E  H  L
   CGGGTCATCGGGGCC GTGGGCATTGGCATC GCCTGTGTGCAGGTC TTCGGCATGATCTTC  720
221 R  V  I  G  A   V  G  I  G  I   A  C  V  Q  V   F  G  M  I  F
   ACCTGCTGCTTGTAC AGAAGCCTCAAGCTG GAGCACTACTGA                     762
241 T  C  C  L  Y   R (S) L  K  L   E  H  Y  *

   CCATGGTGCCAGGCCAGAAGGCCAGCCTCGCGCTGTGTCCCAGCCCCCCACTACTGAACA     822
   CTACTGAGTTAGACTGCTGACTGCCAGAGAGGTAGCTGCCCTCATGCCATTACCATGTCC     882
   CCTGGTGGCTCCACCTCCGTGGTAGCTTCAAGTGCCTTTCCCTGCACACCAAAGTCCCAA     942
   AGCTTGAGGCAGCGTACTTGCCCCAGCATTAACAAGAGGGGGCATGTATGCCAGTGTTGG    1022
   GCTAGGCCTGGCTAGTAGAGTAGGGTTTAGATTACTTGACAGAAGTGCCTCTTATGTAGA    1082
   CTGCTTCCCTTTGGGTAGCTGTGATCTGCAGTAAGTCTGGGGAGAATCCGAGCATACCAC    1142
   TACACGGTGGGTAAGAGCAGGTGGGAAGGGGCCTTTCTGGTTGGGCTAGGCACCTTTCAT    1202
   GCCTGCTGCCAGGAACCCTCAGATAGGGCAAGCTTAGGGGAACTGGAATCCCACACTGTA    1262
   TGCTGTCCTTTCCACACAGGCCTCTTGCTCCTAACGGCCTCAGCCCTGGGCTTTTCCTAC    1322
   TACTGATAACACTATGTAAATGTTCCATGTTCCTGTACACTGTCTCTTTTGGGAACATCT    1382
   CTGAATGGAGCATGGCTGTTTTCATTCACTGCTGTATCCCAGATGCCCACAGATGCTCCT    1422
   GCTGTGTGACAGGTGCTCAATAAATGTATAAGCAGTCAAAAAAAAAAAAAAAAAAAAAA    1482
   AAA                                                             1485
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Figure 32 The nucleotide and deduced amino acid sequence (single letter code) of the cDNA encoding rat CD151. The positions of amino acid are numbered on the left, whereas the positions of DNA nucleotide are numbered on the right. Deduced amino acid sequence is shown below the nucleotide sequence. The predicted termination codon is indicated by an asterisk. Potential phosphorylation sites for PKA is boxed and for PKC is in hexagon. N-linked glycosylation site is circled. Signature for transmembrane 4 family is bolded and leucine zipper pattern is double-underlined, whereas poly(A) signal is underlined. This sequence has been deposited in the GenBank database (Accession number AF192547).

AF275665) and 92% to human CD151 (Fitter et al, 1995). Within the TM4SF motif, the amino acid sequence identity between C1 and mouse was 100%; between C1, monkey and human CD151 was 95% (Fig. 33). The 3' and 5' noncoding sequences of rat CD151 were highly similar to the mouse CD151 cDNA, but were different from those of human CD151. On the basis of sequence analysis, it was concluded that this clone is true orthologue of the human/mouse CD151.

The expression of rCD151 mRNA in rat tissues was examined by northern blot analysis using the radiolabeled C1 cDNA as probe (Fig. 34B). The relative distribution of the 1.6 kb species appears more abundant than the other two species in all the tissues examined, except in cerebellum where the 2.0 kb appears to be predominant. The rCD151 mRNA was observed in various brain regions, with high abundance in cerebellum and midbrain, and lower amounts found in the striatum, hippocampus, and cerebral cortex. In the peripheral tissues, the strongest signal was observed in the heart and lung, followed by kidney, liver, and spleen. The rCD151 mRNA signal was also detectable in primary cultures of rat cerebral cortical neurons (Fig. 34B).

In order to test whether the three mRNA transcripts observed with 5'5EB cDNA probe are splice variants of the same gene, southern blot analysis was performed. Southern blot analysis of rat genomic DNA digested with different restriction enzymes identified a 9.0 kb species with Bam HI, whereas a 3.5 kb species was found with EcoR I, Hind III and Pst I digestion (Fig. 34A). These preliminary results suggest that the three observed mRNA species in northern blot might be splice variants of the same gene.

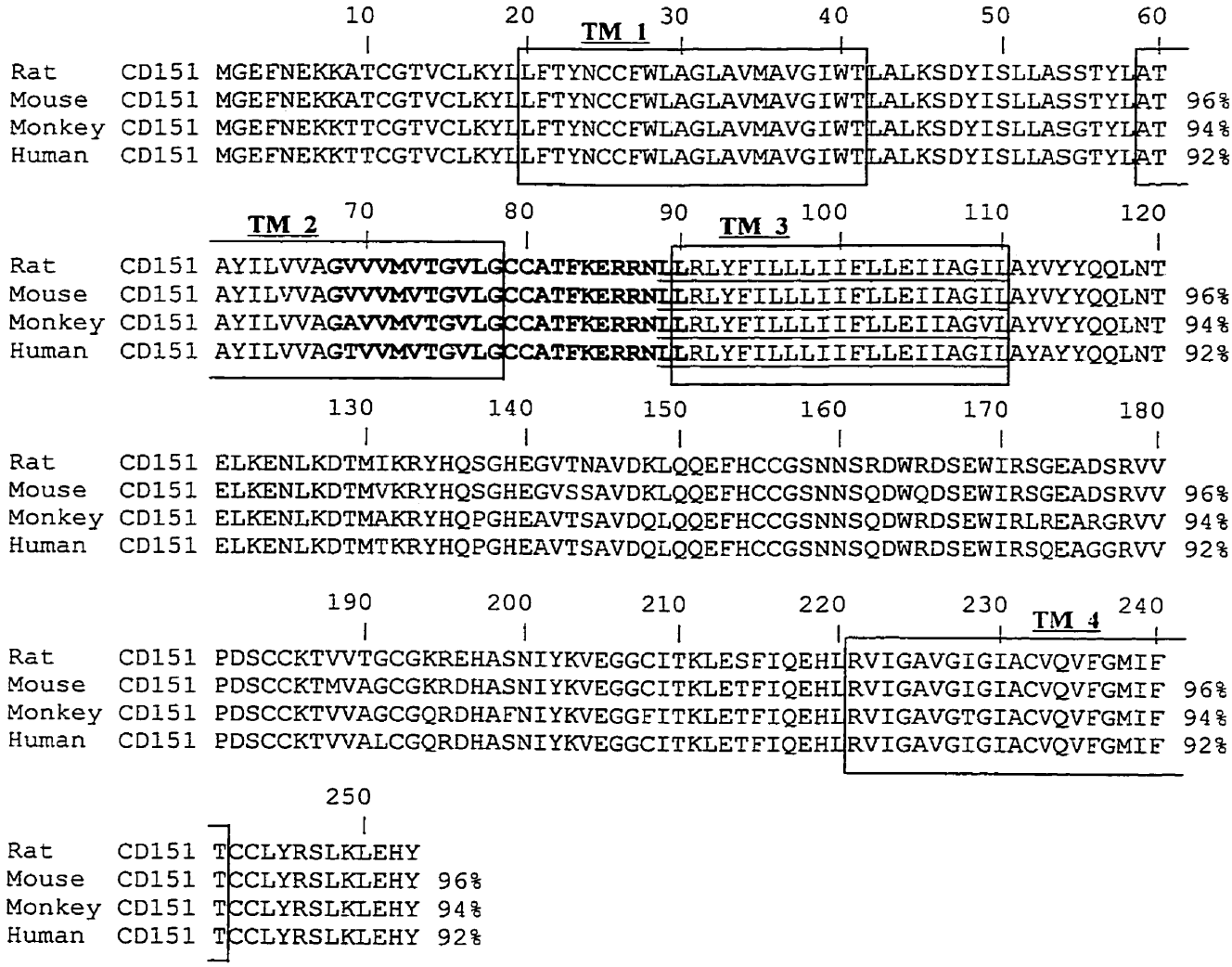

Figure 33 Multiple alignment of deduced amino acid sequence of the rat CD151 with amino acid sequences of human (Accession # NP004348), mouse CD151 (Accession#NP033972), and monkey CD151 (Accession # AF275665). Single-letter amino acid code is used and identical residues between the three sequences are shaded. Putative transmembrane regions are boxed and the leucine zipper pattern is underlined.

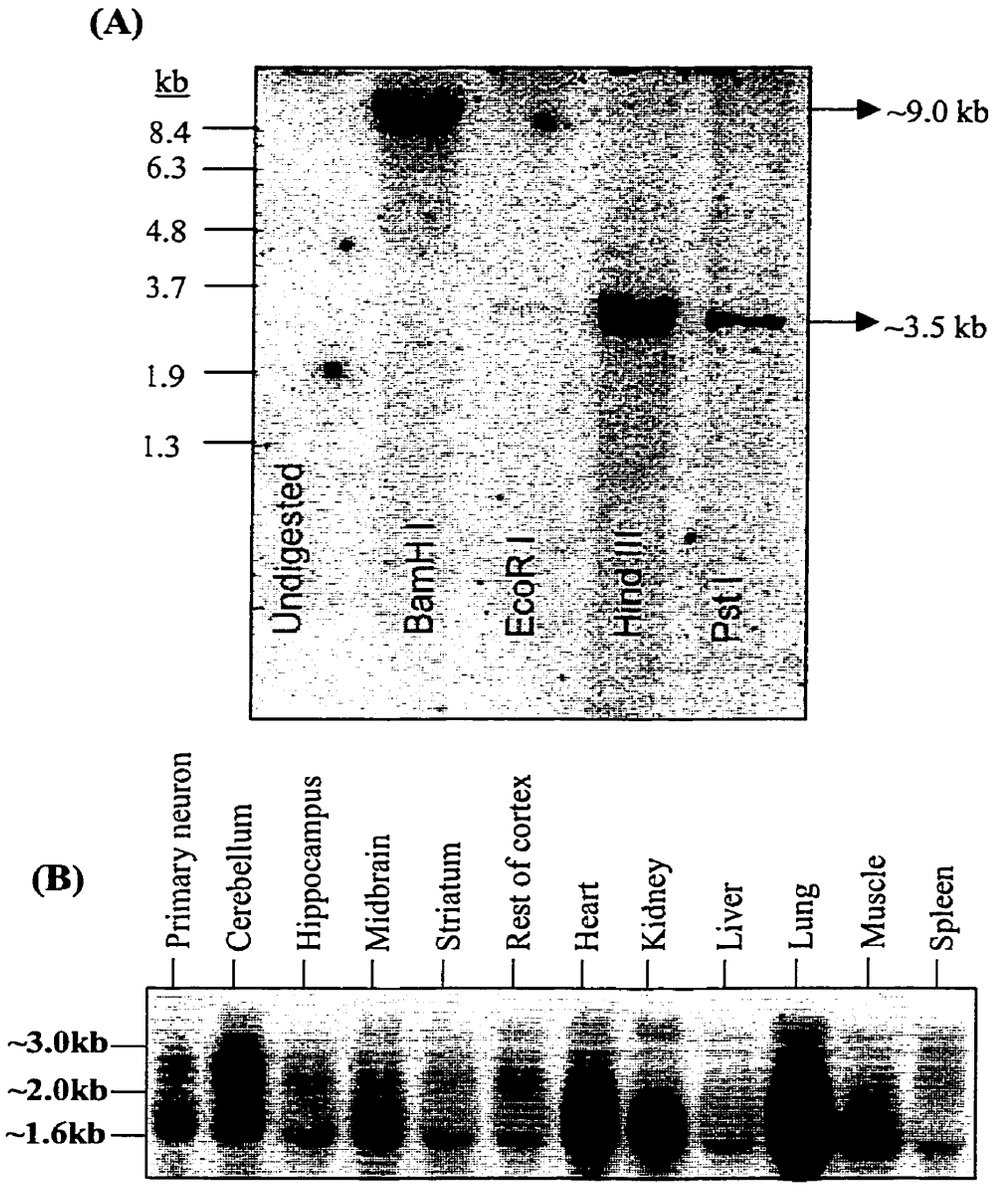

Figure 34 (A) Southern blot analysis of LRG3. 30 μg of rat genomic DNA digested with different restriction enzyme was hybridized with radiolabeled EcoRI/BamHI digest (nt 187-690) of rat (5'5EB). A single band is revealed with Bam HI, Hind III, or Pst I digest. DNA ladder (1kb) was used as size marker. (B) Tissue distribution of CD151 mRNA in the rat. Northern blot of 10 μg total RNA from different rat tissues and brain regions was hybridized with same cDNA probe. Three mRNA species of 1.6 kb, 2.0 kb and 3.0 kb are detected.

CHAPTER 4

DISCUSSION

The clinical response to lithium requires several weeks of administration, suggesting that long-term neuronal adaptations mediated by changes in gene expression may be important to the therapeutic action of lithium treatment. Studies on the alteration of gene expression following chronic administration of lithium to animals or cultured cell lines have received much attention in the last decade. The identification of genes that are modulated by lithium in a therapeutically relevant paradigm is an important step to elucidate the pharmacotherapeutic mechanism of lithium, to develop novel therapeutics for BD, and to better understand the pathophysiology and/or pathogenesis of BD.

In the present study, three candidate lithium-regulated genes were isolated and characterized. The expression of these genes was significantly altered following chronic, but not short-term, administration of lithium. The parallel between the delayed onset of these changes and the onset of clinical efficacy suggests that these genes may represent therapeutically relevant targets for lithium. The identification of these genes and their putative role in the PI signaling pathway further support the notion that modulation of the PI signaling cascade and/or inositol phosphate metabolism may be important in the therapeutic action of lithium.

In the ensuing sections, the results of this project will be discussed in relation to the designs and their potential pharmacotherapeutic significance in BD.

4.1 mRNA DIFFERENTIAL DISPLAY

Currently available data suggest that lithium has the potential to regulate the expression of a large number of genes, and indeed a number of genes have been found to be affected by lithium using the "candidate gene" approach (reviewed in Manji et al, 1995; Jope, 1999b; Li et al, 2000). This approach, however, only permits the examination of a limited number of known genes. Therefore, an experimental approach that allows an integrated view of differentially expressed mRNAs affected by lithium may be crucial to better understand the cellular and

molecular mechanisms by which lithium exerts its therapeutic action. In the 1990s, mRNA differential display was an emerging technique for the rapid screening of candidate genes under the influence of drugs. Wang and Young (1996) were the first to show that chronic exposure to therapeutically relevant concentrations of lithium increased 2'3'-cyclic nucleotide 3'-phosphodiesterase mRNA levels in C6 glioma cells. This was followed by the identification of a transcription factor, β-subunit of the polyomavirus enhancer-binding protein 2, the expression of which was increased by chronically administered lithium or valproate in rat frontal cortex (Manji et al, 1997). A third study by mRNA differential display in rat brain revealed increased expression of a gene that has significant homology with yeast nitrogen permease regulator 2 (Wang et al, 1999). In the present study, two cDNA fragments were identified which were differentially regulated by chronic, but not short-term, lithium treatment. These observations, taken together, support the usefulness of the ddPCR technique to identify transcriptional changes in other genes affected by lithium that would not otherwise be considered with the candidate gene approach.

While the original differential display methodology has significant advantages, some limitations have been recognized with increased use of this technique over the years (Ledakis et al, 1998). The major concerns are the high incidence of false positives, often reaching 50% or more (Debouck, 1995; Wan et al, 1996). In addition the cDNA fragments isolated by this method are typically small, and frequently located in the 3'-untranslated region. Therefore, in order to identify the differentially expressed gene, one may need to screen a cDNA library to isolate the full-length cDNA clone. Moreover, there are indications that the method is biased towards abundant transcripts; the rare mRNA species are mostly under-represented (Bertioli et al, 1995; Graf et al, 1997). Consequently, significant modifications and refinements have been made in recent years to the original method in order to overcome some of the shortcomings inherent in the technique (Liang and Pardee, 1995; Matz and Lukyanov, 1998). Over the years, other gene

expression profiling methods have emerged that include reverse transcription representational difference analysis (RT-RDA) (Hubank and Schatz, 1993), DNA microarray (Schena et al, 1995), and serial analysis of gene expression (SAGE) (Velculescu et al, 1995). While new methods might provide some advantages over mRNA differential display, they are still going through the test of time and have not surpassed the popularity of mRNA differential display. Table 3 summarizes some of the features of the high throughput methods for analysis of differential gene expression.

In this study, contamination from genomic DNA was minimized by treating RNA samples with DNase prior to RT-PCR. Initially, total RNA from two control versus two lithium-treated animals were run in parallel and only bands that appeared differentially on differential display autoradiograms in both lanes of the comparison groups were selected as candidates for further examination to minimize the selection of false positives. Later in the project, total RNA from 5 control versus 5 lithium-treated animals as run in parallel and only bands that showed differences in at least four out of five lanes were selected as candidates. In this project, the differential display was carried out using the 128 pairs of the downstream anchored and upstream arbitrary primers (Appendix I). Thirty-eight putative differentially expressed cDNA fragments were isolated and subcloned; 32 up-regulated and 6 down-regulated by chronic lithium treatment (Appendix II). DNA sequencing and analyses of 32 of the lithium-regulated cDNA fragments detected in the differential display revealed insert sizes ranging from 179 – 630 bp (Appendix III), 12 of which contained the polyadenylation signals that indicate the 3-UTR of the corresponding genes. Nine cDNA fragments contained arbitrary primers at both ends, suggesting that these fragments were amplified only by two arbitrary primers along the 5' end of the transcript and not necessarily restricted to the 3'-UTR. Another observation from the DNA sequences was that 3 of the fragments identified with different combinations of primers showed identical sequence information indicating some redundancy with the method.

Table 3: Methods for high throughput analysis of differential gene expression (Adapted from Carulli et al, 1998)

Feature	**Differential Display**	**cDNA Microarrays**	**RT-RDA[1]**	**SAGE[2]**
Inventors	Liang and Pardee, 1992	Schena et al, 1995	Hubank and Schatz, 1994	Velculescu et al, 1995
Minimum mRNA requirements	10-100 ng	1.0 μg	10-100 ng	1.0-5.0 μg
Throughput	High	High	Medium	High
Sequencing Requirements	Medium	Low	Low	High
Advantages	Simple and sensitive	Multiple assays on the same arrays.	Subtractive cloning	SAGE tag data
Disadvantages	False positives	Large amount of RNA required	Pairwise comparison	Technically difficult
Number of publications (Medline, Dec. 2000)	1598	351	190	58

[1] Reverse transcription representational difference analysis
[2] Serial analysis of gene expression

With large numbers of potential candidates to be confirmed, northern blotting becomes one of the most time consuming aspects of the DD protocol. For this reason, reverse northern blotting was used as an alternative technique (Mou et al, 1994; Zhang et al, 1996) to screen many candidate genes simultaneously using only a small amount of total RNA. However, this technique does not provide information about the size or the multiplicity of the transcripts. Nevertheless, we used this approach to rapidly screen the DD candidates and selected those that were differentially expressed on reverse northern blots for further analysis by northern blotting.

Twenty of the thirty-eight candidates were screened by reverse northern blot. Of the twenty candidates screened by reverse northern blot analyses, ten clones were found to be up-regulated, four clones were down-regulated by chronic lithium treatment, and six clones did not show detectable signals (Appendix II). Fourteen candidate genes that were differentially displayed on reverse northern blots were used as probes for northern blot analyses. Although there were directional trends of up or down regulation, twelve of the fourteen clones analyzed did not show a statistically significant difference between control and lithium-treated animals (Fig. 11C). The expression of two candidate cDNA fragments was significantly altered by chronic lithium treatment (Fig. 11C). Therefore, the rate of false positives was about 88%. High incidence of false positives has been frequently reported and considered as the major limitation of the original DD protocol (Debouck, 1995; Wan et al, 1996). For example, one study reported the identification of one true positive by northern blot analysis out of 30 cDNA candidate fragments selected from DD gel (Chen et al, 1996b). One of the major reasons for the high false positive rate is the presence of multiple cDNA species in the band of interest. We have addressed this potential problem by analyzing ten colonies from the cloning of each band of interest. The different colonies are grouped into homogenous cDNA species by either restriction enzyme digestion or single-nucleotide DNA sequencing prior to cDNA probe synthesis for northern analysis. However, the possibility of missing true DD cDNA candidates within the 10 picked

colonies could not be excluded. This may partly account for the high rate of false positives. Additional conditions could have been incorporated into the experimental design to reduce the high rate of false positives. For instance, DD screening of samples at different time-points during the treatment period (e.g. 7, 14, 21, and 35 days) could be incorporated to allow the examination of the temporal effect of the drug on expression of that particular gene. Using two different reverse transcriptases at the RT step of the reaction could also help to minimize false positives. Different (1:10 and 1:100) dilutions of each sample can be included to minimize the differential PCR amplification rates. Longer primer designs and higher temperature conditions for PCR could help to enhance more specific gene amplifications. Furthermore sensitive mRNA quantitation techniques, such as RNase protection assays, competitive RT-PCR or real-time quantitative PCR, can be used for true-positive confirmation. Table 4 summarizes some of the features of the different methods for mRNA quantitation.

The true positive clones, designated hereafter as LRG1 and LRG2 were further characterized. In addition, an unexpected cDNA product from RACE procedures, designated as LRG3, was also differentially regulated by lithium and was subsequently characterized. The results from these investigations will be discussed in turn.

4.2 EFFECTS OF LITHIUM AND OTHER PSYCHOTROPIC DRUGS ON LRG1, LRG2 AND LRG3 mRNA LEVELS

Three candidate lithium-regulated cDNA fragments (LRG1-3) were isolated and characterized. Northern blot analyses revealed that chronic (5 weeks), but not short-term (1-week), lithium treatment significantly decreased LRG1 and LRG3 mRNA levels, whereas the mRNA levels of LRG2 were significantly increased. Because the alterations in LRG1-3 mRNA levels were detected only after chronic lithium treatment, and at concentrations within the

Table 4: Methods for quantitative mRNA analysis (Adapted from Ambion[1] TechNote 6(3))

Feature	**Northern Blot**	**Nuclease Protection Assays**	**RT-PCR**	**In Situ Hybridization**
Sensitivity (mRNA copies)	10,000	4,000-5000	1 (theoretical)	Medium
Level of Optimization Required	Medium	Medium	High	High
Sample Size	30μg max	100μg max	Not critical	Tissue sections
Detect Multiple Transcripts	Yes, by stripping and reprobing	Yes, in same tube	Yes, in same tube	No
Sizing mRNAs	Yes	No	No	No
Localizes mRNA Within Cell/Tissue	No	No	No	Yes
Mapping Studies	No	Yes	No	No
Tolerates Partially Degraded mRNA	No	Yes	Yes	Yes
Advantages	RNA undergoes minimal processing	Can use up to 100μg of sample RNA	Most sensitive technique	Localizes mRNA within tissue

[1] Ambion: The RNA company, http://www.ambion.com

therapeutically relevant range (0.6 – 1.2 mM), these changes may be associated with and important for the development of its mood stabilizing effects.

The mechanisms by which lithium affects the expression of these candidate genes in brain are largely unknown. The possibility that lithium regulation of these candidate transcripts is secondary to the weight changes commonly observed in rats during lithium treatment appears unlikely, as there were no significant difference in weight gain between control and lithium-treated animals during the treatment period (Fig. 8A).

The findings that lithium modulates the activity of multiple transcription factors (e.g. AP-1, CREB, NF-κB) (Yuan et al, 1998; Wang et al, 1999a; Hoeflich et al, 2000) suggest transcriptional regulation may be involved in the altered expression of the LRGs identified here. However, post-transcriptional modifications, e.g. mRNA export and degradation could also contribute to the altered levels of these candidate transcripts. Future experiments using nuclear runoff assay and mRNA decay studies are necessary to examine whether the rate of transcription of these genes or mRNA stability is affected following chronic administration of lithium.

Regulation of LRG2 expression appears to be specific to lithium, since chronic administration of anticonvulsant mood stabilizing drugs, CBZ and VPA, did not affect LRG2 mRNA levels. Chronic CBZ administration, but not VPA administration, reduced LRG1 expression, whereas chronic VPA or CBZ administration reduced LRG3 transcript levels. Moreover the expression of these lithium-regulated transcripts was not influenced by chronic administration with a typical antidepressant (imipramine) or antipsychotic drug (haloperidol).

Analysis of drug concentrations revealed that the plasma VPA levels in this study were substantially lower (~ 27 μM) than the therapeutic range effective for treatment of BD patients (300 - 600 μM) (Goodwin and Jamison, 1990). While it appears that the lack of effect of VPA on LRG1 and LRG2 expression is related to suboptimal levels of this drug, the significant effect of chronic VPA administration on LRG3 mRNA levels renders this possibility unlikely. The low

plasma levels of VPA likely reflect the short half-life of this drug in rats, which metabolize VPA at an order of magnitude faster than humans (Levy and Kerr, 1989). It is also possible that the levels of VPA are higher in the brain of the rat than the serum data suggest. Recent studies have also shown that chronic VPA treatment (300 mg/kg once daily for 21 days) upregulates the expression of endoplasmic reticulum stress proteins, glucose-regulated protein (GRP) 78, GRP 94 and calreticulin in rat brain (Wang et al, 1999b; Chen et al, 2000b). Interestingly, very low plasma levels (5.3 ± 0.3 μM, n = 10) were also found in a separate group of rats receiving VPA under this dosing regimen. These results, taken together, indicate that very low plasma VPA concentrations are sufficient to induce a highly significant biochemical and/or pharmacological effect. Thus, it is likely that the lack of effect of VPA on LRG1 and LRG2 expression may be related differential sensitivity of these genes to VPA.

Increasing evidence suggests that mood stabilizing agents may exert their therapeutic effects through convergent mechanisms, such as targeting specific signal transduction pathways (Li et al, 2000; Manji et al, 2000). Thus the similar changes in LRG1 mRNA levels induced by chronic lithium or CBZ treatment and the change in LRG3 mRNA levels induced by lithium, VPA or CBZ treatment may represent one of the overlapping molecular/pharmacological mechanisms of these therapeutic agents in the treatment of BD. On the other hand, there is also increasing recognition of differential clinical responses to lithium and other mood stabilizing agents as well as augmenting effects of various combinations of lithium and anticonvulsants in BD patients refractory to monotherapy (Post et al, 1992, 1994). In this respect, the specific action of lithium on LRG2 expression may represent a distinct mode of action unique to this drug.

Taken together, the findings in this study identified shared as well as unique effects for the three mood stabilizing drugs, an observation that is consistent with findings from clinical studies (Post et al, 1996). The common sites of action may account for their utility in the treatment of BD, whereas the distinct mode of action might result in differential clinical

responses in monotherapy and additive responses in combination therapy (Post and Chuang, 1991; Post et al, 1992, 1994).

4.3 CHARACTERIZATION OF LITHIUM-REGULATED cDNA FRAGMENTS

The identity of LRG1-3 was assessed by comparing their respective DNA sequences against those in the Genbank, followed by RACE or cDNA library screening to obtain the full-length cDNA clone.

4.3.1 Identity of LRG1

Comparison of the sequence for LRG1 against those in the GenBank database revealed that this fragment corresponds to aldolase A, a key enzyme in glycolysis. This is further supported by the finding that the LRG1 probe hybridized to a single transcript of 1.4 kb, as previously reported for aldolase A (Joh et al 1985; Mukai et al, 1986).

In vertebrates, three isozymic forms, designated aldolase A (muscle type), aldolase B (liver type) and aldolase C (brain type) are known to exist, and their distribution is specific for tissue type and developmental stage (Horecker et al, 1972). Aldolase C is mainly expressed in the brain and is known to form a hybrid with aldolase A as a heterotetrameric holoenzyme (Penhoet et al, 1966; Lebherz and Rutter, 1969). Thus we also determined its mRNA level in rat frontal cortex following chronic lithium treatment. Interestingly, northern blot analysis revealed that a significant reduction of the mRNA levels of this isozyme following chronic lithium administration. Therefore, chronic lithium administration regulates the expression of both A and C isozymes of aldolase in rat frontal cortex. Western blot analysis showed that the cytosolic, but not particulate, aldolase protein levels were also significantly reduced by chronic lithium administration. These data suggest that both the mRNA and protein levels of aldolase were significantly reduced following chronic lithium treatment in rat frontal cortex.

4.3.2 Identity of LRG2

DNA sequencing of LRG2 revealed a fragment of 630 bp that shared a significant identity to an EST sequence (U95001) in the Genbank. Subsequent screening of a rat cDNA library was performed to isolate the full-length cDNA clone. Comparing the DNA sequence of the isolated clone (C10) to those in the gene bank revealed it to be the rat homologue of DIPP2 recently identified in human (Caffrey et al, 2000). It is likely that the isolated clone represents the full length cDNA sequence encoding LRG2 since the size of the insert (2954 bp) is similar to that of the LRG2/rDIPP2 transcript detected by northern blot analysis (~3 kb; Fig. 16B). Although there are three polyadenylation signals in the 3'-UTR, it appears that only the most distal one, which is 26 bp upstream of the poly(A) tail, is functional. This is in marked contrast to the expression of multiple transcripts of the hDIPP2 due to the use of alternate polyadenylation signals (Caffrey et al, 2000). On the other hand, similar features are evident in the 5'-UTR of the rat and human DIPP cDNAs (Safrany et al, 1998; Caffrey et al, 2000), namely i) the presence of multiple CpG doublets that are characteristic of 'housekeeping' enzymes (Bird, 1986), and ii) the high G/C content, which may be important in regulating translational efficiency (Kozak, 1996).

The rat DIPP2 cDNA encodes a 179 amino acid polypeptide with a deduced molecular weight of $\approx$ 20 kDa whose sequence shows significant homology (95%) with that of the human homologue (Caffrey et al, 2000). It contains two potential sites for N-glycosylation and two putative sites for phosphorylation by PKC. Homologies are especially noteworthy in several regions, including the Nudix motif (Fig. 25), the two glycine rich regions (Gly-49 to Gly-51; Gly-71 to Gly-81) flanking the Nudix motif, and the amino acid residues Phe-83 and His-90, all of which are essential for the catalytic activity of DIPPs (Yang et al, 1999). Thus, the

conservation of amino acid sequence likely reflects the constraints against evolutionary divergence in protein structure dictated by the unique function of this protein.

The database of rat ESTs maintained by the GenBank was queried with the nucleotide sequence of C10 using the BLAST algorithm (Altschul et al, 1990). The returned dbEST sequences that had partial rDIPP2 ORF showed either a perfect match or had an additional CAG codon inserted at nt 362 of the C10 (e.g. GenBank Accession #AA925550, AI170686, AI172168, AI175162, AI599827, AI602300, AI105415, AI411034, and AW433926). Such an in-frame insertion predicts coding for an additional Gln residue. This observation, together with the recent evidence of two isoforms of human DIPP2, which differ only by a Gln residue (Caffrey et al, 2000), suggests that another rDIPP2 variant with an extra amino acid may also occur in the rat.

Northern blot analysis revealed that rDIPP2 mRNA are expressed widely in different adult rat tissues as well as in embryonic rat heart and brain (Fig. 27). The levels of rDIPP2 mRNA in embryonic rat heart were analyzed, because the LRG2 cDNA fragment was initially found to have a high identity to the rat EST known as developmentally-regulated cardiac factor 5, in the Genbank (Fig. 20). The findings of rDIPP2 mRNA in embryonic and adult heart suggest that this gene might play a role in the development of the heart and maintenance of cardiac function. However, the precise role of rDIPP2 in cardiac tissue remains to be elucidated.

The rat recombinant DIPP2 protein was expressed using the pET expression vector in a bacterial host. A band of ~ 25 kDa (compared with protein size markers) was detected on coomassie blue-stained SDS-PAGE and identified as the putative purified recombinant rDIPP2 by western blotting using anti-DIPP2 antibody and functional activity (see below). The molecular size observed is consistent with that found for recombinant human DIPP2 expressed in *E. coli* (Caffrey et al, 2000).

The activity of the expressed protein was analyzed via HPLC measuring the hydrolysis of PP-[^{3}H]$InsP_5$ to [^{3}H]$InsP_6$. PP-[^{3}H]$InsP_5$ was dephosphorylated by the recombinant rat DIPP2 to [^{3}H]$InsP_6$ by removal of the β-phosphate of the diphosphate group. Of note, there was no accumulation of $InsP_5$ that would have been formed if the diphosphate group were hydrolyzed from PP-$InsP_5$, nor was any PP-$InsP_4$ detected, which would have reflected cleavage of a monoesterphosphate from PP-$InsP_5$. However, the ability of rDIPP2 to hydrolyze other known substrates of this family of enzymes, such as diadenosine polyphosphates, $[PP]_2$-$InsP_4$ and PP-$InsP_4$ (Caffrey et al, 2000), remains to be shown. Nevertheless, this observation suggests that the recombinant protein, rDIPP2, expresses enzyme activity towards PP-[^{3}H]$InsP_5$ similar to the DIPP protein family first identified in human (Safrany et al, 1998).

The question of whether the increased DIPP2 mRNA is translated into increased enzyme activity and/or protein levels remains to be demonstrated. The protein levels of rDIPP2 were examined using anti-mouse DIPP2 antibody that was generously provided by Dr. Stephen Shears (Research Triangle Park, NC). Preliminary data, however, revealed multiple nonspecific signals and the band of interest was difficult to identify unequivocally. This is probably due to the fact that the antibody was raised against a short carboxyl-terminal epitope, hence it might cross-react with other related proteins. Alternatively, multiple bands may have resulted from the binding of different proteins by contaminating antibodies in the antiserum that was not subjected to affinity purification. Therefore, a polyclonal antibody specific to rDIPP2 and which is immunoaffinity purified needs to be raised for this analysis. The question of whether lithium affects the activity of this enzyme is more difficult to answer, because the multiple isoforms of the enzyme can not be distinguished individually with current methods. Moreover, the product of rDIPP2, $InsP_6$, is known to act as an inhibitor of the enzyme (Caffrey et al. 2000).

4.3.3 Identity of LRG3

During the course of isolating the full-length cDNA for LRG2, another novel clone was isolated. This novel cDNA detected 3 bands (1.6, 2.0, 3.0 kb) on all northern blots containing total RNA from control and lithium-treated rats. Screening of a rat cDNA library identified a cDNA clone (C1) of ~1.6 kb, similar in size to the smallest transcript detected by northern blot (Fig. 22). Comparison of the amino acid sequence encoded by C1 with those in the Genbank revealed considerable sequence homology to members of the transmembrane 4 superfamily (TM4SF) (Maecker et al, 1997), with a particularly striking sequence homology (> 92% identity) to human platelet-endothelial cell tetraspan antigen 3 (PETA-3) (Fitter et al, 1995), monkey PETA-3 (Genbank accession # AF275665) and mouse PETA-3 (Hasegawa et al, 1997) (Fig. 31). PETA-3 was originally identified as a platelet glycoprotein using a monoclonal antibody (14A2.H1) that induced platelet aggregation (Fitter et al, 1995). The human homolog, independently cloned as SFA-1(adult T-cell leukemia cell line (SF-HT) -activated gene 1), was shown to be up-regulated in human T cells transformed by T cell-leukemia virus type 1 (HTLV-1) (Hasegawa et al, 1996). PETA-3/SFA-1 was later assigned the nomenclature CD151 at the VIth International Leucocyte Differentiation Antigen Workshop (Ashman et al, 1997).

One intriguing observation arising from the analysis of the deduced amino acid sequence of CD151 from all three species using the PROSITE database (Bairoch et al, 1997), is the presence of a leucine zipper (LZ) motif (residue 89-110) within the third putative transmembrane domain (Fig. 32). LZ motifs are found in many eukaryotic transcription factors (Jun, Fos, CREB, etc.) and are involved in multimerization, generally dimer formation to regulate gene transcription (Hurst, 1995). The presence of LZ motif suggests that CD151 might exist as homodimers via LZ – LZ dimerization.

Members of TM4SF, including CD151, have been suggested to play a role as a "molecular facilitator" by interacting with and grouping specific cell-surface proteins, and

consequently increasing the formation and stability of functional signaling complexes (Maecker et al, 1997). CD151, in particular, has been reported to associate with different integrin complexes (Fitter et al, 1999; Serru et al, 1999) and other TM4SF members including CD9, CD63 and CD81 (Fitter et al, 1999). A recent study has demonstrated that the large extracellular loop (EC2) of CD151 is involved in the physical interaction with integrins (Yauch et al, 1998). Therefore, the interaction of CD151 with members of integrins appears to be mediated by a binding region distinct from the LZ motif.

Northern blot analysis, using an EcoR I/BamH I digest corresponding to nt 187-690 of C1 as a probe, identified three species, 3 kb, 2 kb, and 1.6 kb, in rat frontal cortex with the following order of relative abundance, 1.6 > 2.0 > 3.0 kb (Fig. 17B). This finding is in marked contrast to the expression of a single (~1.6 kb) transcript in humans (Fitter et al, 1995) and mouse CD151 (Hasegawa et al, 1997). Although a recent study of the genomic structure of mouse CD151 reported that alternate splicing could generate three transcripts with different 5'-UTRs, the sizes of these transcripts ranged from 1.6 to 1.8 kb (Fitter et al 1998). Therefore, the ~2.0 kb and ~3.0 kb transcripts detected in rat brain might be splice variants of the same gene, immature mRNA, or other closely related genes. Preliminary southern blot analysis of restriction fragments of genomic DNA hybridized with 5'5EB revealed a single band, suggesting that the ~2.0 kb and ~3.0 kb transcripts detected in rat brain might be splice variants of the same gene. However, isolation of the genomic DNA of CD151 is necessary to confirm this possibility. On the other hand, northern blot analysis of cytoplasmic mRNA can be done to determine whether the larger transcripts are the immature mRNA species.

In human and mouse, high levels of CD151 mRNA were found in heart, lung, kidney, stomach, pancreas, whereas levels were low in brain (Fitter et al, 1995, Hasegawa et al, 1997, Fitter et al, 1998). However, CD151 protein was not detectable in nerve bodies in the gray matter and myelinated fibers in the white matter in a recent immunohistochemical study of its

distribution in human tissues, whereas the vascular endothelium of the cerebral cortex exhibited high levels of staining (Sincock et al, 1997). In order to confirm that the CD151 transcript detected in rat brain was present in neuronal cells and did not arise from vascular endothelium contamination, northern blot analysis was performed on primary cultures of cortical neurons devoid of vascular endothelium. The result revealed the presence of the CD151 transcripts in these cultured neurons. The neural localization of CD151 was further substantiated by the recent findings of the presence of CD151 protein in immortalized neuronal precursors (Fitter et al, 1998) and CD151 mRNA in the developing brain (Stipp and Hemler, 2000) and postmortem human brain (unpublished data). Moreover, CD151 is also present in cultured human NT2N neurons (Stipp and Hemler, 2000). Cumulatively, these findings strongly indicate that CD151 is present in neurons, and might have a functional role in the CNS. Other members of TM4SF have been identified in hemopoietic system as well as neural tissues, including CD9 (Kaprielian et al, 1995), CD81 (Geisert et al, 1996) and rat Tspan-2 (Birling et al, 1999).

The protein levels of rCD151 were examined using anti-human CD151 monoclonal antibody (14A2.H1, PharMingen Canada). However, preliminary experiments found no detectable sign for rat brain or human platelet membranes (positive control). These findings suggest that this antibody may not be useful for measuring the levels of CD151 protein. Therefore, a polyclonal antibody specific to rCD151 needs to be raised for this analysis.

4.4 PHARMACOLOGICAL SIGNIFICANCE OF LITHIUM REGULATED GENES

The identities of LRG1, LRG2 and LRG3, are aldolase A, DIPP2 and CD151, respectively. Having presented and discussed of the evidence supporting the identities of the three genes, the potential pharmacological significance of lithium regulation of each gene is discussed in order. Then, a postulated model of how the findings in this study may be involved in the therapeutic mechanism of action of lithium will be presented.

4.4.1 Pharmacological Significance of Lithium Regulation of Aldolase

Whether lithium regulation of aldolase expression contributes to its therapeutic or adverse effects is unclear at present. Aldolase (EC4.1.2.13) is a glycolytic enzyme that catalyzes the reversible cleavage of fructose 1,6-bisphosphate to glyeraldehyde 3-phosphate and dihydroxyacetone phosphate, a key reaction in glycolysis and energy production. The finding that lithium exerts modulatory effects on a metabolic enzyme is not unprecedented. Previous studies have shown that lithium *in vitro* inhibits the activity of regulatory metabolic enzymes such as fructose 1,6-bisphosphatase (Marcus and Hosey 1980) and GSK-3β (Klein and Melton 1996). Thus, the reduction of mRNA and proteins levels of aldolase A and C could conceivably be related to the effect of lithium on carbohydrate metabolism. In this respect, one of the most common undesirable side effects of long-term lithium administration in BD patients has been body weight gain (Schou et al, 1970; Vendsborg et al, 1976; Gopalaswamy and Morgan, 1985; Vestergaard et al, 1988), which could result from lithium-induced metabolic changes. However, the possibility that lithium modulation of aldolase expression may contribute to body weight change observed in BD patients is unlikely, because no significant body weight changes were observed in our studies between lithium-treated and control rats. Furthermore, there was no apparent changes in aldolase expression following chronic administration of imipramine or haloperidol, which have the capacity to induce weight gain in major depressive (Frank et al., 1990), or schizophrenic (Ganguli, 1990) patients, respectively.

The associations between the temporal effect of lithium on aldolase expression and the apparent delay in the clinical response to lithium in BD patients suggest that the changes in aldolase A/C expression may be therapeutically relevant. Indeed, aldolase isozymes have been reported to modulate several other cellular functions that, on closer perusal, suggest effects that could be important to the therapeutic action of lithium. For example, aldolase A has been shown to interact physically with tubulin and actin filaments, an action which may contribute to the

regulation of cytoskeletal structures and cell mobility (Kusakabe et al, 1997; Volker and Knull, 1997; Kao et al, 1999). Secondly, the presence of aldolase A in the nucleus and its ability to interact with certain DNA sequences suggest a potential role of aldolase A in modulating transcriptional activity (Ronai et al, 1992). Furthermore, the finding that aldolase A and C bind $InsP_3$ suggests a potential role for these enzymes in regulating the free intracellular concentrations of this second messenger, and subsequently intracellular calcium dynamics (Koppitz et al, 1986; Baron et al, 1999). This is of particular pathophysiological importance given the well-documented observations of altered calcium homeostasis in BD (Dubvosky et al, 1994; Emamghoreishi et al, 1997). Although it may seem difficult at first to ascribe any direct significance of these diverse cellular functions affected by aldolase isozymes to the therapeutic action of lithium, the observations that chronic lithium treatment exerts modulatory effects on neuronal cytoskeletal architecture, transcription factor activity, $InsP_3$ signaling, and intracellular calcium dynamics (reviewed in Jope, 1999b; Lenox and Hahn, 2000; Li et al, 2000) shed a different light on the role of A and/or C isozymes of aldolase in these cellular changes that are regulated by lithium. Moreover, it has recently been suggested that lithium regulation of cytoskeletal networks and AP-1 transcription factor activity may be mediated via inhibition of GSK-3β, another multifunctional metabolic enzyme (reviewed in Jope, 1999b; Lenox and Hahn, 2000).

Based on the above considerations, it is not unreasonable to suggest that some of these diverse cellular effects of lithium may also involve other metabolic enzymes such as aldolase. The recent suggestion that GSK3β may represent a potential target of lithium in the treatment of BD (Klein and Melton, 1996; Agam and Levine, 1998) underscores the potential importance of lithium regulation of 'multifunctional' metabolic enzyme(s) in its psychotherapeutic mechanisms. Of additional interest in this regard, increased serum aldolase A activity has been reported in a much earlier study of a small group of manic BD patients (Meltzer, 1968, 1970).

While the significance of the increased aldolase A activity was not clear at that time, it was suggested that an increase in cell permeability or decreased clearance of the enzyme from serum might be the peripheral manifestation of a pathologic process occurring in the CNS (Meltzer, 1968). Although it has not yet been proven, our findings of reduced expression of mRNA and protein levels of aldolase A and C following chronic lithium treatment offer a more interesting possibility that dysregulation of aldolase isozymes might occur in the brain of BD patients. Indeed, recent proteomic analysis using two-dimensional gel electrophoresis of postmortem brain reported an increase in the protein level of aldolase C in frontal cortex of BD, schizophrenic, and depressed patients (Johnston-Wilson et al, 2000). While these findings suggest that abnormalities in aldolase protein levels might be common to some psychiatric disorders, the ability of lithium, but not IMI or HAL, to alter the protein and mRNA levels of aldolase strongly suggests that down-regulation of aldolase expression may be of importance in the specific therapeutic action of lithium in BD.

4.4.2 Pharmacological Significance of Lithium Regulation of rDIPP2

Diphosphoinositol polyphosphate phosphohydrolases catalyze the dephosphorylation of the "high energy" diphosphoinositol polyphosphates, PP-$InsP_5$, $[PP]_2$-$InsP_4$, and PP-$InsP_4$ to $InsP_6$ and $InsP_5$, respectively (Safrany et al, 1998; Caffrey et al, 2000). The potential importance of these diphosphoinositol polyphosphates in cellular signaling is underscored by their rapid turnover, responsiveness to receptor-mediated changes in the levels of cAMP and cGMP in a kinase-independent manner, as well as to perturbation of intracellular Ca^{2+} dynamics (for review, see Shears, 1998).

Recent studies have demonstrated the importance of diphosphoinositol polyphosphates in regulating endocytosis, exocytosis, and synaptic vesicle trafficking (De Camilli et al, 1996; Fukuda and Mikoshiba, 1997; Safrany et al, 1999a). Electrophysiological studies in *Drosophila*

have provided evidence of aberrant synaptic transmission in response to lithium exposure, which is due to a marked increase in the probability of synaptic vesicle release (Acharya et al, 1998). In line with the latter observation, lithium has been shown to increase the release of serotonin (Wang and Friedman, 1988) and glutamate (Dixon et al, 1994) in brain cerebral cortical slices. These results, taken together, suggest that the modulation of synaptic function by lithium may be mediated partly by its influence on diphosphoinositol polyphosphate metabolism and/or turnover.

In addition to dephosphorylating diphosphoinositol polyphosphates, DIPP also actively hydrolyzes diadenosine 5',5'''- P^1,P^5-pentaphosphate (Ap_5A) and diadenosine 5',5'''- P^1,P^6-hexaphosphate (Ap_6A) (Yang et al, 1999), a group of putative signaling molecules implicated in regulating a diverse array of cellular functions (Kisselev et al, 1998; Miras-Portugal et al, 1998). It has been reported that the levels of diadenosine polyphosphates were increased in response to heat shock and oxidative stress (Baker and Jacobson, 1986). The accumulation of these compounds could be potentially hazardous to cell function through their inhibitory action on nucleotide kinases, protein kinases and other enzymes (Safrany et al, 1999b). It is of interest in this regard that long-term lithium treatment has been shown to exert neuroprotective effects against neuronal cell death induced by a variety of insults both to cultured neurons and in intact animals (Nonaka and Chuang, 1998, Nonaka et al, 1998a,b). It is tempting to suggest that the regulation of rDIPP2 expression by lithium may also play a role in its neuroprotective actions by regulating the levels of the diadenosine polyphosphates. In this context, the presence in rDIPP2 of the MutT/Nudix motif, a conserved signature sequence implicated in the breakdown of potentially deleterious endogenous metabolites (Bessman et al, 1996), underscores the potential importance of this enzyme in the maintenance of cell integrity and viability.

4.4.3 Pharmacological Significance of Lithium Regulation of rCD151

The pharmacological relevance of the regulation of rCD151 mRNA expression by lithium is presently unclear, since the precise biochemical function of the TM4SF, including CD151, remains to be elucidated. Furthermore, most of the investigations on the biochemical functions of CD151 to date have been performed on haemopoietic cell lines; very little is known about the functional significance of CD151 in the brain. The data currently available, however, largely suggest a role for the TM4SF superfamily in anchoring signal transduction complexes and the regulation of cell development, proliferation, activation and motility (Maecker et al, 1997, Levy et al, 1998).

One of the roles suggested for members of TM4SF, CD151 in particular, is to serve as an anchor for signal transduction complexes, and this function could be as important and relevant to neural as it is to haemopoietic cells. CD151 has been suggested to act as a linker between $\alpha_3\beta_1$ integrin and PI-4K (Yauch et al, 1998). It is hypothesized that $\alpha_3\beta_1$-CD151-PI-4K complexes could localize the production of phosphatidylinositol-4-phosphate (PtdIns-4-P), and subsequently PtdIns-4,5-P (PIP_2). In addition to acting as a linker for integrin and PI4K, CD151 also serves as an anchoring protein for activated PKC (Hemler, 1998). Upon activation and translocation of PKCα and PKCβ from cytoplasm to the membrane, these enzymes can become directly associated with CD151. Thus PKCs can be recruited into complexes with integrin $\alpha_3\beta_1$ via CD151 linkage. One consequence of this complex formation is to facilitate the regulation of integrin function by PKC-dependent phosphorylation of integrin α_3 cytoplasmic tails (Hemler, 1998). In line with a potential role of CD151 in modulating the membrane PIP_2 and PKC levels, elevated membrane PIP_2 in platelets of bipolar patients in the manic state have been reported (Brown et al, 1993), and recent *ex vivo* evidence demonstrated that patients receiving lithium treatment exhibit a decreased relative amount of PIP_2 in the platelet cell membranes compared

with normal controls (Soares et al, 1999). Furthermore, chronic lithium treatment has been shown to decrease the levels of membrane-associated PKCα and PKCε in rat brain (Manji et al, 1993, 1996) and levels of cytosolic PKCα in platelets of BD patients (Soares et al, 2000b) These findings taken together suggest that the decrease in rCD151 expression by lithium may mediate some of the reported effects in BD. However, further analyses of CD151 levels in cellular conditions in which PIP_2 or membrane PKC levels are aberrant is necessary to substantiate this idea.

One very recent study provides the first hint to the potential role of CD151 in the brain. The functional relevance of specific $\alpha_3\beta_1$ – CD151 complex was examined in human NT2N neuron-like cells. Anti-CD151 antibody was found to strongly inhibit the total NT2N neurite outgrowth stimulated by laminin-5, a ligand for $\alpha_3\beta_1$ integrin, suggesting that CD151 contributes towards neurite number, length and rate of growth (Stipp and Hemler, 2000). Of particular relevance, lithium has previously been reported to inhibit neurite outgrowth in rat pheochromocytoma PC12 cells induced by nerve growth factor (NGF) and in mouse sympathetic neurons (Burstein et al, 1985; Harada et al, 1996). Inhibition of microtubule associated protein (MAP) phosphorylation by lithium was suggested as one of the possible biochemical mechanisms by which neurite outgrowth is suppressed (Daniels, 1972; Mitchison and Kirschner, 1988). However, extracellular matrix (ECM) has also been reported to sustain PC12 neurite elongation in the absence of NGF and microtubules, suggesting that ECM can modulate neurite outgrowth via an alternate mechanism (Lamoureux et al, 1990). Interestingly, laminin, a component of ECM and ligand for integrins, has been shown to specifically stimulate growth cone-like behavior of NG108-15 cells (Smalheiser, 1989). These results, taken together, raise the interesting possibility that modulation of the neurite outgrowth in neuronal cells by lithium might be mediated, in part, by its downregulation of rCD151 expression. However, it must be shown

first that lithium mimics the action of anti-CD151 antibody on neurite outgrowth and that overexpression of CD151 could rescue the phenotype in NT2N cells to confirm this hypothesis.

4.4.4 A Postulated Model

Although it was initially thought that the differentially regulated genes identified were unrelated, closer examination of the known and/or suggested roles for these putative lithium-regulated genes suggests an interesting relationship among their cellular functions. The proteins encoded by these three lithium-regulated genes each appear to be involved in some manner in inositol phosphate metabolism and/or the phosphoinositide signaling pathway (Fig. 34). For example, aldolase is known to bind $InsP_3$, and can regulate the free concentration of this second messenger and subsequently intracellular Ca^{2+} dynamics (Koppitz et al, 1986). DIPP2 is an enzyme that catalyzes the dephosphorylation of [PP]-$InsP_5$, $[PP]_2$-$InsP_4$ and PP-$InsP_4$ to $InsP_6$ and $InsP_5$, respectively (Caffrey et al, 2000), a group of highly phosphorylated inositols that have been implicated in the synaptic vesicle trafficking (Voglmaier et al, 1996; Shears, 1998). Similarly CD151 has been suggested to act as an anchoring protein for PI-4K and PKC, both of which are key components in the PI signaling system (Hemler, 1998). This observation is intriguing particularly when considered against the large body of data implicating abnormalities in the PI/PKC signaling system in BD and modulatory effects of lithium on PI/PKC signalling system (reviewed in Manji et al, 1999; Li et al., 2000; Warsh and Li, 2000). For instance, the relative percentage of platelet membrane PIP_2 was significantly higher in unmedicated manic patients than in control subjects, but was decreased and normalized after lithium treatment (Brown et al, 1993; Soares et al, 1997, 2000a,b). Disturbances of intracellular Ca^{2+} homeostasis have been well documented in peripheral blood cells and transformed B lymphoblasts derived from bipolar patients (Dubvosky et al 1992; Emamghoreishi et al 1997). Elevated levels of cytosolic PKCα and membrane-associated PKCγ were found in postmortem brains of BD

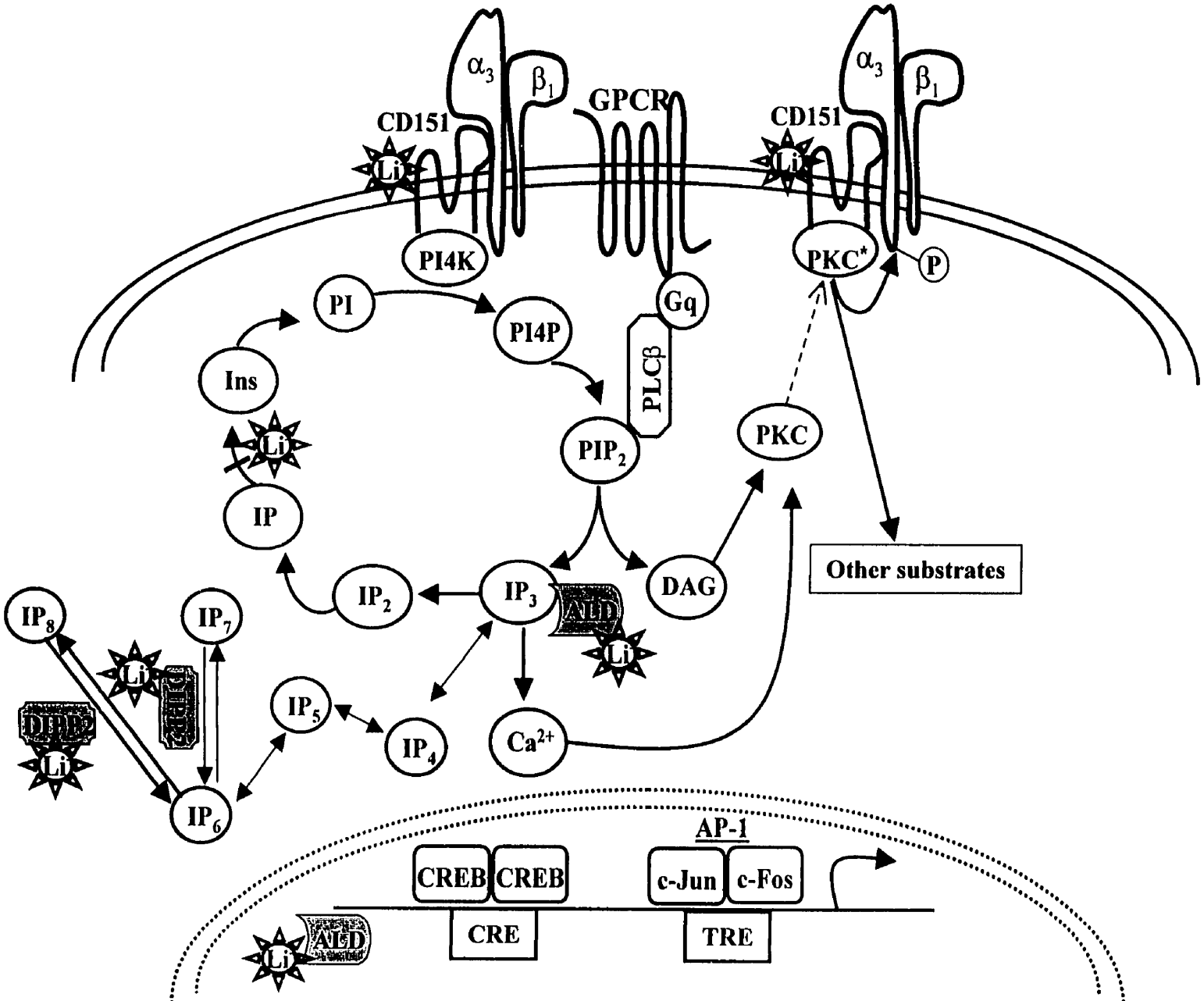

Figure 35 Postulated model of lithium's action. Lithium downregulation of aldolase A may causes a decrease in the level of IP_3. Down regulation of CD151 might account for the reduction of level PIP_2 and membrane PKC observed with lithium. Lithium increase of DIPP2 could affect levels of inositol phosphates in cell. Taken together, lithium might modulate the PI signaling via these three proteins in additionto its well known inhibition of inositol monophosphatase. * activated PKC.

patients compared with matched control subjects (Wang and Friedman, 1996). Moreover, chronic lithium treatment decreased the levels of specific PKC isozymes in a number of cell systems including the brain (Jope and Williams, 1994; Manji et al, 1993, 1995, 1996) and in platelets of BD patients (Soares et al, 2000b). In the absence of a definitive explanation of the mechanism by which lithium produces the above changes, our findings of three novel lithium targets that are involved in one way or another in PI signaling suggest an appealing model to explain the mechanisms underlying some of the above findings. Most of the studies on the effects of lithium on PI signaling pathways stemmed from the inhibitory effect of lithium on IMPase and accordingly the results were interpreted with respect to possible consequences of IMPase inhibition. However, it has been increasingly appreciated in recent years that the therapeutic effects of lithium cannot be accounted for solely by a reduction of *myo*-inositol as a result of IMPase inhibition. In line with this notion, our findings suggest that in addition to regulating the activities of IMPase (Haller and Sherman 1980), inositol polyphosphate 1-polyphosphate (Inhorn and Majerus 1987), *myo*-inositol uptake (Lubrich and van Calker 1999), and $G_{q/11}$ (Jope, 1999), chronic lithium treatment also regulates the expression of genes that may be involved in modulation of PI signaling.

In addition to regulating the expression of aldolase A/C, DIPP2, and CD151, chronic lithium treatment also alters the gene expression of IMPase (Sharmir et al, 1998), sodium/*myo*-inositol cotransporter (Lubrich and van Calker, 1999) and prolyl oligopeptidase (Williams et al., 1999), all of which play an important role in inositol phosphate metabolism and/or PI signaling. Such a cluster of changes is unlikely a chance event. A similar phenomenon is also evident in the cAMP signaling cascade, in which the expression of another subset of genes (e.g. Gαs, Gαi1, Gαi2, and AC subtypes) is altered by chronic lithium administration (Colin et al, 1991; Li et al, 1991; 1993). The changes of a spectrum of genes within a signaling cascade by lithium may represent sequential adaptive changes normalizing the putative disturbances of the intracellular

signaling cascades in the disease process. Alternatively, such alterations may reflect compensatory mechanisms operative within or between different intracellular signaling pathways to maintain intracellular signaling homeostasis in response to lithium treatment. Nevertheless, these observations clearly support the potential psychotherapeutic relevance of this effect of lithium to regulate the expression of a distinctive set of genes in signaling pathways known to be dysregulated in BD (Li et al., 2000; Warsh and Li, 1996; 2000).

4.5 FUTURE STUDIES

The data presented in this thesis were derived from the initial set of experiments identifying and characterizing lithium regulated genes. A number of questions remain to be addressed. Firstly, the regional and cell-specific effects of lithium on LRG1-3 mRNA expression remain to be established. This could be achieved by in-situ hybridization. Secondly, the molecular mechanisms by which lithium modulates the expression of aldolase A/C, DIPP2 and CD151 remain to be further investigated. The upstream regulatory regions for CD151 and DIPP2 have not been characterized, therefore, cloning the promoter regions is necessary for characterization of transcriptional regulation of these genes. On the other hand, the upstream promoter regions of the rat aldolase A and C genes have been shown to contain binding sites for Sp1, AP1 and AP2 transcription factors (Vibert et al, 1989; Hermfisse et al, 1996). These findings suggest that lithium may modulate the expression of aldolase genes by affecting the activities of these transcription factors. However reporter gene assays for specific transcription factor are necessary to address these hypotheses. Another related question is whether the effects of lithium on the expression of these genes are mediated by transcriptional or post-transcriptional mechanism. Transcriptional regulation could be examined by nuclear runoff assay, whereas post-transcriptional regulation, such as mRNA stability, could also be performed by measuring the decay of the genes of interest over time after treatment with an inhibitor of mRNA synthesis,

actinomycin D. Thirdly, anti-rDIPP2 and anti-rCD151 antibodies need to be raised to measure the levels of their respective proteins after chronic lithium treatment, by western blot analysis. And finally, the most challenging question that needs to be addressed is the pharmacological relevance of lithium's effects on these genes in relation to its therapeutic action.

In an attempt to elucidate the pathophysiology of bipolar disorder and the mechanisms by which mood stabilizing drugs act, biomedical researchers face a number of difficulties. BD is a complex disorder, comprising several subtypes distinguished by phenomenology, natural history, response to pharmacotherapies and prognosis (Goodwin and Jamison, 1990). Furthermore, the relative inaccessibility of brain tissues for direct study and the lack of a suitable animal model for BD have prompted search for alternative cellular models as indirect approach for the direct study of the affected brain. A good model should exhibit the disease in a tissue-specific and species-specific manner, and the disease could be biochemically and genetically analyzed. One cellular model appears to meet most of the above criteria is the B lymphoblast cell lines (BLCL), which has extensively been used in our laboratory as a disease model of BD (Emamghoreishi et al, 1997, 2000). B-lymphocytes can be obtained from patients with bipolar disorder and healthy volunteers and virally transformed into BLCL and used to examine the effects of mood stabilizing drugs, which is of particular importance to evaluate the therapeutic relevance of the pharmacological effects observed in rat models. The relevance of BCLC models for studies of BD has been supported by a number of earlier findings. For example, altered β-AR density and functionality (Kay et al, 1993, 1994), Na^+/K^+-ATPase activity (Cherry and Swann, 1994), and intracellular calcium concentrations (Emamghoreishi et al, 1997) have been found in BLCLs from bipolar patients. In addition, the mRNA and/or protein levels of these genes can be measured in different postmortem brain regions of bipolar patients, compared with controls matched with age, gender, pH and postmortem delay. The examination of different regions of the brain is important, because earlier findings suggest that lithium acts specifically in selective brain

regions including frontal cortex, hippocampus, and striatum (Manji et al, 1993; Chen et al, 1998), areas of the brain which have been implicated in the neuropathology of BD (Drevets et al, 1997; Ketter et al, 1997; Rajkowska et al, 1999).

The ultimate goal of any analysis of differential gene expression is to decipher the relevance of a responsive gene in relation to the paradigms being studied, but in order to make this connection the biochemical roles of the responsive gene must be well characterized. In this study, we have identified three genes differentially regulated by lithium. The biological functions of two of these gene products, DIPP2 and CD151, are poorly understood especially in the brain and need to be further characterized. Empirically, the most definitive means to assign a function to a gene is to artificially augment its expression by transiently transfecting an expression vector containing the cloned gene. On the other hand, the expression of the gene can be decreased substantially with antisense oligonucleotides or abrogated with knock – out approaches.

DIPP2 dephosphorylates diphosphoinositol polyphosphates, a group of enzymes that might play a role in synaptic vesicle trafficking and maintenance of cell integrity and viability. Since lithium was found to increase the expression of this enzyme, it would be of interest to examine the effect of overexpression of this enzyme on the rate of transmitter release as well as on the integrity of neurons following chemical insults. Similarly, the level of CD151 protein can be suppressed in cells by antisense oligonucleotides and the effect of its underexpression on the activity of PI-4K and the levels of PIP_2 and membrane associated PKCα could be examined. In the case of aldolase, the underexpression of aldolase by antisense oligonucleotide will be used to examine the impact of reduced aldolase expression on the free concentration of IP_3, basal intracellular Ca^{2+} or agonist-stimulated increase in $[Ca^{2+}]$. Adenovirus probably is the vector of choice for overexpression studies, since a number of features inherent to the vector make it rather suitable for protein expression studies in cell biology, including ability to infect a wide variety of nondividing cells, large capacity for insertion of DNA, ease of production and stability of viral

particles and high viral titers. A protein of interest can be introduced readily into most or all cells in an experimental setting for further characterization (Von and Nemerow, 1999).

CHAPTER 5

CONCLUSIONS

5.1 CONCLUSIONS

In this project, I have provided the first line of evidence that chronic lithium administration reduces the expression of A and C isozymes of aldolase, a multifunctional enzyme, in brain. In addition to being a glycolytic enzyme, aldolase can be found in the nucleus suggesting a possible role in transcriptional regulation, and it binds to various cytoskeletal proteins and $InsP_3$. These findings, in conjunction with evidence from clinical (Meltzer 1968) and postmortem brain (Johnston-Wilson et al, 2000) studies, strongly suggest that down-regulation of aldolase expression may be of importance in the therapeutic action of lithium.

I also report herein the novel observations that lithium, when administered in a therapeutically relevant paradigm, increases the expression of DIPP2, an enzyme involved in the metabolism of diphosphoinositol polyphosphates, in rat frontal cortex. My results suggest that one of the DIPP isoforms (i.e. DIPP2) may represent a biologically relevant target of lithium therapy, further supporting the notion that abnormalities in the inositol phosphate metabolism and/or the phosphoinositide signaling pathway may be significant in the pathophysiology and pharmacotherapy of BD (Jope, 1999b; Lenox and Hahn, 2000; Li et al, 2000). In addition, regulation of DIPP2 by lithium might also have a protective effect against neuronal cell death; that is also consistent with the current notion of lithium's role in neuroprotection (Manji et al, 1999, 2000).

Finally, the expression of CD151, a multifunctional membrane integral protein, is decreased following chronic lithium, VPA or CBZ treatment in rat frontal cortex. Regulation of CD151 expression by lithium may play a role in regulating the levels of PIP_2 and membrane PKC, both of which are critical components in PI signaling.

In addition to regulating the activity of IMPase (Hallcher and Sherman, 1980), inositol-polyphosphate 1-phosphatase (Inhorn and Majerus, 1987), 3'-phosphoadenosine 5'-phosphate phosphatase (Lopez-Coronado et al, 1999), *myo*-inositol uptake (Lubrich and van Calker, 1999)

and $G\alpha_{q/11}$(Avissar et al, 1988), the present results indicate that chronic lithium administration has the potential to modulate the expression of cellular proteins that may be involved in modulation of PI signaling. Taken together the above observations are consistent with the notion that modulation of critical cellular components in the PI signaling cascade by lithium may be important to its mood stabilizing properties. Finally, the findings further support the usefulness of the mRNA differential display in identifying pharmacologically relevant targets that would not otherwise be considered with the "candidate" gene approach.

In summary, the major findings in this study are the identification and molecular cloning of three novel lithium-regulated genes (aldolase A, CD151 and DIPP2), the expression of which is affected in a time-dependent and pharmacologically specific manner. The potential pharmacological significance of lithium regulation of these genes appears to be related to their involvement in the pathway of inositol metabolism and PI signaling, which are consistent with the current notion that alterations in PI signaling may underlie in part the pathophysiology and pharmacotherapy of BD.

CHAPTER 6

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6.1 REFERENCES

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CHAPTER 7

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Hua LV, Green M and Li PP: Differential display of altered gene expression in rat frontal cortex following chronic lithium treatment. *Fifth Annual Vision in Pharmacology Research Symposium.* University of Toronto, May 1997.

Hua LV, Green M, Warsh JJ, Li PP: Identification of lithium regulated genes in rat brain by differential display RT-PCR. *Southern Ontario Neuroscience Association and Program in Neuroscience Annual Meeting.* University of Toronto, May 1999.

Hua LV, Warsh JJ and Li PP: Lithium regulation of aldolase A expression in rat frontal cortex: Identification by differential display. *Eastern Student Research Forum 2000*, Miami, Florida, Feb. 23rd-26th .

ORAL PRESENTATIONS:

Hua LV, Green M, Warsh JJ and Li PP: Molecular cloning and characterization of a novel lithium-reguated gene in rat brain, *55th Annual Scientific Convention and Program for the Society of Biological Psychiatry*, Chicago, Illnois, May 11-13, 2000.

Hua LV, Green M, Warsh JJ and Li PP: Molecular cloning of a novel lithium-regulated gene in rat frontal cortex identified by differential display, *26th Annual Harvey Stancer Research Day*, Toronto, Ontario, June 15th, 2000.

Hua LV: Identification of novel lithium regulated genes in rat brain. *Neuroscience Serires Seminar*, CAMH, Toronto, Ontario, January 22nd, 2001

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Hua LV, Green M, Warsh JJ and Li PP (2000): Lithium regulation of aldolase A expression in rat frontal cortex: Identification by differential display, *Biological Psychiatry* **48**: 58-64.

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CHAPTER 8

APPENDICES

Appendix I: Primers for PCR and DNA sequencing .

Primers use for Rapid Amplification of cDNA Ends (RACE) and Gene Walking			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
AP1	27	CCA TCC TAA TAC GAC TCA CTATAG GGC	RACE/Sequencing
AP2	23	ACT CAC TAT AGG GCT CGA GCG GC	RACE/Sequencing
AP-oligod(T)	52	TTC TAG AAT TCA GCG GCC GC $(T)_{30}N_{-1}N$	RACE
3HC3-4	26	CGC CTG CAG CCT GCT GTG TGT GCT TA	RACE/Sequencing
3NHC3-4	27	CGC CTG CAG CCT GCT GTG TGT GCT TA	RACE/Sequencing
NP-RACE1	21	GTA CAT GAC CGT CTT CTT GTC	Sequecing
NP-RACE2	21	TCT CGC TGG TAG ACA TCT CTC	Sequencing
5HC3-4	27	CCA TTT CTT TAC GCC GCC ACA CAA GTC	RACE/Sequencing
5NHC3-4	27	CAG GCG GCT GGG CTG GTA GAC ATC TCG	RACE/Sequencing
AP-RACE1	20	AAG CAC CTA CCT AGC AAC AG	Sequencing
AP-RACE2	21	CAG GTC TTC GGC ATG ATC TTC	Sequencing
AP-RACE3	21	AGA ATC CGA GCA TAC CAC TAC	Sequencing
AP-RACE4	20	CTC CGT GGT AGC TTC AAG TG	Sequencing
M_{13} reverse	17	CAG GAA ACA GCT ATG AC	Sequencing
T_7	20	TAA TAC GAC TCA CTA TAG GG	Sequencing
M_{13} forward (-40)	23	GTT TTC CCA GTC ACG ACG TTG TA	Sequencing
UP4	21	GAG TCT TCC CAG TCT TCC ATT	Sequencing
UP3	22	GAG ACC CAA AGT AAT GGG GTA G	Sequencing
RP 0	21	TAG GGA GGA AGA GAG AGT GGT	Sequencing

Primers for mRNA Differential Display (RNAmap Kit)			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
AP-1	10	AGC CAG CGA A	DD
AP-2	10	GAC CGC TTG T	DD
AP-3	10	AGG TGA CCG T	DD
AP-4	10	GGT ACT CCA C	DD
AP-5	10	GTT GCG ATC C	DD
AP-6	10	GCA ATC GAT G	DD
AP-7	10	CCG AAG GAA T	DD
AP-8	10	GGA TTG TGC G	DD
AP-9	10	CGT GGC AAT A	DD
AP-10	10	TAG CAA GTG C	DD
AP-11	10	CAG ACC GTT C	DD
AP-12	10	TGC TGA CCT G	DD
AP-13	10	AGT TAG GCA C	DD
AP-14	10	AAT GGG CTG A	DD
AP-15	10	AGG GCC TGT T	DD
AP-16	10	CGT CAG TGA C	DD
AP-17	10	GCA AGG AGT C	DD
AP-18	10	CTG AGC TAG G	DD
AP-19	10	GGC TAA TGC C	DD
AP-20	10	GTG ATC GGA C	DD
$T_{12}VA$	14	TTT TTT TTT TTT VA	DD
$T_{12}VC$	14	TTT TTT TTT TTT VC	DD
$T_{12}VG$	14	TTT TTT TTT TTT VG	DD
$T_{12}VT$	14	TTT TTT TTT TTT VT	DD
Lseq	17	ATC ACG AGG CCC TTT CG	Sequencing
Rseq	17	GAT ACT GGA CGC GAG CC	Sequencing

Primers for mRNA Differential Display (RNAimage Kit)			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
H-T_{11}G	16	AAG CTT TTT TTT TTT G	DD
H-T_{11}A	16	AAG CTT TTT TTT TTT A	DD
H-T_{11}C	16	AAG CTT TTT TTT TTT C	DD
H-AP1	13	AAG CTT GAT TGC C	DD
H-AP2	13	AAG CTT CGA CTG T	DD
H-AP3	13	AAG CTT TGG TCA G	DD
H-AP4	13	AAG CTT CTC AAC G	DD
H-AP5	13	AAG CTT AGT AGG C	DD
H-AP6	13	AAG CTT GCA CCA T	DD
H-AP7	13	AAG CTT AAC GAG G	DD
H-AP8	13	AAG CTT TTA CCG C	DD
H-AP9	13	AAG CTT CAT TCC G	DD
H-AP10	13	AAG CTT CCA CGT A	DD
H-AP11	13	AAG CTT CGG GTA A	DD
H-AP12	13	AAG CTT GAG TGC T	DD
H-AP13	13	AAG CTT CGG CAT A	DD
H-AP14	13	AAG CTT GGA GCT T	DD
H-AP15	13	AAG CTT ACG CAA C	DD
H-AP16	13	AAG CTT TAG AGC G	DD

Primers for Amplification of Rat Aldolase C			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
5RALDOC	20	ATA TGA AGG CAG TGG AGA TG	cDNA probe
3RALDOC	21	GGA AAG GTT CTG TTC TGA CTT	cDNA probe

Primers for Amplification of Human Aldolase A			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
HALDO5	21	CTG TCT ACA AGG CTC TGA GTG	cDNA probe
HALDO3	20	GTG TAC TTT CCT TGA CAG GC	cDNA probe

Primers for Amplification of Rat CD151			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
5CD151	20	ATT GCT GGC ATC CTG GCC TA	PCR screening/cDNA probe
3CD151	20	GAA GAC CTG CAC ACA GGC GA	PCR screening
3C1	20	CTT CTG TAC AAG CAG CAG GT	cDNA Probe

Primers for Protein Expression			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
C10-U1	30	CCG CTC GAG ATG AAG TTC AAG CCC AAC CA	Recombination
C10-L1	30	CGC GGA TCC TAT CTT ATA CTG GAT GGC AC	Recombination

Primers for Rapid Screening of cDNA Library			
Name	Size (bp)	Primer sequence (5' → 3')	Usage
VSP	21	GCA GAG CTC GTT TAG TGA ACC	Full length cloning
L2SP	27	CCA TTT CTT TAC GCC GCC ACA CAA GTC	Full length cloning
L3SP	20	GAA GAC CTG CAC ACA GGC GA	Full length cloning

Appendix II: Summary of sequence analyses of lithium regulated genes.

LDR	Reverse Northern	Size (bp)	Identity/Homology	Accession #	mRNA (bp)	Overlap	Northern Blot (t-test, P value)
1	Down	540	98% rat aldolase A mRNA	M14420	1339	6-527/819-1339	0.03
2	Up	630	98% rat DRCF-5 (EST)	U95001	624	1-615/11-622	0.02
3	Down	503	95% mouse CD151 mRNA	NM 009842	1819	3-503/334-834	0.03
4	No signal	312	No match				0.39
5	Up	450	95% mouse capping protein mRNA	U10407	1014	9-443/505-941	0.43
6	Weak signal	193	96% rat rS-Rex-b mRNA	U17604	3502	7-192/1240-1429	0.28
7	Up	230	93% bovine vacuolar H^+ ATPase mRNA	J03244	1219	7-95/166-254	0.29
8	Down	177	No match				
9	Up	325	87% human DKFZp 761J1810	AL191985	3304	191-314/3157-3282	0.37
10	Up	179	NO MATCH				
11	Weak signal	274	97% Rat cca2 mRNA	AB000199	1795	13-250/1557-1795	
12	Up	337	94% Rat ribosomal protein L13a	X68282	653	82-331/29-285	0.44
13	Up	233	94% Mouse DNA for SMEC66	D12713	1889	8-231/1583-1805	0.42
14	Up	292	96% mouse ELKL motif kinase 2 (EMK2) mRNA	AF240783	2884	76-291/382-596	0.36
15	Down	340	95% human KIAA0669 mRNA	AB014569	4550	171-339/3098-3266	0.25
16	Up	496	No match				

LDR	Reverse Northern	Size (bp)	Identity/Homology	Accession #	mRNA (bp)	Overlap	Northern Blot (t-test, P value)
17	No signal	243	No match	17	No signal		
18	No signal	186	No match				
19	No signal	205	No match				
20	Up	427	100% Rat EST	AI137766	422	13-409/19-415	
21	Up	322	No match				
22	Up	>314	No match				0.45
23	Up	427	96% mouse Ppar binding protein mRNA	NM013634	5796	2-427/761-1187	0.20
24	Up	302	96% rat phosphoglycerate mutase B mRNA	S63233	1670	15-229/1387-1667	0.35
25	Up	382	93% mouse EST	AI835433	326	148-378/8-238	
26	Up	256	97% rat EST	AI454282	411	20-256/24-261	
27	Up	241	No match				
28	Up	322	93% rat EST	BE117074	400	1-320/7-325	
29	Up	299	97% rat EST	AI575082	541	42-145/404-509	
30	Down	438	89% mouse non-muscle myosin light chain 3 mRNA	U04443	613	21-421/209-607	
31	Down	444	90% rat ribosomal protein S3a	X75161	880	7-440/430-872	

LDR	Differential Display	Size (bp)	Identity/Homology	Accession #	mRNA (bp)	Overlap	Sequence Similarity
32	Up	>149	97% mouse proteinase-3 mRNA	AX001322	5856	1-144/2251-2394	
33	Up	201	No match				
34	Up						
35	Up						
36	Down						
37	Up						
38	Up						
39	Up						

Appendix III: DNA sequences of lithium regulated gene candidates identified by mRNA differential display

LRG	Length (bp)	Sequence
1	540	AGGTGACCGTCACAGCACTTCGTCGAACAGTGCCCCCTGCCGT CCCTGGGGTCACTTTCCTGTCTGGAGGGCAGAGTTGAGGAAGA GGCATCCATCAACTCAATGCTATCAACAAGTGTCCCCTGCTGA AGCCATGGGCCTTGACTTTCTCCTATGGCCGAGCCCTGCAGGC CTCTGCTCTAAAGGCTTGGGGTGGGAAGAAGGAAACCTGAAG GCAGCCCAGGAGGAGTACATCAAGCAAGCCCTGGCCAACAGC CTCGCTTGTCAAGGAAAGTACACTCCAAGTGGCCAGTCTGGAG CCGCAGCCAGTGATCTCTCTTCATCTCTAACCATGCCTACTAA CCAGAGCTGATCTAAGGCTGCTCCATCGACACGCCAGGCCCCT GCCTACCVACTTGCTATTGAAGAGGGGCCGTCAGGCTCTTTCC CATCACTCTTGCTGCCCTCGTGTGTGCAGTGTTGTCTGTGAATG CTAAATCTGCCATCCCTTCCAGCCSACTGCC**AATAAA**CAGCTA TTTAAGGGGAAAAAAAAAAAAA
2	630	AAGCTTTGGTCAGAWACATTTGGACTAAGTCATGTCACCTGG GTCAGGATTTTCTTCAATGCCGTGTAGCAAAACTATCTTTAGT CTATGCAAATAGCGAGTCACTGAGTGTGACAGAATGCAACTTC ACTGTTAAACTTCACCTGAGGGTTCCTCATTCTCCTGGAATCC AGACTGCAAGATTATAAAGGAAAAGACCTAAGGCAATTCAGT TCTTTTTGCAAATCAATTGAATCCACGAGAGATGTCTACCAGC GAGATGTCTACCAGCCCAGCCGCCTGCAGCCTGCTGTGTGTGC TTATTTGTGCGCTGAATAAAATGGGGCAGCTAAATTCTCCAGT TCCATATGCCTCCGAAGTTCAAAGAAAAAAAGCAAAGTAACA TGTTAGACTTGACTTGTGTGGCGGCGTAAAGAAATGGCATCTT CCCACTAAGAACGAACCATCCAGTTCTTTTGTCAGTCACACTA TGAAACAGGGAAGGTGAAGGGAAGAAATGGTTATGTGTGCAC GAATCGCTTTGCATGGTCTCATGAGATGGCTGCATTCGAACTG TTTTAAGAATTGTAAGGATCTTGACTTTTTTACATTTGGAAACA TCA**AATAAA**AACAAACATA ATCTGAAAAAAAAA
3	503	GGGAATTCAACGAGAAGAAGGCAACATGTGGCACAGTCTGCC TCAAGTATCTGCTGTTCACCTACAACTGCTGCTTCTGGCTGGCT GGCCTGGCCGTCATGGCAGTGGGCATTTGGACACTGGCCCTCA AGAGTGACTACATTAGTCTGCTGGCTTCAAGCACCTACCTAGC AACAGCCTACATCTTAGTGGTGGCTGGTGTTGTTGTCATGGTG ACTGGAGTCCTGGGCTGCTGTGCCACTTTCAAGGAGCGACGGA ACCTGTTACGCTTGTACTTCATCCTGCTCCTCATCATCTTCCTT CTGGAGATCATTGCTGGCATCCTGGCCTATGTCTACTATCAGC AGCTCAACACCGAGCTCAAGGAGAATCTGAAGGACACCATGA TCAAGAGATACCACCAGTCAGGCCATGAGGGTGTGACCAACG CTGTAGACAAGCTGCAGCAAGAGTTCCACTGCTGTGGCAGCA ACAACTCTCGAGACTGGAGAGACAGCGAGTGG

LRG	Length (bp)	Sequence
4	312	AGCCAGCGAAGAAAGCAGAAGCTCTGGAAGCAGCCATTCAGA ACCTCACTGAAGCCAAGAACTACTTTGCAAAAGTCGACTGTAG AGAGCGCATCAGGGATGTGTCGTACTTCCACGCCAGGCTGTAC CACGCCCTCGGCAAGACCCAGGAGAGGAACCACTGTGCCATG GTCTTCCGGCAGCTGCACCAGGAGCTGCCTTCCCATGGGGTGC CCCTGATTAACCATCTCTAGAAAAGGCTCCCTGCTTGATGCTC TGTAGTGTCTCTATATGCTATCTTGT**CAATAAA**CTGTNCTGGTC AAAAAAAAAAAA
5	450	AGGTGACCGTCAGGGAGGGAAAGGAAGAGGGAAAAGGAGCA GAAAACGAGTTTTCTAAAGAAAGGAACCTAACGAGTGCATGG CGTGTCCGGTTAGCGTGAAGCAGGTCTTTAACACTGCTGCTTT CTCTTCAAGGCTTCCACCAGGTCATTCTTAAGGGCTTCCTGCTT TGATTTGTCTGCAAACGTCTGCACAGACCTCAGCCCGTTGACG ATGTCCTTTGTTTTTCCAAAGTAGATCTCATTCAGCGTGCTTCT GATTTGTTTTCCATGTCCTCCACCAGGCGCCCAATGTTGGCTAT GTGTGGGGAACAGTCACTCACAGTTTCGTCCTTCTCCATCTGT CTGGTTAGGCTGCCTCCCAGGTTCATGGTGCCATGAGCCGGAT CTGTTGGTTTGCAGCCATAGCATCACTGTGGAGGTCAACTAGA AATGGGCAGTACGCTCACCT
6	195	AGGTGACCGTGTATATGGTGATCCGTTTTGTATAACTAAGAAT GATCGAATTAAAGAATAAATCCAAAAATGAACGCACAGTACT TTTATTGTATGGGATGCCTGGGCTGATTTACATGTATGGTAAC TAAAGTGCCAGCATGTAGACTTTATTACGATTTGTATTTCTTCC TCTGTAGTTCCTAACGGTCACCT
7	230	GCAATCGATGCTAAGGCAGAAGAAGAGTTCAGCATTGAGAAA GGTCGTCTTGTGCAAACCCAGAGATTGAAGATTATGGAATACT ATGAGAAGAAGGCATCATGACCCCTGACACTGGAGTTAGAGA TTAGTTATCAGACCCCATGTGAGGACTGAGAACCAAACCTGA GTCTTCTGTAAGAGCAGCCAATGCTCTTAACCCCAGAGCCATC TCTCCAGCCATCGATTGC
8	175	AGGTGACCGTGCAAAGGAACGCCTAGACGAGGAACTCCGAAT ACAAGCCCAGGAAGAGAGAGAAGGGCAGACGCCCAATAC GTGACTGCTGGGCTCTCCATACCCGCCCATTGCCTCATAATTT ATAGCCTAGT**AATAAA**TGTCTTTTCGTGTTTCTCCGCAAAAAA AAAAAA

LRG	Length (bp)	Sequence
9	325	CCGAAGGAATGGTGGCAAAAGAAAATAGTATCGCTCATAAGT TAGTGNATTTTATTATTGGACCCACCTACTCGGAAGCTAGGTC ACCCCAGACCTCTGTCCCATCCACACCGTTGTCCATCTGGGGG ACTAACCCTGATTCAGAGCCAGTACTTAGAGCCAGTCCATACC TACTAGTCCCATCCATGCCACCTACATCCAAGGTCTTCAGCTTT AGACTGCTGGGGCCAGGCCCCAAGGAGGGTTTACTGAGGGGT CCATGGGTGTGACTAAAAT**AATAAA**TGATACCCGTGTTTGATG CGCTTTTAACTTTGACTAAAAAAAAAA
10	179	AGCCAGCGAACGGTAGTGATGACCAGCCTGGTGACAGTCGTT GTCCCTCTGGTTTTCTGGGTTTGACCCATTATGTTCCATCATCT TTGTTTTGACTTTTATGCAGCTGAGCAATTTGAACTGTTCTAAA CCATTTCACTCCCACGTTTCTTGGTTAGTGTACCCCCAAAAAA AAAAA
11	274	TTTTTTTTTTTTGGGGGGGGGGGAGCTGGGGACCGAACCCAGG GCCTTGCGCTTACTAGGCAAGCGCTCTACCACTGAGCTAAATC GTTTTCTCAAGTGTATCTCTTTTAGACATTTTTCTTTTAATCTGC TTTAAAGAAAACTGAAGGAATGAATGGTTTTCCATGTCTTGCC TTCTTACAAATTCAGCTATGATATTGTTAGCTTCCTCCCTGAGG ATGCCAACCTGTATCTGGCCACACCTGGATTAAAATCCTTTGC CAAAAAAAAAAAA
12	337	GGATTGGTGCGGCACATGCCTCGCACAGTGCGCCAAAAAATG CGGCTTGGGGCTCGGGAAGTGGGTAGGAATCTCGAGACGGGT TGTTGTTCATCCGCTTTCGGAGAAAGGCCAGATATTTAACTTG TTTCTGTAGAAATTTCCAGAAATGTTGATGCCCTCACAGCGTA CAACCACCACCTTTCCGCCAGAGTACCTGCTTGGCCACAATCG CCAGGCGGCCCAGAAGATGCCCCGGCCATCCAATACTAGAAC CTAAAGACTGCCGCGGCTGGTTATGCCCACGTTACACAACCCC CTCGCGTCAAAACGTTTGCTTAAGTCCCATCGCACAATCC
13	233	GTCGGCAATATACAGAGCGGAGACAGAGGAAGGGCCAGACCT GCTTAGGTGGTTGGACAGACAGCTCATTCGAATGTGTCAGAAA TTTGGAGAGTATCACAAAGATGATCCAAATTCCTTCCGGTTTT CAGAAACATTTTCCCTTTATCCACAGTTTATGTTTCATTTAAGA AGATCTCCCTTCTTGCAAGTTTTTAACAACAGTCCTGATGAGA GTTCATACTATTGCCACA

LRG	Length (bp)	Sequence
14	292	CGTGGCAATAAAGATAATTGACAAAACTCAGTTGAATCCAAC AAGTCTACAGAAGGGTTTGTCTTTTGCTTTTTTAGCTCTTCAGA GAAGTAAGAATAATGAAGATTTTAAATCACCCAAACATAGTG TTATTGTTCGAGGTCATTGAAACGGAAAAAACACTCTACTTAA TCATGGAATATGCAAGTGGAGGTGAAGTATTTGACTATTTGGT TGCACATTGGAAGAATGAAGGAAAAAGAAGCAAGAGCTAAGT TTAGACAGATTGTGTCCGCAGTACAGTATTGCCACG
15	340	GGAGCCTGTTTAGATATCACAGAAACATTAAAAAGCAAGAAG GAGAAAACTGCTTTTAAAAAACAAAAATATAGAAACCATTTA GCGTTTATAAGGCCAGATAGCCTAGAACAATGTAGCTCTTAAC TCTTCTCAACTCTTTTTTGATGTTCCTAATTTTGTTCTTCACAGG ATCTGGTGAAAAGCCATTTGATGTATGCAGTAAGAGAAGAAG TGGAAGTTCTAAAGGAGCAGATAAAAGAATTAGTGGAAAGAA ACTCTTTACTTGAACGAGAAAATGCACTGTTAAAATCTCTTTC AAACAATGATCAGTTGTCCCAACTCCCAGCCCAACAGGCCCT
16	496	AGGGCCTGTTAAATAAGTGTGCTTATGTGCTTAGTGACCTGAG TTTGAGTCCTGTAACACACATGGAAGAAAGGTGTCCTCTGGCT TCCACATGCACACCATGGTACATATGTGCATATGAAGAAGATA AGTAAGTGTACATTTTTAAAGATGTTTAAATCAAGTCTGGGAA TTGTTAATCTAATGCTTTCTGCCTAATTAAAACAAATAAAGTG GGGCTGGAGAGACGACTCAGCAGTTAGGAGCATATATGGTTC TTCCAGAAGACCAAAGTTAAGTATCGACATCCGATGGCTCACA ACTTCTGGTAACTCAAACTCCTGCAGAACCCGACTCCTGTGGC CTCTACGGGATCCTGCACCAGTGTGCATGCACCTACACGCACA GAAATGTAATCTTAAGAAAAGAGAGAGAGCACCGCTCTGACA GTGAGCCCTGCCCATAGAGACCAACTACACAAGCTACTCCAGT TACACTGCCAACAGCTCAACAGGCCCT
17	243	CGTGGCAATACTATCAGACTGGATGAATTAGACAATGAAATTT TGTGCAAATAAAGGCAATAAAGAATGAAAAAGTCACTATCTC AGTAAAACTTTAGACAGAACTCAACATTCCAGAAAGACCCTG AGGTCTCACCAGTAAGAGAATTATGGAAGGGAACCATATATC TTTCCAAAGATTCCCACCACCACTTCTCTTCCTCTTGAAATGAA **AATAAA**CTGTGGACCTCCTATTGCCACG

LRG	Length (bp)	Sequence
18	186	AGGTGACCGTGCAAAGGAACGCCTAGACGAGGAACTCCGAAT ACAAGCCCAGGAAGAGAGAGAGAAGGGCAGACGCCCAATAC GTGACTGCTGGGCTCTCCATACCCGCCCATTGCCTCATAATTT ATAGCCTAGT**AATAAA**TGTCTTTTCGTGTTTCTCCGCAAAAAA AAAAAA
19	205	CGTGGCAATAGGTCACCAGTCATATAAACTCACATACCCTGCA CAAAATAAAGTCACTATCAATTATAAGACCCTCCCCAAGGAG AAGCGATCACTAAAGTCTCTAAGAGCCTGCCACCCATCCCATA CTGTCCTTGATAGACCAAGCCCTTTAAGTGTATGCATCACGCA TACACTCATGTTACATCCGCCTATTATTGCCACG
20	427	TTTTTTTTTTTTGCAGGAGATGGGATCTTTATTAGCAGCTAGAG CTATGAGCTACAAATAAATCAAAAACATTGACACTGAAGTTG CTGGAAACAGGTCTGTACAGAAATATTACAAAAATCTCATCTT CCAATCCCACAGCTAACCTCTTGGGGAAGGGCAGCCTTGGCCA GGGCAAAGCAGcACAGaAGTTATCCTAAGAAAGTCTCATCCAC CAGGAAAGCAGTGAACTACAGCTACATGGCACTAAAACTCAA GATAGCAGTTTGTAAAGAGTCCCTTAATCATAAAAGTTTTCGA ATGAAAACACGTGCCCCTCCTGTCCCGGGGGCTTCCAGATCTC TACCCAAGAGACTTGAGTACTTCTATTAAGGCAACTGGAAGCC CAGCACGTCCTTCCTAACATGCTATGGGGAAAAAAAAAAAA
21	322	CGTGGCAATAGCAATTAAAGATGTAAGAATTTTCACATTAAAA CCTGTGAGGTAAGTTGTAGAGAAATGCAGAATTTGAAAGTAC TCTTTCCTTCTGCTTCTCTTCCTCTGAGAGAACAGTAAGGGCTC AGGTCTGCAGACTGAGCCTCTTCTGAATGTTGTATGTGATCTTT GGAGTGAGGATTTAGTTAATTGTTCCAGTTGCTTTCCTTGTTTC TCTCGTTCCTTTCTGCTACTCTGTTGGATTTTGAACCTCGTTTA GGAGAATAATGTGCTCATTTGTGTCTTCTCCCCCTGCTCCCCCC CCCACAAAAAAAAAAAA
22	>314	GTGATCGGACCACACACCACATCCTGCAGAAGCAAGAAGGCT TCTTCAAATCTAGATTGACATGGAGCTTTATTTGCTAGACCAA AGTGTACGTGTAATCAGCTGTTATTAACCCCAGACTCTTGATT AAAGTCCTAAAAGTTTCGTACAGGAATTGAGAGTAGCTGTTTC ATTGGCAGTATCCATCCTGCAAAACATGCCACAGCCAACGCAT GAGGTATGATGTCTGAGGGGTTGCCCGGGTTTAGGACATCATG ACCATGGTCCAACGTACCTGCGTCTTCTCATACTTTACATTTTC TTTCTTCTGGAAT

LRG	Length (bp)	Sequence
23	427	TGCTGACCTGCTGCATGATACAACTGCCTCTCCTATCACTTTGC ATGAAAAGAATGTTAATCGGTCTTTGGGAATGAATGCCTCAGT GACAATTGAAGGAACCTCTGCTATGTACAAACTCCCAATTGCC CCATTAATTATGGGGTCTCTCCCAGCTGACAACAAATGGACCC CTTCTTTCTCTGCAGTCACTAGTGCCAACAGTGTTGATCTTCCT GCGTGTTTCTTCTTGAAATTTCCCCAGCCAATCCCAGTATCTAA AGCATTTGTTCAGAACTGCAAATTGCACGGGAATCCCGTTGTT TGAGACTCCGCCTACTTACCTGCCCCTCTATGAACTCATCACTC AGTTTGAGCTGTCAAAGGATCCTGACCCTTTACCTTTGAATCA CAACATGCGATTTTACGCTGCTCTTCCAGGTCAGCA
24	302	GTGATGGATATCTGCNGAATTCGGCTTTTTTTTTTTTTTGCACA TCACTCCTTTATTATACTGATCTGGAAAGAGACTTAGTTGTTAT GCCCAAACGAGTTCTGAACCCACACAGTAGGGTCAAGCAGCT GGAACATGATTCAGACAGTGAAAGACAAAACCTTGGACATGA TCAAGGGGCTTCACCTCTACAAAACAAGGGCAAGGGGATCCA AACACAGCAAGAGGTACAAGGAGTTTATCCCATTTTGATATGA GGGAATGGCTTTTTTTCTGGTGACCACTTAGGACTCTCAACTG CCACTAGCAACCTTGTTGCTTTTTGG
25	382	AAGCTTAGTAGGCCTAGTGTAAAAACAGAATGCTTGAGTTTTC AGCGTGAGGDCACGCGGATGCCAAAGGTCTGGGCTTTGACGT TGACTGGTGGGCGAGTCTCCAGCCCTTCCTTGACTCTTATTGGT GGACTCCATCTCCACCGTTTATGAGGDAAATTTCACACCACTT CACATCGTATATCTCAGGCCCATACTGTGAATTTAAAAATGGG GAATTAATAATTACTTATAACTCCCTGCAACTGTGGCTGGCAG ATAAAATGCCATCTGCCAGACAAGATTTATTTATAATGGGGTT TATACATATTTATAAGTTTGCAAGCDACGAATGCACAGTTTTR AAACTGGCACATTTTATATTGGCTAAAAAAAAAAAGCTT
26	256	GGGVCGCCATGGCGCGGGATTATTGTTAAACAGAGAAGTCAC AAAGCAAAAGTGAAATGGGACACAAATCCAAGATGATTTTAA AACATTTCTTTTGGTGTGTGTCAAATATTTTTCTAAGTTTAANA AGGAGGNAAAGTGACAAACGAAAGAKACTTTGTTCAAATGAA GTCTTTCATTGGAAAGGAAAACCACTGGTCTTTAGAACCTAGA TCATGACCCACAGCCCTTACTTAAGAGATGGCGGTAAAAGCTT

LRG	Length (bp)	Sequence
27	241	CGTGGCAATAGGAGGTCCACAGTTTATTTTCATTTCAAGTGGA AGAGAAGTGGTGGTGGGAATCTTTGGAAAGATATATGGTTCC CTTCCATAATTCTCTTACTTGGTGAGACCTCAGGGTCTTTCTGG AATGTTGAGTTCTGTCTAAAGTTTTACTGAGATAGTGACTTTTT CATTCTTTATTGCCTTATTTGCACAAAATTTCATTGTCTAATTC ATCCAGTCTGATAGTATTGCCACG
28	322	TTTTTTTTTTAGTCAAAGTTAAAAGCGCATCAAACACGCCTAG CATTTATTATTTTAGTCACACCTACGGACCCCTCAGTAAACCCT CCTTGGGGCCTGGCCCCAGCAGTCTAAAGCTGAAGACCTTGGA TGTAGGTGGCATGGATGGGACTAGTAGGTATGGACTGGCTCTA AGGACTGGCTCTGAATCAGGGTTAGTCCCCCAGATGGACAAC GGTGTGGATGGGACAGAGGTCTGGGGTGACCTAGCTTCCGAG TAGGTGGGTCCAAT**AATAAA**ATNCACTAACTTATGGAGCGAT ACTATTTTCTTTTGCCACCATCC
29	299	ATTACTTGGYtCTCTCGgWCTGAACWTCTGTWATMTGAATACC ACACTAGACcGgAATGTACTTGTAAGADMAAGTCCAGTGTAAT GGATAGATTAAACCACAGGAATGCTTCCTCAGCACTTCYCCAG GGGGGCGGGRAGAACACTCTGCGTGTCTMAAaDWATCCCCAT GCACACCCAATDTAGACYCWCAKKRaBBGMYAKGGGGgAMA RTAGGGGTGSGYTTTGTtCTCTCWAGAKTGTtATAKGTATTCGC TCTCGGGSGMTACTCTAAaTAACTACATCGWGTTTTWTAyTAC
30	438	AAGCTTCGACTGTBGCCAAGACAAGGACCAAGGAACCTACGA GGANTTATGTTGAAGGCCTTCGTGTGTTTGACAAANAAGGGA ATGGCACTGTCATGGGTGCTGAAATCCGTCATGTCCTAGTCAC ACTGGGGGAGAAGATGACAGABGAAGAAGTCGAGATGCTAGT GGCGGGGCATGAGGACAGCAACGGTTGCATTAACTATGaAGC ATTTGTGAGGCATATCCTGTCGGGGTGACGGGCVCATGGGGC GGASTTGTCCGGATGGTGVTGAATGGCTGAGGACATTTACGTG TCTCGAGCCTGCTCCTGCCCAGTGTGAGATTCCTGTGTGGCCCc AGAGGCTCCCTAATCACAGCACCTTGCCTGTCTGGTTTCTTTTG GATAATGTTTGCCTTCACCA**AATAAA**ACTTGCTCTCTGTGAAA AAAAAAAGCTT
31	>149	AATTCTGCAGATATCCATCACACTGNNNGNCGCTCGAGCATGC ATCTAGAGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAAT TCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG GCGTTACCCAACTTAANNGCT

LRG	Length (bp)	Sequence
32	>201	AGGGTTCCAGCAAGCTAGATTCCAGTTAGTCAGGCTTTACTTC ACAGCATTCTTTTTTAAAAAATGCTTTATATTTATTGGTAAGAT GAGCCCCTCCCCCTCCTAAACAGGAAGTGGGATATTTCTAATC AGCCTCAAAGTTTAAAGGTTGCGAATGGTCATATAATCTGCTC AAAAATCACCTCAATTTCTAGTCTAAAG
33	444	AAGCTTCGACTGTTCTGTGTTGGTTTCACTAAAAAGTACAACA ACCAGATTAGAAAGATATCCTATTCGCAGGTCTACCAGATCCA GAAGAAGATGATGGAAATCATGACCCGAGAAGTGCAGACAAA TGACTTGAAGGAAGTAGTTAATAAACTGATTTTAGGCAGCATT GGGAAAGACATAGAGAAGGTTTGCCAGTCCATCCATCCTCTCC ATGACGTCTTTGTTAGGAAAGTCAAAATGCTGAAGAAACCCA AGTTTGAATTGGGAAAACCCATGGAGCTCCATGGTGAAGGCG GTGGTTCTCACAAAACAACTGGTGAGGAGACAAGTGCTAAAG TTGAACGAGCTGATGGCTATGAACCACCAGTCCAGGAATCAG TTTAAACTCCAGATTTTTAATAGTGACA**AATAAA**AAGTCCTAT TTGTGAAAAAAAAAAGCTT